APC

FIFTH EDITION

TEXTBOOK OF MICROBIOLOGY

Dr. C P Baveja

ARYA PUBLICATIONS

Contents

UNIT I

GENERAL MICROBIOLOGY

Chapter 1

HISTORICAL INTRODUCTION

I. INTRODUCTION

Microbiology is the science of living organisms that are only visible under the microscope. *Medical microbiology* deals with the causative agents of infectious diseases of man, his reaction to such infections, the ways in which they produce disease and the methods for their diagnosis.

The disease is transmitted by contact, from man to man, was known since biblical times. *Varo and Columella* (first century B.C.) postulated that diseases were caused by invisible organisms which they called '*Animalia minuta*'. *Fracastorius of Verona* (1546) proposed a *contagium vivum* (living germs) as a possible cause of infectious disease. *Von Plenciz* (1762) suggested that each disease was caused by a separate agent. *Kircher* (1659) reported finding minute worms in the blood of plague patients, but it is more likely that he observed only blood cells with the equipment available to him.

As microbes are invisible to the unaided eye, definitive knowledge about them had to await till microscopes were developed. The credit for observation and description of bacteria goes to *Antony van Leeuwenhoek* (1632-1723), a draper from Delft, Holland, whose hobby was grinding lenses. He described three major morphological forms of bacteria and communicated his findings to the Royal Society of London. The significance of these observations was not then realised. In 1678, *Robert Hook* developed compound microscope and confirmed Leeuwenhoek's observations.

To Leeuwenhoek the world of those "little animalcules" represented only a curiosity of nature and their role in disease was not realised. Their observations lay dormant almost for a century. It was only some two centuries later that the importance of microorganisms in medicine was recognised.

The earliest discovery of pathogenic role of microorganisms probably goes to *Augustino Bassi* (1835), who showed that the muscardine disease of silk worms was caused by a fungus. *Oliver Wendell Holmes* (1840) and *Ignaz Semmelweis* (1846) had independently concluded that puerperial sepsis was transmitted by the contaminated hands of obstetricians, nurses and medical students and this could be prevented by washing hands in antiseptic solution.

Needham (1745), an Irish priest, believed in spontaneous generation (abiogenesis) of microorganisms from the decomposing organic matter. He had shown in his experiments that microbes developed in closed

flasks of previously heated putrescible fluids. This view was opposed by *Spallanzani* (1769) who found no such generation of microorganism after long heating of the putrescible fluids. He proved that organic contents of flask supported the microbial growth when exposed to air. Later on, Pasteur proved conclusively that all forms of life, even microbes, arose only from their like and not *de novo*.

II. SCIENTIFIC DEVELOPMENT OF MICROBIOLOGY

Scientific development of microbiology was ushered by *Louis Pasteur*, perfection on microbiological studies by *Robert Koch,* the introduction of antiseptic surgery by *Lord Lister* and the contributions of *Paul Ehrlich* in chemotherapy.

A. Louis Pasteur (1822-1895)

He was a trained chemist of France. His studies on fermentation led him to take interest in microbiology. He established that fermentation was caused by microbial agents. He further noted that different types of fermentations were associated with different kinds of microorganisms. He is also known as *father of microbiology*.

Fig. 1.1 Louis Pasteur

Important Contributions of Louis Pasteur in Microbiology

1. Development of methods and techniques of bacteriology.
2. Proved conclusively that all forms of life, even microbes, arose only from their like and not *de novo*.
3. Pasteur also had to face challenge from *Pouchet*, who was a proponent of *spontaneous generation* theory of microbe. Pasteur disproved the view by demonstrating the ubiquity of microorganisms in the air by his experiments performed in the swan-necked flasks.
4. Introduction of sterilisation techniques and development of steam steriliser, autoclave and hot-air oven.
5. Studies on anthrax, chicken cholera and hydrophobia. During studies on rabies, though Pasteur could not isolate any microorganism from man and dog but suggested that the causative agent of rabies was too small to be seen by microscope.
6. Live vaccine: He introduced attenuated live vaccine for prophylactic use. An accidental observation that chicken cholera bacillus cultures left for several weeks lost their pathogenicity but retained their ability to protect the birds against subsequent infection by them led to the concept of attenuation and development of live vaccines. He attenuated the anthrax bacillus by incubation at high temperature (42°–43°C) and proved that inoculation of such bacilli in animals induced specific protection against anthrax. He convincingly demonstrated the protective role of anthrax vaccine in a public experiment (1881) where vaccinated sheep, goats and cows were challenged with a virulent anthrax bacillus culture. All the vaccinated animals survived while an equal number of unvaccinated control animals died. Pasteur coined the term *vaccine* for such prophylactic preparations to commemorate the first of such preparations, namely, cowpox, employed by Jenner for protection against smallpox.

   Pasteur's development of a vaccine for hydrophobia marked a milestone in development of immunisation in medicine. He obtained the fixed virus of rabies by serial intracerebral passage in rabbits. The rabies vaccine was prepared by drying pieces of spinal cord from rabbits infected with fixed virus. This work was acclaimed throughout the world. The Pasteur Institute, Paris, was built by public contribution for the preparation of vaccines and for the investigation of infectious diseases.

B. Joseph Lister (1827-1912)

He was a Professor of Surgery in Glasgow Royal Infirmary. He applied Pasteur's work and introduced antiseptic techniques in surgery (1867) effecting a pronounced drop in mortality and morbidity due to surgical sepsis. It was a milestone in the evolution of surgical practice from the era of 'laudable pus' to modern aseptic techniques. His antiseptic surgery involved the use of carbolic acid. He is known as *father of antiseptic surgery*.

C. Robert Koch (1843-1910)

Robert Koch was a German general practitioner. He is also known as the *father of bacteriology*. His contributions are as follows:

1. Perfected bacteriological techniques and introduced methods for isolation of pure strains of bacteria.

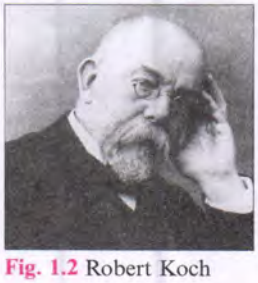


Fig. 1.2 Robert Koch

2. Introduced methods of obtaining bacteria in pure cultures using solid media.
3. Introduced staining techniques.
4. Discovered the anthrax bacillus (1876), tubercle bacillus (1882) and the cholera vibrios (1883).
5. ***Koch's postulates***: According to Koch's postulates, a microorganism can be accepted as the causative agent of an infectious disease only if the following conditions are fulfilled.
   (*i*) The organism should be constantly associated with the lesions of the disease.
   (*ii*) It should be possible to isolate the organism in pure culture from the lesions of the disease.
   (*iii*) The isolated organism (in pure culture) when inoculated in suitable laboratory animals should produce a similar disease.
   (*iv*) It should be possible to re-isolate the organism in pure culture from the lesions produced in the experimental animals.

   An additional criterion introduced subsequently requires that specific antibodies to the organisms should be demonstrable in the serum of patients. These postulates have proved to be useful in confirming doubtful claims made regarding the causative agents of infectious diseases. However, it may not always be possible to satisfy all the postulates in every case. An important example of not fulfilling the Koch's postulates is lepra bacillus.
6. ***Koch's phenomenon***: Robert Koch observed that guineapigs already infected with tubercle bacillus responded with an exaggerated inflammatory response when injected with the tubercle bacillus or its protein. This hypersensitivity reaction is called *Koch's phenomenon*.

D. Paul Ehrlich (1854-1915)

Paul Ehrlich was a German scientist and is also known as *father of chemotherapy*. His contributions are as follows:

1. Applied stains to cells and tissues for study of their functions.
2. Reported the acid-fast nature of tubercle bacillus.
3. Proposed '*side chain theory*' of antibody production.
4. Discovered *salvarsan*, an arsenical compound, sometimes called the '*magic bullet*'. It was capable of destroying the spirochaete of syphilis. Later on he discovered '*neosalvarsan*' and thus created a new branch of medicine known as *chemotherapy*.
5. Introduced methods of standardising toxin and antitoxin.

Important Discoveries by other Scientists

Lepra bacillus	:	*Hansen* (1874)
Gonococcus	:	*Neisser* (1881)
Diphtheria bacillus	:	*Klebs* (1883), *Loeffler* (1884)
Pneumococcus	:	*Frankel* (1886)
Meningococcus	:	*Weichselbum* (1887)
Diphtheria toxin	:	*Roux and Yersin* (1888)
Tetanus bacillus	:	*Kitasato* (1889)
Plague bacillus	:	*Yersin* (1890)

III. VIROLOGY

The existence of viruses became evident during the closing years of nineteenth century, when many infectious diseases had been proved to be caused by bacteria. But there remained a large number of infectious diseases for which no bacterial cause could be established. During studies on rabies, Pasteur suspected that the causative agent could be too small to be seen under microscope. The existence of such ultramicroscopic microbes was proved when *Ivanovosky* (1892) reproduced mosaic disease in tobacco plant by applying the juice of diseased plants to the healthy leaves. *Loeffler and Frosch* (1898) observed that the causative agent of foot and mouth disease in cattle could also pass through a bacterial filter. *The first human disease proved to be of viral origin was yellow fever*. It was discovered by *Walter Reed* (1902) in Cuba and he established that the disease is transmitted through the bite of infected mosquitoes. *Landsteiner* and *Popper* (1909) demonstrated that poliomyelitis was due to filterable virus and they also transmitted the disease experimentally to monkeys. *Goodpasture* (1930) developed techniques of viral cultivation in chick embryos. The application of tissue culture expanded the scope of virological techniques considerably. Though the larger viruses could be seen under light microscope after appropriate staining, detailed morphological study of viruses became possible only with the introduction of electron microscope by *Ruska* (1934).

The possibility of virus causing cancer was first put forth by *Ellerman* and *Bang* (1908) in fowl leukaemia. *Rous* (1911) isolated a virus causing fowl sarcoma. Several viruses have since been isolated which cause tumours in animals and birds. Viruses can also cause malignant transformation of infected cells in tissue cultures. The discovery of viral and cellular oncogenes was a breakthrough for possible mechanisms of viral oncogenesis. The positive proof of viral causation of human malignancy was established when the human T-cell leukaemia virus was isolated in 1980.

KEY POINTS

1. *Microbiology* is the science of living organisms that are only visible under microscope.
2. Scientific development of microbiology is contributed by several scientists.
3. A microorganism can be accepted as the causative agent of infectious disease only if it fulfills the *Koch's postulates*.
4. Nobel prize has been awarded to various scientists for their discoveries.

YOU MUST KNOW

1. Contributions of Robert Koch, Louis Pasteur, Joseph Lister and Paul Ehrlich.
2. Koch's postulates and Koch's phenomenon.

STUDY QUESTIONS

1. Write short notes on:
   (a) Robert Koch
   (b) Louis Pasteur
   (c) Koch's postulates
   (d) Koch's phenomenon
   (e) Paul Ehrlich

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Who is known as the Father of Microbiology?
   (a) Paul Ehrlich (b) Joseph Lister (c) Louis Pasteur (d) Kitasato
2. Who introduced the techniques of sterilisation?
   (a) Robert Koch (b) Louis Pasteur (c) John Needham (d) Joseph Lister
3. The term vaccine was coined by:
   (a) Louis Pasteur (b) Robert Koch (c) Joseph Lister (d) Edward Jenner
4. Who introduced methods of obtaining bacteria in pure cultures using solid media?
   (a) Robert Koch (b) Louis Pasteur (c) Joseph Lister (d) Paul Ehrlich
5. Who discovered *Mycobacterium tuberculosis*?
   (a) Louis Pasteur (b) Robert Koch (c) Loeffler (d) Yersin
6. Who is known as the '*Father of Chemotherapy*'?
   (a) Robert Koch (b) Louis Pasteur (c) Paul Ehrlich (d) Joseph Lister
7. Who proposed 'side chain theory' of antibody production?
   (a) Paul Ehrlich (b) Edward Jenner (c) Robert Koch (d) Joseph Lister
8. Who introduced vaccination method for prevention of small pox?
   (a) Edward Jenner (b) Joseph Lister (c) Robert Koch (d) Louis Pasteur
9. Which is the first human disease proved to be of viral origin?
   (a) Japanese encephalitis (b) Yellow fever (c) Dengue (d) Small pox

Answers (MCQs):

1. (c) 2. (b) 3. (a) 4. (a) 5. (b)
6. (c) 7. (a) 8. (a) 9. (b)

Chapter 2

MICROSCOPY AND MORPHOLOGY OF BACTERIA

I. INTRODUCTION

Microorganisms are living structures of microscopical size. These were originally classified under plant and animal kingdoms. This classification proved unsatisfactory, therefore, a third kingdom *Protista* was formed.

Protista

It is divided into two groups—*prokaryotes* and *eukaryotes* (Table 2.1).

1. *Eukaryotes*

Fungi, algae (except blue green algae), protozoa and slime moulds are included in this group.

2. *Prokaryotes*

Bacteria and blue green algae belong to this group. Bacteria are unicellular without any true branching except in the higher bacteria (*Actinomycetales*). They don't contain chlorophyll but in contrast blue green algae possess chlorophyll.

Bacteria

The size of bacteria is measured in unit of a micron (micrometre).

Table 2.1 Differences between Prokaryotic and Eukaryotic Cells

Structure	Prokaryotes	Eukaryotes
Nucleus		
Nuclear membrane	Absent	Present
Nucleolus	Absent	Present
Chromosome	One	More than one
Deoxyribonucleoprotein	Absent	Present
Division	By binary fission	By mitosis
Cytoplasm		
Mitochondria, Golgi apparatus, Lysosomes, Pinocytosis, Endoplasmic reticulum	All are absent	All are present
Chemical Composition		
Sterols	Absent	Present
Muramic acid	Present	Absent

1 micron (μ) or micrometre (μm) = 1/1000 millimetre

1 millimicron (mμ) or nanometre (nm) = 1/1000 μm (micron) or one millionth of a millimetre

1 Angstrom unit (A°) = 1/10 nm (nanometre)

Bacteria of medical importance measure 2-5 μm (L) × 0.2-1.5 μm (W). The resolution power of an unaided eye is about 200 microns. Bacteria, being much smaller than the resolution limit, can be visualised only under magnification. Hence, the study of bacteria requires the use of microscopes.

II. MICROSCOPY

Microscopy is done for following purposes:

1. magnification of an object
2. maximisation of resolution
3. optimisation of the contrast between structures, organisms and background.

The following types of microscope are being employed for the study of bacteria.

A. Optical or Light Microscope

The light microscope contains a light source and a compound lens system. The lens system consists of a number of *objective lenses* in conjunction with a fixed (usually X10), ocular (*eye piece*) lens. Light microscopes are equipped with objective lenses of low power (X10), high power (X40) and oil immersion (X100), which will result in final magnifications, in conjunction with the ocular lens, of X100, X400, and X1,000, respectively. Besides magnification, resolving power is another essential component of microscopy. *Resolving power is the ability of the lens system to distinguish two objects as separate, rather than one.* Resolving power is dependent on the wavelength of the light used to illuminate the object and the numerical aperture of the microscope. *Numerical aperture is defined as a light gathering power of microscope.* Resolution power can be optimised through proper use of the condenser, which focuses light into the plane of the object. The commonly used condensers can produce a numerical aperture of 1.25. Resolution power can be further increased by adjusting the medium through which light passes between the object and the objective lens. Special oils, termed *immersion oils*, have a refractive index similar to glass, thus, use of these oils permit more light to be incorporated in the image resulting in improving the resolution power. Visualisation of bacteria generally requires the use of immersion oil with X100 objective. This combination results in resolution of approximately 0.2 microns. However, X40 objective is used for examination of wet preparations e.g. hanging drop, stool for ova and cyst.

B. Phase Contrast Microscope

Phase contrast microscopy improves the contrast and makes evident the internal structures of cells which differ in thickness or refractive index. The different parts of a cell and its surrounding medium have got different refractive indices. When rays of light are passed through an object, they emerge in different phases depending on the difference of the refractive indices between the object and its surrounding medium. A special optical system (special condenser and objective lens) converts difference in phase into difference in intensity of light, producing light and dark contrast in the image. A light microscope can be converted into phase contrast microscope by using a special condenser and objective lens.

C. Dark Field (Dark Ground) Microscope

Reflected light is used instead of the transmitted light used in the light microscope. A dark field condenser with a circular stop is fitted with a light microscope. This condenser lens system is arranged in such a way that no light reaches the eye, unless reflected from the object. The object or bacterium appears self-luminous against a dark background. The contrast gives an illusion of increased resolution, so that extremely slender organisms such as spirochaetes, not visible under ordinary microscope, can be seen under the dark field microscope.

D. Interference Microscope

It is useful for revealing cell organelles and for quantitative measurements of the chemical constituents of cell such as, lipids, proteins and nucleic acids.

E. Fluorescent Microscope

Fluorescence is a phenomenon that occurs when an object is impacted by a given wavelength of light and emits light at a wavelength longer than the one to which it was exposed. In fluorescent microscope, specimens are exposed to a light of shorter wavelength (ultraviolet light), which results in emission of longer wavelength visible light. Due to shorter wavelength of UV light, the resolving power can be proportionately extended. Bacteria stained with fluorescent dye (e.g. auramine, rhodamine) become visible as brightly glowing objects in a dark background.

Fluorescent microscopy has also been employed for detection of antigen (direct fluorescent antibody technique) and antibodies (indirect fluorescent antibody methods). These methods are described in Chapter 14.

F. Electron Microscope

A beam of electrons is employed instead of the beam of light used in optical microscope. The electron beam is

focussed by circular electromagnets (magnetic condenser), which are analogous to the lenses of light microscope. The wavelength of electrons is approximately 0.005 nm, as compared to 500 nm with visible light. The resolving power of any microscope is directly related to the wavelength, thus, the resolving power of the electron microscopes should be theoretically 100,000 times that of light microscopes but in practice, it is about 0.1 nm.

Shadow casting is an important technique in electron microscopy. This is achieved by depositing a thin layer of metal (e.g. platinum) on the object. Such metal-coated object held in the path of the beam, scatters the electrons and produces an image which is focussed on a fluorescent viewing screen. Another technique in studying the fine structures of the object is negative staining with phosphotungstic acid.

The scanning electron microscope is a recent development which provides a higher resolution and three dimensional image of the object.

Although the substantial increase in resolution power of electron microscope has led to significant discoveries, a major disadvantage is the inability to examine living cells. A method introduced to overcome this disadvantage is freeze-etching. This method involves rapid cooling of specimens by deep-freezing in liquid gas and the subsequent formation of carbon-platinum replicas of the material. Since such frozen cells may remain viable, it enables the study of cellular ultra structure as it appears in the living state.

Electron microscopy is especially used for rapid detection of viruses in clinical samples. It is particularly useful for detecting non-cultivable viruses. Ultrastructural study of various microorganisms may also be done by this method.

III. STUDY OF BACTERIA

A. Unstained (Wet) Preparations

Unstained preparations are examined mainly for bacterial motility (e.g. hanging drop preparation) and for demonstration of spirochaetes (e.g. dark ground microscopy).

B. Stained Preparations

Structural detail of bacteria cannot be seen under light microscope due to lack of contrast. Hence it is necessary to use staining methods to produce colour contrast.

Smear made from bacterial culture or specimen is first dried and then fixed with heat by flaming the slide from underneath. Heat kills and fixes the bacteria on slide due to coagulation of bacterial proteins. The fixed smear is stained by appropriate staining technique.

Common Staining Techniques

1. *Simple stains*
   Basic dyes such as methylene blue or basic fuchsin are used as simple stains. They provide the colour contrast, but impart the same colour to all the bacteria in a smear.
2. *Negative staining*
   Bacteria are mixed with dyes such as India ink or nigrosin. The background gets stained and unstained bacteria stand out in contrast. This is very useful in the demonstration of bacterial capsules which do not take simple stains.
3. *Impregnation methods*
   Bacterial cells and structures that are too thin to be seen under the light microscope, are thickened by impregnation of silver on the surface to make them visible e.g. demonstration of bacterial flagella and spirochaetes.
4. *Differential stains*
   They impart different colours to different bacteria or bacterial structures. The most commonly employed differential stains are the *Gram stain*, the *acid-fast stain* and the *Albert stain*.

GRAM STAIN

It is the most widely used stain in bacteriology. The stain was originally devised by the histologist Christian Gram (1884) as a technique of staining bacteria in tissues. Nowadays, modification of original Gram stain is used.

Method

1. Heat fixed smear of specimen or bacterial culture is stained with crystal violet (primary stain) for one minute. Other pararosaniline dyes such as gentian violet or methyl violet may also be used as primary stain.
2. Pour Gram's iodine (dilute solution of iodine) over the slide for one minute.
3. Wash the smear with water.
4. Decolourise with acetone for 10-30 seconds. Alcohol can be substituted for acetone.
5. Wash the smear with water.
6. Counterstain with a dye safranin for 30 seconds. Dilute carbol fuchsin or neutral red may also be used as counterstain.

Differentiation on Gram Staining

Two broad groups

- Gram positive
- Gram negative

Gram positive

– resist decolourisation and retain the colour of primary stain i.e. violet

Gram negative

– are decolourised by acetone/alcohol and, therefore, take counterstain and appear red.

Mechanism of Gram Staining

The exact mechanism is not understood. It may, however, be attributed to following.

1. *Permeability of bacterial cell wall and cytoplasmic membrane*

The Gram positive cells have a more acidic protoplasm, which accounts for retaining the basic dye more strongly than the Gram negative bacteria. Iodine makes the protoplasm more acidic and serves as mordant, i.e. iodine combines with dye to form a dye-iodine complex and fixes the dye in bacterial cell. The Gram positive cell wall or cytoplasmic membrane being less permeable, the dye-iodine complex gets trapped within the cell. The Gram negative cell wall has increased permeability to acetone or alcohol, permitting the outflow of complex during decolourisaton. This is not all-or-none phenomenon. The Gram positive cells may be decolourised by prolonged treatment with acetone/alcohol. In contrast, inadequate decolourisation may cause cells to appear Gram positive.

2. *Integrity of cell wall*

The integrity of the cell wall is essential for a positive stain. The Gram positive bacteria become Gram negative when cell wall is damaged.

ACID-FAST STAIN (ZIEHL-NEELSEN STAIN)

The acid-fast stain was discovered by Ehrlich and subsequently modified by Ziehl and Neelsen. Staining of Mycobacteria (usually tubercle and lepra bacilli) is done by this technique.

Method

1. The carbol fuchsin stain is poured on a slide containing fixed smear. Gentle heat is applied to the underside of the slide, by means of a spirit flame, until the stain just commences to steam. The carbol fuchsin is left on the slide for 5-10 minutes with intermittent heating during that period. Care must be taken to ensure that the stain does not dry out, to counteract drying more solution of stain is added to the slide and the slide reheated. Heating of the stain is required for penetration of the dye into the cell wall.
2. Wash in tap water.
3. The stained smear is decolourised with 20% sulphuric acid and washed with water. This step should be repeated till the pink/red colour stops coming out. In case of lepra bacilli 5% sulphuric acid is used as *M. leprae* is less acid-fast. Another alternative for decolourisation is acid-alcohol (3 ml HCl and 97 ml ethanol).
4. The smear is counterstained with 2% methylene blue for 1-2 minute. Malachite green can also be used as counterstain instead of methylene blue.
5. Wash with water and air dry.

Microscopic Examination of the Smear

Acid-fast bacilli appear red (colour of carbol fuchsin) in blue (colour of methylene blue) background of pus cells and epithelial cells.

Principle

Acid-fastness is due to the high content of lipids, fatty acids and higher alcohols found in the cell wall of mycobacterium. Mycolic acid (a wax), acid-fast in the free state, is found in all acid-fast bacteria. Besides lipid contents, acid-fastness depends also on the integrity of the cell wall.

ALBERT'S STAIN

Staining of Corynebacteria (*Corynebacterium diphtheriae* and other corynebacteria is done by this technique.

Method

1. The smear is heated gently by flaming the slide from underneath. It will fix the smear. Do not overheat.
2. Cover the smear with Albert I (Albert's stain) for 5 minutes.
3. Drain off the whole stain without washing.
4. Pour Albert II (iodine solution) over the smear so as to cover it completely, leave it for 2 minutes.
5. Drain off the Albert II solution without washing.
6. Blot dry the smear with the help of filter paper.

Reagents

A. Albert I or Albert stain

1. Toludine blue	..	0.15 gm
2. Malachite green	..	0.20 gm
3. Glacial acetic acid	..	1 ml
4. Alcohol (95 per cent ethanol)	..	2 ml
5. Distilled water to make	..	100 ml

B. Albert II or Albert's iodine solution

1. Iodine	..	2 gm
2. Potassium iodide	..	3 gm
3. Distilled water to make	..	300 ml

Microscopic Examination of the Smear

Corynebacterium diphtheriae appear as green coloured bacilli with bluish black metachromatic granules. These bacilli are arranged in *Chinese letter* or *cuneiform arrangement*.

C. Morphology of Bacteria

Depending on their shape, bacteria are classified into several types (Fig. 2.1):

1. Cocci (from kokkos, meaning berry): These are oval or spherical cells. These cocci may be arranged in pairs (diplococci), chains (streptococci), clusters (staphylococci) and groups of four (tetrads) or eight (sarcina).
2. Bacilli (bacillus, meaning rod): These are rod shaped cells. Some of these bacilli may be having peculiar arrangement or shape as follows:
   (*i*) Coccobacilli—length of bacteria is approximately same as its width e.g. Brucella.
   (*ii*) Streptobacilli—These bacilli are arranged in chains e.g. Streptobacillus.
   (*iii*) Chinese letter or cuneiform pattern—arranged at angles to each other e.g. Corynebacterium.
   (*iv*) Comma-shaped—curved appearance e.g. Vibrio.
   (*v*) Spirilla—rigid spiral forms e.g. *Spirillum*.
3. Spirochaetes (from spiera meaning coil; chaite meaning hair): These are slender, flexous spiral forms e.g. Treponema.
4. Actinomycetes (from actis, meaning ray, mykes meaning fungus): These are branching filamentous bacteria resembling fungi. They have a rigid cell wall.
5. Mycoplasmas: These bacteria are cell wall deficient and hence do not possess a stable shape. They may occur as round or oval bodies and as interlacing filaments. They are very small in size (50-300 nm in diameter). They can reproduce in cell-free medium.
6. Rickettsiae and Chlamydiae: These are very small, obligate parasites. Due to their inability to grow outside living cells, they were previously considered as viruses. Now they are classified as bacteria because of typical bacterial cell wall, possession of various bacterial enzymes and structural similarities with bacteria.

D. Bacterial Anatomy

The outer layer or cell envelope of a bacterial cell consists of two components—

1. a rigid cell wall.
2. underlying cytoplasmic or plasma membrane.

The cell envelope encloses the protoplasm which comprises cytoplasm, cytoplasmic inclusions (mesosomes, ribosomes, inclusion granules, vacuoles) and a single circular chromosome of deoxyribonucleic acid (DNA) (Fig. 2.2). Besides these essential components, some bacteria may possess additional structures, such as, capsule, flagella and fimbriae.

1. THE CELL WALL (TABLE 2.2)

The cell wall is a tough and rigid structure, surrounding the bacterium like a shell. It weighs about 20-25% of the dry weight of the cell. The thickness of Gram negative cell wall is 10-25 nm. The cell wall has following functions:

1. Accounts for the shape of the cell.
2. Provides protection to the cell against osmotic damage.
3. Confers rigidity upon bacteria.
4. It takes part in cell division.
5. It possesses target site for antibiotics, lysozymes and

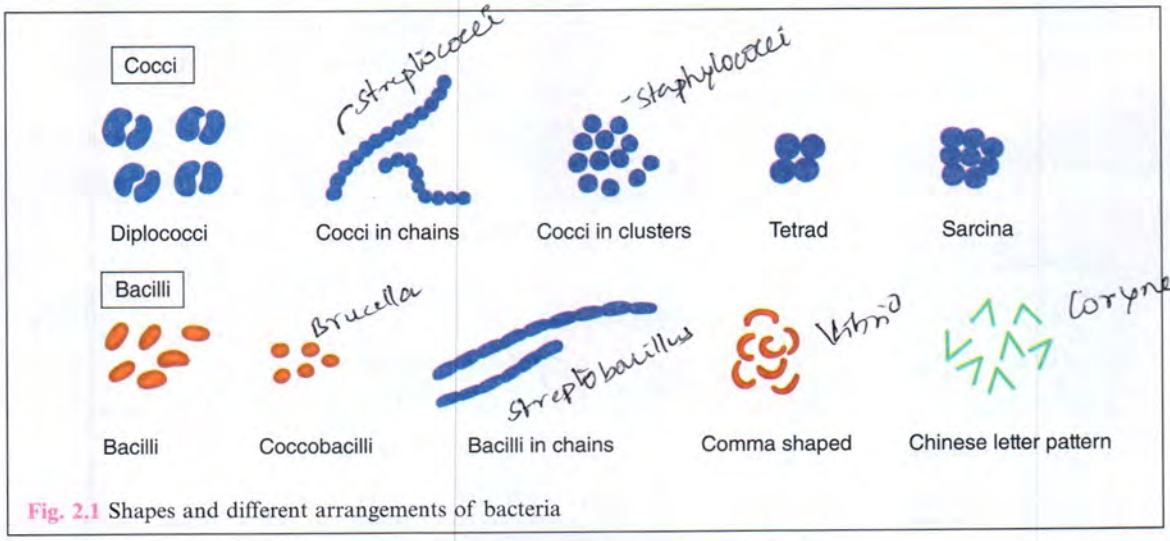


Fig. 2.1 Shapes and different arrangements of bacteria

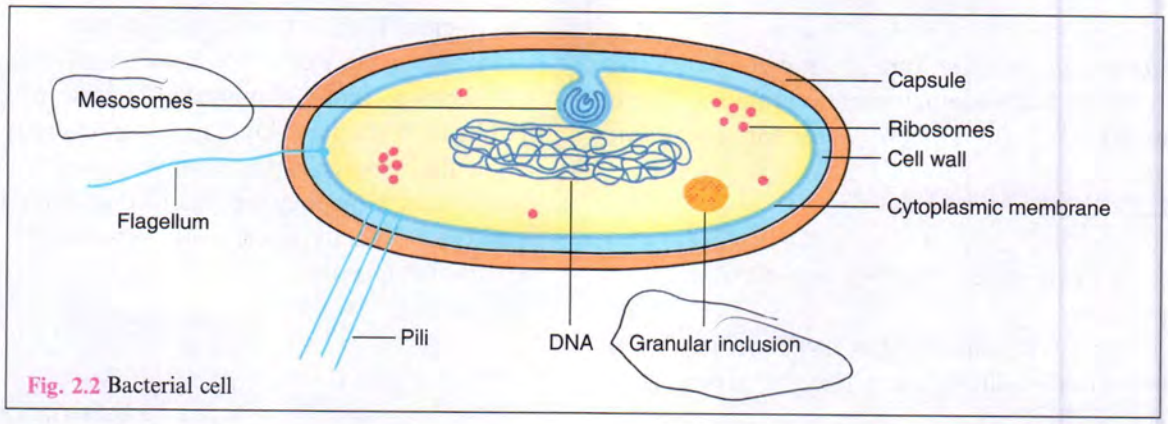


Fig. 2.2 Bacterial cell

bacteriophages. It carries bacterial antigens that are important in virulence and immunity.

Table 2.2 Differences between Cell Wall of Gram positive and Gram negative Bacteria

Character	Gram positive	Gram negative
Thickness	Thicker	Thinner
Periplasmic space	Absent	Present
Lipids	Absent or small	Present
Teichoic acid	Present	Absent
Peptidoglycan	16-80 nm	2 nm

The rigid part of the cell wall is a peptidoglycan which is a mucopeptide (murein) composed of N-acetyl muramic acid and N-acetyl glucosamine molecules alternating in chains, cross linked by peptide subunits (Fig. 2.3). The cell walls of Gram positive bacteria have simpler chemical nature than those of Gram negative bacteria.

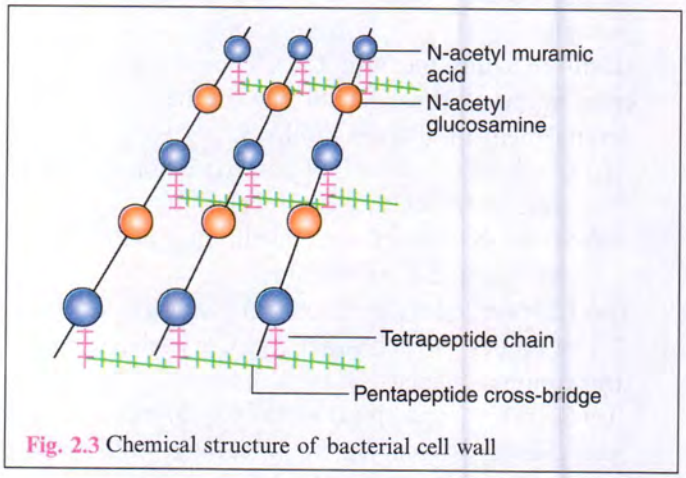


Fig. 2.3 Chemical structure of bacterial cell wall

(i) Gram-negative Cell Wall

The Gram negative cell wall is a complex structure with the following components (Fig. 2.4).

(*a*) Lipoprotein layer: It connects the peptidoglycan to outer membrane.

(*b*) Outer membrane: This contains certain proteins named as outer membrane proteins (OMP). These are target sites for phages, antibiotics and bacteriocins.

(*c*) Lipopolysaccharide (LPS): This layer consists of lipid A, to which is attached a polysaccharide. LPS constitutes the endotoxin of gram-negative bacteria. The polysaccharide determines a major surface antigen, the O antigen. The toxicity (pyrogenicity, lethal effect, tissue necrosis) of bacteria is associated with lipid A.

(*d*) The periplasmic space: It is the space in between the inner and outer membranes. It contains various binding proteins for specific substrates.

(*e*) Peptidoglycan.

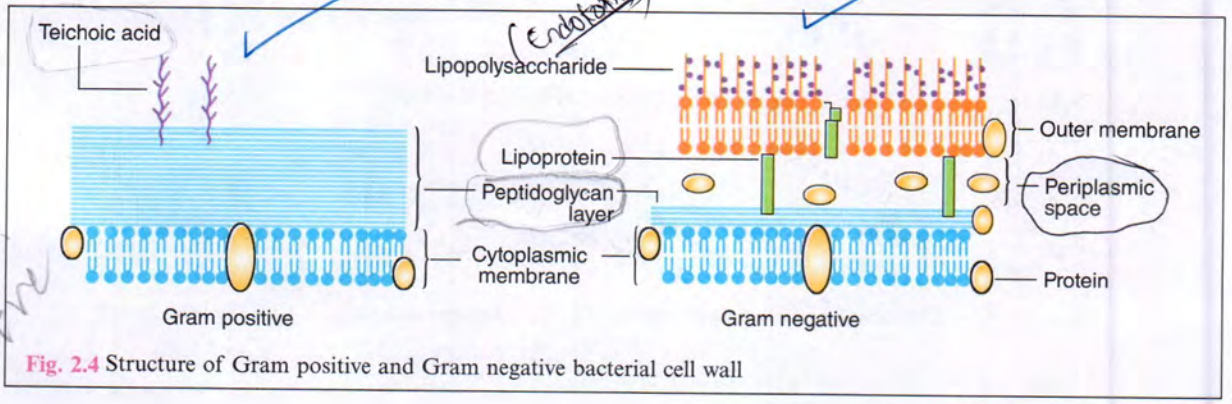


Fig. 2.4 Structure of Gram positive and Gram negative bacterial cell wall

(ii) Gram-positive Cell Wall

(*a*) Peptidoglycan: This layer in Gram positive bacteria is thicker (16-80 nm) than that in Gram negative bacteria (2 nm) (Fig. 2.4).

(*b*) Teichoic acid: Gram positive cell wall contains significant amount of teichoic acid which is absent in Gram negative bacteria. The teichoic acids constitute major surface antigens of Gram positive bacteria. They are water soluble polymers, containing ribitol or glycerol polymers. Teichoic acids are of two types, cell wall teichoic acid and membrane teichoic acid. The cell wall teichoic acid is covalently linked to peptidoglycan; and the membrane teichoic acid to cytoplasmic membrane.

(*c*) Other components: Certain Gram positive cells also contain antigens such as protein and polysaccharides.

Demonstration of Cell Wall

The cell wall cannot be seen by light microscope and does not stain with simple dyes. Demonstration of cell wall can be done by following methods:

(*a*) Plasmolysis—When bacteria is placed in a hypertonic saline, shrinkage of the cytoplasm occurs, while cell wall retains original shape and size.

(*b*) Microdissection

(*c*) Differential staining

(*d*) Reaction with specific antibody

(*e*) Electron microscopy.

Bacteria with Defective Cell Wall

The synthesis of cell wall may be inhibited or interfered by many factors such as, antibiotics, bacteriophages and lysozyme. Lysozyme, an enzyme normally present in many tissue fluids, lyses susceptible bacteria by splitting the linkage of peptidoglycan in the cell wall. When lysozyme acts on a Gram positive bacterium in a hypertonic solution, a protoplast is formed. In case of Gram negative bacteria, the result is a spheroplast. Bacteria with defective cell wall may probably have a role in the persistence of certain chronic infections such as pyelonephritis. Bacteria without cell walls or with deficient cell walls are of four types:

(*i*) *Mycoplasma*: This is a naturally occurring bacteria without cell walls. Mycoplasma is classified as an independent bacterial genus. They don't require hypertonic environment for maintenance and are stable in culture medium.

(*ii*) *L-forms*: Kleineberger-Nobel, while studying *Streptobacillus moniliformis* in the Lister Institute, London, observed abnormal forms of the bacteria and named them L-forms after the Lister Institute.
L-forms develop either spontaneously or in the presence of penicillin or other agents that interfere with synthesis of cell wall. These are difficult to cultivate and require agar containing solid medium having right osmotic strength. L-forms are sometimes spontaneously formed in patients treated with penicillin. L-forms are more stable than protoplasts and spheroplasts.

(*iii*) *Protoplasts*: These are derived from Gram positive bacteria. They contain cytoplasmic membrane and cell wall is totally lacking. These are produced artificially by lysozyme in a hypertonic medium. These are unstable. Hypertonic condition is necessary for their maintenance.

(*iv*) *Spheroplasts*: These are derived from Gram negative bacteria. They are produced in presence of penicillin. They are osmotically fragile and must be maintained in hypertonic culture medium. They differ from the protoplast in that some cell wall material is retained.

Pleomorphism and Involution Forms

Certain species of bacteria which exhibit great variation in shape and size of individual cells are called pleomorphism. Some bacteria show swollen and aberrant forms in ageing laboratory cultures and are known as involution forms. Defective cell wall synthesis is often responsible for development of these two abnormal forms.

2. CYTOPLASMIC MEMBRANE

It is 5-10 nm thick elastic semipermeable layer which lies beneath the cell wall separating it from the cell cytoplasm (Fig. 2.2). Electron microscopy shows the presence of three layers constituting a unit membrane. Chemically, it consists of lipids and protein molecules. Sterols are absent, except in mycoplasma.

Cytoplasmic membrane act as an osmotic barrier. It is the site of numerous enzymes (permease, oxidase, polymerase) involved in the active transport of selective nutrients. It acts as a semipermeable membrane controlling the inflow and outflow of metabolites to and from the protoplasm. It also contains cytochrome oxidase, enzymes of tricarboxylic acid cycle and enzymes necessary for the cell wall synthesis.

3. CYTOPLASM

The bacterial cytoplasm is a colloidal system containing a variety of organic and inorganic solutes in a viscous watery solution. It lacks mitochondria and endoplasmic reticulum of eukaryotic cell. It contains ribosomes, mesosomes, vacuoles and inclusions. The cytoplasm stains uniformly with basic dyes in young cultures.

(i) Ribosomes

These are the centres of protein synthesis. These are composed of ribosomal RNA (rRNA) and ribosomal proteins. Ribosomes are integrated in linear strands of mRNA to form polysomes and it is at this site that code of mRNA is translated into peptide sequences. They are10-20 nm in size with a sedimentation constant of 70 S (S for Svedberg units). Each 70 S unit consists of a 30 S and a 50 S subunit.

(ii) Intracytoplasmic inclusions

These are sources of stored energy and present in some species of bacteria. Their function and significance is uncertain. They may be present as polymetaphosphate (volutin), lipid, polysaccharide (starch or glycogen) and granules of sulphur. They are most frequent in bacteria grown under conditions of nutritional deficiency and disappear when the deficient nutrients are supplied.

(iii) Mesosomes (chondroids)

Mesosomes are vesicular, multilaminated or convoluted structures formed as invaginations of the plasma membrane into the cytoplasm (Fig. 2.2). They are the principal centres of respiratory enzyme and are analogous to mitochondria of eukaryotes. These are more prominent in Gram positive bacteria. There are two types of mesosomes—septal and lateral. The septal mesosome is attached to bacterial chromosome and is involved in DNA segregation and in the formation of cross-walls during binary fission.

4. NUCLEUS

Bacterial nucleus has no nuclear membrane or nucleolus. It cannot be demonstrated by ordinary microscope but needs an electron microscope. The nuclear deoxyribonucleic acid (DNA) doesn't appear to contain any basic protein. The genomic DNA is double stranded in the form of a circle. It measures about 1 mm (1000 μm) when straightened. The bacterial DNA is haploid, replicates by simple fission and maintains bacterial genetic characteristics.

Some bacteria may possess extranuclear genetic material in the cytoplasm consisting of DNA named as plasmids or episomes. The plasmid replicates autonomously. They are not essential for the life of the cell, but may confer on the bacteria certain properties, such as drug resistance and toxigenicity which constitute a survival advantage to the bacteria. These plasmids can be transmitted from one bacterium to another either by conjugation or by the agency of bacteriophage. Besides these methods, plasmids may be transferred to daughter cells during cell division by binary fission.

5. BACTERIAL CAPSULE AND SLIME LAYER

It is the amorphous viscid bacterial secretion which surrounds some bacteria as their outermost layer (Fig. 2.2). When it diffuses into the surrounding medium and remains as a loose undemarcated secretion as in *Leuconostoc*, it is known as slime layer. When this secretion is organised into a sharply defined structure, as in *Streptococcus pneumoniae* (pneumococcus), it is known as the capsule. Capsules which are very thin and cannot be demonstrated under the light microscope are called microcapsules e.g. *Neisseria meningitidis*. The slime layer or capsule is generally polysaccharide in nature but it is polypeptide in anthrax bacillus. Some bacteria may have both a capsule and a slime layer (e.g. *Streptococcus salivarius*). Bacteria like *Klebsiella* secreting large amount of slime produces mucoid growth on agar, with a stringy consistency when touched with the loop.

Functions

(*i*) Capsule enhances bacterial virulence by inhibiting phagocytosis. Loss of capsule may render the bacterium avirulent. Bacteria tend to lose capsules on repeated subcultures.

(*ii*) It acts as protective covering against antibacterial substances such as, bacteriophages, phagocytes, enzymes.

(*iii*) Capsular antigen is specific for bacteria and can be used for identification and typing of bacteria.

Capsulated Organisms

Streptococcus pneumoniae, Klebsiella sp., Bacillus anthracis, Haemophilus influenzae, Cryptococcus neoformans (a fungus).

Demonstration of Capsule

Capsule has little affinity for basic dyes, therefore, it can't be stained by Gram staining. The following methods have been used for demonstration of capsule.

(i) India ink staining (negative staining)

Capsule appears as a clear halo around bacterium as the ink can't penetrate the capsule (Fig. 2.5).

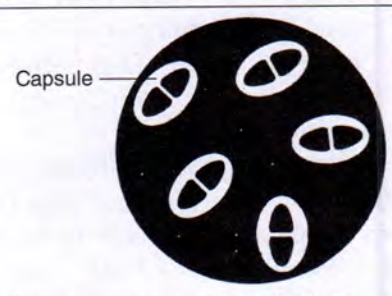


Fig. 2.5 Pneumococci stained with India ink to show capsule

(ii) Serological methods

Capsular material is antigenic and can be demonstrated by mixing it with a specific anticapsular serum. When a suspension of capsulated bacterium is mixed with its specific anticapsular serum and examined under the microscope, the capsule appears 'swollen' due to increase in its refractivity. This phenomenon is called as capsule swelling reaction or Quellung phenomenon. This is also named as Neufeld reaction after the person who described it. This was widely employed for the typing of pneumococci.

(iii) Special capsule staining

These techniques employ copper salts as mordants for staining of capsule.

6. FLAGELLA

Flagella are cytoplasmic appendages protruding through cell wall. These are thread like structures composed of a protein (flagellin), 5-20 µm in length and 0.01-0.02 µm in diameter. They are organ of locomotion. All motile bacteria, except spirochaetes, possess one or more flagella.

Parts and Composition

Each flagellum consists of three parts (Fig. 2.6).

(*i*) filament
(*ii*) hook
(*iii*) basal body

The filament lies external to the cell and is connected to the hook at the cell surface. The hook-basal body portion is embedded in the cell envelope. The basal body contains outer and inner rings by which the basal body is attached to the cytoplasmic membrane. Outer rings are absent in Gram positive bacteria.

The flagella is made up of protein (flagellin) which is similar to myosin. Although chemical composition of different genera of bacteria is similar, they are antigenically different. Specific flagellar antibodies are produced in high titres in response to antigenic stimulation of flagella. These antibodies are useful in serodiagnosis but are not protective.

Arrangement/Types (Fig. 2.7)

(*i*) Monotrichous—Single polar flagellum (at one end) e.g. *Vibrio cholerae*.
(*ii*) Amphitrichous—Single flagellum at both the ends e.g. *Alcaligenes faecalis*.
(*iii*) Lophotrichous—Tuft of flagella at one or both ends e.g. *Spirilla*.
(*iv*) Peritrichous—Flagella arranged all round the cell e.g. *Salmonella typhi*.

Demonstration

Flagella are about 0.02 µm in thickness and hence beyond the resolution limit of the light microscope. The following methods are used for its demonstration:

(*i*) Dark ground illumination.
(*ii*) Special staining techniques in which thickness of flagella is increased by mordanting.
(*iii*) Electron microscopy.
(*iv*) Indirect methods by which motility of bacteria can be seen or demonstrated.
  (*a*) Spreading type of growth on a medium e.g. swarming growth of Proteus sp.
  (*b*) Motility under the microscope e.g. hanging drop preparation.

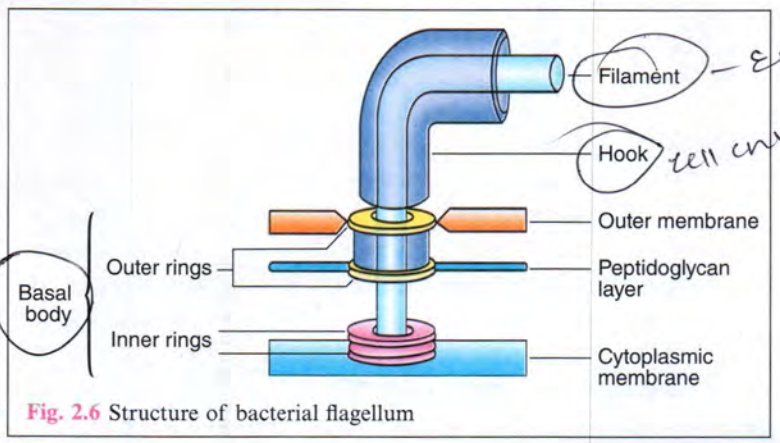


Fig. 2.6 Structure of bacterial flagellum

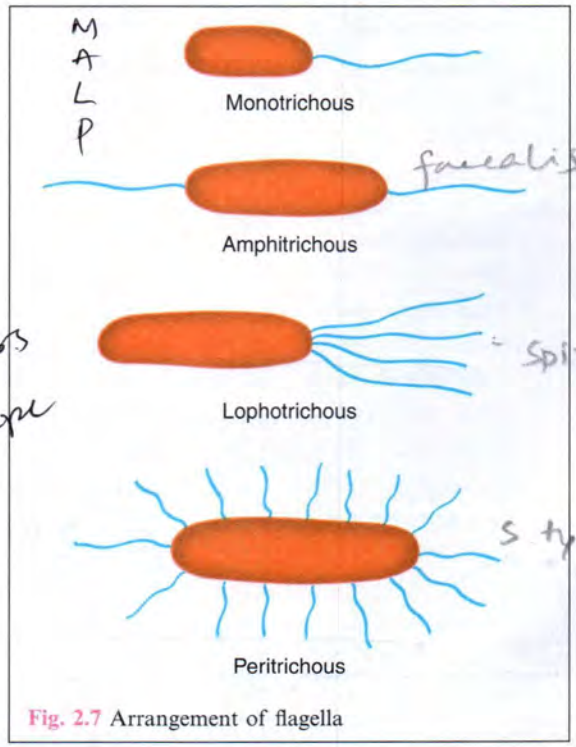


Fig. 2.7 Arrangement of flagella

(c) Spreading of bacteria in semisolid agar e.g. Craigie's tube method.

7. FIMBRIAE

These are hair-like appendages projecting from the cell surface as straight filaments. They are also called pili. They are 0.1 to 1.0 μm in length and less than 10 nm thick (shorter and thinner than flagella). Fimbriae are found in some Gram negative bacteria. Each bacterium possesses 100-500 fimbriae peritrichously. They are more numerous than flagella. They are antigenic. They are best developed in freshly isolated strains and in liquid cultures. They tend to disappear when subcultures are made on solid media. Fimbria is composed of protein called pilin. They are unrelated to motility and are found on motile as well as non-motile bacteria.

Types

There are three main types of fimbriae.

(*i*) Common pili—These are of six types depending on their morphology, number per cell, adhesive properties and antigenic nature.
(*ii*) Sex or F (fertility) pili
(*iii*) Col I (colicin) pili

Functions

(*i*) Adhesion—Fimbriae are organs of adhesion. This property enhances the virulence of bacteria.
(*ii*) Transfer of genetic material—Sex pili are present in male bacteria. These pili are longer (18-20 μm) and 1-4 in number. They help the male cells to attach with non-male (female) cells in forming "conjugation tubes" through which genetic material is believed to be transferred from the donor (male) to the recipient (female) cell.

Detection of Fimbriae

(*i*) Electron microscopy.
(*ii*) Haemagglutination—Many fimbriated bacteria (e.g. *Escherichia coli, Klebsiella*) strongly agglutinate red blood cells of guinea pigs, fowl, horses and pigs; human and sheep cells weakly and ox cells scarcely. This property of haemagglutination is a simple method for detecting the presence of fimbriae. The haemagglutination can be specifically inhibited by D-mannose.

8. BACTERIAL SPORE

Spores are highly resistant resting stage formed in unfavourable environmental conditions presumed to be related to the depletion of exogenous nutrients. As bacterial spores are formed within the parent cell, these are called endospores. Sporulation is not a method of reproduction. In the process of sporulation, each vegetative cell forms only one spore and during subsequent germination, each spore gives rise to only one vegetative bacterium.

Sporulation (Fig. 2.8)

Bacterial cell undergoes spore formation in nutritionally deprived conditions and this process is called *sporulation*. Spore develops from a portion of protoplasm (*forespore*) near one end of the cell. The remaining part of cell is called *sporangium*. Bacterial DNA replicates and divides into two DNA molecules. One of these is incorporated into forespore and other into sporangium. A transverse septum grows across the cell from the cell membrane. It divides forespore and sporangium. The forespore is completely encircled by this septum as a double layered membrane. The inner layer becomes the *spore membrane* and the outer layer becomes thickened *spore coat*. Between the two layers is *spore cortex*.

Morphology of Spore

The clear area in the protoplasm of cell becomes gradually more opaque with condensation of nuclear chromatin forming the forespore. The cell membrane grows inward and forms spore wall around the core (forespore). The inner-most layer of the spore wall forms the spore membrane from which the cell wall of future vegetative bacterium develops. Outside this membrane is thick layer, the cortex and a multilayered tough spore coat. Some spores have an additional apparently rather loose, outercovering called exosporium (Fig. 2.9).

Shape and Position of Spores

The precise position, shape and relative size of spore are constant within a particular species. Spores may be central, subterminal or terminal (Fig. 2.10). They may be oval or spherical in shape. The diameter of spore may be same or less than the width of bacteria (*Bacillus*), or may be wider than the bacillary body producing a distension or bulge in the cell (*Clostridium*).

Resistance

Bacterial spores are extremely resistant to ordinary boiling, disinfectants and heating. The high resistance of spores is due to high content of *calcium* and *dipicolinic acid*; low water content; the thick impervious cortex and spore coats; their low metabolic and enzymatic activity. However, spores of all medically important bacteria are destroyed by autoclaving at 121°C for 15 minutes.

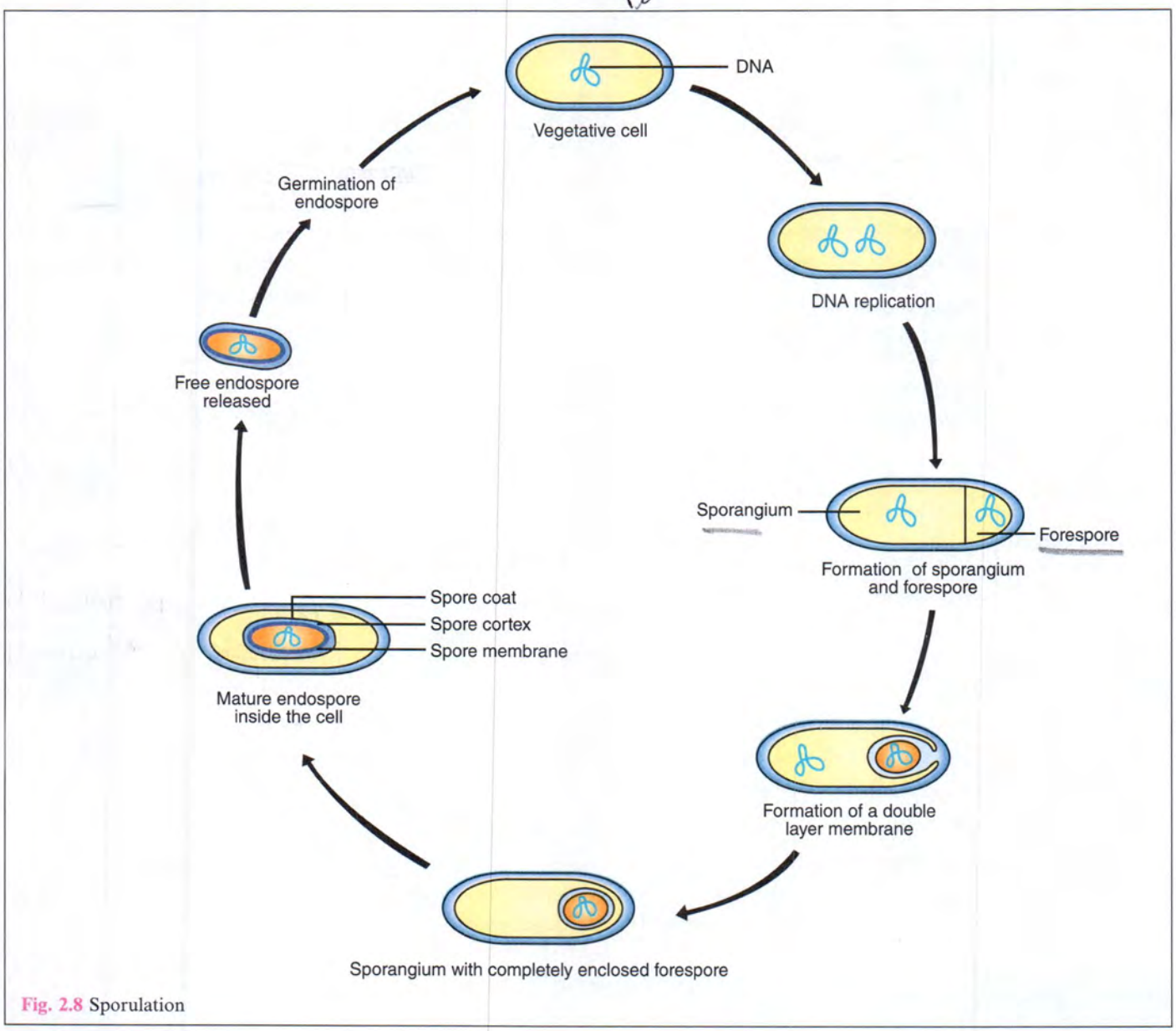


Fig. 2.8 Sporulation

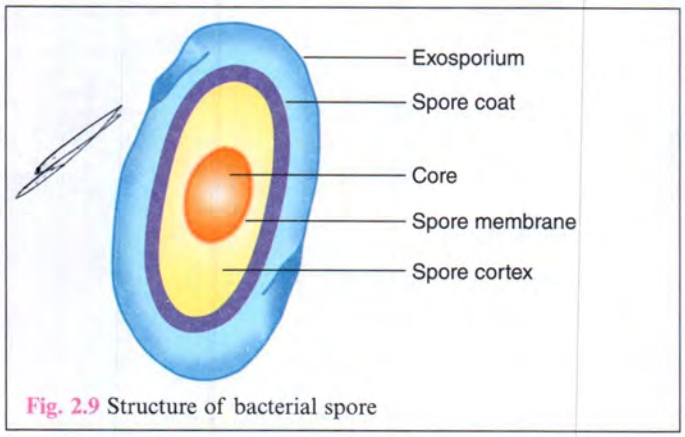


Fig. 2.9 Structure of bacterial spore

Methods of disinfection and sterilization should ensure destruction of spores also.

Germination

The process of conversion of a spore into vegetative cell under suitable conditions is known as germination. The spore loses its refractivity, and swells when transferred to conditions conducive to vegetative growth. The spore wall is shed and the germ cell appears by rupturing the spore coat. The formation of vegetative bacterium occurs by elongation of the germ cell.

Demonstration

(*i*) Gram staining—Spores appear as an unstained refractile body within the cell.

(*ii*) Modified Ziehl-Neelsen (ZN) staining—Spores appear as acid-fast (red colour). Ziehl-Neelsen staining with 0.25-0.5% sulphuric acid (instead of

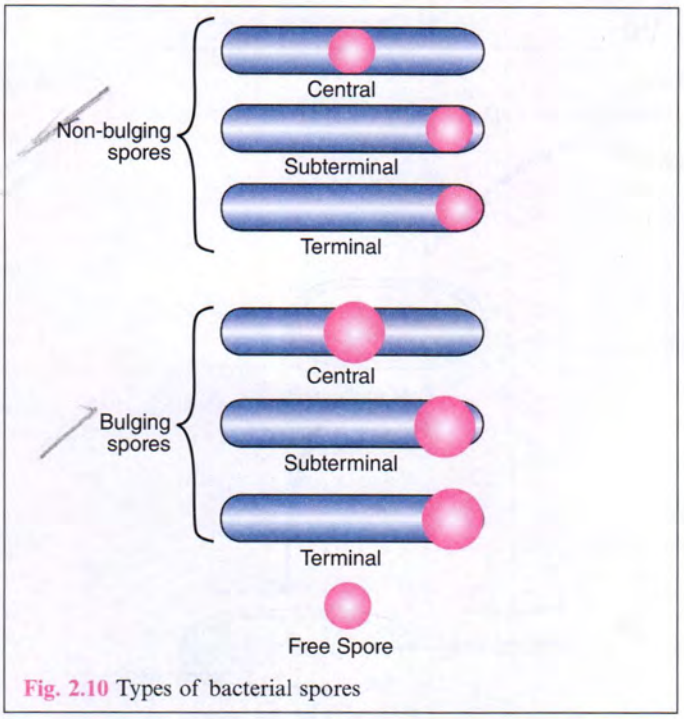


Fig. 2.10 Types of bacterial spores

20% sulphuric acid as used in conventional method) as decolouring agent is used for spore staining.

Uses of Spores

Spores of certain species of bacteria are employed as indicator for proper sterilisation e.g. *Bacillus stearothermophilus* which is destroyed at a temperature of 121°C for 10-20 minutes (same temperature and time as used in autoclaving). These spores may be kept in autoclave prior to its use. Absence of the spores after autoclaving indicates proper sterilisation.

Spore Forming Bacteria

Obligate aerobes—genus *Bacillus* e.g. *B. anthracis* and *B. subtilis*

Obligate anaerobes—genus *Clostridia e.g. Cl. tetani, Cl. welchii, Cl. botulinum*

Both Bacillus and Clostridia are Gram positive bacilli.

KEY POINTS

1. Microorganisms are living structures of microscopical size. They belong to *prokaryotes*.
2. Microscopy can be done to observe these microorganisms.
3. Unstained (wet) and stained preparations are examined for the study of bacteria.
4. Bacteria are either *Gram positive* or *Gram negative* on Gram staining.
5. Broadly bacteria are classified into *cocci* or *bacilli*.
6. Bacterial cell possesses several components such as *the cell wall*, *the cytoplasmic membrane*, *the cytoplasm*, *the nucleus*, *the bacterial capsule*, *the flagella*, *the fimbriae* and *the bacterial spore*.

YOU MUST KNOW

1. Differences between prokaryotes and eukaryotes.
2. Principles of different microscopes.
3. Principles of various staining techniques.
4. Differences between cell wall of Gram positive and Gram negative bacteria.
5. Bacterial capsule and its functions.
6. Types of flagella.
7. Functions of fimbriae.
8. Different types of bacterial spores.

STUDY QUESTIONS

1. Write short notes on:
   (a) Phase contrast microscope
   (b) Dark field microscope
   (c) Fluorescent microscope
   (d) Electron microscope.

2. Draw a labelled diagram of bacterial cell.
3. Write short notes on:
   (a) Bacterial cell wall
   (b) Cytoplasmic membrane
   (c) Bacterial capsule
   (d) Flagella
   (e) Fimbriae
   (f) Bacterial spore.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Peptidoglycan layer of cell wall is thicker in:
   (a) Gram positive bacteria
   (b) Gram negative bacteria
   (c) Fungi
   (d) Parasites
2. Lipopolysaccharide is a major component of cell wall in:
   (a) Gram positive bacteria
   (b) Gram negative bacteria
   (c) Fungi
   (d) Parasites
3. A tuft of flagella present at one or both ends of bacterial cell, is known as:
   (a) Monotrichous
   (b) Amphitrichous
   (c) Lophotrichous
   (d) Peritrichous
4. Which one of the following bacteria is cell wall deficient?
   (a) *Escherichia coli*
   (b) *Salmonella typhi*
   (c) *Mycoplasma*
   (d) *Treponema pallidum*
5. All of the following are spore forming bacteria except:
   (a) *Clostridium botulinum*
   (b) *Bacillus subtilis*
   (c) *Bacillus anthracis*
   (d) *Pseudomonas aeruginosa*
6. Indian ink staining is used to demonstrate:
   (a) Cell wall
   (b) Bacterial capsule
   (c) Bacterial flagella
   (d) Bacterial spore

Answers (MCQs):

1. (a) 2. (b) 3. (c) 4. (c) 5. (d) 6. (b)

Chapter 3

GROWTH, NUTRITION AND METABOLISM OF BACTERIA

The minimum nutritional requirement for growth and multiplication of bacteria includes sources of carbon, nitrogen, hydrogen, oxygen and some inorganic salts. Bacteria can be classified on the basis of nutrition as follows:

1. ***Autotrophs*** : Bacteria which can synthesise their essential metabolites (organic compounds) from the atmospheric carbon dioxide and nitrogen are known as autotrophs or lithotrophs. Autotrophs obtain their energy either photosynthetically (*photolithotrophs* or *photoautotrophs*) or by oxidation of chemical compounds (*chemolithotrophs* or *chemoautotrophs*).
2. ***Heterotrophs*** : These bacteria are unable to synthesise their own metabolites. They depend on preformed organic compounds. Majority of the pathogenic bacteria are heterotrophs and parasitic. They have evolved with the animal body where readymade nutrients are freely available.

I. BACTERIAL GROWTH

A. Bacterial Cell Division

Bacteria divide by binary fission (Fig. 3.1). When a bacterial cell reaches a critical mass in its cellular constituents, the cell division starts. Bacterial nucleus or chromosome is a circular double stranded DNA molecule. The nuclear division precedes bacterial cell division. During replication, the two strands of DNA are separated and new complementary strands are formed. Thus two identical double stranded DNA molecules are formed.

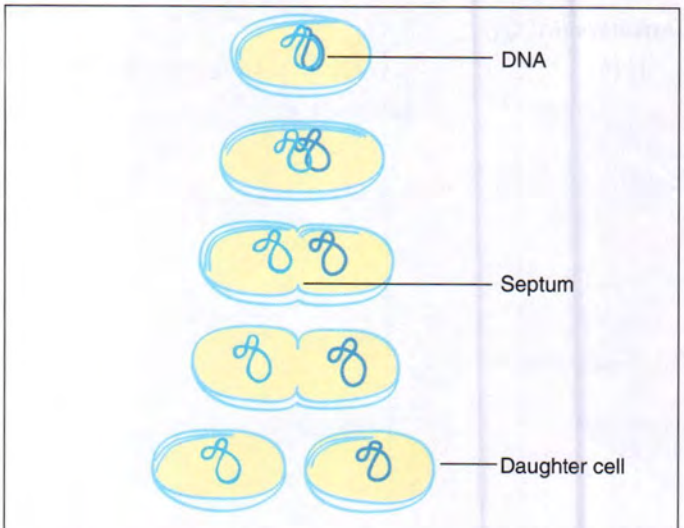


Fig. 3.1 Binary fission

The replicated DNA molecules are distributed to the two daughter cells. A transverse septum grows across the cell from the cell membrane. This is followed by the deposition of cell wall material and the two daughter cells separate. In some bacterial species, the daughter cells may remain partially attached even after cell division.

Clinical Significance

Some bacteria are present as 'diplococci' (Neisseria, Pneumococcus) and others in chains (Streptococcus). Morphology of 'diplococci' and 'in chains' are due to non-separation of daughter cells after cell division. These morphological features are very useful in identification of these bacteria in the laboratory.

B. Generation Time

The time required for a bacterium to give rise to two daughter cells under optimum conditions, is known as the **generation time**. The generation time in most of the medically important bacteria is about 20 minutes. In *Mycobacterium tuberculosis* it is about 20 hours and in lepra bacilli it is 20 days.

As bacteria grow so rapidly and by geometric progression, a single bacterium can theoretically give rise to 10^{21} progeny in 24 hours. In actual practice, bacterial multiplication is arrested after a few cell divisions due to exhaustion of nutrients and accumulation of toxic products.

Clinical Significance

When generation time is about 20 minutes (most of the medically, important bacteria), bacteria take 18-24 hrs to grow on culture media. In contrast *M.tuberculosis* take longer duration (in weeks) to grow as its generation time is longer i.e. 20 hrs. *M.leprae* takes months to grow in animal models as its generation time is 20 days.

C. Bacterial Count

1. **Total count:** This indicates total number of bacteria in the specimen, irrespective of whether they are living or dead. Total count may be made by counting the bacteria under microscope using counting chamber.
2. **Viable count:** It measures the number of living (viable) cells. Viable counts are obtained by dilution or plating methods. In the dilution method, several tubes with liquid culture media are inoculated with varying dilutions of sample and the viable count calculated statistically from the number of tubes showing growth. Though this method does not give accurate values, but is still used widely for finding out presumptive coliform count in drinking water.

In the plating method, appropriate dilutions of the specimen are inoculated on solid media, either on the surface of plates or as pour plates. The number of colonies that grow after incubation gives an estimate of the viable count. Each colony represents a clone of bacteria derived from a single parent cell. Therefore one colony is equivalent to one bacterium. Number of colonies are equal to number of bacteria in the specimen.

Clinical Significance

Viable count is useful in clinical microbiology for testing of water by presumptive coliform count (Ref. Chapter 80) and also in diagnosis of urinary tract infection (Ref. Chapter 69).

D. Bacterial Growth Curve

When a bacterium is inoculated into a suitable culture liquid medium and incubated, its growth follows a definite course. When bacterial count of such culture is determined at different intervals and plotted in relation to time, a growth curve is obtained (Fig. 3.2). The growth curve has four phases.

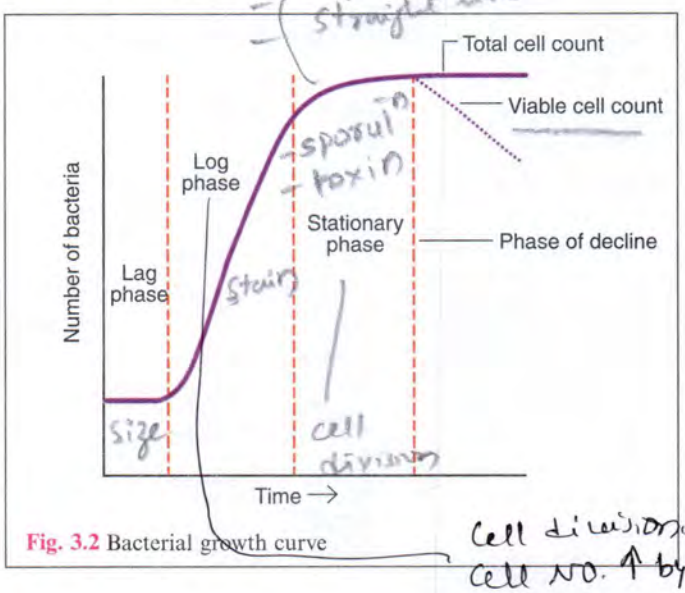


Fig. 3.2 Bacterial growth curve

1. *Lag Phase*

After inoculation of the culture medium, multiplication usually does not begin immediately. The period between inoculation and beginning of multiplication is known as lag phase. During this period the organisms adapt to the new environment, during which necessary enzymes and intermediate metabolites are built up in adequate quantities for multiplication to proceed. There is increase in the size of the cells but there is no appreciable increase in numbers. The duration of lag phase varies with the species, nature of culture medium and temperature etc.

2. *Log (Logarithmic) or Exponential Phase*

The cell division starts and their numbers increase exponentially or by geometric progression with time. If the logarithm of the viable count is plotted against time, a straight line is obtained.

3. *Stationary Phase*

After log phase, the bacterial growth ceases almost completely due to exhaustion of nutrients and accumulation of toxic products. The number of progeny cells formed is just enough to replace the number of cells that die. The number of viable cells remain stationary as there is almost a balance between the dying cells and the newly-formed cells.

4. *Phase of Decline*

After a period of stationary phase, the bacterial population decreases due to the death of cells. The decline phase starts due to exhaustion of nutrients, accumulation of toxic products and autolytic enzymes. There is decline in viable count and not in total count. With autolytic bacteria, even the total count shows a phase of decline.

Morphological and physiological alterations of cells during growth curve

The end of the lag phase	—	Bacteria have maximum cell size.
In the log phase	—	Cells are smaller and stain uniformly.
In the stationary phase	—	• Cells are Gram-variable and show irregular staining (due to intracellular storage granules). • Sporulation occurs. • Many bacteria produce exotoxins.
In the phase of decline	—	Involution forms are common.

E. Batch Culture and Continuous Culture

When bacteria are grown in a vessel of liquid medium (**batch culture**), after sometime the cell division ceases and multiplication is arrested due to depletion of nutrients or accumulation of toxic products. By the use of special devices for replenishing nutrients and removing bacterial cells, **continuous culture** of bacteria is maintained. The techniques namely **chemostat** and **turbidostat** are in use. Maintenance of continuous culture of bacteria is sometimes required for industrial and research purposes.

F. Bacterial Growth in Vivo

There is a significant difference of bacterial growth in human body and artificial culture medium. When bacteria multiply in host tissues, the situation may be intermediate between a batch culture and a continuous culture. The source of nutrients may be plenty, but the defence mechanisms of the body influence the bacterial growth in-vivo.

II. BACTERIAL NUTRITION

The principal constituent of bacterial cell is water, which is about 80% of the total weight. For growth of bacteria, the minimum nutritional requirement is water, a source of carbon, a source of nitrogen and some inorganic salts. These nutritional elements may be supplied in suitable artificial culture media. In addition to these, bacteria require sulphur, phosphorus and other elements such as sodium, potassium, magnesium, iron and manganese in small amounts.

Some bacteria are fastidious and will not grow unless certain organic compounds are added to the medium. These are known as growth factors or bacterial vitamins. In many cases, bacterial vitamins are same as the vitamins necessary for mammalian nutrition, particularly those belonging to the vitamin B group—thiamine, nicotinic acid, riboflavine, pyridoxine, folic acid and vitamin B_{12}. These growth factors may be 'essential' for some bacteria when growth does not occur in their absence, or 'accessory' when they enhance growth, without being absolutely necessary for it.

Significance

Knowledge of compounds required by different bacteria is helpful in preparation of culture media for growing that particular bacterium. Some bacteria may require some special nutrients to be incorporated in the culture media.

ENVIRONMENTAL FACTORS AFFECTING GROWTH

1. *Moisture and Desiccation*

Moisture is an essential requirement for the growth of bacteria because 80% of the bacterial cell consists of water. However, the drying has varying effects in different organisms. Some organisms like *Treponema pallidum* and *N. gonorrhoeae* die quickly after drying while *M. tuberculosis* and *Staph. aureus* may survive drying for several weeks. Drying in cold and vacuum (**lyophilisation or freeze drying**) is used for preservation of microorganisms.

Clinical Significance

Transport media are used for transport of those clinical specimens which may contain delicate organisms such

as *Neisseria gonorrhoeae*. In contrast, specimens of pus suspected to have *Staph. aureus* do not require any transport medium. Lyophilisation is an important method of preservation of microorganisms and many biological materials such as vaccines, antibiotics etc.

2. *Oxygen*

On the basis of requirement of oxygen, bacteria are divided into *aerobes* and *anaerobes*.

(*a*) **Aerobes:** They require oxygen for their growth. They may be **obligate aerobes**, which grow only in the presence of oxygen, or **facultative anaerobes** that are ordinarily aerobes but can also grow without oxygen. Most of the pathogenic bacteria are facultative anaerobes. Example of obligate aerobe is *Pseudomonas aeruginosa*. Microaerophilic bacteria (**microaerophiles**) can grow in the presence of traces of oxygen. *Campylobacter* and *Helicobacter pylori* are examples of microaerophilic bacteria.

(*b*) **Anaerobes:** The **obligate or strict** anaerobes can grow only in the absence of oxygen. *Clostridium tetani* is one such example. Obligate anaerobes cannot grow even in the presence of traces of oxygen.

3. *Carbon Dioxide*

Small amount of carbon dioxide is required by all bacteria. This requirement is usually made available by the carbon dioxide present in the atmosphere, or produced endogenously as a product of cellular metabolism. Some organisms such as *Brucella abortus* require much higher level of carbon dioxide (5-10 per cent) for their growth, especially for primary isolation. These bacteria are called *capnophilic bacteria*.

4. *Temperature*

The temperature at which growth occurs best, is known as the *optimum temperature*. The optimum temperature range varies with different bacterial species. Most of the pathogenic bacteria grow optimally at 37°C (our body temperature). On the basis of optimum temperature range for growth, bacteria are grouped as follows:

(*a*) **Mesophiles** – between 25°C and 40°C e.g. majority of the pathogenic bacteria.

(*b*) **Psychrophiles** – below 20°C e.g. soil and water saprophytes.

(*c*) **Thermophiles** – between 55°C and 80°C e.g. *Geobacillus stearothermophilus* (formerly *Bacillus stearothermophilus*).

Clinical Significance

Most of the pathogenic bacteria die between 55°C to 65°C and most spores between 100°C to 120°C. This knowledge is useful for sterilisation (destruction of microorganisms) by heat e.g. autoclave (121°C) and hot-air oven (160°C). In contrast, some bacteria die rapidly at low temperature (e.g. *Haemophilus influenzae*) but most survive well. Low temperature is used for preservation of bacterial cultures in refrigerator (3°C to 5°C) or the deep freezer (–30°C to –70°C).

5. *pH*

Most of the medically important bacteria can grow at pH 7.2-7.6. However, some bacteria such as lactobacilli and cholera vibrio grow at acidic and alkaline pH respectively. Strong solutions of acid or alkali (5% hydrochloric acid or sodium hydroxide) kill most bacteria but mycobacteria are resistant to these agents.

Clinical Significance

Culture media having alkaline pH are used for growing *Vibrio cholerae* (Ref. Chapter 34). Strong solutions of acid or alkali are used in Petroff's concentration method for sputum specimen for *M.tuberculosis*). (Ref. Chapter 42)

6. *Light*

Bacteria (except phototrophic species) grow well in darkness. They are sensitive to ultraviolet rays and other radiations. Photochromogenic mycobacteria produce pigment only on exposure to light.

7. *Osmotic Effect*

Bacteria are able to withstand a wide range of external osmotic variation because of the mechanical strength of the cell wall. Sudden exposure of bacteria to hypertonic solutions may cause *plasmolysis*—osmotic withdrawal of water leading to shrinkage of protoplasm. This occurs more readily in Gram-negative than in Gram-positive bacteria. Sudden transfer of bacteria from concentrated solution to distilled water may cause *plasmoptysis*—excessive osmotic imbibition of water leading to swelling and rupture of the cell.

8. *Mechanical and Sonic Stresses*

Though bacteria have tough cell walls, they may be ruptured and disintegrated by vigorous shaking with glass beads and by exposure to ultrasonic vibrations.

III. BACTERIAL METABOLISM

The series of changes of substance (carbohydrate, protein or fat) within the bacterial cell from absorption to elimination is known as metabolism of the substance. Aerobic bacteria obtain their energy only through oxidation involving oxygen as the ultimate hydrogen

acceptors, while the anaerobes use hydrogen acceptors other than oxygen.

A. Oxidation

In case of aerobes, where the ultimate hydrogen acceptor is oxygen, the carbon and energy source may be completely oxidised to carbon dioxide and water. The conversion of adenosine diphosphate (ADP) to adenosine triphosphate (ATP) provides energy in this process. This process is called **oxidative phosphorylation**. Small amount of hydrogen peroxide produced in the process is disposed off by enzymes catalase or peroxidase present in aerobes.

B. Fermentation

In anaerobic respiration, hydrogen acceptor compounds are nitrates or sulphates, instead of oxygen. In fermentation, a series of oxidoreductions occur in which the carbon and energy source acts as both the electron donor and electron acceptor. The organic end products of fermentation include acids (lactic acid, formic acid, pyruvic acid), gas (hydrogen, carbon dioxide) and alcohols.

During fermentation, energy rich phosphate bonds are produced by the introduction of organic phosphate into intermediate metabolite. This process is known as **substrate-level phosphorylation**. These energy-rich phosphate groups so formed help in conversion of ADP to ATP. Fermentation is carried out by both obligate and facultative anaerobes.

The oxygen and hydrogen peroxide are toxic to anaerobes. The reason for this toxicity is not well understood. It is suggested that in the presence of oxygen, hydrogen peroxide accumulates in the media and inhibits the growth of anaerobes The enzyme catalase, which splits hydrogen peroxide, is present in aerobic bacteria but is absent in the anaerobes. Another reason might be that anaerobes possess essential enzymes that are active only in the reduced state.

The facultative anaerobes may act in both ways i.e. by the process of fermentation and aerobic respiration. They derive their energy by fermentation in the absence of oxygen but switch over to aerobic respiration in the presence of oxygen.

Clinical Significance

Biochemical reactions are used for identification of Gram negative bacilli. Carbohydrates are fermented to produce acid (lactic acid, pyruvic acid etc.) and gas (hydrogen, carbon dioxide).

C. Oxidation-Reduction (O-R) Potential (Redox potential)

Oxidising agent is a substance which can accept electrons while reducing agent is able to part with electrons (Fig. 3.3). The oxidising or reducing condition of a system is indicated by the net readiness of all the components in that system to accept or to part with the electrons. This is called oxidation-reduction potential or **redox potential**. The redox potential of a medium is determined by recording the electrical potential difference set up between the medium and an 'unattackable' electrode immersed in it. The electrode potential (Eh) can be measured in millivolts. This depends upon the state of oxidation or reduction of the solution.The more oxidised the system, the higher is the potential and in reduced system potential is lower. The redox-potential of the medium can also be assessed by the use of certain indicator dyes such as methylene blue or resazurin, which become coloured in oxidised state. It is a simple but less accurate method. Anaerobic organisms require low redox potential for their growth.

Clinical Significance

Anaerobic jar is used for growing anaerobic bacteria in it. Methylene blue solution is kept in the anaerobic jar to acertain anaerobic conditions in the jar. Methylene blue solution is colourless in anaerobic conditions and blue in aerobic conditions. (Ref. Chapter 6).

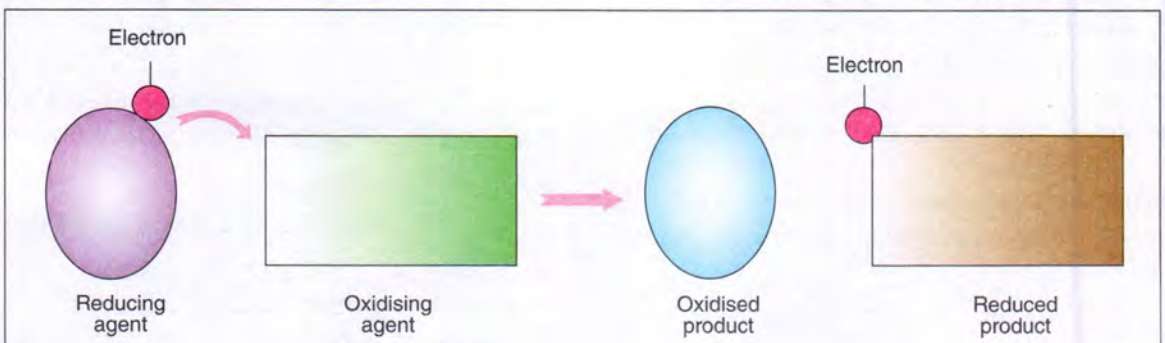


Fig. 3.3 Exchange of electrons in an oxidation – reduction process

KEY POINTS

1. The minimum nutritional requirement for growth and multiplication of bacteria includes sources of *carbon*, *nitrogen*, *hydrogen*, *oxygen* and some *inorganic salts*.
2. Bacterial cell division occurs by binary fission.
3. The time required for the bacterium to give rise to two daughter cells under optimum conditions is known as the *generation time*.
4. *Viable count* measures the number of living (viable) bacteria while *total count* indicates total number of bacteria in the specimen, irrespective of whether they are living or dead.
5. Bacterial growth curve has four phases namely *lag phase*, *log phase*, *stationary phase* and *phase of decline*.
6. On the basis of requirement of oxygen, bacteria are divided into *aerobes* and *anaerobes*.
7. Some organisms require higher level of carbon dioxide (5-10%), for their growth, they are named as *capnophilic* bacteria.
8. Majority of bacteria grow between temperature range of 25°C to 40°C i.e. *mesophiles*.
9. The oxidising or reducing condition of a system is indicated by the net readiness of all the components in that system to accept or to part with the electrons. This is called oxidation reduction potential or *redox potential*.
10. Anaerobic organisms require low redox potential for their growth.

YOU MUST KNOW

1. Generation time.
2. Various phases of bacterial growth curve.
3. Microaerophilic bacteria, capnophilic bacteria and their examples.
4. Redox potential.

STUDY QUESTIONS

1. Write short notes on:
   (a) Bacterial growth curve (b) Redox potential.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Generation time of *Mycobacterium tuberculosis* is about:
   (a) 20 seconds (b) 20 minutes (c) 20 hours (d) 20 days
2. Bacteria which can grow at temperature between 25°C and 40°C are known as:
   (a) Mesophiles (b) Psychrophiles (c) Thermophiles (d) None of the above
3. Which one of the following bacteria can grow in acidic pH?
   (a) Klebsiella sp. (b) Lactobacilli (c) *Pseudomonas aeruginosa* (d) *Vibrio cholerae*
4. The bacteria which require higher level of carbon dioxide for their growth are known as:
   (a) Microaerophilic bacteria (b) Capnophilic bacteria (c) Aerobic bacteria (d) None of the above
5. Low redox potential of culture medium is required for growth of:
   (a) Anaerobic bacteria (b) *Escherichia coli* (c) *Pseudomonas aeruginosa* (d) None of the above

Answers (MCQs):

1. (c) 2. (a) 3. (b) 4. (b) 5. (a)

Chapter 4

STERILISATION AND DISINFECTION

I. INTRODUCTION: SOME DEFINITIONS

Microorganisms are responsible for contamination and infection. They are present all around. The aim of sterilisation is to remove or destroy them from materials or from surfaces.

Sterilisation

It is a process by which an article, surface or medium is made free of all microorganisms either in the vegetative or spore form.

Disinfection

It means the destruction of all pathogens or organisms capable of producing infections but not necessarily spores. All organisms may not be killed but the number is reduced to a level that is no longer harmful to health.

Antiseptics

These are chemical disinfectants which can safely be applied to living tissues and are used to prevent infection by inhibiting the growth of microorganisms.

Asepsis

The technique by which, the occurrence of infection into an uninfected tissue is prevented.

Uses of Sterilisation/Disinfection

1. Sterilisation of materials, instruments used in surgical and diagnostic procedures.
2. For media and reagents used in the microbiology laboratory.
3. In food and drug manufacturing to ensure safety from contaminating organisms.

II. METHODS OF STERILISATION

A. Physical methods

1. Sunlight
2. Heat
   (*a*) Dry heat
   (*b*) Moist heat
3. Ozone
4. Filtration
5. Radiation

B. Chemical methods

1. Alcohols
2. Aldehydes
3. Phenols
4. Halogens
5. Oxidising agents
6. Salts
7. Surface active agents
8. Dyes
9. Vapour phase disinfectants

A. Physical Methods

1. *Sunlight*

Sunlight has an active germicidal effect due to its content

of ultraviolet rays. It is a natural method of sterilisation in cases of water in tanks, rivers and lakes.

2. Heat

Heat is the most reliable and commonly employed method of sterilisation. It should be the method of choice unless contraindicated. Two types of heat are used, dry heat and moist heat.

Principle

(*i*) Dry heat kills the organisms by denaturation of bacterial protein, oxidative damage and by the toxic effect of elevated levels of electrolytes. However, the possibility of DNA damage is also incriminated as one of the mechanisms of inactivations of microbes.

(*ii*) Moist heat kills the microorganisms by denaturation and coagulation of proteins.

Factors influencing

(*i*) *Nature of heat:* Dry heat or moist heat

(*ii*) *Temperature and duration:* The time required for sterilisation is inversely proportional to temperature to which organisms are exposed.

(*iii*) *Characteristic of microorganisms and spores present:* Bacterial spores are killed by moist heat at 121°C for 15 minutes. Most vegetative bacteria, fungi and viruses are killed in 30 minutes at 65°C by moist heat.

(*iv*) *Type of material:* A high content of organic substances tend to protect the vegetative form and spores against the lethal action of heat. Materials containing organic substances require more time for sterilisation. Proteins, sugars, fats and starch are some of the organic substances.

DRY HEAT STERILISATION

The following procedures are used for sterilisation by dry heat.

(*i*) Red heat
(*ii*) Flaming
(*iii*) Incineration
(*iv*) Hot air oven

(i) Red heat

Inoculating wires or loops, tips of forceps and needles are held in the flame of a Bunsen burner till they become red hot.

(ii) Flaming

Glass slides, scalpels and mouths of culture tubes are passed through bunsen flame without allowing them to become red hot.

(iii) Incineration

By this method, infective material is reduced to ashes by burning. Instrument named incinerator may be used for this purpose. Soiled dressings, animal carcasses, bedding and pathological materials are dealt with this method.

(iv) Hot Air Oven

It is the most widely used method of sterilisation by dry heat. The oven is electrically heated and is fitted with a fan to ensure adequate and even distribution of hot air in the chamber (Figs. 4.1 and 4.2). It is also fitted with a thermostat that maintains the chamber air at a chosen temperature.

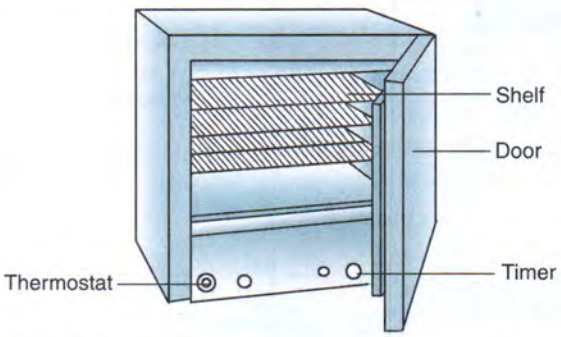


Fig. 4.1 Hot air oven–Diagramatic

Fig. 4.2 Hot air oven

Temperature and time

160°C for two hours (holding time) is required for sterilisation (previously it was 160°C for one hour). However, alternative temperatures and holding time include 170°C for one hour and 180°C for 30 minutes.

Uses

It is used for sterilisation of

(*i*) Glasswares like glass syringes, petridishes, flasks, pipettes and test tubes.

(*ii*) Surgical instruments like scalpels, scissors, forceps etc.

(*iii*) Chemicals such as liquid paraffin, fats, sulphonamides powders etc.

Precautions

(*i*) It should not be overloaded.

(*ii*) The material should be arranged in a manner which allows free circulation of air.

(*iii*) Material to be sterilised should be perfectly dry.

(*iv*) Test tubes, flasks etc. should be fitted with cotton plugs.

(*v*) Petridishes and pipettes should be wrapped in paper.

(*vi*) Rubber materials (except silicone rubber) or any inflammable material should not be kept inside the oven.

(*vii*) The oven must be allowed to cool for two hours before opening the doors, since the glasswares may crack by sudden cooling.

Sterilisation control

(*i*) The spores of *Bacillus subtilis subsp. niger* (NCTC 10075 or ATCC 9372) are kept inside the oven. These spores should be destroyed if the sterilisation is proper.

(*ii*) Thermocouples may also be used.

(*iii*) Browne's tube with green spot is available. After proper sterilisation a green colour is produced (after two hours at 160°C).

MOIST HEAT STERILISATION

This method of sterilisation may be used at different temperatures as follows.

(*i*) At a temperature below 100°C

(*ii*) At a temperature of 100°C

(*iii*) At a temperature above 100°C

(i) At a Temperature below 100°C

(*a*) *Pasteurisation of milk*—Two types of method, holder method (63°C for 30 minutes) and flash method (72°C for 20 seconds followed by cooling quickly to 13°C or lower) are used. All nonsporing pathogens such as mycobacteria, brucellae and salmonellae are killed except *Coxiella burnetii* which being relatively heat resistant may survive in holder method.

(*b*) *Inspissation*—Some serum or egg media, such as Lowenstein-Jensen's and Loeffler's serum, are rendered sterile by heating at 80-85°C temperature for half an hour daily on three consecutive days. This process of sterilisation is called inspissation. The instrument used is called inspissator.

(*c*) *Vaccine bath*—Bacterial vaccines are sterilised in special vaccine baths at 60°C for one hour. Serum or body fluids can be sterilised by heating for one hour at 56°C in a water bath on several successive days.

(*d*) *Low temperature steam formaldehyde (LTSF) sterilisation*—Items which cannot withstand the temperature of 100°C may be sterilised by a method known as low temperature steam-formaldehyde (LTSF) sterilisation. In this method steam at subatmospheric pressure at the temperature of 75°C with formaldehyde vapour is used. *Bacillus stearothermophilus* has been used as biological control to test the efficacy of LTSF sterilisers.

(ii) At a Temperature of 100°C

(*a*) *Boiling*—Boiling for 10 to 30 minutes may kill most of the vegetative forms but many spores withstand boiling for a considerable time. When better methods are not available, boiling may be used for glass syringes and rubber stoppers. It is not recommended for the sterilisation of instruments used for surgical procedures.

(*b*) *Tyndallisation*—Steam at 100°C for 20 minutes on three successive days is used. This is known as tyndallisation or intermittent sterlisation. The principle is that the first exposure kills all the vegetative forms, and in the intervals between the heatings the remaining spores germinate into vegetative forms which are killed on subsequent heating. It is used for sterilisation of egg, serum or sugar containing media which are damaged at higher temperature of autoclave. The instrument commonly used is Koch's or Arnold's steam steriliser.

(*c*) *Steam steriliser at 100°C for 90 minutes*—Koch's or Arnold's steam steriliser (Fig. 4.3) is usually used for media which are decomposed at high temperature of autoclave. The articles are kept on a perforated tray through which steam can pass. They are exposed to steam at atmospheric pressure for 90 minutes. Most of the vegetative forms are killed by this method except thermophiles.

(iii) At a Temperature above 100°C (under pressure)

Water boils when its vapour pressure equals that of the surrounding atmosphere. When the atmospheric pressure is raised then the boiling temperature is also raised. At

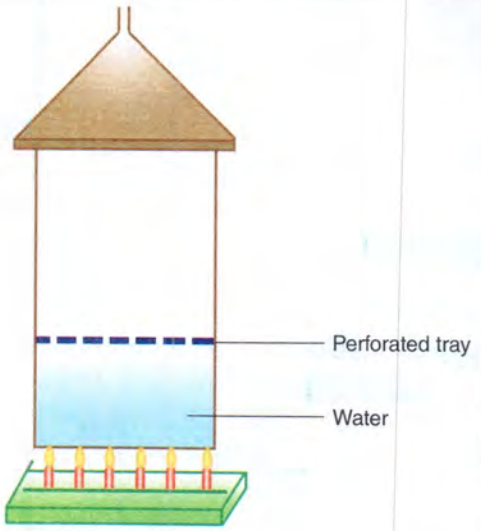


Fig. 4.3 Steam steriliser

normal pressure water boils at 100°C but when pressure inside a closed vessel increases, the temperature at which water boils also increases. This principle has been applied in *autoclave* and *pressure cooker*.

AUTOCLAVE

Principle

Steam above 100°C or saturated steam has a better killing power than dry heat. Bacteria are more susceptible to moist heat as bacterial protein coagulates rapidly. Saturated steam can penetrate porous material easily. When steam comes into contact with a cooler surface it condenses to water and liberates its latent heat to that surface, for example, 1600 ml of steam at 100°C and at atmospheric pressure condenses into one ml of water at 100°C and releases 518 calories of heat. The large reduction in volume sucks in more steam to the same site and the process continues till the temperature of the article is raised to that of steam. The condensed water produces moist conditions for killing the microbes present.

Components of autoclave

Autoclave is a modified pressure cooker or boiler. It consists of a vertical or horizontal cylinder of gunmetal or stainless steel in a supporting iron case. The lid is fastened by screw clamps and rendered air tight by an asbestos washer. The lid bears a discharge tap for air and steam, a pressure gauge and a safety valve. Heating is generally done by electricity. The steam circulates within the jacket and is supplied under high pressure to the inner chamber where materials are kept for sterilisation (Figs. 4.4 and 4.5).

Fig. 4.4 Vertical autoclave

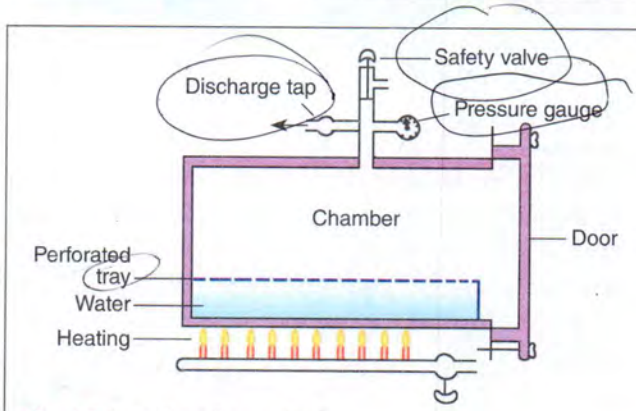


Fig. 4.5 Autoclave (inner view)

Procedure

The cylinder is filled with sufficient water and the material to be sterilised is placed on the tray. The lid is closed with the discharge tap open. The heater is put on. Safety valve is adjusted to the required pressure. After boiling of water, the steam and air mixture is allowed to escape till all the air has been displaced. This can be tested by leading the escaping steam-air mixture into a pail of water through rubber tubing. When the air bubbles stop coming in the pail it indicates that all the air has been displaced. The discharge tap is now closed. The steam pressure rises inside and when it reaches the desired set level (15 pounds (lbs) per square inch), the safety valve opens and the excess steam escapes. From this point, the holding period (15 minutes) is counted. After the holding period, the heating is stopped and autoclave allowed to cool till the pressure gauge indicates that the pressure inside is equal to the atmospheric pressure. The discharge tap is opened slowly and air is allowed to enter the autoclave. The lid is now opened and the sterilised material removed.

Note: The domestic pressure cooker works on the same principle and may be used for sterilisation of small articles in clinics.

Sterilisation conditions

Temperature — 121°C
Chamber pressure — 15 lb per square inch
Holding time — 15 minutes

These conditions are generally used, however, sterilisation can also be done at higher temperatures, at 126°C (20 lbs/square inch) for 10 minutes or at 133°C (30 lbs/square inch) for 3 minutes.

Uses

(*i*) To sterilise culture media, rubber material, gowns, dressing, gloves etc.
(*ii*) It is particularly useful for materials which cannot withstand the higher temperature of hot air oven.
(*iii*) For all glass syringes, hot air oven is a better sterilising method.

Precautions

(*i*) The air must be allowed to escape from the chamber as temperature of air-steam mixture is lower than that of pure steam.
(*ii*) Materials should be arranged in such a manner as to ensure free circulation of steam inside the chamber.

Sterilisation control

(*i*) *Thermocouple*—It is to record the temperature directly by a potentiometer.
(*ii*) *Bacterial spores*—Spores of *Bacillus stearothermophilus* are used as the test organism. This organism has an optimum growth temperature of 55-60°C and its spores are killed in 12 minutes at 121°C. An envelope containing a filter paper strip impregnated with 10^6 spores is placed inside the autoclave during sterilisation. After sterilisation is over the strip is inoculated into a suitable recovery medium and incubated at 55°C for five days. Spores are destroyed if sterilising conditions of autoclave are proper.
(*iii*) *Chemical indicators*—Browne's tube contains red solution which turns green, when exposed to temperature of 121°C for 15 minutes in autoclave.
(*iv*) *Autoclave tapes*

3. Ozone

Low Temperature sterilisation by ozone

Ozone steriliser uses oxygen, water and electricity to produce ozone within the steriliser and provide sterilisation without producing toxic chemicals. It runs at lower temperature i.e. 25°C-35°C. In this device, oxygen molecules (O_2) are separated into atomic oxygen (O) in the presence of intense electrical field. This atomic oxygen (O) combines with other oxygen molecules (O_2) to form ozone (O_3). The ozone provides a sterility assurance of 10^{-6} in approximately 4 hours.

4. Filtration

This method of sterilisation is useful for substances which get damaged by heat process e.g. sera, sugars, antibiotic solutions etc.

Uses of filtration

(*i*) To sterilise sera, sugars and antibiotic solutions.
(*ii*) Separation of toxins and bacteriophages from bacteria.
(*iii*) To obtain bacteria-free filtrates of clinical samples for virus isolation.
(*iv*) Sterilisation of hydatid fluid.
(*v*) Filter discs retain the organism which can then be cultured e.g. testing of water samples for cholera vibrios or typhoid bacilli.
(*vi*) Purification of water.

Limitation

The pore size of filter is around 0.75 µm in diameter which retains bacteria but allows viruses and mycoplasma to pass through filter, therefore, filtered preparations are not safe for clinical use.

Types of filters

(*i*) Candle filters e.g. Berkefeld, Chamberland filter
(*ii*) Asbestos disc filters e.g. Seitz filter
(*iii*) Sintered glass filters
(*iv*) Membrane filters
(*v*) Air filters
(*vi*) Syringe filters

It is normal practice to use positive or more usually negative pressure by suction pump to enhance filtration.

(*i*) *Candle filters*—These have been used widely for purification of water for industrial and drinking purposes. These filters are usually made in the form of hollow candles. The liquid to be filtered is passed through the candle. They are available in different grades of porosity. Candle filters are of two types:
(*a*) Diatomaceous earth filters e.g., Berkefeld
(*b*) Unglazed porcelain filters e.g., Chamberland.

(*ii*) *Asbestos disc filters (Seitz filter)*—These are made up of asbestos (magnesium silicate). The filter disc is supported on a metal mount. The filter is attached to a vacuum flask through a silicone

rubber bung. After use, the filter disc is discarded. Each time a fresh disc is used and the outfit is sterilised by autoclaving. These filter discs are available in different grades.

(*iii*) *Sintered glass filters*—These are prepared by fusing finely powdered glass particles. These are available in different pore sizes.

(*iv*) *Membrane filters*—Membrane filters are made up of cellulose esters. These are routinely used in water analysis, sterility testing, and for the preparation of solutions for parenteral use. Nitrocellulose (Millipore) membrane filters are widely used. Membrane filters are available in pore sizes of 0.015 to 12 µm. The 0.22 µm filter is the most commonly used because the pore size is smaller than that of bacteria. For bacterial counts of water, a known amount of water is filtered through the membrane filter disc. The upper side of filter disc is placed on a culture medium and incubated. The colonies develop on the medium. Viable counts can be calculated by counting the colonies.

(v) *Air filters*—These filters are used to deliver clean bacteria-free air to a cubicle or a room. High efficiency particulate air (HEPA) filters are used in air filtration in laminar air flow system in microbiology laboratories. HEPA filters can remove particles of 0.3 µm or larger.

(*vi*) *Syringe filters*—Syringes fitted with membrane of different diameters are available. For sterilisation, the fluid is forced through the disc (membrane) by pressing the piston of the syringe.

5. *Radiations*

Two types of radiations are used for sterilisation: ionising and non-ionising.

(*i*) *Ionising radiations* — Ionising radiations include gamma rays, X-rays and cosmic rays. They have very high penetrating power. They are highly lethal to all cells including bacteria. They damage DNA by various mechanisms. Gamma radiations from a Cobalt 60 source are commercially used for sterilisation of disposable items such as plastic syringes, swabs, culture plates, cannulas, catheters etc. This method is also known as *cold sterilisation* because there is no appreciable increase in temperature. The advantages of this method include speed, high penetrating power (it can sterilise materials, through outer packages and wrappings), and the absence of heat. *Bacillus pumilis* has been used for testing the efficacy of ionising radiations.

High energy electron radiation is not used widely in medicine.

(*ii*) *Non-ionising radiations*—These include infrared and ultraviolet (UV) radiations. Infrared is used for rapid mass sterilisation of syringes and catheters. Ultraviolet radiation with wavelength of 240 to 280 nm has marked bactericidal activity. It acts by denaturation of bacterial protein and interference with DNA replication. UV radiation is used for disinfecting enclosed areas such as bacteriological laboratory, inoculation hoods, laminar flow and operation theatres. Most vegetative bacteria are susceptible to UV radiation but spores are highly resistant. Susceptibility of viruses is variable.

B. Chemical Methods

A variety of chemical agents are used as antiseptics and disinfectants. An ideal antiseptic or disinfectant should

(*i*) have wide spectrum of activity and be effective against all microorganisms including bacteria (both vegetative and spore forms), viruses, protozoa and fungi,
(*ii*) act in the presence of organic matters,
(*iii*) have high penetration power and quick action,
(*iv*) be stable and effective in acidic as well as in alkaline conditions,
(*v*) not corrode metals,
(*vi*) be compatible with other disinfectants,
(*vii*) not cause local irritation,
(*viii*) not be toxic if absorbed into circulation,
(*ix*) be safe and easy to use, and
(*x*) be easily available and cheap.

The disinfectant which possesses all the above criteria is yet to be found.

The following factors influence the potency of a disinfectant :

(*i*) concentration
(*ii*) time of action
(*iii*) pH
(*iv*) temperature
(*v*) nature of organism and
(*vi*) presence of organic matter.

Disinfectants can be divided into three groups:

1. High level disinfectants.
2. Intermediate level disinfectants.
3. Low level disinfectants.

1. *High level disinfectants*

The effectiveness of high level disinfectants may be equivalent to that of sterilisation. These disinfectants are used for certain types of endoscopes, cystoscopes

and surgical instruments with plastic components which cannot withstand the sterilisation procedures such as autoclaving. The high level disinfectants include glutaraldehyde, hydrogen peroxide, peracetic acid and chlorine compounds.

2. *Intermediate level disinfectants*

Intermediate level disinfectants may not be effective against bacterial spores, hence these disinfectants are used for instruments (e.g. laryngoscopes, fiberoptic endoscopes) where contamination with spores and other highly resistant organisms is unlikely. The intermediate level disinfectants include alcohols, iodophores and phenolic compounds.

3. *Low level disinfectants*

Many organisms can survive on exposure to low level disinfectants. These disinfectants are used for items which come in contact with the patients but they do not penetrate into tissue. Stethoscopes, electrocardiogram electrodes etc. are examples of such items.

1. *Alcohols*

Ethyl alcohol and isopropyl alcohol are the most frequently used. They act by denaturing bacterial proteins. They rapidly kill bacteria including tubercle bacilli but they have no sporicidal or virucidal activity. However, human immunodeficiency virus (HIV) is susceptible to 70% ethyl alcohol and 35% isopropyl alcohol in the absence of organic matter. They are used mainly as skin antiseptics. To be effective, they should be used at a concentration of 60-70 per cent in water. Isopropyl alcohol is preferred to ethyl alcohol as it is a better fat solvent, more bactericidal and less volatile.

Methyl alcohol is effective against fungal spores and is used for treating cabinets affected by them. Methyl alcohol vapour is toxic and inflammable.

2. *Aldehydes*

FORMALDEHYDE

It is markedly bactericidal, sporicidal and virucidal. It is used both as aqueous solution and in gaseous form. A 10% aqueous solution of formalin is routinely used. It is active against amino group in the protein molecule.

Uses

(*i*) Preservation of tissue for histological examination.
(*ii*) To sterilise bacterial vaccines.
(*iii*) To prepare toxoid from toxin.
(*iv*) For killing of bacterial cultures and suspensions.
(*v*) For destroying anthrax spores in hair and wool.

Formalin gas has been described under "Vapour phase disinfectants".

GLUTARALDEHYDE

It is effective against bacteria (including *M. tuberculosis*), fungi and viruses (including human immunodeficiency viruses, hepatitis B virus and enteroviruses). It also kills spores. It is less toxic and irritant to the eyes and skin than formaldehyde. It is rapid, broad spectrum, and is one of the few chemicals accepted as a sterilant and high level disinfectant. It can act in the presence of organic matter and does not damage plastics. It is used as 2% buffered solution. It is available commercially as 'cidex'. It can be used for delicate instruments having lenses.

Uses

(*i*) For sterilisation of cystoscopes, endoscopes and bronchoscope.
(*ii*) To sterilise plastic endotracheal tubes, face masks, corrugated rubber anaesthetic tubes and metal instruments.

ORTHO-PHTHALALDEHYDE

Ortho-phthalaladehyde (OPA) is a high level disinfectant. It is more stable during storage and more rapidly mycobactericidal than glutaraldehyde. 0.5% OPA is slowly sporicidal. OPA vapours may irritate the respiratory tract and eyes, therefore, it must be handled with appropriate safety precautions. It can be used for materials where glutaraldehyde is used such as endoscopes. This new disinfectant may be useful for glutaraldehyde resistant mycobacteria.

3. *Phenols*

Lister, the father of antiseptic surgery, first introduced use of phenol (carbolic acid) in surgery (1867). Phenols are produced by distillation of coal tar between temperatures of 170°C and 270°C. The lethal effect of phenols is due to cell membrane damage, thus releasing cell contents and causing lysis. Phenol (1%) has bactericidal action. It is readily absorbed by skin and causes toxicity. Certain phenol derivatives like cresol, chlorhexidine, chloroxylenol and hexachlorophane are commonly used as antiseptics.

(*i*) *Cresols*—Lysol is a solution of cresols in soap. It is most commonly used for sterilisation of infected glasswares, cleaning floors, disinfection of excreta. They are not readily inactivated by the presence of organic matter. They are active against a wide range of organisms.
(*ii*) *Chlorhexidine*—Savlon (chlorhexidine and cetrimide) is widely used in wounds, pre-operative disinfection of skin, as bladder irrigant etc. It is bactericidal at a high dilution. They are more active against Gram

positive than Gram negative bacteria with no action against tubercle bacilli or spores and have very little activity against viruses. They have a good fungicidal activity.

(*iii*) *Chloroxylenol*—It is an active ingredient of dettol. It is less toxic and less irritant. It is readily inactivated by presence of organic matter. It is inactive against Pseudomonas.

(*iv*) *Hexachlorophane*—It is more active against Gram positive than Gram negative bacteria. It is applied on skin as prophylaxis against staphylococcal infections. It is bacteriostatic at very high dilutions. It is potentially toxic and should be used with care.

4. *Halogens*

Chlorine and iodine are two commonly used disinfectants. These are bactericidal and are effective against sporing bacteria and viruses.

Chlorine is used in water supplies, swimming pools, food and dairy industries. Chlorine compounds in the form of bleaching powder, sodium hypochlorite and chloramine are also used. The disinfection action of all the chlorine compounds is due to release of free chlorine. When hypochlorites are added to water, the chlorine reacts with water to form hypochlorous acid which is a strong oxidising agent and effective disinfectant. The activity of chlorine is markedly influenced by the presence of organic matter. Bleaching powder or hypochlorite solution are the most widely used for human immunodeficiency virus (HIV) infected material. The hypochlorites have a bactericidal, fungicidal, virucidal and rapidly sporicidal action. Hypochlorite solution decays rapidly and should be prepared daily. It should not be used in the presence of formaldehyde as one of the reaction products is found to be carcinogenic. Chloramines are used as antiseptics for dressing wounds.

Iodine in alcoholic and aqueous solutions is used as a skin disinfectant. It is actively bactericidal, with moderate action against spores. It is active against *M. tuberculosis* and viruses. Like chlorine, it is also inactivated by organic matter. Compounds of iodine with surface active agents known as iodophores, are claimed to be more active than the aqueous or alcoholic solutions of iodine. Betadine is one example of commonly used iodophores.

5. *Oxidising Agents*

(*i*) *Hydrogen peroxide*

Hydrogen peroxide (H_2O_2) is effective against most organisms at concentration of 3-6%, while it kills all organisms including spores at higher concentration (10-25%). Mode of action is by liberation of free hydroxyl radical on decomposition of H_2O_2. These free radicals are the active ingredient in the disinfection process. H_2O_2 is used to disinfect contact lenses, surgical prostheses and plastic implants. It is used for high level disinfection and sterilisation.

(*ii*) *Peracetic acid*

Peracetic acid is an oxidising agent. It is one of the high level disinfectants. It is effective in the presence of organic matter. It is a more potent germicidal agent than hydrogen peroxide. The end products (acetic acid and oxygen) of this agent are non-toxic. It is also used in plasma sterilisation procedure.

(*iii*) *Plasma sterilisation*

Plasma refers to any gas which consists of electrons, ions or neutral particles. The formation of a low temperature plasma requires several devices. Chemical disinfectant such as H_2O_2 alone or a mixture of H_2O_2 and peracetic acid is used in these devices to induce the plasma. The resulting UV radiation causes destruction of vegetative organisms and spores. Two commercial plasma sterilisers available are *Sterrad 100S steriliser* and *Plazlyte steriliser*. It is used for sterilisation of surgical instruments.

6. *Salts*

Salts of heavy metals have toxic effect on bacteria. The salts of copper, silver and mercury are used as disinfectant. They are protein coagulants and act by combining with sulphydryl (SH) groups of bacterial proteins and other essential intracellular compounds. Merthiolate (sodium ethyl mercurithiosalicylate) is used in a dilution of 1: 10,000 for preservation of sera.

Mercuric chloride, once used as a disinfectant is highly toxic. Thiomersal and mercurochrome are less toxic and are used as mild antiseptics. They have a marked bacteriostatic but limited fungicidal action. Copper salts are used as fungicides.

7. *Surface Active Agents*

Substances which alter energy relationships at interfaces, producing a reduction of surface tension, are known as surface active agents or surfactants. They are classified into anionic, cationic, nonionic and amphoteric compounds. Of these, the cationic surfactants have been the most important antibacterial agents. These act on the phosphate groups of the cell membrane and also enter the cell. This results in loss of membrane semipermeability and the cell proteins are denatured. Quaternary ammonium compounds are the most important cationic surfactants. Although these compounds are bactericidal for a wide

range of organisms, Gram positive species are more susceptible. They have no action on spores and tubercle bacilli. They are active against viruses with lipid envelopes (e.g. rabies virus) and much less against non-enveloped viruses (e.g. enteroviruses). The common cationic compounds are acetyl trimethyl ammonium bromide (cetavalon or cetrimide) and benzalkonium chloride. The activity of these compounds is greatest at an alkaline pH. Acid inactivates them. Antibacterial activity is reduced in the presence of organic matter. Anionic surface active agents, like ordinary soaps, render them inactive.

The anionic compounds, e.g. common soaps, have strong detergent but weak antimicrobial properties. These agents are most active at acidic pH. They are effective against Gram positive organisms but are relatively ineffective against Gram negative species.

The amphoteric compounds, known as 'Tego' compounds possess detergent properties of anionic and antimicrobial activity of cationic compounds. They are active against a wide range of Gram positive and Gram negative organisms and some viruses. They are active over a wide range of pH. The presence of organic matter reduces their activity.

8. *Dyes*

Two groups of dyes, aniline dyes and acridine dyes have been used extensively as skin and wound antiseptics. Both are bacteriostatic in high dilution but have low bactericidal action. Aniline dyes include crystal violet, brilliant green and malachite green. They are more active against Gram positive bacteria than Gram negative bacteria. They have no activity against *M. tuberculosis*, and hence the use of malachite green in the Lowenstein-Jensen medium makes it selective for isolation of tubercle bacilli. They are non-toxic and non-irritant to the tissues. Their activity is inhibited by organic material such as pus. They interfere with the synthesis of peptidoglycan component of the cell wall. These dyes are used in the laboratory as selective agents in culture media.

Acridine dyes include acriflavine, euflavine, proflavine and aminacrine. They are affected very little by the presence of organic material. They are also more active against Gram positive bacteria than Gram negative bacteria but are not as selective as the aniline dyes. These dyes are used in clinical medicine. They interfere with the synthesis of nucleic acids and proteins in bacterial cells.

Gentian violet and acriflavine are two widely used dyes for skin disinfection especially in Gram positive bacterial infections.

9. *Vapour Phase Disinfectants*

FORMALDEHYDE GAS

This is employed for fumigation of operation theatres, wards laboratories etc. Formaldehyde gas is generated by adding 150 gm of $KMnO_4$ to 280 ml formalin for 1000 cu. feet of room volume. This reaction produces considerable heat, and so heat resistant containers should be used. The doors should be sealed and left unopened for 48 hours. Sterilisation is achieved by condensation of gas on exposed surfaces. The gas is irritant and toxic when inhaled. After completion of disinfection, the effect of irritant vapours should be nullified by exposure to ammonia vapour.

ETHYLENE OXIDE (ETO)

It is a colourless liquid with a boiling point of 10.7°C. It is effective against all types of microorganisms including viruses and spores. It is a highly penetrating gas and it readily penetrates some plastics. It acts by alkylating the amino, carboxyl, hydoxyl and sulphydryl groups in protein molecules. In addition, it reacts with DNA and RNA. It has a potential toxicity to human beings, including mutagenicity and carcinogenicity. It is highly inflammable. It forms explosive mixture when its concentration in air is greater than 3 per cent. By mixing it with inert gases such as carbon dioxide, its explosive tendency can be eliminated.

It is specially used for sterilising plastic and rubber articles, respirators, heart-lung machines, sutures, dental equipments and clothing. It is commercially used to sterilise disposable plastic syringes, petridishes etc. It has a high penetrating power and thus can sterilise prepackaged materials. Ethylene oxide steriliser called a *chemiclave* is used for sterilisation. It is unsuitable for fumigation of rooms because of its explosive nature. It is irritant, and personnel working with it have to take precautions.

Bacillus globigi (a red pigmented variant of *Bacillus subtilis*) has been used as biological control for testing of ethylene oxide sterilisers.

BETAPROPIOLACTONE (BPL)

This is a condensation product of ketane and formaldehyde. It has a boiling point of 163°C. Though it is a gas, it has a low penetrating power. It has a rapid action and is used in 0.2 per cent. It is effective against all microorganisms including viruses. It is more efficient for fumigation than formaldehyde. Unfortunately it has some carcinogenic activity. BPL is used for inactivation of vaccines.

Methods of sterilisation and disinfection of some important materials are shown in Table 4.1.

Gaseous sterilisation

Table 4.1 Methods of Sterilisation and Disinfection of Some Important Materials

	Materials	Methods
1.	Inoculating wires or loops	Red heat
2.	Glasswares—syringes, petridishes, test tubes, flasks, universal container, oily fluids (Paraffin)	Hot-air oven
3.	Disposable syringes and other disposable items	Gamma radiations
4.	Culture media	Autoclaving
5.	Culture media containing serum, egg	Tyndallisation
6.	Toxin, serum, sugar and antibiotic solutions	Filtration
7.	Milk	Pasteurisation
8.	Cystoscope and endoscope	Glutaraldehyde
9.	Sterilisation of operation theatre 3M RS	Formaldehyde gas
10.	Infective material like soiled dressings, beddings, animal carcasses	Incineration
11.	Skin	Tincture iodine, 70% ethanol, savlon
12.	Aprons, gloves, catheters, surgical instruments except sharp instruments	Autoclaving
13.	Sharp instruments	5% Cresol
14.	Rubber, plastic and polythene tubes	Glutaraldehyde

Recommended concentrations of some commonly used disinfectants are given in Table 4.2.

Table 4.2 Recommended Concentrations of Some Disinfectants

Disinfectant	Concentration
Ethyl alcohol	70%
Glutaraldehyde	2%
Lysol	2.5%
Savlon (chlorhexidine and cetrimide)	2%, 5%
Dettol (chloroxylenol)	4%
Bleaching powder (calcium hypochlorite)	14 gm in one litre of water
Sodium hypochlorite	1%, 0.1%
Betadine (Iodophore)	2%

III. TESTING OF DISINFECTANTS

1. *Rideal Walker test*
2. *Chick Martin test*
3. *Kelsey-Sykes test*
4. *In-Use tests*

1. Rideal Walker Test

In Rideal Walker test, suspensions containing similar quantities of organisms are submitted to the action of varying concentrations of phenol and of the disinfectant to be tested. The dilution of the test disinfectant which sterilises the suspension in a given time is divided by the corresponding dilution of phenol. This gives the phenol coefficient. The phenol coefficient of 1.0 means that the test disinfectant has been as effective as phenol. Higher the phenol coefficient, more effective is the disinfectant. The phenol coefficient does not, however, give any indication of how the test disinfectant will function practically in the presence of organic matters.

2. Chick Martin Test

Chick Martin test is a modification of Rideal Walker test. In this test , disinfectant acts in the presence of organic matter to simulate natural conditions. Organic matter, in the form of dried yeast or faeces, is included.

3. Kelsey-Sykes Test

This test gives a measure of the capacity of a disinfectant to retain its activity when repeatedly used microbiologically. It is also named as *capacity test*. The standard organism (*Staphylococcus aureus, Esch. coli, Ps. aeruginosa*) is added to the disinfectant in three successive lots at 0, 10 and 20 minutes. These three lots are in contact with disinfectant for eight minutes and samples are transferred at 8, 18 and 28 minutes respectively to a recovery medium. The disinfectant is judged by its ability to kill bacteria (growth or no growth on recovery medium) and not by comparison with phenol. The test is carried out under both 'clean' and 'dirty' conditions. Thus it also measures the effectiveness of a disinfectant in the presence of an organic matter.

4. In-Use Tests

The liquid phase of disinfectant solutions in actual use in hospital practice is examined quantitatively for viable organisms. A use-dilution is then determined which only very rarely yields a positive culture. The efficiency of a new disinfectant is determined by its ability to inactivate a known number of a standard strain of a pathogenic staphylococcus on a given surface within a certain time. The results of such tests are generally more useful than those of the phenol coefficient test and its modifications.

STERILISATION OF PRIONS

Prions are infectious proteins without any detectable nucleic acid. They are highly resistant to physical and chemical agents. They produce slow infections with long incubation period (Refer 'Slow Viral Diseases' in Chapter 64).

1. *Heat*: They are extremely resistant to dry heat. A temperature of 360°C for one hour has not been found to be effective completely. They are more resistant to moist heat than bacteria, spores, fungi and viruses. Steam at a temperature of 134-138°C for 18 minutes is found to be effective.

2. *Chemicals*: Prions are inactivated by sodium hypochlorite (25% available chlorine) if treated for one hour. They are also sensitive to household bleach, phenol (90%) and iodine disinfectants. Chemicals such as aldehydes, hydrogen peroxide, ethylene oxide, ethanol and ionising radiations are found to be ineffective.

KEY POINTS

1. *Sterilisation* is a process by which an article, surface or medium is made free of all microorganisms either in the vegetative or spore form.
2. *Disinfection* means the destruction of all pathogens or organisms capable of producing infections but not necessarily spores.
3. *Asepsis* is the technique by which, the occurrence of infection into an uninfected tissue is prevented.
4. Methods of sterilisation include *physical methods* and *chemical methods*.
5. *Hot-air oven* and *autoclave* are two commonly used methods of sterilisation. Hot-air oven is a *dry heat sterilisation* method while autoclave is a *moist heat sterilisation*.
6. Temperature and time period for hot-air oven is *160°C for two hours* (*holding time*) whereas it is *121°C for 15 minutes at pressure of 15 pounds per square inch* for autoclave.
7. Other important methods of sterilisation include *filtration, radiations*, *glutaraldehyde* and *ethylene oxide*.
8. Filtration is used to sterilise sera, sugars and antibiotic solutions.
9. *Gamma radiations* and *ethylene oxide* (ETO) are the methods commercially used for sterilisation of disposable syringes and other disposable items.
10. *Glutaraldehyde* is used for sterilisation of cystoscopes, endoscopes and bronchoscopes. It is effective against bacteria (including *M. tuberculosis*), fungi and viruses (including human immunodeficiency viruses and hepatitis B virus).

YOU MUST KNOW

1. Difference between sterilisation and disinfection.
2. List of physical methods and chemical methods of sterilisation.
3. Principle, temperature and time period; uses of hot-air oven, autoclave, pasteurisation, inspissation and tyndallisation.
4. Uses of filtration and types of filters.
5. Types of radiations and their uses.
6. Uses of formaldehyde and glutaraldehyde as disinfectants.
7. Uses of gases as chemical method of sterilisation and role of ethylene oxide in sterilisation of disposable items.
8. Methods used for testing of disinfectants.

STUDY QUESTIONS

1. Define sterilisation. How does it differ from disinfection? Classify the various agents used in sterilisation. Add a note on the principle and functioning of autoclave.
2. Write short notes on:
   (a) Hot air oven (b) Autoclave
   (c) Filtration (d) Sterilisation by radiation
   (e) Vapour phase disinfectants or Gaseous sterilisation.
3. Write briefly about:
   (a) Sterilisation by moist heat (b) Tyndallisation
   (c) Inspissation (d) Pasteurisation.
   (e) Sterilisation of prions

MULTIPLE CHOICE QUESTIONS (MCQs)

1. The process by which an article, surface or medium is made free of all microorganisms either in the vegetative or spore form is known as:
   (a) Sterilisation (b) Disinfection (c) Asepsis (d) None of the above
2. Chemical disinfectants which can be safely applied to living tissues to prevent infection are known as:
   (a) Antiseptics (b) Formaldehyde (c) Ethylene oxide (d) None of the above
3. Temperature and time period used in hot air oven is:
   (a) 160°C for two hours (b) 140°C for two hours (c) 160°C for one hour (d) None of the above
4. Temperature and time period used in holder method of Pasteurisation is:
   (a) 63°C for 30 minutes (b) 63°C for 50 minutes (c) 72°C for 20 seconds (c) 72°C for 40 seconds
5. Which of the following bacteria can survive in holder method of Pasteurisation?
   (a) *Bordetella pertussis* (b) *Coxiella burnetii* (c) *Salmonella typhi* (d) *Mycobacterium bovis*
6. Steam sterilisation at 100°C for 20 minutes on three successive days is known as:
   (a) Tyndallisation (b) Inspissation (c) Pasteurisation (d) Vaccine bath
7. Which of the following sterilisation conditions are used in autoclave?
   (a) 121°C, 15 pounds pressure/sq inch, 15 minutes (b) 160°C, 20 pounds pressure/sq inch, 20 minutes
   (c) 140°C, 10 pounds pressure/sq inch, 15 minutes (d) None of the above
8. Which bacterial spores are used as sterilisation control in autoclave?
   (a) *Clostridium perfringens* (b) *Bacillus cereus* (c) *Bacillus stearothermophilus* (d) *Pseudomonas aeruginosa*
9. Gamma radiations can be used for sterilisation of:
   (a) Plastic syringes (b) Catheters (c) Swabs (d) All of the above
10. Glutaraldehyde is used for sterilisation of:
    (a) Cystoscopes (b) Endoscopes (c) Bronchoscopes (d) All of the above
11. The most widely used disinfectant for human immunodeficiency virus (HIV) infected material is:
    (a) Hypochlorite solution (b) Lysol (c) Formaldehyde (d) Mercuric chloride
12. Which of the following is used commercially for sterilisation of disposable plastic items?
    (a) Ethylene oxide (b) Autoclave (c) Glutaraldehyde (d) Ethyl alcohol

Answers (MCQs):

1. (a) 2. (a) 3. (a) 4. (a) 5. (b) 6. (a) 7. (a) 8. (c) 9. (d) 10. (d)
11. (a) 12. (a)

Chapter 5

CULTURE MEDIA

I. Introduction
II. Types of Media

I. INTRODUCTION

Culture media are required to grow the organisms from infected material to identify the causative agent. The basic constituents of culture media are:

1. **Water**: Source of hydrogen and oxygen.
2. **Electrolyte**: Sodium chloride or other electrolytes.
3. **Peptone**: It is a complex mixture of partially digested proteins. It is obtained from lean meat or other protein material such as heart muscle, casein or fibrin, usually by digestion with proteolytic enzymes. It contains proteoses, amino-acids, polypeptides, phosphates, minerals (K, Mg), and accessary growth factors like nicotinic acid and riboflavin. Special brands of peptone such as neopeptone, proteose peptone are available for special uses.
4. **Meat extract**: It is available commercially as 'Lab-lemeo'. It contains protein degradation products, inorganic salts, carbohydrates and growth factors.
5. **Blood or serum**: These are used for enriching culture media. Usually 5-10% defibrinated sheep blood is used. In certain media, serum is used.
6. **Agar**: It is prepared from sea weed (Algae—geladium species). It contains mainly long-chain polysaccharide, a small amount of protein-like material and a variety of inorganic salts. It is available either in long shreds or powder form. It is used in concentration of 2-3%. It melts at 98°C and usually solidifies at 42°C. Agar does not provide any nutrition to the bacteria but acts as a solidifying agent only. New Zealand agar has twice the jellifying capacity than that of Japanese agar.

II. TYPES OF MEDIA

Media are classified in many ways:

1. Based on physical state
   - (*i*) Liquid media
   - (*ii*) Semisolid media
   - (*iii*) Solid media
2. On the basis of presence of molecular oxygen and reducing substances in the media
   - (*i*) Aerobic media
   - (*ii*) Anaerobic media
3. Based on nutritional factors
   - (*i*) Simple media
   - (*ii*) Complex media
   - (*iii*) Synthetic media
   - (*iv*) Special media
     - (*a*) Enriched media
     - (*b*) Enrichment media
     - (*c*) Selective media
     - (*d*) Differential media
     - (*e*) Indicator media
     - (*f*) Transport media
     - (*g*) Sugar media

Simple Media

Nutrient broth is an example of simple medium. It contains peptone water and meat extract 1%. When glucose (0.5%) is added to nutrient broth, it becomes glucose broth. Peptone water and glucose broth are shown in Figs. 5.1 and 5.2.

Fig. 5.1 Peptone water

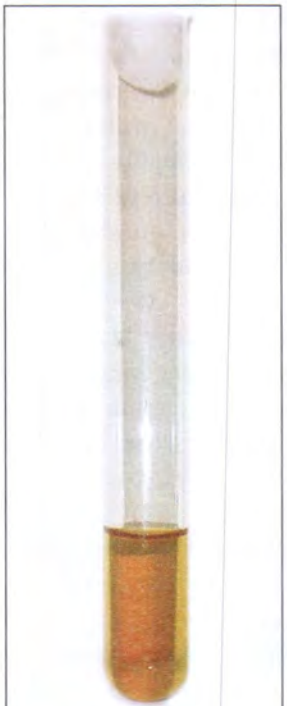


Fig. 5.2 Glucose broth

Fig. 5.3 Nutrient agar

When 2-3% agar is added to nutrient broth, it becomes nutrient agar (Fig. 5.3). This is the simplest and routinely employed medium in laboratory for diagnostic purposes. If concentration of agar is reduced (0.2 to 0.4%), semisolid medium is obtained which enables motile bacteria to spread.

Complex Media

All media other than simple media are complex. Complex media have added ingredients for bringing out certain properties or providing special nutrients required for the growth of the bacterium in question.

Synthetic Media

These are prepared from pure chemicals and the exact composition of the medium is known. These are used for special studies such as metabolic requirements. Dubo's medium with tween 80 is one example of a synthetic medium.

Special Media

(a) Enriched Media

When basal medium is added with some nutrients such as blood, serum or egg, it is called enriched medium. Some examples of enriched media are:

Blood agar (Fig. 5.4) – Blood is added to nutrient agar. It may be used for growing a number of bacteria but one specific example is *Streptococcus* which requires blood for its growth.

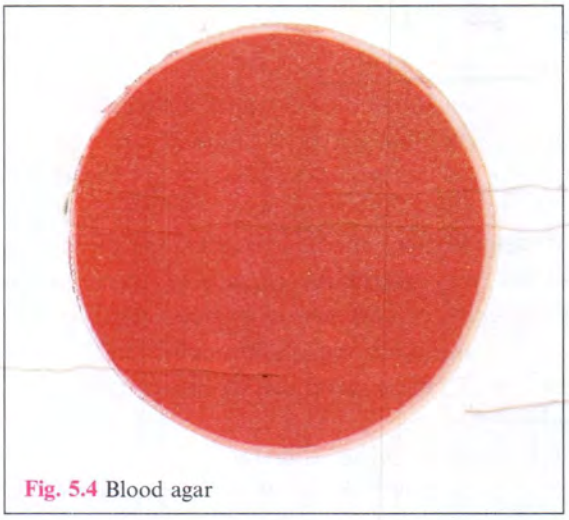


Fig. 5.4 Blood agar

Chocolate agar (Fig. 5.5) – It is a heated blood agar used for isolation of *Neisseria* and *Haemophilus influenzae*.

Fig. 5.5 Chocolate agar

Loeffler's serum slope (Fig. 5.6) – Serum is added for enriching the medium. This medium is used for grouping *Corynebacterium diphtheriae.*

These media are employed to grow organisms which are more exacting in their nutritional needs.

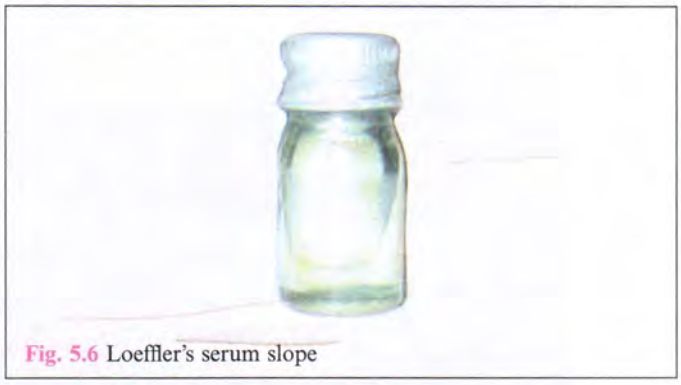


Fig. 5.6 Loeffler's serum slope

(b) Enrichment Media

Some substances are incorporated in the liquid medium which have a stimulating effect on the bacteria to be grown or inhibits its competitors. This results in an absolute increase in the number of wanted bacteria related to other bacteria. Such media are called enrichment media. These are very useful for culture of faeces where the nonpathogenic or commensal bacteria tend to overgrow the pathogenic ones, e.g., Salmonella being overgrown by *Esch. coli.* Some examples of enrichment media are as follows:

Tetrathionate broth – Tetrathionate is added which inhibits coliforms while allows typhoid-paratyphoid bacilli to grow.

Selenite 'F' broth – Selenite has similar action as that of tetrathionate in tetrathionate broth.

Alkaline peptone water – It is used to grow *Vibrio cholerae.*

(c) Selective Media

Selective media contain substances that inhibit all but a few types of bacteria and facilitate the isolation of a particular species. These media are used to isolate a particular bacteria from specimens where mixed bacterial flora is expected. Selective media are solid in contrast to enrichment media which are liquid. Examples of selective media are:

Deoxycholate citrate agar (DCA) – Addition of deoxycholate acts as a selective agent for enteric bacilli (Salmonella, Shigella).

Bile salt agar (BSA) – Bile salt is a selective agent. It favours the growth of only *Vibrio cholerae* whereas inhibits the growth of other intestinal organisms.

(d) Differential Media

When a medium contains substances which help to distinguish differing characteristics of bacteria, it is called "differential medium" e.g. MacConkey's medium, which contains peptone, lactose, agar, sodium taurocholate and neutral red. The lactose fermenters (LF) form pink coloured colonies whereas non-lactose fermenters (NLF) produce colourless or pale colonies.

(e) Indicator Media

These media contain an indicator which changes colour when a bacterium grows in them. *Salmonella typhi* grow as black colonies on Wilson and Blair medium containing sulphite.

MacConkey's agar (Fig. 5.7) is also an indicator medium. Due to fermentation of lactose, there is acidic pH which forms the pink colonies in the presence of neutral red indicator. The blood agar is an enriched medium but bacteria lysing red blood cells give clearing (haemolysis) around their colonies, thus, it is also an indicator medium. Therefore, some of these terms like indicator, differential, selective or enriched are interchangeable and more than one term may be applied for a single medium.

Fig. 5.7 MacConkey's agar

(f) Transport Media

These are used in the case of delicate organisms (e.g. gonococci) which may not survive the time taken for transit or may be overgrown by nonpathogenic bacteria (e.g. cholera organisms). For transport of specimens to the laboratory, special media are devised and these are termed transport media. Examples of transport media are:

(*a*) Stuart's transport medium is a non-nutrient soft agar gel containing a reducing agent to prevent oxidation,

and charcoal to neutralise bacterial inhibitors. It may be used for organisms such as gonococci.

(*b*) Buffered glycerol saline transport medium for enteric bacilli.

(*g*) *Sugar Media*

Sugar media (Fig. 5.8) help in identification of bacteria. The term sugar in microbiology denotes any fermentable substance. Glucose, lactose, sucrose and mannitol are routinely employed for fermentation tests. Sugar media contain 1% sugar in peptone water alongwith an indicator (Andrade's indicator—0.005% acid fuchsin in 1N NaOH). A small tube (Durham's tube) is kept inverted in the larger sugar tube to detect gas production. The colourless medium turns pink with production of acid by bacteria and gas production is indicated by gas bubbles accumulated in Durham's tube. Certain bacteria are exacting in their growth requirements and need serum for their growth e.g. Hiss's serum sugars for pneumococcus.

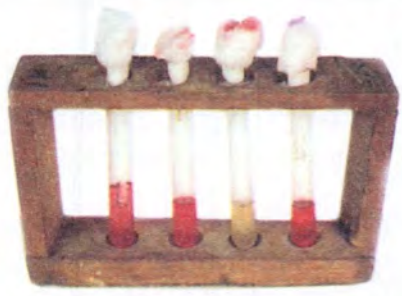


Fig. 5.8 Sugar media

ANAEROBIC MEDIA

These are used for cultivation of anaerobic bacteria e.g.

(*a*) Cooked meat broth (CMB)

(*b*) Thioglycollate broth.

For more details about these media, refer to Chapter 6.

MEDIA FOR TESTING SPECIAL PROPERTIES

Urea medium is used to test the property of urease production. Phenylpyruvic acid (PPA) is employed for identification of Proteus sp. These miscellaneous tests are discussed in Chapter 7.

DEHYDRATED MEDIA

Dehydrated media are commercially available. These are simply reconstituted in distilled water and sterilised before use. With dehydrated media, the process of media making has become simpler and its quality more uniform.

Table 5.1 and Table 5.2 show some of the liquid media and solid media respectively.

Table 5.1 Composition and Uses of Liquid Media

	Medium	Composition	Uses
1.	Peptone water	Peptone – 1% NaCl – 0.5% Water – 100 ml (pH 7.4)	Routine culture, Sugar fermentation tests
2.	Nutrient broth	Peptone water + Meat extract—1%	Routine culture
3.	Glucose broth	Nutrient broth + Glucose – 0.5%	Blood culture, Culture of certain organisms e.g. Streptococci
4.	Enrichment media		
	(*i*) Alkaline peptone water	Peptone water (pH 9.0)	Culture of Vibrio
	(*ii*) Selenite F broth	Peptone water, Sodium selenite	Culture of faeces for salmonellae and shigellae
	(*iii*) Tetrathionate broth	Nutrient broth, Sodium thiosulphate, Calcium carbonate, Iodine solution	Culture of faeces especially for salmonellae
	(*iv*) Cooked meat broth (CMB)/ Robertson's cooked meat broth (RCM)	Nutrient broth, Predigested cooked meat of ox heart	Culture of anaerobic bacteria

Table 5.2 Composition and Uses of Solid Media

	Medium	Composition	Uses
1.	Simple medium		
	(*i*) Nutrient agar	Nutrient broth, Agar (2-3%)	Routine culture
2.	Enriched media		
	(*i*) Blood agar	Nutrient agar, Sheep blood (5-10%)	Routine culture
	(*ii*) Chocolate agar	Heated blood agar (55°C × 2hr)	Culture of Neisseria, *H. influenzae*
	(*iii*) Loeffler's serum slope (LSS)	Nutrient broth, Glucose, Horse serum	Culture of *C. diphtheriae*
3.	Indicator medium		
	(*i*) MacConkey's medium	Peptone Lactose Sodium taurocholate Agar Neutral red	Culture of Gram negative bacilli
4.	Selective media		
	(*i*) Deoxycholate citrate agar (DCA)	Nutrient agar, Sodium deoxycholate, Sodium citrate, Lactose, Neutral red	Culture of *Salmonellae* and *Shigellae*
	(*ii*) Bile salt agar (BSA)	Nutrient agar, Sodium taurocholate (0.5%) pH 8.2	Culture of *Vibrio cholerae*

KEY POINTS

1. *Culture media* are required to grow the organisms from infected material to identify the causative agent.
2. The basic constituents of culture media are water, electrolyte, peptone, meat extract, blood or serum and agar.
3. When basal medium is added with some nutrients such as blood, serum or egg, it is called *enriched medium*.
4. Some substances are incorporated in the liquid medium which have a stimulating effect on the bacteria to be grown or inhibits the competitors. Such media are called *enrichment media*, e.g., selenite broth.
5. *Selective media* contain substances that inhibit all but a few types of bacteria and facilitate the isolation of a particular species. Example is *bile salt agar* (BSA).
6. *Differential medium* contains substances which help to distinguish differing characteristics of bacteria. Example is MacConkey's medium.
7. *Indicator media* contain an indicator which changes colour when bacterium grows in them. MacConkey's medium is differential as well as an indicator medium.
8. *Anaerobic media* are used for cultivation of anaerobic bacteria, e.g., *cooked meat broth* (*CMB*).

YOU MUST KNOW

1. List of types of media.
2. Basic differences between enriched media, enrichment media, selective media, differential media, indicator media and examples of these media.
3. Composition and uses of different liquid media and solid media.

STUDY QUESTIONS

1. Write short notes on:
   (a) Enriched media
   (b) Enrichment media
   (c) Selective media
   (d) Differential media
   (e) Indicator media
   (f) Transport media.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. All of the following are examples of enriched media except:
   (a) Blood agar
   (b) Chocolate agar
   (c) Loeffler's serum slope
   (d) Bile salt agar
2. When a solid medium contains a substance that inhibits all unwanted bacteria but facilitates the isolation of particular bacteria, it is known as:
   (a) Selective medium
   (b) Enriched medium
   (c) Indicator medium
   (d) None of the above
3. When a liquid medium contains a substance that inhibits all unwanted bacteria but stimulates the growth of particular bacteria, it is known as:
   (a) Selective medium
   (b) Enriched medium
   (c) Enrichment medium
   (d) None of the above
4. Which enrichment medium is preferred to grow *Vibrio cholerae*?
   (a) Tetrathionate broth
   (b) Selenite F broth
   (c) Alkaline peptone water
   (d) All of the above

Answers (MCQs):

1. (d) 2. (a) 3. (c) 4. (c)

Chapter 6

CULTURE METHODS

In the clinical laboratory, bacterial cultures are indicated to

1. isolate bacteria in pure culture from the clinical specimens and their identification by various tests,
2. determine antibiotic susceptibility,
3. prepare antigens for serodiagnosis of infective diseases,
4. maintain stock cultures.

I. METHODS OF CULTURE

A. Streak culture
B. Lawn culture
C. Stroke culture
D. Stab culture
E. Pour plate culture
F. Liquid culture

A. Streak Culture (Surface Plating)

It is the routine method employed for bacterial isolation in pure culture. A platinum or nichrome wire loop of 2-4 mm internal diameter is used (Fig. 6.1). Due to high cost of platinum, loops for routine laboratory work are made of nichrome wire (24 S.W.G. size). This loop is first sterilised in the bunsen flame by making it red hot and cooled by touching an uninoculated part of the medium. Then a loopful of specimen is smeared onto the surface

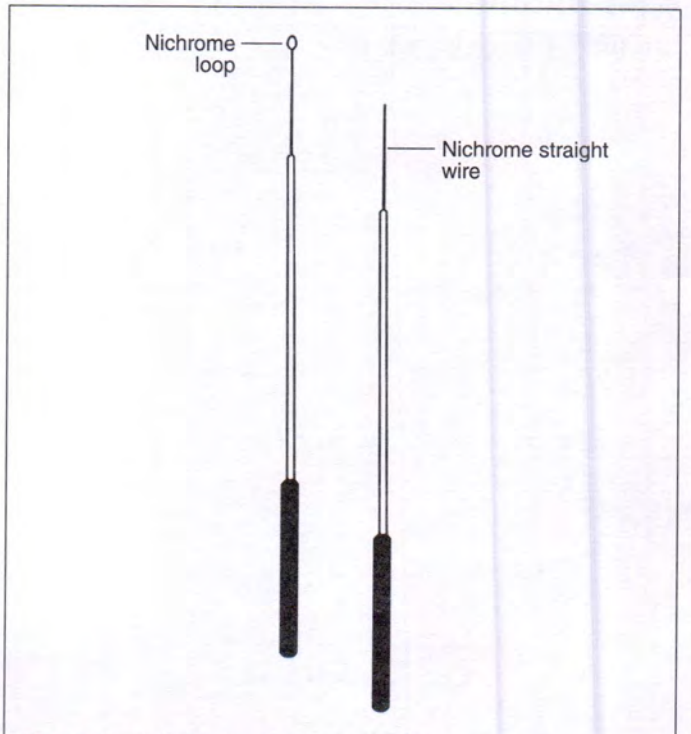


Fig. 6.1 Inoculating loop and straight wire

of a dried plate near the peripheral area. This is named as primary inoculum. From the primary inoculum, it is spread thinly over the plate by streaking with the

loop in parallel lines (Fig. 6.2). The loop is flamed and cooled in between the different set of streaks. It is done to obtain isolated colonies over the final series of streaks. The culture plate is incubated at 37°C for overnight. Confluent growth occurs at the primary inoculum and well separated colonies are obtained on the final streaks. Single isolated colony is the best to study the various properties of bacteria.

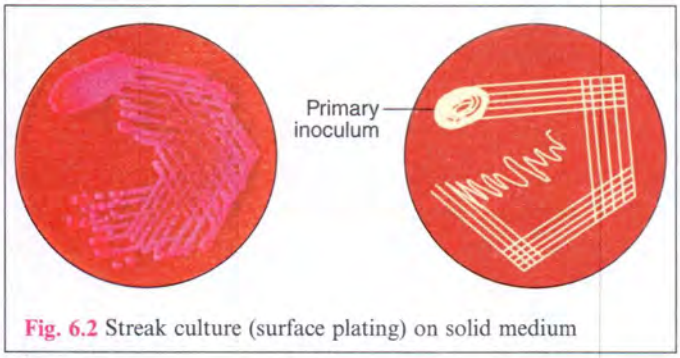


Fig. 6.2 Streak culture (surface plating) on solid medium

B. Lawn Culture

This type of culture method is employed in antibiotic sensitivity testing (disc diffusion method) and in bacteriophage typing. It may also be employed for preparation of bacterial antigens and vaccines where a large amount of bacterial growth is required. Lawn cultures are obtained by flooding the surface of the plate with a liquid culture or suspension of the bacterium. Culture plate is kept for a minute and then excess material is poured off. Alternatively, the culture plate may be inoculated by a sterile swab soaked in liquid bacterial culture or suspension. Plate is then incubated at 37°C overnight to obtain bacterial colonies.

C. Stroke Culture

Stroke culture is done in tubes containing agar slope and is employed for providing a pure growth of the bacterium for slide agglutination and other diagnostic tests. Commonly used agar slope is Nutrient agar slope.

D. Stab Culture

Stab culture is performed by a straight wire, charged with culture material (bacteria), by puncturing deep inside the agar. This technique is employed to demonstrate gelatin liquefaction, oxygen requirement of the bacterium and to maintain stock cultures for preservation of bacteria.

E. Pour Plate Culture

Tubes containing 15 ml of agar medium in each are melted and kept to cool in a water bath at 45-50°C. The inoculum to be tested is diluted in serial dilutions. One ml of each diluted inoculum is added to each tube of molten agar, mixed well and the contents of the tube poured into a sterile petridish and allowed to solidify. These plates are incubated at 37°C for overnight. Colonies will be seen throughout the depth of the medium and can be counted using colony counter.

Uses

(a) To estimate viable bacterial count in a suspension.
(b) To quantitate bacteria in urine cultures.

F. Liquid Culture

Liquid cultures in test tubes, screw-capped bottles or flasks may be inoculated by touching with a charged loop or by adding the inoculum with pipettes or syringes. This type of culture method is adopted for blood culture and for sterility tests, where the number of bacteria in the inocula are expected to be small. Liquid cultures are preferable for specimens containing antibiotics and other antibacterial substances, as these become ineffective by dilution in the medium. Liquid cultures are also preferred when large yields of bacteria are required.

Disadvantages

(a) It does not provide a pure culture from mixed inocula.
(b) Identification of bacteria is not possible.

Incubation of Culture Media

Most of the pathogenic organisms grow best at 37°C i.e. body temperature of human beings. The inoculated culture media are incubated at 37°C in an incubator (Fig. 6.3).

II. ANAEROBIC CULTURE METHODS

Anaerobic bacteria grow only in the absence of oxygen (anaerobic conditions). These anaerobic conditions or anaerobiosis can be established by various methods.

Methods of Anaerobiosis

1. Production of a vacuum.
2. Displacement of oxygen.
3. By displacement and combustion of oxygen.
4. Absorption of oxygen by chemical or biological methods.
5. By reducing agents.
6. Anaerobic chamber.

1. *Production of a Vacuum*

Cultivation in vacuum was attempted by incubating cultures in a vacuum desiccator, but it proved to be unsatisfactory. This method is not in use now.

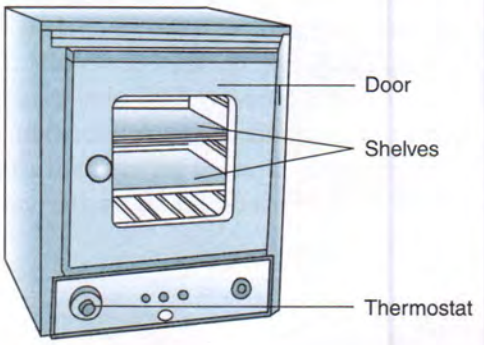


Fig. 6.3 Incubator

2. *Displacement of Oxygen*

Displacement of oxygen by inert gases like hydrogen, nitrogen, carbon dioxide or helium is sometimes employed. Oxygen can never be removed completely by this method. A popular, but ineffective, method is the use of candle. A lighted candle is kept in a large air-tight container loaded with inoculated plates. It is expected that burning candle will use up all the oxygen inside before it is extinguished but some amount of oxygen is always left behind.

3. *By Displacement and Combustion of Oxygen*

Anaerobiosis obtained by "McIntosh and Filde's" anaerobic jar (Fig. 6.4) is the most reliable and widely used method.

Procedure

McIntosh and Filde's anaerobic jar consists of a stout glass or metal jar with a metal lid which can be clamped air-tight with a screw. The lid is fitted with two tubes with taps, one acting as inlet for introduction of gas and the other as the outlet. The lid also contains two terminals which can be connected to an electrical supply. A catalyst (alumina pellets coated with palladium) is suspended under the lid by stout wires which are connected with the terminals to heat the catalyst for its activity. Nowadays catalyst (without heating) at room temperature is used.

Culture plates inoculated with specimens are placed inside the anaerobic jar with an indicator. The lid is clamped tight. The outlet tube is connected to a vacuum pump while inlet tube is closed. The air inside is evacuated. The outlet tube is then closed and hydrogen gas is passed through inlet tube till reduced atmospheric pressure is brought to normal atmospheric pressure (i.e. 760 mm Hg) which is monitored on the vacuum gauze as zero. Electric terminals are switched on to heat the catalyst and if 'room temperature catalyst' is used, heating is not required. The catalyst helps to combine hydrogen and residual oxygen to form water.

Reduced methylene blue is generally used as indicator of anaerobiosis in the jar. It remains colourless in anaerobic conditions, but turns blue on exposure to oxygen.

4. *Absorption of Oxygen by Chemical or Biological Methods*

CHEMICAL METHODS

(i) Pyrogallol

Alkaline pyrogallol absorbs oxygen. This method was first

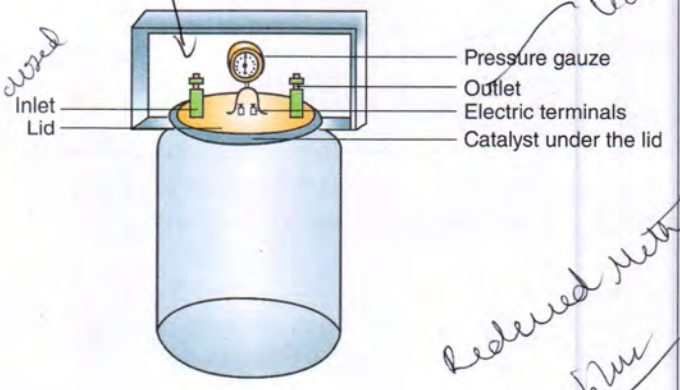


Fig. 6.4 McIntosh and Filde's anaerobic jar

introduced by *Buchner* (1888). A large tube containing solution of sodium hydroxide and pyrogallic acid placed inside air-tight jar produces anaerobic conditions.

(ii) Chromium and sulphuric acid

A mixture of chromium and sulphuric acid is used for producing anaerobiosis. The two chemicals react in the presence of oxygen and produce chromous sulphate.

(iii) Gas-pak

It is available commercially as a disposable packet containing pellets of sodium borohydride, cobalt chloride, citric acid and sodium bicarbonate. It is now widely used for preparing anaerobic jars. These chemicals generate hydrogen and carbon dioxide when water is added. Hydrogen combines with oxygen in the presence of a catalyst.

After the inoculated plates are placed inside an air-tight jar, the packet of "Gas-pak" with water added, is kept inside and the lid is tightly closed. The "Gas-pak" technique is simple as compared to filling of gases by cylinders.

BIOLOGICAL METHODS

This has been attempted by incubating aerobic organisms along with anaerobic bacteria. Two blood agar plates are taken—one is inoculated with *Pseudomonas aeruginosa* (aerobic bacteria) and the other with specimen of anaerobic bacteria. Two plates are placed one over the other and sealed along the rims and are incubated. This method is slow and ineffective.

5. By Reducing Agents

Oxygen in culture media can be reduced by various agents such as glucose, thioglycollate, cooked meat pieces, cysteine and ascorbic acid. Based on this principle, the two most widely employed anaerobic liquid culture media are:

THIOGLYCOLLATE BROTH

It contains nutrient broth and 1% thioglycollate.

COOKED MEAT BROTH (CMB)

It is also known as Robertson's cooked meat (RCM) medium.

It contains nutrient broth and pieces of fat-free minced cooked meat of ox heart.

Principle

1. Unsaturated fatty acids present in meat utilise oxygen for autooxidation, this reaction is catalysed by haematin in the meat.
2. Glutathione and cysteine (both are reducing agents) present in meat also utilize oxygen.
3. Sulphydryl compounds (present in cysteine) also contribute for a reduced oxidation-reduction (OR) potential.

Procedure

Before inoculation, the medium is boiled in water bath at 80°C for 30 minutes to make it oxygen free. For strict anaerobiosis the surface of CMB medium may be covered with a layer of sterile liquid paraffin.

Interpretation

Saccharolytic anaerobes (*Cl. perfringens*) turn the colour of meat pieces into red while it becomes black in case of proteolytic anaerobes (*Cl. tetani*).

6. Anaerobic Chamber

It is an anaerobic incubation system. It provides oxygen-free environment for inoculating culture media and for their incubation. It is fitted with airtight rubber gloves to insert hands for working with specimens. These anaerobic chambers contain a catalyst, dessicant, hydrogen gas, carbon dioxide gas, nitrogen gas and an indicator.

III. METHODS OF ISOLATING PURE CULTURES

1. *Surface plating*
   It is routinely employed in the laboratory.
2. *Use of enriched and selective media*
   It is employed for isolating pathogens from specimens with varied flora e.g. faeces.
3. *Pretreatment of specimens*
   Suitable bactericidal substances are used for pre-treatment of specimens to isolate a particular bacteria e.g. concentration and decontamination of sputum before culture for *Mycobacterium tuberculosis*.
4. *By heating liquid medium*
   Specimens are heated at 80°C to destroy vegetative forms of bacteria but spore bearing bacteria survive e.g. isolation of tetanus bacilli from dust and similar sources.

KEY POINTS

1. Various culture methods namely *streak culture*, *lawn culture*, *stroke culture*, *stab culture*, *pour plate culture* and *liquid culture* are used in microbiology laboratory.
2. *Streak* culture is the routine method employed for bacterial isolation in pure culture.
3. Anaerobic bacteria grow only in the absence of oxygen (anaerobic conditions) and hence require anaerobic culture methods for their growth.
4. *McIntosh and Filde's* anaerobic jar is the most reliable and widely used method for obtaining anaerobiasis. *Gas-pak* is now widely used for preparing anaerobic jars.
5. Commonly used anaerobic culture medium is *cooked meat broth* (CMB).

YOU MUST KNOW

1. Methods of anaerobic culture.
2. Principle and use of McIntosh and Filde's anaerobic jar.
3. Principle and uses of cooked meat broth (CMB).

STUDY QUESTIONS

1. Write briefly about anaerobic culture methods.
2. Write short notes on:
   (a) Streak culture
   (b) Lawn culture
   (c) Pour plate culture
   (d) Cooked meat broth (CMB) or RCM medium
   (e) Methods of isolating pure cultures.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Anaerobic jar is commonly used to grow:
   (a) *Clostridium tetani*
   (b) *Klebsiella sp.*
   (c) *Pseudomonas aeruginosa*
   (d) None of the above
2. Which of the following bacteria produce saccharolytic reaction in cooked meat broth (CMB)?
   (a) *Clostridium perfringens*
   (b) *Clostridium tetani*
   (c) *Pseudomonas aeruginosa*
   (d) None of the above

Answers (MCQs):

1. (a) 2. (a)

Chapter 7

IDENTIFICATION OF BACTERIA

Identification and Characterisation of a Bacterial Strain

I. Conventional Methods

- A. Morphology of Bacterial Colony
- B. Growth in Liquid Media
- C. Staining
- D. Hanging Drop Preparation
- E. Biochemical Tests
- F. Antigenic Structure
- G. Typing Methods
- H. Pathogenicity Tests
- I. Antibiotic Sensitivity Tests

II. Rapid Methods

After isolation of bacteria in pure culture from a specimen, it has to be identified. The following studies are necessary to characterise a bacterial strain.

A. Morphology of bacterial colony
B. Growth in liquid media
C. Staining
D. Hanging drop preparation
E. Biochemical tests
F. Antigenic structure
G. Typing methods
H. Pathogenicity tests
I. Antibiotic sensitivity tests

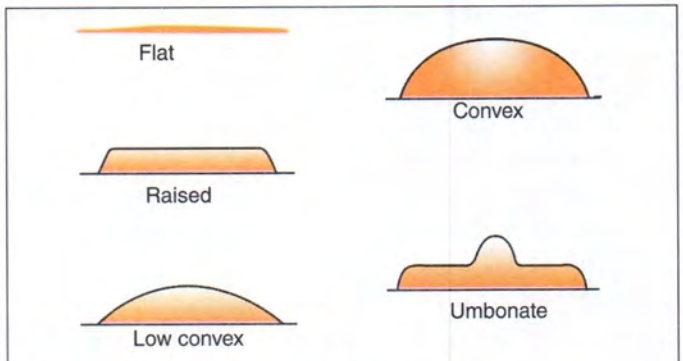


Fig. 7.1 Different elevation of colonies

I. CONVENTIONAL METHODS

A. Morphology of Bacterial Colony

The appearance of bacterial colony on culture medium is usually characteristic. The following features of the colony are studied:

(*i*) Size—in millimetres e.g. pin head size is characteristic of staphylococci whereas pin point size is a feature of streptococci colonies.
(*ii*) Shape — Circular, irregular
(*iii*) Surface — Smooth, rough, granular
(*iv*) Elevation — Colony may be flat, raised, low convex, convex or umbonate (Fig. 7.1).
(*v*) Edge—Entire, crenated, lobate, undulate, ciliate (Fig. 7.2)

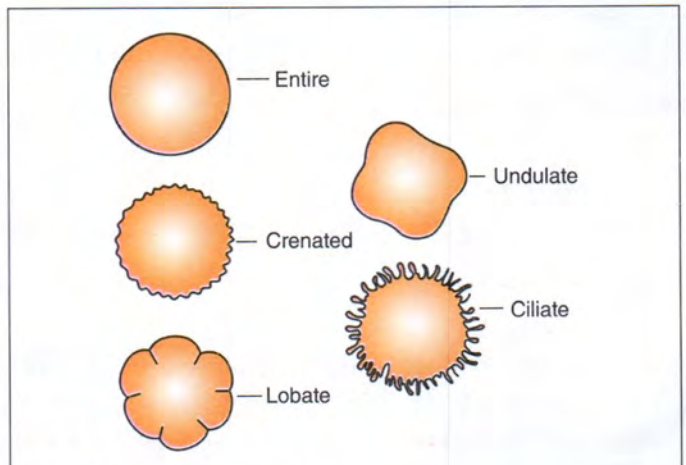


Fig. 7.2 Various types of edges of colony

(*vi*) Opacity—Translucent, transparent or opaque
(*vii*) Colour—Pigment may be produced by certain bacteria.
(*viii*) Haemolysis—Haemolysin produced by some bacteria leads to haemolysis around the colony.
(*ix*) Consistency—Mucoid, friable, firm, butyrous

B. Growth in Liquid Media

Commonly used liquid media are peptone water and nutrient broth. Bacterial growth appears in following forms:

(*i*) Uniform turbidity—Most of the Gram negative bacteria grow in this form.
(*ii*) Deposit at bottom—This occurs in growth of streptococci, chains of this bacteria being heavier may settle down as deposit.
(*iii*) Surface pellicle formation—All aerobes have tendency to grow on surface of media due to more content of oxygen present on the surface e.g. *Pseudomonas* sp.

C. Staining

Staining methods are employed to examine smears prepared from the bacterial colony or liquid culture. Commonly used stains are as follows:

(*i*) Gram's staining—It divides bacteria into Gram positive and Gram negative. It may be used for almost all the bacteria.
(*ii*) Albert's staining—It is employed to identify *Corynebacterium diphtheriae*, a causative agent of diphtheria.
(*iii*) Ziehl-Neelsen (ZN) staining—It differentiates acid-fast bacilli (AFB) from non-acid-fast bacilli. This stain is commonly employed for identification of *Mycobacterium tuberculosis* and *M. leprae* which are causative agents of tuberculosis and leprosy respectively. Both these bacteria are acid-fast in nature.

D. Hanging Drop Preparation

It helps to distinguish motile bacteria from non-motile ones.

E. Biochemical Tests

Bacterial species differ in their capacity to break down different sugars. Some biochemical tests are based on presence of specific enzymes such as catalase, coagulase, oxidase, urease, gelatinase etc. Some of the widely used biochemical tests are described below.

1. *Sugar Fermentation*

Principle

To determine the ability of an organism to ferment a specific carbohydrate (sugar) incorporated in a medium producing acid or acid with gas.

Procedure

Test organism is inoculated in a sugar medium and incubated at 37°C for 18-24 hours. Glucose, lactose, sucrose and mannitol are widely used sugars. Sugar media contain 1% sugar. Indicator used is Andrade's indicator (a solution of acid fuchsin to which is added sodium hydroxide).

Interpretation

Positive—Pinkish-red (acidic)
Negative—Yellow to colourless (alkaline)
Gas production can be seen as bubbles in Durham's tube (Figs. 7.3 and 7.4).

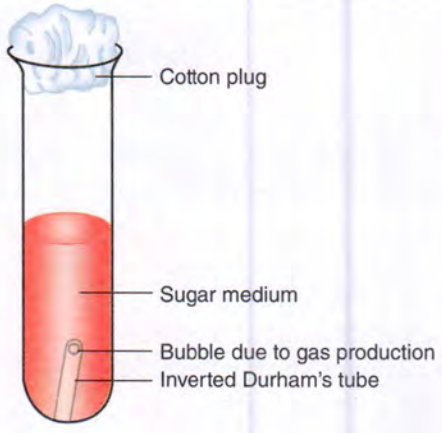


Fig. 7.3 Inverted Durham's tube showing gas production

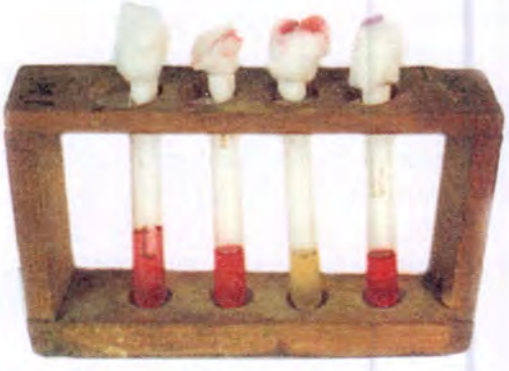


Fig. 7.4 Sugar fermentation tests

Examples of fermentative bacteria

Glucose fermenters—All members of the *Enterobacteriaceae*

Glucose and lactose fermenters—*Escherichia coli, Klebsiella sp.*

Glucose and mannitol fermenter—*Salmonella sp.*

2. Indole Production

Principle

To determine the ability of an organism to decompose aminoacid tryptophan into indole. Tryptophan is decomposed by an enzyme tryptophanase produced by certain bacteria.

Procedure

Indole production is detected by inoculating the test bacterium into peptone water (tryptophan rich) and incubating it at 37°C for 48-96 hours. 0.5 ml of Kovac's reagent is added to the bacterial growth and gently shaken.

Kovac's reagent contains

Paradimethyl amino-benzaldehyde	—	10 g
Amyl or isoamyl alcohol	—	150 ml
Conc. HCl.	—	50 ml

Interpretation

Indole positive (Fig. 7.5)—A red coloured ring near the surface of the medium

Indole negative (Fig. 7.5) — Yellow coloured ring near the surface of the medium

Positive and negative bacteria

Indole positive—*Esch. coli, Proteus sp.* other than *P. mirabilis, Edwardsiella*

Indole negative—*Klebsiella sp., Proteus mirabilis*

3. Urease Production

Principle

To determine the ability of an organism to produce an enzyme urease which splits urea to ammonia. Ammonia makes the medium alkaline and thus phenol red indicator changes to pink/red in colour.

Procedure

The test is done in Christensen's urease medium. The test organism is inoculated on the entire slope of medium and incubated at 37°C. It is examined after 4 hours and after overnight incubation.

Christensen's urease medium contains

Peptone water
Urea (20%)
Agar
Phenol red

Interpretation

Positive (Fig. 7.6)—Pink colour.

Negative (Fig. 7.6)—Pale yellow colour.

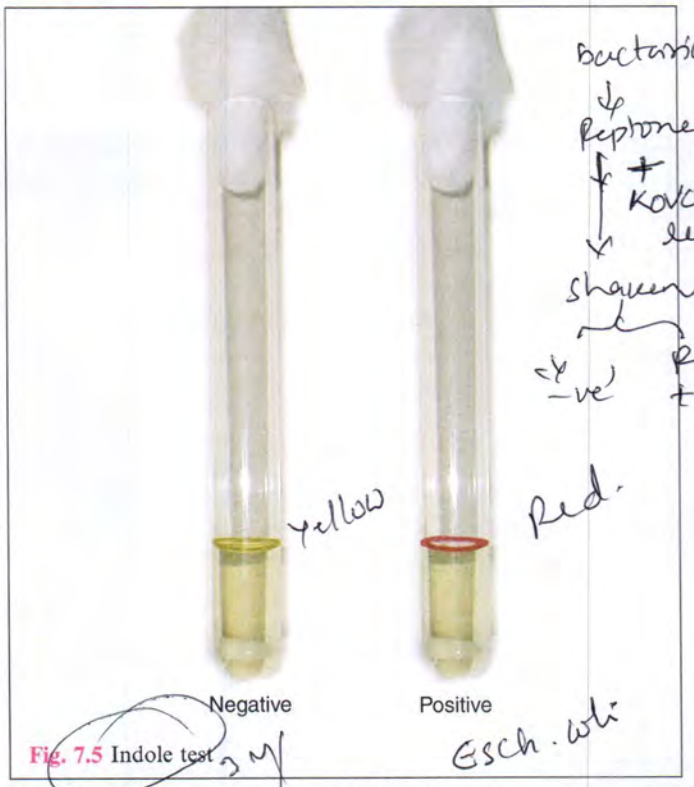


Fig. 7.5 Indole test

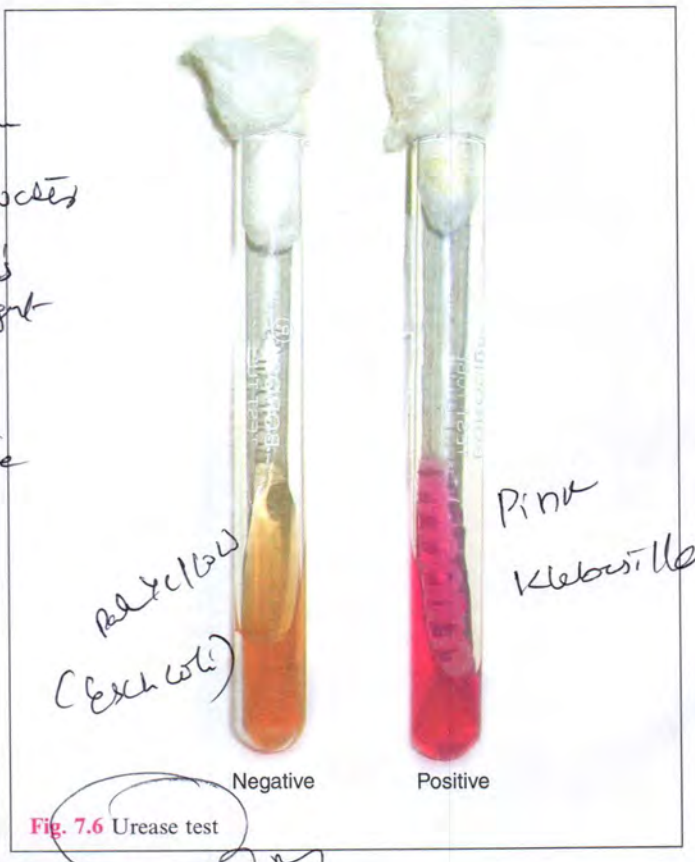


Fig. 7.6 Urease test

Positive and negative bacteria

Urease positive—*Klebsiella sp., Proteus sp., Yersinia enterocolitica, Helicobacter pylori*

Urease negative—*Esch. coli, Providencia sp., Yersinia pestis.*

4. *Citrate Utilisation Test*

Principle

It is the ability of an organism to utilize citrate as the sole source of carbon for its growth, with resulting alkalinity.

Procedure

Solid (Simmon's) or liquid (Koser's) media can be used. A bacterial colony is picked up by a straight wire and inoculated into either of these media. These inoculated media are incubated at 37°C for overnight. Simmon's citrate medium contains agar, citrate and bromothymol blue as an indicator. Original colour of the medium is green.

Interpretation

Simmon's citrate medium

Positive (Fig.7.7)—Growth with an intense blue colour on the slant. Blue colour is due to the alkaline pH which results from utilization of citrate. Bromothymol blue (indicator) is blue in alkaline conditions.

Negative (Fig. 7.7)—No growth with no change in colour (green).

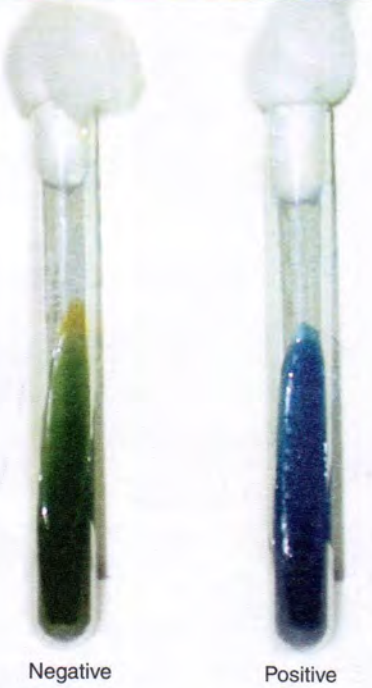


Fig. 7.7 Citrate utilisation test

Koser's citrate medium

Positive—Turbidity due to growth of bacteria

Negative—No turbidity

Positive and negative bacteria

Citrate positive—*Klebsiella sp., Salmonella sp.* except *S. typhi, Citrobacter sp., Enterobacter sp.*

Citrate negative—*Esch. coli, Edwardsiella, Salmonella typhi*

5. *Catalase Test*

Principle

Certain bacteria have an enzyme catalase which acts on hydrogen peroxide to release oxygen.

$$H_2O_2 \xrightarrow{\text{catalase}} H_2O + O$$

Nascent oxygen

Procedure

Pick up a few colonies of test bacteria with platinum loop from nutrient agar slope/plate and mix it in a drop of H_2O_2 (10 volumes) on a clean glass slide. A positive catalase reaction produces gas bubbles immediately. Since blood contains catalase, culture on blood containing media may result in false positive reaction. Use of iron wireloop for picking up bacterial colonies may be another cause of false positive test.

Interpretation

Positive test—Immediate bubbling, easily observed (O_2 formed)

Negative test—No bubbling (no O_2 formed)

Positive and negative bacteria

Catalase positive—All members of enterobacteriaceae except *Shigella dysenteriae type 1; Staphylococcus, Micrococcus, Bacillus*

Catalase negative—*Shigella dysenteriae type 1, Streptococcus, Clostridium*

Catalase test is primarily used to differentiate between genera *Staphylococcus* from *Streptococcus*

6. *Oxidase Test*

Principle

To determine the presence of an enzyme cytochrome oxidase which catalyses the oxidation of reduced cytochrome by molecular oxygen.

Procedure

Freshly prepared solution of 1% tetra-methyl paraphenylene diamine dihydrochloride (oxidase reagent) is used. There are different methods to perform this test.

(*i*) A filter paper strip, soaked in the oxidase reagent,

is smeared with test organism. In a positive oxidase reaction the smeared area turns deep purple within 10 seconds. Oxidase positive control (Pseudomonas sp.) should always be included to find out the working of oxidase strip.

(*ii*) Another method is to pour oxidase reagent on the surface of colonies, the colonies become purple within 10-30 minutes. This technique is useful to pick up Neisseria colonies from mixed growth on culture media. Purple coloured colonies can be subcultured to get pure cultures.

Interpretation

Positive—Deep purple within 10 seconds
Negative—No colour change

Positive and negative bacteria

Oxidase positive—*Pseudomonas sp., Vibrio sp., Neisseria sp., Aeromonas sp., Alcaligenes sp., Plesiomonas sp., Moraxella sp.*

Oxidase negative—All members of enterobacteriaceae, *Stenotrophomonas maltophila*

7. *Phenylalanine Deaminase Test*

To determine the ability of an organism to deaminate phenylalanine to phenyl pyruvic acid (PPA). This test is also commonly called as *PPA* test.

Procedure

A medium containing phenylalanine is inoculated with a growth of bacterial culture and incubated at 37°C for overnight. A few drops of 10% ferric chloride solution is added. If PPA is formed, green colour is produced due to reaction of ferric chloride with PPA.

Interpretation

Positive—Green colour
Negative—No colour change

Positive and negative bacteria

PPA positive (Fig. 7.8)—*Proteus sp., Morganella sp., Providencia sp.*

PPA negative—All members of enterobacteriaceae.

8. *Methyl Red (MR) Test*

Principle

This test detects the production of sufficient acid during fermentation of glucose by bacteria and sustained maintenance of a pH below 4.5.

Fig. 7.8 Phenylpyruvic acid (PPA) test

Procedure

The test organism is inoculated in glucose phosphate broth and incubated at 37°C for 2-5 days. Then add five drops of 0.04% solution of methyl red, mix well and read the results immediately.

Interpretation

Positive—Red colour
Negative—Yellow colour

Positive and negative bacteria

MR Positive—*Esch. coli, Yersinia sp., Listeria monocytogenes*

MR negative—*Klebsiella sp., Enterobacter sp.*

9. *Voges-Proskauer (VP) Test or Acetoin Production Test*

Principle

This test depends on the production of acetyl methyl carbinol (acetoin) from pyruvic acid in the media. In the presence of alkali and atmospheric oxygen, acetoin is oxidised to diacetyl which reacts with α-naphthol to give red colour.

Procedure

Test organism is inoculated in glucose phosphate broth and incubated at 37°C for 48 hours. Then add 1 ml of 40% KOH and 3 ml of a 5% solution of α-naphthol in absolute alcohol.

Interpretation

Positive—Pink colour within 2-5 minutes, deepening to crimson colour in 30 minutes.

Negative—Colourless for 30 minutes.

Positive and negative bacteria

VP positive—*Klebsiella sp., Enterobacter sp., ElTor vibrios, Staphylococcus*

VP negative—*Esch. coli, Micrococcus*

Indole, MR, VP and citrate tests are very useful in the identification of enteric Gram negative bacteria. These tests are commonly referred to by the sigla 'IMViC' tests.

10. *Nitrate Reduction Test*

Principle

This test depends upon presence of an enzyme nitrate reductase, which reduces nitrate to nitrite or free nitrogen gas.

Procedure

Test organism is grown for five days at 37°C in a broth containing 1% KNO_3. Then 0.1 ml of the reagent (sulphanilic acid and α-naphthylamine) is added.

Interpretation

Positive—Red colour develops within few minutes

Negative—No colour development

In case of no colour development, it indicates either nitrate has not been reduced or nitrate has been reduced beyond nitrite to nitrogen gas, which reagents will not be able to detect. To interpret this, zinc dust is added to the test. Zinc reduces nitrate to nitrite, so development of red colour after addition of zinc dust means that the organism was unable to reduce the nitrate to nitrite (negative test). However, no colour development after addition of zinc dust indicates positive test as nitrate has been reduced to nitrite and further nitrogen gas.

Positive and negative bacteria

NO_3 reduction positive—All members of enterobacteriaceae, *Branhamella catarrhalis*

NO_3 reduction negative—*Haemophilus ducreyi*

11. *Triple-Sugar Iron (TSI) Agar*

Principle

To determine the ability of an organism to attack specific carbohydrates incorporated in a growth medium, with or without the production of gas, along with the determination of possible hydrogen sulphide (H_2S) production.

Procedure

TSI is a composite medium which contains three carbohydrates namely glucose, lactose, sucrose and also ferric salts for testing H_2S production. The concentration of lactose and sucrose is 10 times that of glucose in the medium. Phenol red is incorporated as an indicator. This medium is widely used and is in the form of a butt and slant in the test tube. Medium is inoculated with bacterial culture by a straight wire pierced deep in the butt (stab culture). It is incubated at 37°C for overnight.

Bacterium attacks simple sugar (glucose) first and then the lactose and the sucrose. The test is read after 18 to 24 hours and not before 18 hours. If the test is read before 18 hours, the glucose fermentation produces sufficient acid to make both slant and butt yellow (acidic) thus giving a wrong interpretation of A/A (refer Interpretation below). After 18 to 24 hours the glucose concentration is depleted in the slant and the butt. The organism begins oxidative degradation of the peptones present in the slant, resulting in alkaline byproducts which change the indicator to a red colour. Fermentation (anaerobic) of glucose in the butt produces a larger amount of acid, overcoming the alkaline effect of peptone degradation, therefore the butt remains yellow (acidic).

Certain bacteria produce H_2S which is a colourless gas. H_2S combines with ferric ions (from ferric salts) to form ferous sulphide as black precipitate and thus blackening of the medium.

Interpretation

Yellow colour (acidic)—Fermentation of carbohydrate

Red colour (alkaline)—No fermentation

Bubbles in the butt—Gas is also produced during fermentation of carbohydrate.

Blackening of the medium (Fig. 7.9)—H_2S production

Various possible combinations of the different TSI reactions are listed below; remembering that the slant is first, followed by the butt reaction.

K/A (red/yellow)—Glucose only fermented.

A/A (yellow/yellow)—Glucose fermented, lactose and/or sucrose fermented.

K/K (red/red)—Neither glucose, lactose, nor sucrose fermented

K—Alkaline

A—Acidic

Fig. 7.9 Triple sugar iron (TSI) agar

12. *Hydrogen Sulphide Production*

Principle

To determine whether hydrogen sulphide (H_2S) has been liberated, by enzymatic action, from sulphur containing amino acids producing a visible, black colour reaction.

Procedure

Organisms are cultured in media containing lead acetate, ferric ammonium citrate or ferrous acetate, they turn them black or brown. Another method is to grow the organisms in culture tubes, inserting a filter paper strip impregnated with lead acetate between the cotton plug and the tube. Blackening of the paper indicates H_2S production.

Interpretation

Positive — Black colour
Negative — No change in colour

Positive and negative bacteria

H_2S positive—*Proteus mirabilis, Proteus vulgaris, Salmonella sp.* with some exceptions

H_2S negative—*Morganella sp., Salmonella paratyphi A, S. cholerae-suis*

13. *Growth in KCN*

Principle

To determine the ability of an organism to grow in a medium containing potassium cyanide.

Procedure

Inoculate peptone water containing 1 in 13,000 concentration of potassium cyanide, with test organism. Incubate at 37°C for 24-48 hours.

Interpretation

Positive — Turbidity due to growth
Negative — Clear (no growth)

Positive and negative bacteria

Positive KCN test—*Klebsiella sp., Citrobacter freundii, Pseudomonas aeruginosa*

Negative KCN test—*Salmonella sp., Esch. coli, Alkaligenes faecalis*

F. Antigenic Structure

Biochemically identified organisms are further confirmed by agglutination or precipitation reaction. Unknown bacterial antigen is identified by known specific antisera by slide agglutination test.

Salmonella typhi or other salmonella can be confirmed by agglutination with the specific antisera.

G. Typing Methods

This is useful for epidemiological studies. All the above mentioned tests can identify up to species level though intraspecies differentiation is not possible. Hence, typing methods like bacteriophage typing, bacteriocin typing and others are very useful for intraspecies differentiation. Typing methods help to find out the source of infection in epidemics or sometimes in outbreak of food poisoning.

H. Pathogenicity Tests

Guinea pigs, rabbits and mice are mostly used for pathogenicity tests. These animals may be injected by subcutaneous, intramuscular, intraperitoneal, intravenous or intracerebral routes depending upon the organism to be tested. The identification of the organism is carried out on the basis of postmortem findings and cultural characteristics.

I. Antibiotic Sensitivity Tests

The isolated bacterium is subjected to antibiotic sensitivity tests in-vitro for selecting appropriate antibiotic for therapeutic use. Different techniques for antibiotic sensitivity testing are described in Chapter 78.

II. RAPID METHODS

Conventional methods take days to identify and characterise the isolates while rapid automated methods take only hours. Detection of specific enzymes, toxins, antigens or metabolic end products of the isolates make

the identification simpler and rapid. For example, gas liquid chromatography (GLC) is based on identification of specific fatty acids (metabolic end products) produced by the organisms. This technique is very useful in identifying obligate anaerobes. Polymerase chain reaction (PCR) and nucleic acid probes carrying specific DNA or RNA base sequences are some molecular methods being widely used for identifying microbes.

KEY POINTS

1. After isolation of bacteria in pure culture from a specimen, it can be identified by *morphology* of bacterial colony, *staining*, *hanging drop* preparation and various *biochemical tests*.
2. Biochemically identified organisms are further confirmed by *slide agglutination* with the specific antisera.
3. Typing methods are useful for epidemidogical studies.
4. Antibiotic sensitivity tests are done to select appropriate antibiotic for therapeutic use.
5. Polymerase chain reaction (PCR) and nucleic acid probes are rapid methods for identifying microbes.

YOU MUST KNOW

1. Principle and interpretation of biochemical tests such as sugar fermentation, indole production, urease production, citrate utilisation test, catalase test, oxidase test phenylalanine deaminase test, triple-sugar iron (TSI) agar.
2. Examples of bacteria which give positive tests for above mentioned biochemical tests.

STUDY QUESTIONS

1. Write short notes on:
   (a) Indole production (b) Citrate utilisation test (c) Urease test (d) Catalase test (e) TSI
   (f) PPA test (g) Nitrate reduction test (h) VP test (i) Oxidase test (j) MR test.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. All of the following bacteria are urease test positive except:
   (a) *Klebsiella sp.* (b) *Yersinia enterocolitica* (c) *Yersinia pestis* (d) *Helicobacter pylori*
2. Catalase test is negative in:
   (a) *Staphylococcus aureus* (b) *Salmonella typhi* (c) *Streptococcus pyogenes* (d) *Klebsiella sp.*
3. All of the following bacteria are oxidase test positive except:
   (a) *Neisseria meningitidis* (b) *Vibrio cholerae* (c) *Escherichia coli* (d) *Pleisiomonas sp.*
4. Phenylalanine deaminase test is negative in:
   (a) *Proteus vulgaris* (b) *Providencia sp.* (c) *Morganella sp.* (d) *Klebsiella sp.*
5. Which of the following tests detects the production of acetyl methyl carbinol from pyruvic acid in the media?
   (a) Methyl red test (b) Voges-Proskauer test (c) Urease test (d) Citrate utilisation test
6. Triple sugar iron (TSI) agar medium contains all the following carbohydrates except:
   (a) Glucose (b) Lactose (c) Sucrose (d) Mannitol

Answers (MCQs):

1. (c) 2. (c) 3. (c) 4. (d) 5. (b) 6. (d)

Chapter 8

BACTERIAL TAXONOMY

I. Phylogenetic Classification
II. Adansonian Classification
III. Genetic Classification
IV. Intraspecies Classification

Taxonomy is the classification of organisms into ordered groups. Organisms are divided into three main Kingdoms:

1. Animals
2. Plants
3. Protista—contains unicellular organisms including eukaryotes and prokaryotes (bacteria).

There is no universally accepted bacterial classification. There are mainly three approaches, Phylogenetic, Adansonian and Genetic. However, bacterial species can be further sub-classified by intraspecies classification.

I. PHYLOGENETIC CLASSIFICATION

Phylogenetic classification groups together types that are related on evolutionary basis where several ranks are used: *Divisions, Classes, Orders, Families, Tribes, Genera and Species*. Major groups are differentiated by some characters of special importance such as Gram reaction, spore formation etc. The genera and species are distinguished by less important properties, such as fermentation reactions, nutritional requirement etc. The full taxonomical position of *Salmonella typhi* is as follows:

Division : *Protophyta*
Class : *Schizomycetes*
Order : *Eubacteriales*
Family : *Enterobacteriaceae*
Tribe : *Salmonellae*
Genus : *Salmonella*
Species : *Salmonella typhi*

A phylogenetic classification of bacteria has been published in USA as Bergey's Manual of Systematic Bacteriology. The manual is very useful in compilation of names and descriptions and also as an aid to identification of newly isolated bacterial types.

For identification and classification of bacteria, a minimum number of important characters are selected so that various members can be distinguished. These important characters include:

1. Morphology
2. Staining
3. Cultural characteristics
4. Biochemical reactions
5. Antigenic structure
6. Guanine: Cytosine ratio of DNA.

Nomenclature

It is the labelling of the groups and of individual members. Scientific name of bacteria usually consists of two words—first is the name of the genus and second is that of the species (e.g. *Proteus vulgaris*). The generic name is usually a Latin noun and second name indicates some property of the species (e.g. *albus*, meaning white), the disease it causes (e.g., *diphtheriae*, from diphtheria), the person who discovered it (e.g., *welchii*, after Welch), or the animal in which it is found (e.g., *suis*, meaning pig). The generic name always begins with a capital letter

and the second name with a small letter, even if it refers to a person or place (e.g. *Salmonella london*).

II. ADANSONIAN CLASSIFICATION

The Adansonian classification was originally proposed by Michael Adanson in the 18th century. It makes no phylogenetic assumption, but takes into account all the characters with equal importance. It groups organisms on the basis of similarities in large number of characters. The computer has extended the scope of this classification by permitting comparison of very large number of properties of several organisms at the same time. The computer analyses the observations and prints out a list of strains on the basis of degree of similarity in their properties. Thus several broad subgroups of bacterial strains are identified, which are further divided into species. This is also known as Numerical classification.

III. GENETIC CLASSIFICATION

It is based on the degree of genetic relatedness of different organisms. This classification is the most natural or fundamental since all properties of bacteria are regulated by their genes. DNA is extracted from pure bacterial culture and their G + C (guanine + cytosine) content is estimated. The nucleotide base composition and base ratio (adenine—thymine: guanine—cytosine) varies widely among different groups of microorganisms, but for any one species, it is constant.

IV. INTRASPECIES CLASSIFICATION

For diagnostic or epidemiological purposes, it is often necessary to subclassify bacterial species on the basis of biochemical properties (biotypes), antigenic structure (serotypes), bacteriophage susceptibility (phage types) or production of bacteriocins (colicin types). A species may be divided first into groups and then into types.

The application of newer molecular techniques has led to greater discrimination in intraspecies typing. These molecular techniques are of two types, phenotypic and genotypic. Phenotypic methods include electrophoretic typing of bacterial protein and immunoblotting. Genotypic methods include Southern blotting, PCR, and nucleotide sequence analysis. Some of these methods are described in Chapter 9.

KEY POINTS

1. *Taxonomy* is the classification of organisms into ordered groups.
2. There are mainly three approaches of bacterial classification namely *Phylogenetic*, *Adansonian* and *Genetic*. Bacterial species can be further subclassified by *intraspecies classification*.

YOU MUST KNOW

1. Approaches of bacterial classification.

STUDY QUESTIONS

1. Write briefly about bacterial taxonomy.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Which of the following methods may be used in bacteriology for epidemiological purposes?
   (a) Biotyping
   (b) Phage typing
   (c) Serotyping
   (d) All of the above
2. Molecular techniques employed for intraspecies typing of bacteria include:
   (a) Southern blotting
   (b) Polymerase chain reaction
   (c) Nucleotide sequence analysis
   (d) All of the above

Answers (MCQs):

1. (d) 2. (d)

Chapter 9

BACTERIAL GENETICS

Genetics is the study of heredity and variation. All hereditary characteristics are encoded in DNA. The chromosomal DNA plays a major role in the maintenance of characters constant from generation to generation. This is accomplished by most accurate replication of its DNA, yet, heritable variations in small proportion of daughter cells occur.

I. BASIC PRINCIPLES

The DNA molecule is composed of two strands of complementary nucleotides wound together in the form of a double helix (Fig. 9.1) as deduced by Watson & Crick. The bacterial nucleus contains a circular chromosome of a double stranded DNA molecule of approximately 1000 μm (1 mm) long when straightened.

Each strand has a backbone of de-oxyribose (sugar) and phosphate groups. There are four nitrogenous bases, two purines : adenine (A) and guanine (G) and two pyrimidines : thymine (T) and cytosine (C). One of these four nitrogenous bases is attached to each deoxyribose. The two strands are held together by hydrogen bonds between the nitrogenous bases on the opposite strands. This bonding is in such a specific manner that hydrogen bonds can only be formed between adenine and thymine (A-T) and between guanine and cytosine (G-C) (Fig. 9.2). Adenine and thymine thus form one complementary

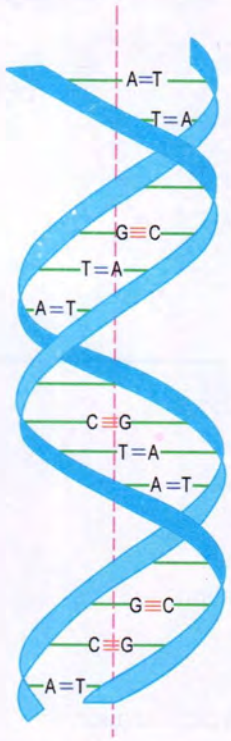


Fig. 9.1 DNA double helix (Watson-Crick model)

base pair, and guanine and cytosine form another. Thus, when the arrangement of bases along one strand is T-C-G-A-T-C, the arrangement on the other strand will be A-G-C-T-A-G. Double strand of this DNA will be as follows:

T — C — G — A — T — C
| | | | | |
A — G — C — T — A — G

Hence, a molecule of DNA contains as many numbers of adenine as thymine, and of guanine as cytosine. The ratio of A + T to G + C is constant for each species but varies widely from one bacterial species to another. During replication of the DNA molecule, the two strands separate at one end, and each strand then acts as a template for the synthesis of a complementary strand, with which it then forms a double helix.

RNA is structurally similar to DNA, except for two differences, sugar ribose is present instead of deoxyribose and the nitrogenous base uracil replaces thymine. There are three different types of RNA in a cell, messenger RNA (mRNA), ribosomal RNA (rRNA) and transfer RNA (tRNA).

The Important Definitions in Genetics

Gene—A segment of DNA that specifies for a particular polypeptide is called a gene.

Codon—Genetic information is stored in the DNA as a code. Codon consists of a sequence of three nucleotide bases i.e. the code is triplet. Each codon specifies for production of a single aminoacid but more than one codon may exist for the same aminoacid e.g. AGA codes for arginine but CGU, CGC, CGG, CGA and AGG also code for the same aminoacid, arginine. This is also applicable to other aminoacids as well.

Non-sense codons—Three codons (UAA, UGA and UAG) do not code for any aminoacid and act as 'stop codons' for terminating the message for the synthesis of a polypeptide. These are called nonsense codons.

II. SYNTHESIS OF POLYPEPTIDE

Genetic information in DNA, is transcribed on to RNA and then translated as the particular polypeptide (Fig. 9.3).

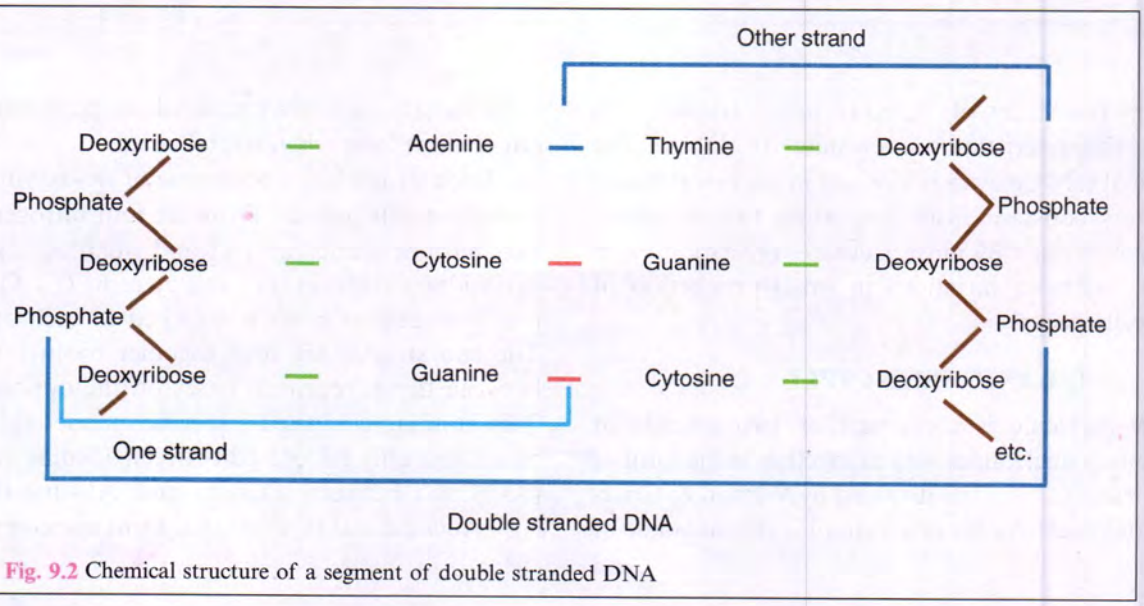


Fig. 9.2 Chemical structure of a segment of double stranded DNA

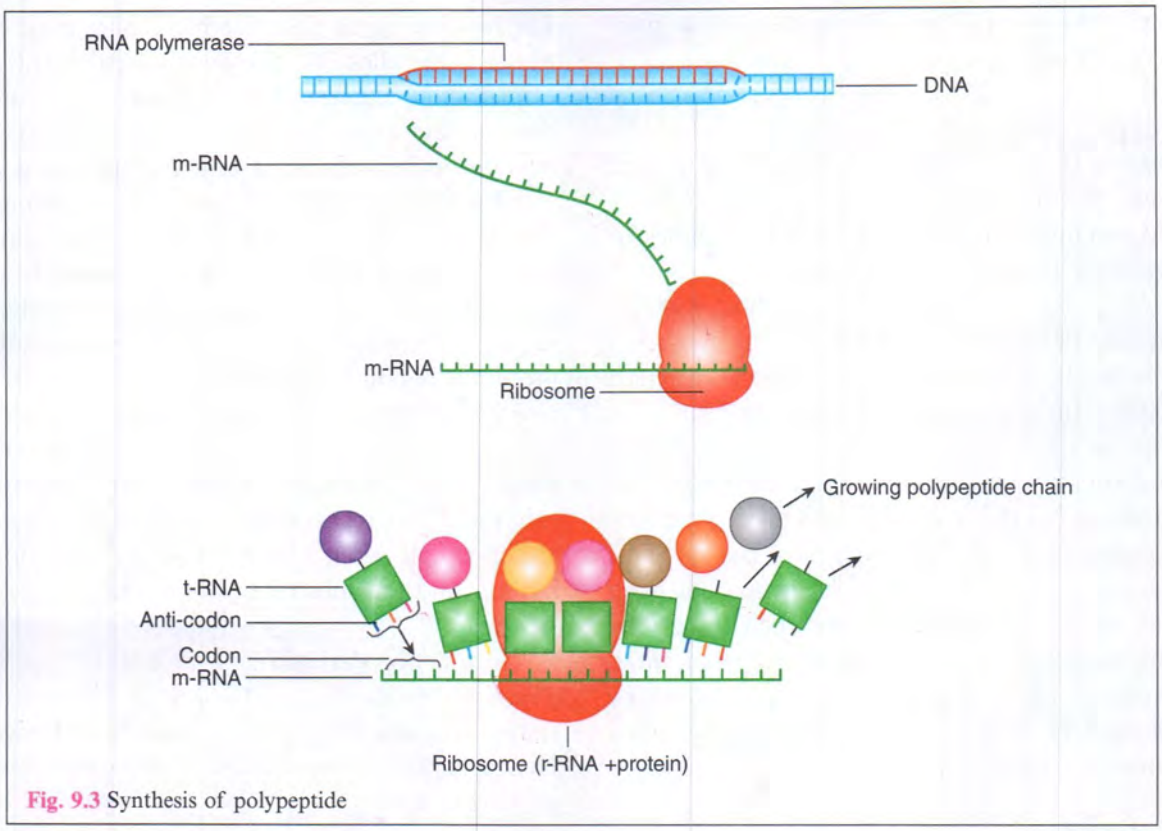


Fig. 9.3 Synthesis of polypeptide

A. Transcription

RNA polymerase attaches itself to the beginning of a gene on DNA and synthesises m-RNA, using one of the strands in DNA as a template. This process is known as *transcription*. DNA acts as a template for synthesis of m-RNA, therefore, the bases in m-RNA will be complementary to one strand of DNA.

B. Translation

The m-RNA passes into cytoplasm and then m-RNA and t-RNA come together on the surface of ribosome containing r-RNA. The triplet base sequence on m-RNA is known as *codon*. The base sequences on m-RNA are recognised by the anti-codon (corresponding complementary) sequences of t-RNA. The t-RNA molecule contains a triplet at one end and aminoacid at the other end. The ribosome moves along the m-RNA until the entire m-RNA molecule has been translated into corresponding sequences of aminoacids. This process is called *translation*.

III. EXTRACHROMOSOMAL GENETIC ELEMENTS

Besides the chromosomal DNA, some bacteria may also possess extrachromosomal DNA which may be *plasmids* (when these are situated in the cytoplasm as free state) or *episomes* (when integrated with chromosomal DNA of bacteria). Plasmids are circular DNA molecules and can replicate autonomously (independent replicons). It is often not possible to differentiate between plasmids and episomes, therefore, the two terms are frequently used synonymously. Plasmids and *episomes* are not essential for function of the host bacterium but they may carry properties of drug resistance, toxigenicity, conjugation and others.

Some plasmids have an ability to transfer themselves to other bacteria of the same and also of different species. These are called *self transmissible* plasmids. This transfer occurs usually by method of conjugation. There are some other non-conjugative (non-transmissible) plasmids which cannot transfer themselves but can be transduced.

By their ability to transfer DNA from one cell to another, plasmids have become important vectors in recombinant DNA technology or genetic engineering.

IV. BACTERIAL VARIATION

There are two types of variation

A. Phenotypic variation
B. Genotypic variation

The *phenotype* ('Phaeno'; display) is the expression of various characters by bacterial cells in a given environment.

The *genotype* is the sum total of the genes. It is the genetic constitution of an organism. All the genes of the

bacterial cell may or may not be expressed in a given environmental situation.

A. Phenotypic Variation

A bacterial cell may exhibit different phenotypic appearances in different environmental conditions. Phenotypic variations are reversible. Some examples of environmental influence on bacteria are:

1. *Synthesis of flagella*: *Salmonella typhi* (typhoid bacillus) is generally flagellated, but when grown in phenol agar, the flagella are not formed. This effect is reversed when subcultured from phenol agar into broth.
2. *Synthesis of enzyme*: *E. coli* possesses the genetic information for the synthesis of the enzyme, beta-galactosidase, but the actual synthesis takes place only when grown in a medium containing lactose. This enzyme is necessary for lactose fermentation. Such enzymes which are synthesised only when induced by the substrate (lactose) are called *inducible enzymes*. The enzymes which are synthesised irrespective of the presence or absence of the substrate are called *constitutive enzymes*.

Gene Regulation ('Operon' concept by Jacob and Monod)

Lactose fermentation of *Esch.coli* requires three enzymes, beta-galactosidase, galactoside permease and transacetylase. These enzymes are coded by the structural genes *lac* Z, *lac* Y and *lac* A respectively. These genes are arranged in sequence, forming a functional unit, *lac operon*. The regulator gene is *lac* I, which codes for a *'repressor'*, which is an allosteric protein molecule. This repressor can combine either with the operator region or with the inducer (lactose in this case). The promoter and operator regions lie between *lac* I and the structural *lac* genes.

The RNA polymerase has to attach to promoter region and travel along the structural genes in sequence, for transcription of RNA for enzyme synthesis. In the resting stage, the repressor molecule is bound to the operator region, preventing the travel of RNA polymerase from the promoter region to the operon. The repressor molecule also has affinity for lactose, in the presence of which, it leaves the operator free so that transcription can take place. When the lactose is completely metabolised and exhausted, the repressor again attaches to the operator, switching off transcription. Lactose acts both as the inducer and the substrate for enzyme (Fig. 9.4).

B. Genotypic Variation

These variations occur in the genetic material. These are stable, heritable and not influenced by the environment. Genotypic variations may occur in bacteria by mutation or gene transfer.

Mutation

Mutation is a random, undirected heritable variation caused by change in nucleotide sequence of the DNA of the cell. It can involve any of the numerous genes present in bacterial chromosome. The frequency of mutation ranges from 10^{-2} to 10^{-10} per bacterium per division.

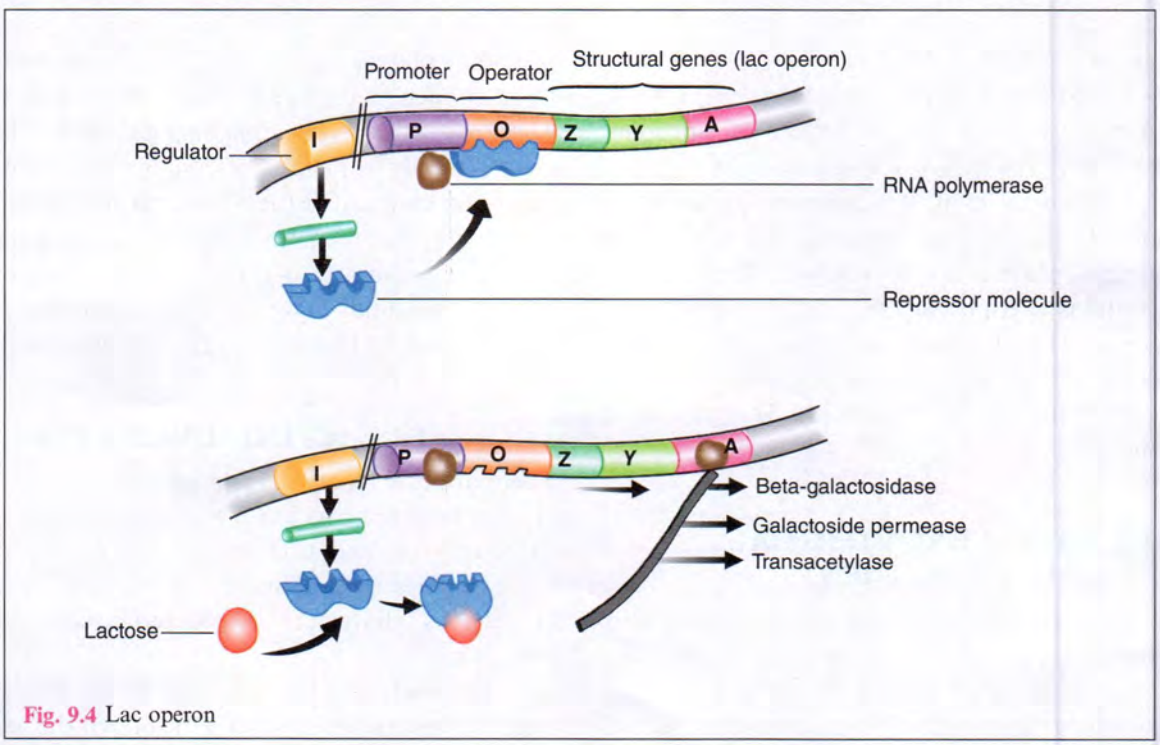


Fig. 9.4 Lac operon

Mutation occurs spontaneously but its frequency may be enhanced by mutagens such as UV rays, alkylating agents, 5-bromouracil and acridine dyes.

Mutation is a natural event, taking place all the time, in all dividing cells. Most mutants go unrecognised as the mutation may be lethal or may involve some minor function that may not be expressed. Mutation is best appreciated when it involves a function which can be readily observed by experimental methods. For example *Esch. coli* mutant that loses its ability to ferment lactose can be readily detected on MacConkey's agar.

Mutation can affect any gene and hence may modify any characteristic of the bacterium e.g. sensitivity to bacteriophages, loss of ability to produce capsule or flagella, loss of virulence, alteration in colony morphology, pigment production, drug susceptibility, biochemical reactions, antigenic structure. The practical importance of bacterial mutation is mainly in the field of drug resistance and the development of live vaccines.

Some mutations involve vital functions producing nonviable mutants (*lethal mutation*). An important type of lethal mutation is 'conditional mutation'. A *conditional lethal mutant* may be able to live under certain conditions (permissive conditions) but not under restrictive or non-permissive conditions. One example of such type of mutant is the temperature-sensitive (*ts*) mutant, which is able to live at the permissive temperature (say, 35°C), but not at the restrictive temperature (say, 39°C).

TYPES OF MUTATION

There are broadly two types of mutations:

1. Point mutation
2. Multisite mutations

Point mutation

It may occur by

(*i*) Base pair substitution
(*ii*) Frame shift mutations

Base pair substitution

A single base pair has been substituted for another pair. It can be subdivided into

(*a*) Transition
(*b*) Transversion

(*a*) Transition

This happens by replacement of one pyrimidine by another pyrimidine, and one purine by another purine ie. AT replaced by GC. Transition is the most frequently occurring mutation.

(*b*) Transversion

When a purine is replaced by pyrimidine and vice versa, it is named transversion, e.g. GC changes to CG.

Frame shift mutations

Sometimes, during DNA replication, one or a few base pairs are inserted into or deleted from the DNA. This shifts the normal 'reading frame' of the coded message forming new set of triplet codon. The coded message is read correctly up to the point of addition or deletion, but the subsequent codons will specify the incorrect aminoacids (Fig. 9.5).

Multisite mutations

Large number of base pairs are altered in DNA (Fig. 9.6). This may be of four types such as

(*i*) Addition (gain) (*ii*) Deletion (loss)
(*iii*) Duplication (*iv*) Inversion

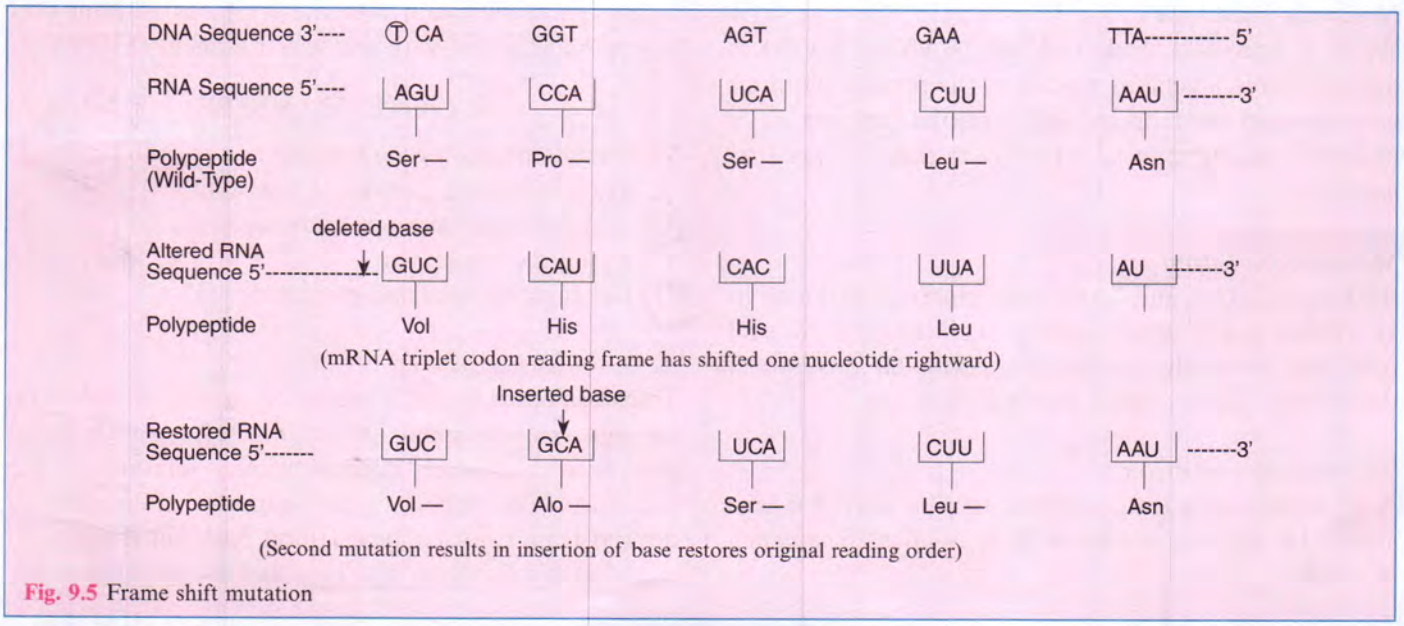


Fig. 9.5 Frame shift mutation

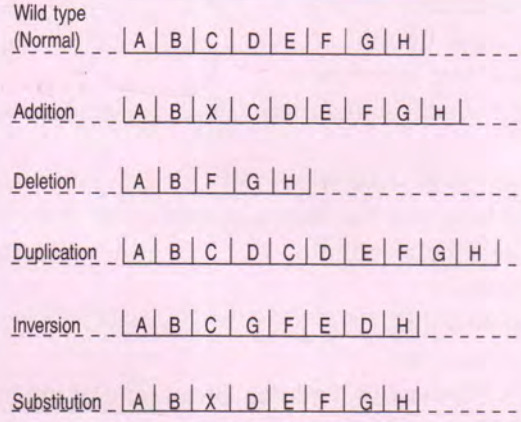


Fig. 9.6 Examples of types of mutations

OTHER TYPES OF MUTATIONS

Induced mutation

Although mutation occurs spontaneously, its frequency is greatly enhanced by certain agents called mutagens. These may be physical or chemical agents.

1. Physical agents

(*i*) UV light
(*ii*) Ionising radiation e.g. X-rays
(*iii*) Visible light
(*iv*) Heat

2. Chemical agents

(*i*) 5-Bromouracil
(*ii*) 2-Aminopurine
(*iii*) Nitrous acid
(*iv*) Acridine dyes

Nonsense mutation

When a non-sense codon (UAG, UAA or UGA) is formed within a gene by mutation, the protein synthesis is terminated prematurely and a partial polypeptide is produced during translation. This is called a *nonsense mutation*.

Missense mutation

Both substitution and frame shift mutation give rise to an altered codon which specifies a different aminoacid from that normally located at a particular position in the protein. This is called *missense mutation*.

Suppression mutation

A mutation (second) that restores the function of a gene altered by previous mutation (first) is called *suppression mutation*.

DEMONSTRATION OF MUTATION IN LABORATORY

Mutation can be recognised by both gene sequencing as well as by observing phenotypic changes.

Fluctuation test and replica plating methods are useful for demonstration of large number of mutant colonies.

Fluctuation test

Luria and Delbruck (1943) demonstrated that bacteria undergo spontaneous mutation independent of environment. They studied bacteriophage sensitive *Esch. coli*. Samples from small volume cultures and a single large volume culture were plated on solid media containing bacteriophage. The mutant bacteria (resistant) formed colonies. Colony count was compared. There was wide fluctuations in the number of bacteriophage resistant colonies in small volume cultures, as compared to a single large volume culture. On statistical analysis it was observed that mutation occurred randomly, some early and some late, which lead to wide fluctuation. However, in large volume cultures, fluctuations were within limits (Fig. 9.7). This experiment was not widely appreciated, probably due to the complicated statistical evaluation. It was the simple but elegant 'replica-plating' technique that proved the point beyond doubt.

Replica plating method (Lederberg and Lederberg, 1952)

Colonies of bacteria were transferred from master plate, on to a number of other plates, using a velvet template. Relative position of all the colonies was retained. By replica plating on culture plates with and without bacteriophages, they were able to demonstrate that bacteriophage resistant mutants appeared without ever having had contact with the selective agent (Fig. 9.8).

V. GENE TRANSFER

Various methods of gene transfer are as follows:

A. Transformation (uptake of naked DNA)
B. Transduction (through bacteriophage)
C. Lysogenic conversion
D. Conjugation (plasmid-mediated)

A. Transformation

Transformation is the transfer of genetic information through free or naked DNA. In 1928, Griffith found that mice died when injected with a mixture of live noncapsulated (rough: no capsule) pneumococci derived from capsular type II and heat killed (smooth: capsulated) strain from type I, neither of which separately

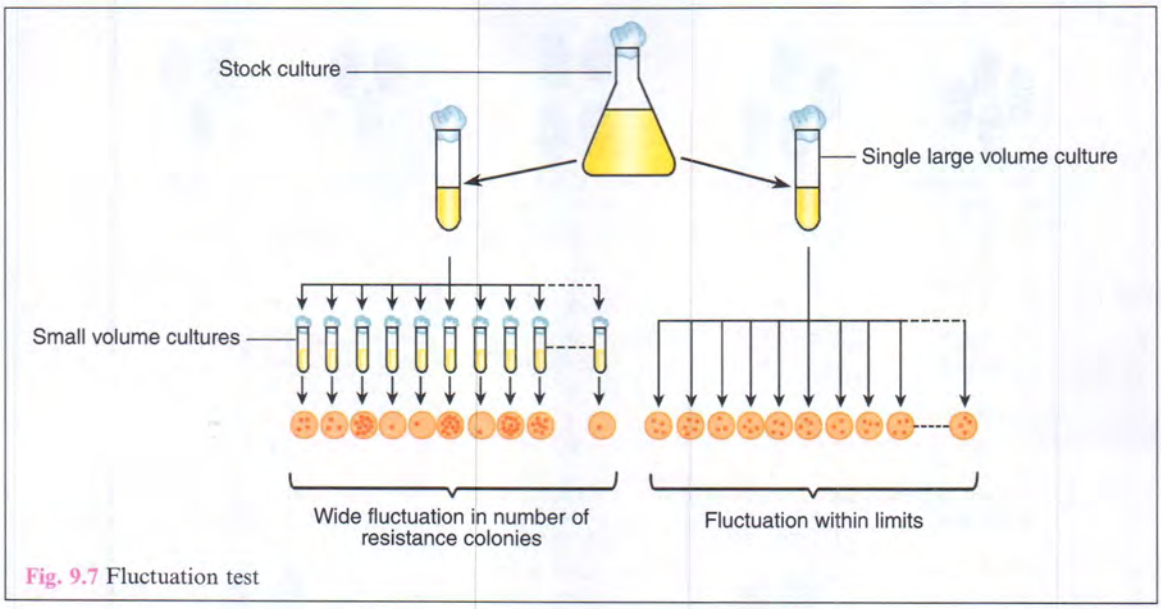


Fig. 9.7 Fluctuation test

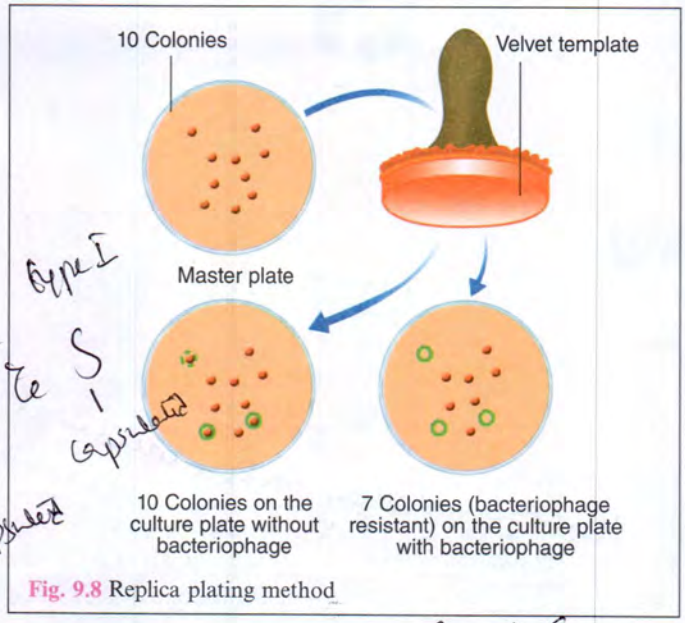


Fig. 9.8 Replica plating method

proved fatal. The live type I capsulated pneumococcus could be isolated from mice that died. It showed that rough forms (noncapsulated) were transformed into the smooth forms (Fig. 9.9). It demonstrated that some factor in heat-killed type I pneumococcus had transferred the information for capsule synthesis in the noncapsulated (rough) strain. Later on, this transforming factor (agent) was identified as free DNA by Avery, Macleod and McCarty in 1944. Transformation has been studied mainly in bacteria like pneumococci, *H. influenzae* and *Bacillus species*.

B. Transduction

Transmission of a portion of DNA from one bacterium to another by a bacteriophage is known as transduction (Fig. 9.10). Bacteriophages are viruses that parasitise bacteria and multiply in it. During the assembly of bacteriophage progeny inside infected bacteria, errors may happen occasionally. Besides its own nucleic acid, host DNA may accidentally be incorporated into the bacteriophage. This is known as 'packaging error'. Hence, when this bacteriophage infects another bacterium, host DNA is transferred and the recipient cell acquires new characters coded by donor DNA.

Types of Transduction

1. Generalised
2. Restricted

1. Generalised transduction

Transduction may be 'generalised', when it involves any segment of donor DNA at random.

2. Restricted transduction

Transduction may be 'restricted', when a specific bacteriophage transduces only a particular genetic trait. It has been studied intensively in the 'lambda' phage of *Esch. coli*.

Role of Transduction

Transduction is not only confined to transfer of chromosomal DNA but episomes and plasmids may also be transduced. Penicillin resistance in staphylococci is due to the plasmids transferred from one bacterium to another by transduction.

Transduction provides an excellent tool for the genetic mapping of bacteria. Any bacteria for which bacteriophage exists can be subject to transduction. Transduction has also been proposed as a method of genetic engineering in the treatment of some inborn metabolic defects.

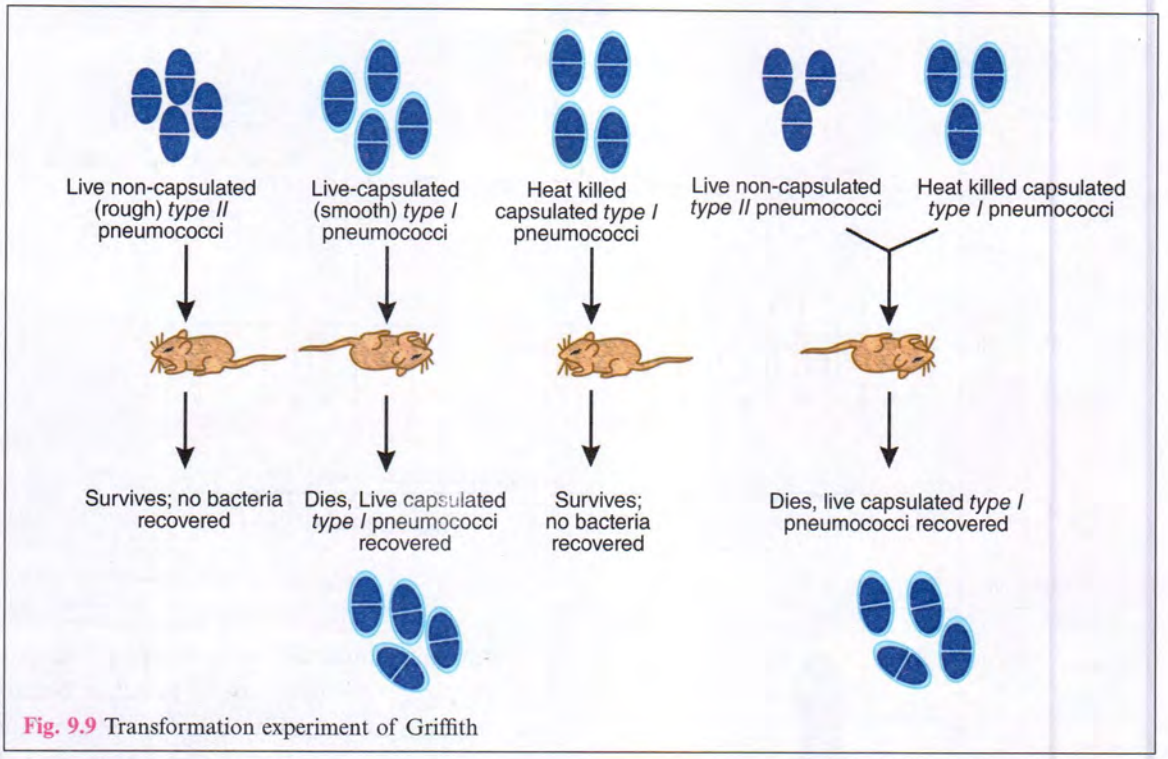


Fig. 9.9 Transformation experiment of Griffith

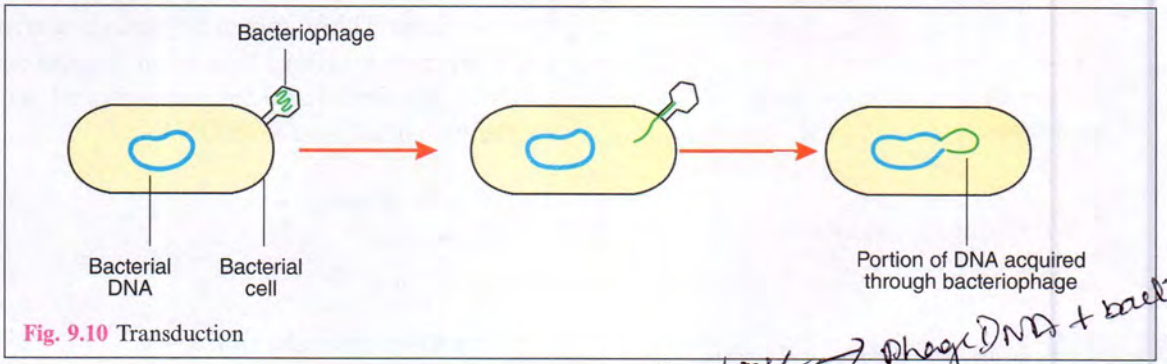


Fig. 9.10 Transduction

C. Lysogenic Conversion

Bacteriophages have two types of life cycle inside the host bacterium.

(*i*) Virulent or lytic cycle

(*ii*) Temperate or nonlytic cycle

(i) Virulent or lytic cycle

Large number of progeny phages are formed and subsequently, these progeny phages are released causing death and lysis of the host cell.

(ii) Temperate or nonlytic cycle

In contrast to virulent cycle, the host bacterium is unharmed. The phage DNA remains integrated with the bacterial chromosome as the *prophage*, which multiplies synchronously with bacterial DNA. The prophage acts as an additional chromosomal element which encodes for new characters and is transferred to the daughter cells. This process is known as *lysogeny or lysogenic conversion* and bacteria harbouring prophages are known as *lysogenic* bacteria. In lysogenic conversion the phage DNA itself is the new genetic element in contrast to transduction where the phage acts only as a vehicle carrying bacterial genes.

Example of lysogenic conversion: Diphtheria bacillus produces toxin (and therefore virulence) only when lysogenisation with the phage beta occurs. Elimination of the phage from a toxigenic strain renders it nontoxigenic.

D. Conjugation

The transfer of genetic material from one bacterium (donor or male) to another (recipient or female) by mating or contact is called conjugation (Fig. 9.11). Donors are those bacteria that contain F plasmid (F^+ or male cells) while cells lacking F plasmid (F^- or female cells) act as recipients. F plasmid is conjugative plasmid which encodes for sex pilus (in F^+ cells) necessary for conjugation. This plasmid is also named as 'sex factor'

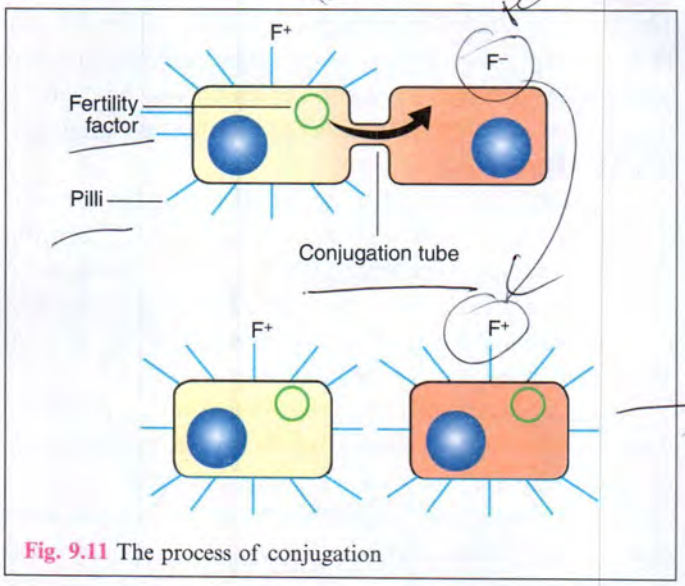


Fig. 9.11 The process of conjugation

or 'fertility' (F) factor. When other similar plasmids were also discovered, a name of 'transfer factor' was given to all such plasmids which conferred on their host cells the ability to act as donors or male cells in conjugation. Bacterial conjugation was first discovered by Lederberg and Tatum in 1946 in *Esch. coli* K12 strains.

During conjugation, the plasmid DNA replicates and copy of it passes from donor to the recipient cell probably along the sex pilus (conjugation tube). As a result, recipient (F^-) becomes donor (F^+) and can in turn conjugate with other female cells (F^-). This character of maleness (F^+) in bacteria is transmissible or infectious.

Hfr cell and F′ cell

F factor or plasmid has the ability to exist in some cells in the integrated state with the host chromosome (same as episome). These cells are able to transfer chromosomal DNA to recipient cells with high frequency, therefore, named as Hfr cells. Conjugation with an Hfr cell, an F^- rarely becomes F^+ but it receives chromosomal DNA from the donor.

The conversion of an F^+ cell into an Hfr cell is reversible.

$$F^+ \rightleftharpoons Hfr$$

When the F factor reverts from the integrated to free state, it may sometimes carry with it some chromosomal DNA from adjacent site of its attachment. Such an F factor carrying some chromosomal DNA is named as F' factor (F prime factor). When F' cell conjugate with a recipient (F^-), it transfers, along with the F factor, the host DNA incorporated with it. This process is called *sexduction* (Fig. 9.12).

$$F' + F^- \longrightarrow \text{Sexduction}$$

Colicogenic (Col) factor and resistance transfer factor (RTF) are two medically important factors which can be transferred by conjugation.

Colicogenic (Col) Factor

Some strains of coliform bacteria produce colicins which are antibiotic like substances lethal to other enterobacteria. Bacteria other than coliforms also produce similar kind of substances e.g. pyocin by *Pseudomonas pyocyanea*, diphthericin by *Corynebacterium diphtheriae*, hence, a general term bacteriocin has been given to these substances. These bacteriocins are useful for intraspecies classification of certain bacteria (e.g. *Shigella sonnei*).

Colicin production is encoded by a plasmid called the *col* factor, which may be transferred by conjugation.

Resistance Transfer Factor (RTF)

This plasmid is responsible for the spread of multiple drug resistance among bacteria. It was first reported by Japanese workers (1959) in shigella strains, resistant simultaneously to four drugs. They observed that patients excreting such shigella strains also shed *Esch. coli* strains resistant to same drugs. Transfer of multiple drug resistance from *Esch. coli* to shigella strains was demonstrated. The resistance is plasmid mediated and is transferred by conjugation. This mechanism of drug resistance is known as transferable or infectious drug resistance.

Resistance transfer factor (RTF) and resistance determinant (r) are two components of the plasmid known as an R factor (RTF + r determinants). The RTF is responsible for conjugational transfer while each r determinant carries resistance for one of the several drugs. An R factor can have several r determinants and resistance to many drugs can be transferred

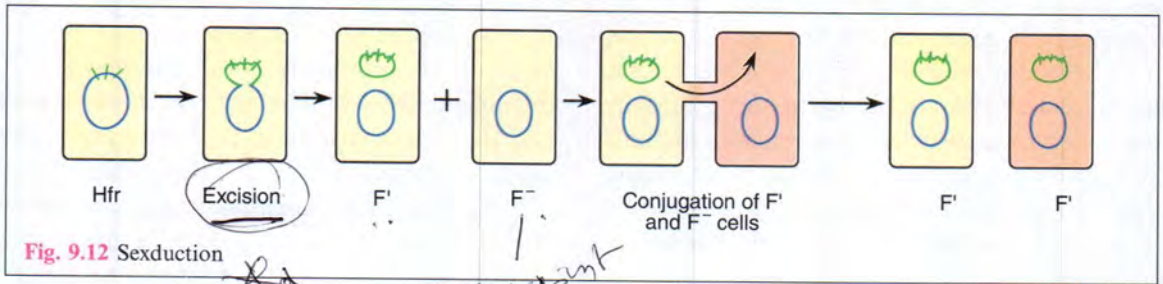


Fig. 9.12 Sexduction

simultaneously (Fig. 9.13). Sometimes RTF dissociates from the r determinants and both exist as separate plasmids. In such cases, the resistance is not transferable though the host cell remains drug resistant. The RTF can also attach to its determinants other than those for drug resistance. Enterotoxin and haemolysin production in some enteropathogenic *Esch. coli* are shown to be transmitted by this transfer factor (RTF).

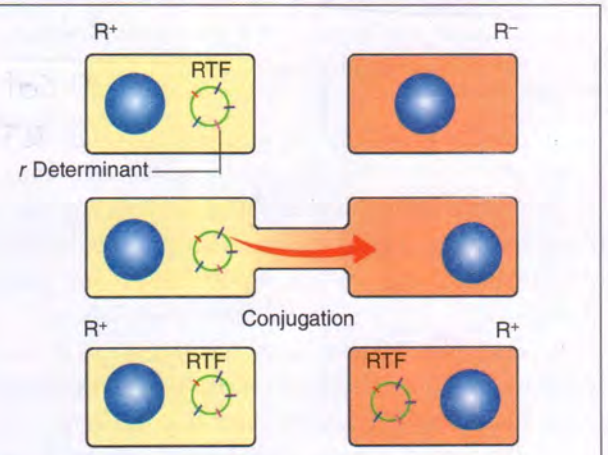


Fig. 9.13 Resistance transfer factor and drug resistance

Transferable drug resistance is seen in various pathogenic and commensal bacteria of man and animals such as *Esch. coli, Klebsiella, Proteus, Vibrio, Pseudomonas*. Transfer of drug resistance occurs readily in vitro but in the normal intestines, it is inhibited by several factors such as anaerobic conditions, bile salts, alkaline pH and abundance of anaerobic bacteria. But in the intestines of persons on oral antibiotic therapy, transfer occurs readily due to the selection pressure produced by the drug.

Transferable drug resistance involves all antibiotics in common use. Bacteria carrying R factors can be transmitted from animals to man. Hence indiscriminate use of antibiotics in animals or in animal feeds can lead to an increase of multiple drug resistance in the community.

VI. GENETIC MECHANISMS OF DRUG RESISTANCE

Bacteria may acquire drug resistance by mutation or by one of the methods of gene transfer. In clinical practice, mutational resistance is very important in tuberculosis. If a patient is treated with streptomycin alone, initially organisms die in large numbers but soon resistant mutants appear and multiply unchecked. By use of two or more drugs, 'a mutant resistant' to one drug will be killed by the other drug. The possibility of a mutant developing resistance to more than one drug at one time is remote. This is the rationale of combined treatment in tuberculosis. However, in spite of this knowledge, inappropriate or inadequate treatment has caused extensive resistance in *M. tuberculosis*, leading to 'multidrug resistant tuberculosis' (MDR TB) cases.

Transferable drug resistance mediated by the R factor is an important method of drug resistance. Acquisition of an R factor simultaneously confers resistance to several drugs, therefore, there is no use of combined therapy. Table 9.1 shows the differences of mutational and transferable drug resistance.

Resistance transfer by transformation can also be demonstrated experimentally, but its significance in nature is unknown. The plasmid carrying the gene for beta-lactamase production can be transferred from a penicillin resistant *Staphylococcus* to a susceptible *Staphylococcus* by transduction.

Table 9.1 Differences between Mutational and Transferable Drug Resistance

	Mutational drug resistance	Transferable drug resistance
1.	Mutational resistance	Transferable resistance
2.	Resistance to one drug at a time	Multiple drug resistance
3.	Low degree resistance	High degree resistance
4.	Resistance is not transferable to other organisms	Resistance is transferable to other organisms
5.	Mutants may be defective	Not defective
6.	Virulence of resistant mutants may be lowered	Virulence not decreased
7.	Resistance can be prevented by treatment with combination of drugs.	Cannot be prevented by treatment with combination of drugs.

Transposable Genetic Elements

Transposable genetic elements are specific sequences of DNA segments that have the ability to move from one plasmid to another plasmid or from plasmid to chromosome and *vice versa* and also within the chromosome. Because of their ability to insert into many sites both on plasmid and chromosome, they have assumed the popular name of *jumping genes*.

The transfer of genetic material from one DNA molecule to another is called *transposition*. It does not require any DNA homology between transposable element and the site of insertion. It is, therefore, different from recombination.

Transposons are larger (4–25 Kb) transposable genetic elements and contain additional genes that encode for at least one function (often conferment of antibiotic

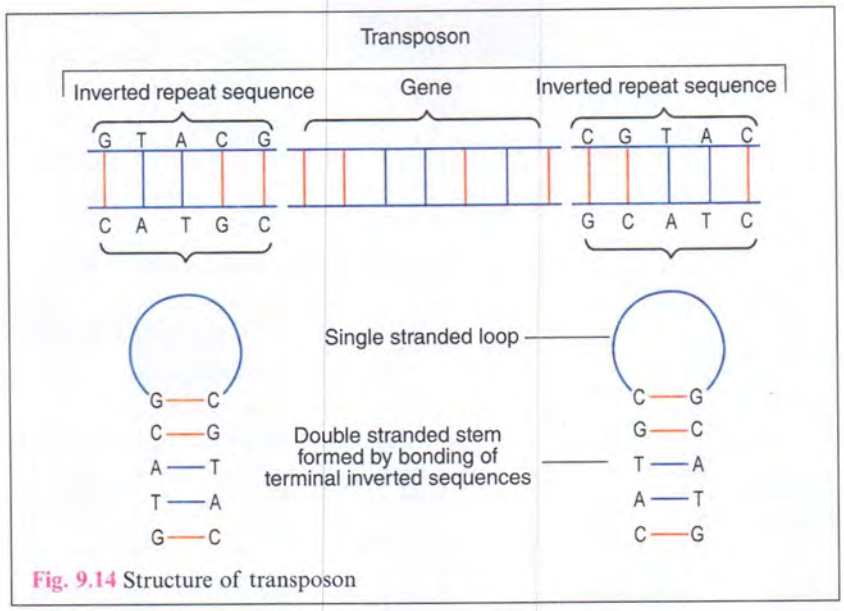


Fig. 9.14 Structure of transposon

resistance). They may also encode for toxin production. Transposon is defined as a segment of DNA with one or more genes in the centre and the two ends carrying inverted repeat sequences of nucleotides—nucleotide sequences complementary to each other but in the reverse order. Because of this feature, each strand of the transposon can form a single-stranded loop carrying the gene or genes, and a double stranded stem formed by hydrogen bonding between the terminal inverted repeat sequences (Fig. 9.14).

Small transposons (1-2 kb) are known as 'insertion sequences' or IS elements. Transposons attach at certain regions of chromosomal, plasmid or phage DNA. Bacteria acquire new characters by the insertion of transposable elements. Unlike plasmids, transposons are not self replicating and are dependent on chromosomal or plasmid DNA for replication.

It has been suggested that R plasmids may have evolved as collections of transposons each carrying a gene that confers resistance to one or several antibiotics. Transposition is a mechanism of amplifying genetic transfers in nature.

VII. GENETIC ENGINEERING

By genetic engineering or recombinant DNA technology, it is now possible to isolate the genes coding for any desired protein from microorganisms or from cells of higher form of life including man, and to insert them into suitable microorganisms in such a way so that it can be expressed in the formation of specific (desired) protein. Such cloning of genes in microorganisms enables the preparation of the desired protein in pure form and in large quantities at a reasonable cost.

The DNA can be cleaved by enzymes called *restriction endonucleases* and the fragments containing the desired genes are isolated. These desired DNA fragments are covalently bound to vectors or carriers, such as plasmids or temperate bacteriophages. The recombinant DNA molecule is sealed by an enzyme DNA ligase and is then introduced into a bacterial host, usually *Esch. coli*, by transformation. Many other bacteria and yeasts have also been employed for introduction of recombinant DNA molecule. *Esch. coli* containing recombinant molecule is grown on a suitable medium and bacterial colonies carrying specified genes are produced (Fig. 9.15). Thus desired protein is obtained in large quantities by growing these *Esch. coli*.

Application of Genetic Engineering

1. *Production of vaccines*: Preparation of certain vaccines is done by producing specific antigen against which antibody is required e.g. hepatitis B, rabies and *B. pertusis* vaccines.
2. *Production of proteins*: Genetic engineering has also been used for the production of proteins of therapeutic interest. These include human growth hormone, insulin, interferons, interleukin-2, tumour necrosis factor and factor VIII.
3. Gene therapy: Genetic diseases can be cured by introducing normal genes into the patient.

Restriction Endonculeases

These are microbial enzymes which split DNA into fragments of varying lengths. They cleave double stranded DNA at specific oligonucleotide sequences. Many such enzymes (for example, *Eco* RI, *Hind* III) which act at different nucleotide sequences have been recognised. The

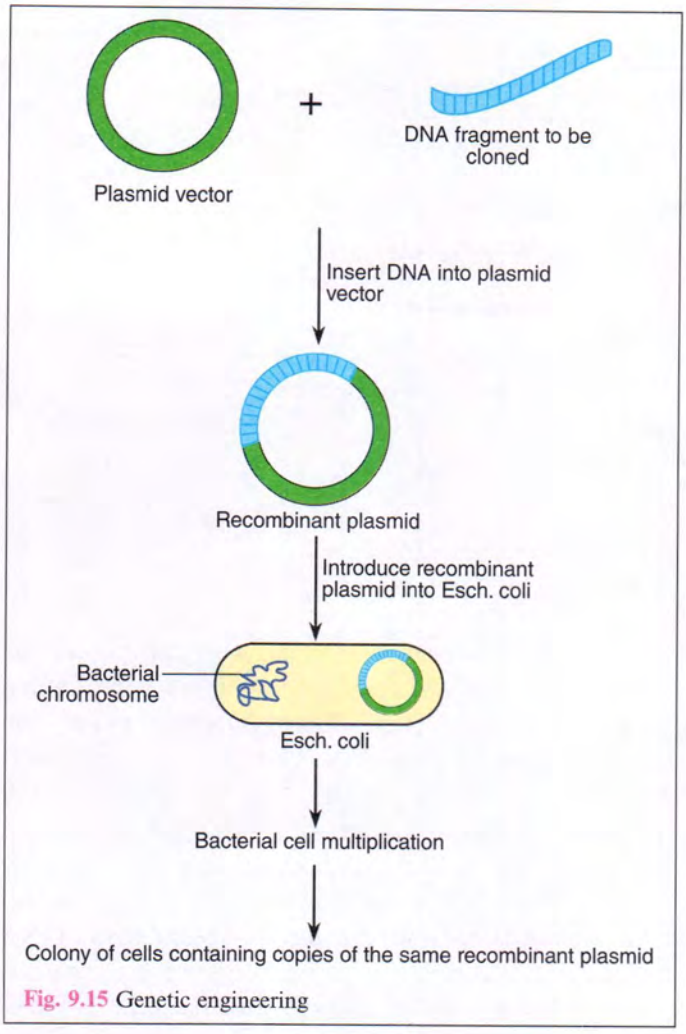


Fig. 9.15 Genetic engineering

natural function of these enzymes in bacteria may be the destruction of foreign DNA which may enter the bacterial cell.

VIII. DNA PROBES

DNA probes are radiolabelled or chromogenically labelled pieces of single stranded DNA which can be used for the detection of homologous DNA by hybridisation. Hybridisation is the technique in which two single-strands of nucleic acid come together to form a stable double-stranded molecule.

All microorganisms contain some unique sequences of nucleic acid within their genome that distinguish them from all other organisms. This unique sequence of nucleic acid can be recognised by hybridisation with a DNA probe (Fig. 9.16). Various diagnostic DNA probes have been developed for identification of different microorganisms. Probes containing sequences unique to the microbe can be added to microbial cultures, body fluids or other specimens suspected to contain the microbe or its DNA. The DNA probe hybridises with the complementary sequences on the microbe's DNA. The advantages of DNA probes are their high degree of specificity and ability to detect minute quantities of DNA. They are also very useful in diagnosis of microbes that are either difficult or impossible to culture. DNA probes which bind to RNA can also be designed and has been particularly used to locate ribosomal RNA.

Application of DNA Probes

Applications of DNA probe technology in microbiology are shown in Table 9.2.

Table 9.2 Applications of DNA Probe Technology

- In clinical microbiology:
  - Direct detection of microbes in specimens.
  - To detect microbes which are either difficult or impossible to culture.
- Identification of culture isolates
- Strain identification
- To identify toxins, virulence factors
- Identification of resistant markers

Diagnostic DNA probes to recognise *Legionella pneumophila, Campylobacter jejuni, M. tuberculosis,*

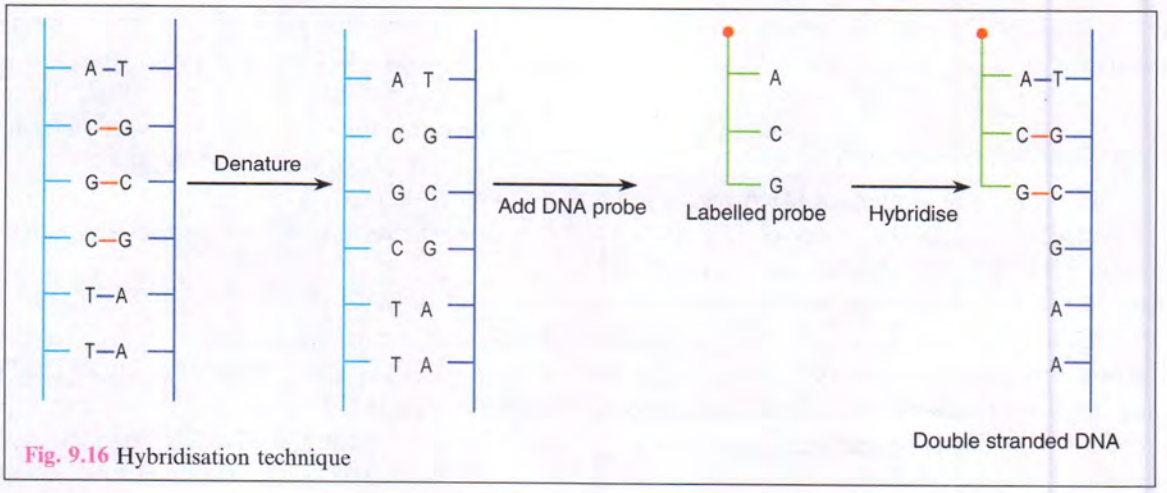


Fig. 9.16 Hybridisation technique

Helicobacter pylori, Esch. coli (LT and ST toxins), hepatitis B virus, *Plasmodium falciparum,* rotavirus and human immunodeficiency virus have been developed.

Blotting Techniques

DNA fragments are obtained by restriction enzyme digestion and separated by gel electrophoresis. These fragments can be transferred from the gel by blotting to nitrocellulose or nylon membranes that bind the DNA. The DNA bound to the membrane is first denatured (converted to single stranded DNA) and then hybridised with radioactive single stranded DNA probes. Hybridisation results in formation of radioactive double-stranded segments, which can be detected on X-ray film. This technique of identifying DNA fragments by DNA:DNA hybridisation is called *Southern blotting*, after E.M.Southern who developed it. This is a highly sensitive technique.

A similar procedure for analysis of RNA has been called *northern blotting*. Here the RNA is separated by gel electrophoresis, blotted and identified using labelled probes.

A technique for identification of proteins is called immunoblotting. The Western blot test is one example, which has been used as confirmatory test for the diagnosis of HIV antibody in sera.

IX. POLYMERASE CHAIN REACTION (PCR)

Polymerase chain reaction (PCR) was first developed in 1983 at Cetus Corporation, USA. It has made a revolutionary impact in molecular biology. In 1993, *Kary Mullis* was awarded the Nobel prize for this work.

A. Principle

It is a DNA amplification system that produces a large amount of DNA *in vitro* from small amounts of starting material. It amplifies a specific DNA sequence (or gene) of interest.

B. Procedure

It involves four main stages which are as follows:

(i) Denaturation

The double stranded DNA is dissociated to a single stranded DNA at 94°C (denaturing temperature).

(ii) Primer Annealing

The temperature is reduced to 50-60°C, then oligonucleotide primers attach to target DNA. This temperature is called annealing temperature and the process is known as *annealing of primers*.

(iii) DNA Synthesis

Polymerase enzyme derived from *Thermus aquaticus* (Taq) triggers the formation of new DNA strand from the free nucleotides. Taq polymerase and nucleotides are added in the tube for formation of new strands of DNA.

These three steps are repeated again and again, a process that is automated by the thermocycler or PCR machine. The products of first cycle become the template for the next cycle. After 20-30 cycles or more, an exponential increase in the amount of DNA occurs (Fig. 9.17).

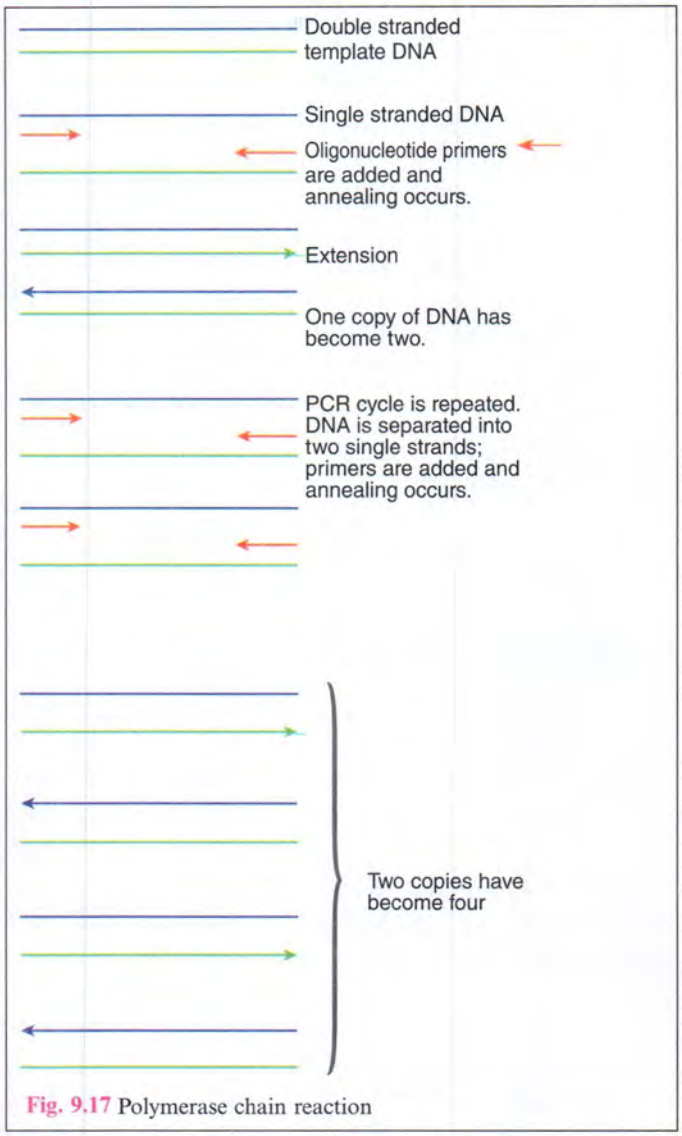


Fig. 9.17 Polymerase chain reaction

(iv) Detection of Amplified Product

Amplified DNA can be detected by Gel electrophoresis.

C. Application of PCR

The PCR provides extremely rapid analysis (one day). PCR is a versatile tool useful in infectious, genetic or neoplastic diseases, in forensic investigations and in the

examination of phylogenetic relationships in evolution. It has been applied in clinical laboratory for diagnosis of various infectious agents (Table 9.3). A specific DNA sequence of a particular infectious agent is amplified with the specific primers.

D. Types of PCR

Besides originally described PCR, other types of PCR include reverse transcriptase PCR (RT-PCR), nested PCR, multiplex PCR and real time PCR. These have been described in chapter 79.

Table 9.3 PCR in Diagnosis of Infectious Agents

Bacteria
M. tuberculosis, Legionella pneumophila, Helicobacter pylori, Chlamydia trachomatis, Mycoplasma pneumoniae
Viruses
Cytomegalovirus, Herpes simplex virus, Hepatitis B virus, Hepatitis C virus, Coxsackie virus, Measles virus, Human immunodeficiency virus (HIV-1 and HIV-2), Human papilloma virus, Rotavirus, Rubella virus, Human herpes virus-6 (HHV-6), rhinovirus, parvovirus, adenovirus.
Fungi
Candida albicans, Cryptococcus neoformans, Pneumocystis jiroveci.
Protozoa
Toxoplasma gondii, Trypanosoma cruzi, Plasmodium sp.

X. GENETICALLY MODIFIED ORGANISMS

The process of artificially introducing foreign DNA into organisms is called *transfection*. The recombinant organisms produced in this way are named *transgenic* or genetically modified organisms. Foreign DNA have been inserted into a variety of microbes, plants and animals through recombinant DNA technology. Transgenic organisms are available for a variety of biotechnological applications.

XI. GENE THERAPY

Gene therapy is a technique by which a faulty gene is replaced with a normal gene in persons with fatal or extremely debilitating genetic diseases. The benefit of this therapy is to permanently cure the physiological dysfunction by repairing the genetic defect.

1. Types

i. *Ex vivo* gene therapy
ii. *In vivo* gene therapy

Ex vivo gene therapy

In this type of therapy, the normal gene is cloned in vectors such as retroviruses (mouse leukaemia virus) or adenoviruses which are infectious but relatively harmless. Tissues removed from the patient are incubated with these genetically modified viruses to transfect them with the normal gene. The transfected cells are then reintroduced into the patient by transfusion.

In vivo gene therapy

Cloning of vector is similar to that of *ex vivo* gene therapy. However, the step of incubating the excised patient tissue with genetically modified viruses is absent in this type of therapy. Thus the naked DNA or a virus vector is directly introduced into the patient's tissues.

2. Applications

The first gene therapy experiment in humans was initiated in 1990 at the National Institutes of Health (NIH), USA. This was done on a four year old girl suffering from a severe immunodeficiency disease caused by the lack of enzyme adenosine deaminase (ADA). She was transfused with her own blood cells which had been engineered to contain a functional ADA gene. Later, other children were given similar treatment. So far, the children have shown marked improvement and continue to be healthy. However, the treatment is not permanent and must be repeated. Single gene defects such as haemophilia and sickle cell anaemia are possible candidates for therapy. Many other clinical trials with gene therapy are also in progress.

3. Gene line therapy

It is the ultimate sort of gene therapy, in which gene are inserted into an egg, sperm or early embryo. Although tests in animals seems it to be effective, its use on humans has been rather controversial.

KEY POINTS

1. *Genetics* is the study of heredity and variation.
2. All hereditary characteristics are encoded in DNA.
3. Genetic information is stored in the DNA as a code. *Codon* consists of a sequence of three nucleotide bases, i.e., the code is triplet.

4. Besides the chromosomal DNA, some bacteria may also possess extrachromosomal DNA such as *plasmids*. These plasmids are circular DNA molecules and can replicate autonomously. They may carry properties of *drug resistance*, *toxigenicity*, *conjugation* and others.
5. Some plasmids have an ability to transfer themselves to other bacteria of the same and also of different species. These are called *self transmissible plasmids*. This transfer occurs usually by method of conjugation.
6. *Mutation* is a random, undirected heritable variation caused by change in nucleotide sequence of the DNA of the cell. The frequency of mutation ranges from 10^{-2} to 10^{-10} per bacterium per division.
7. Mutation occurs spontaneously but its frequency may be enhanced by *mutagens* such as UV rays, alkylating agents, 5-bromouracil and acridine dyes.
8. *Gene transfer* can occur in bacteria. Various methods of gene transfer include *transformation*, *transduction*, *lysogenic conversion* and *conjugation*.
9. *R. factor* is a plasmid responsible for the spread of drug resistance among bacteria. This plasmid is transferred from one bacterium to other by conjugation. R factor has two components, *resistance transfer factor* (RTF) and *resistance determinant* (r). This mechanism of drug resistance is known as *transferable drug resistance*.
10. Some bacteria may acquire drug resistance by mutation. It is known as *mutational drug resistance*. This type of reistance occurs in *M. tuberculosis*.
11. *Transposable genetic elements* are specific sequences of DNA segments that have the ability to move from one plasmid to another plasmid or from plasmid to chromosome and *vice versa* and also within the chromosome. They are also named as *jumping genes*.
12. By *genetic engineering* or *recombinant DNA technology*, it is possible to isolate the genes coding for any desired protein from microorganisms, and to insert them into suitable microorganisms in such a way so that it can be expressed in the formation of specific (desired) protein. This technique has been used for *production of vaccines*, *insulin*, *interferons* and *interleukin-2*. *Gene therapy* is another application based on genetic engineering.
13. *DNA probes* are radiolabelled or chromogenically labelled pieces of single stranded DNA which can be used for the detection of homologous DNA by hybridisation. Various diagnostic DNA probes have been developed for identification of different microorganisms.
14. *Polymerase chain reaction* (PCR) is a DNA amplification system that produces a large amount of DNA *in vitro* from small amounts of starting material. The PCR provides extremely rapid method for diagnosis of various infectious agents.

YOU MUST KNOW

1. Functioning and importance of Lac operon.
2. Types of mutation.
3. Principles and uses of various methods of gene transfer.
4. Resistance transfer factor and its role in drug resistance.
5. Differences between mutational drug resistance and transferable drug resistance.
6. Transposable genetic elements and their importance.
7. Principles and applications of genetic engineering, DNA probes, polymerase chain reaction (PCR).
8. Gene therapy and its importance.

STUDY QUESTIONS

1. Write short notes on:
   (a) Transformation (b) Transduction (c) Conjugation (d) F factor
   (e) R factor (f) Extrachromosomal genetic elements (g) Lac operon.
2. Write short notes on:
   (a) Transposable genetic elements (b) Genetic engineering
   (c) Restriction endonucleases (d) DNA probes and their clinical application
   (e) PCR and its application in clinical microbiology (f) Gene therapy

3. Define mutation. Describe various types of mutation.
4. Name the various methods of gene transfer. Discuss any one of these in detail.
5. Tabulate the differences between mutational drug resistance and transferable drug resistance.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. All the following are non-sense codons except:
   (a) UAG (b) UGA (c) UAA (d) AAG
2. The process of transfer of genetic information from DNA to RNA is known as:
   (a) Transformation (b) Transduction (c) Transcription (d) Translation
3. Which of the following properties may be plasmid mediated?
   (a) Resistance to drugs (b) Enterotoxin production
   (c) Lactose fermentation (d) All of the above
4. Which of the following enzymes are required for lactose fermentation?
   (a) Beta-galactosidase (b) Galactoside permease
   (c) Transacetylase (d) All of the above
5. During mutation, when a purine is replaced by pyrimidine and vice-versa, it is named:
   (a) Transversion (b) Transition (c) Induced mutation (d) None of the above
6. During mutation, when one pyrimidine is replaced by another pyrimidine and one purine by another purine, it is named:
   (a) Transversion (b) Transition (c) Induced mutation (d) None of the above
7. Transfer of genetic information through free or naked DNA is called:
   (a) Transformation (b) Transduction (c) Conjugation (d) Lysogenic conversion
8. Transmission of a portion of DNA from one bacterium to another by a bacteriophage is known:
   (a) Transformation (b) Transduction (c) Conjugation (d) Lysogenic conversion
9. The transfer of genetic material from one bacterium to another by mating or contact is called:
   (a) Transformation (b) Transduction (c) Conjugation (d) Lysogenic conversion
10. F factor carrying some chromosomal DNA is named:
    (a) F factor (b) F prime factor
    (c) Hfr (d) Resistance transfer factor
11. Which of the following factors is responsible for transferable drug resistance in bacteria?
    (a) Resistance transfer factor (b) F factor
    (c) Colicogenic factor (d) All of the above
12. Resistance transfer factor can be transferred from one bacterium to another by:
    (a) Transformation (b) Transduction
    (c) Conjugation (d) None of the above
13. Drug resistance in *Mycobacterium tuberculosis* is due to:
    (a) Transformation (b) Transduction
    (c) Conjugation (d) Mutation
14. Penicillin resistance in *Staphylococcus aureus* may be acquired by:
    (a) Transformation (b) Transduction
    (c) Conjugation (d) Mutation
15. The technique of identifying DNA fragments by DNA: DNA hybridisation is called:
    (a) Southern blotting (b) Northern blotting
    (c) Western blotting (d) None of the above

Answers (MCQs):

1. (d) 2. (c) 3. (d) 4. (d) 5. (a) 6. (b) 7. (a) 8. (b) 9. (c) 10. (b)
11. (a) 12. (c) 13. (d) 14. (b) 15. (a)

Chapter 10

MICROBIAL PATHOGENICITY

I. Microbes and Related Terms
- A. Microbes
- B. Related Terms

II. Infection
- A. Types of Infection
- B. Transmission of Infection
- C. Factors Predisposing to Microbial Pathogenicity

III. Types of Infectious Diseases
- A. Localised
- B. Generalised

The host-parasite relationship is determined by the interaction between host factors and the infecting microorganism. Health or disease depends on the equilibrium between the two.

I. MICROBES AND RELATED TERMS

A. Microbes

Microbes can be divided into saprophytes and parasites, based on their relationship to their hosts.

1. *Saprophytes*: These are free-living microbes that live on dead or decaying organic matter. They are found in soil and water. They are generally unable to invade the living body. However, when host resistance is lowered, some saprophytes like *Bacillus subtilis* may cause infection.
2. *Parasite*: A parasite is a microorganism which lives on a living host and derives nutrition from the host, without any benefit to the host.

B. Related Terms

1. *Commensals*: Commensals live in complete harmony with the host without causing any harm to it. They constitute the normal flora of the body such as *Staphylococcus epidermidis* of skin and *Escherichia coli* of gastrointestinal tract.
2. *Pathogen*: Microorganism capable of producing disease in the host is called pathogen (*pathos*, suffering and *gen*, disease).
3. *Opportunistic pathogens*: Some commensals or saprophytes can produce disease when host resistance is lowered.
4. *Pathogenicity*: It refers to the ability of a class of microbes to produce disease.
5. *Virulence*: It is the degree of pathogenicity of a microbe.

II. INFECTION

Infection: The lodgement and multiplication of a parasite in the body is known as infection. All infections do not invariably result in disease.

A. Types of Infection

1. *Primary infection*: Initial infection with a parasite in a host is termed *primary infection*.
2. *Reinfection*: Subsequent infection with the same parasite in the same host is called *reinfection*.
3. *Secondary infection*: When the body resistance is lowered by a preexisting infectious disease, a new parasite sets up an infection, this is termed *secondary infection*.

4. *Cross infection*: When a patient already suffering from a disease acquires a new infection from another host or another external source, it is known as *cross infection*.
5. *Nosocomial infection*: Cross infection acquired in hospitals is called *hospital-acquired* or *hospital associated* or *nosocomial infection*.
6. *Iatrogenic infection*: An iatrogenic infection is defined as physician induced infection resulting from drug therapy or investigative procedures.
7. *Subclinical infection*: When clinical symptoms of an infection are not apparent, it is known as *subclinical infection*.
8. *Latent infection*: Following infection, some parasites may remain in a latent or hidden form in host tissues and they proliferate and produce clinical disease when the host resistance is lowered. It is known as *latent infection*.
9. *Atypical infection*: In this type of infection, the typical or characteristic clinical manifestations of the particular infectious disease are not present.

B. Transmission of Infection

There are three links in the chain of transmission of communicable diseases. These links include the *reservoir*, *mode of transmission* and the *susceptible host*.

1. Reservoir

It refers to any human being, animal, plant, soil or inanimate matter in which parasite normally lives, multiplies and depends primarily for its survival. The reservoir and source of infection are one and the same in most instances, sometimes they may be different e.g. reservoir of *Cl. botulinum* is the soil but the source of infection is contaminated food.

Sources of infection

These may be endogenous sources or exogenous sources.

(i) Endogenous sources

Organisms of normal flora are usually non-pathogenic but occasionally they behave as pathogens outside their habitat. *Esch. coli* which are the normal flora of the intestines may cause urinary tract infection. Another example is viridans streptococci which are the normal flora of the mouth may cause infective endocarditis.

(ii) Exogenous sources

Most of the infections are exogenous in origin. Exogenous sources include:

(*a*) Human cases and carriers
(*b*) Animals
(*c*) Insects
(*d*) Soil and Water
(*e*) Food.

(*a*) *Human cases and carriers*—The commonest source of human infection is man himself. The parasite may originate from a patient or a carrier.

- *Carrier*: A carrier is a person who harbours the pathogenic microorganism without suffering from its ill effects. Several types of carrier are known.
- *Healthy carrier*: One who harbours the pathogen but has never suffered from the disease caused by that particular pathogen.
- *Convalescent carrier*: One who has recovered from the disease but continues to harbour the pathogen in his body.
- *Temporary carrier*: It depends on the duration of carriage. The temporary carrier state lasts for less than six months.
- *Chronic carrier*: When carrier state lasts for several years and sometimes even for the rest of one's life, it is called chronic carrier.
- *Paradoxical carrier*: It refers to a carrier who acquires the pathogen from another carrier.
- *Contact carrier*: A person who acquires the pathogen from a patient.

Carriers are very important source of infection to spread the disease in a community.

(*b*) *Animals*—Many pathogens are capable of causing infection in both man and animal. Animals may, therefore, act as source of human infection. The infection in animals may be asymptomatic and these animals act as the *reservoir* of human infections. These are called *reservoir hosts*. Infectious diseases transmitted from animals to man are called *zoonoses*.

Zoonotic diseases may be

- *Bacterial*: Bovine tuberculosis, *Salmonella* food poisoning.
- *Viral*: Rabies from dogs.
- *Protozoal*: Leishmaniasis.
- *Helminthic*: Hydatid disease from dogs.
- *Fungal*: Dermatophytes from cats and dogs.

(*c*) *Insects*—Blood sucking insects such as mosquitoes, ticks, mites, flies, fleas and lice may transmit a number of pathogens to man. The diseases so caused are called *arthropod borne diseases*. Insects transmitting these pathogens are known as vectors. They are of two types: mechanical vectors and biological vectors.

Mechanical vectors: These carry the organisms on their legs, wings and body and transmit them to the eatables e.g. transmission of dysentery or typhoid bacilli

to man, through food, by domestic fly.

Biological vectors: The pathogen multiplies in the body of the vector, often undergoing part of a development cycle in it. Such vectors are named *biological* vectors (e.g. *female Anopheles* mosquito in malarial parasite; *Culex* mosquito in filarial parasite).

Extrinsic incubation period: After the entry of pathogen into the vector, the time required for the vector to become infective is called extrinsic incubation period.

Besides acting as vectors, some insects may also act as reservoir hosts (e.g. ticks in relapsing fever). Infection is maintained in such insects by transovarial passage.

(*d*) *Soil and water*—Some pathogens may survive in the soil for very long periods. Spores of tetanus and gas gangrene bacilli remain viable in the soil for several decades and serve as the source of infection. Fungi causing histoplasmosis and parasites such as roundworm and hookworm also survive in soil and cause human infection.

Contamination of water with pathogenic microorganisms (e.g. *Vibrio cholerae*, hepatitis virus) may act as the source of infection.

(*e*) *Food*—Contaminated food acts as a source of infection in cases of food poisoning, gastroenteritis, diarrhoea and dysentery.

2. Modes of Transmission

Pathogenic organisms can spread from one host to another by a variety of mechanisms.

(i) Contact

Contact transmission may be direct or indirect.

(*a*) *Direct contact*—Sexually transmitted diseases (STD) such as syphilis, gonorrhoea, herpex simplex type 2 and AIDS are acquired by direct contact.

(*b*) *Indirect contact*—It may be through the agency of *fomites*, which are inanimate objects such as clothing, toys etc. which may be contaminated by a pathogen and act as a vehicle for its transmission. Face towels shared by various persons may lead to spread of trachoma.

Contagious disease is term used for the disease acquired by direct contact.

Infectious disease is the disease acquired by other modes such as inanimate objects. The distinction between contagious and infectious disease is now not generally employed.

(ii) Inhalation

Respiratory infections such as common cold, influenza, whooping cough and tuberculosis are acquired by inhalation. These organisms are shed into the environment by patients in secretions from the nose or throat during sneezing, coughing or speaking. Large drops of such secretions fall to the ground and dry there. Small droplets, less than 0.1 mm in diameter, evaporate immediately to become minute particles or *droplet nuclei* (1-10 μm in diameter) which remain suspended in air for long periods, acting as sources of infection.

(iii) Ingestion

Intestinal infections like cholera, dysentery, food poisoning and most of the parasitic infections are acquired by ingestion of food or drink contaminated by pathogens. Food borne infections occur mostly through carriers engaged in handling or preparation of food and contaminating the food stuffs. The water supply may get contaminated with the faeces of the patients or carriers. All these may transmit infection.

(iv) Inoculation

Pathogens, in some instances, may be inoculated directly into the tissues of the host, for example, rabies virus is inoculated directly by bite of a rabid animal. Spores of *Cl. tetani* are present in the soil. They get deposited into the host tissue following severe wounds leading to tetanus.

Infection by inoculation may be *iatrogenic* when unsterile syringes and equipments are employed. Hepatitis B and Human Immunodeficiency Virus (HIV) may be transmitted by use of contaminated syringes and through transfusion of infected blood.

(v) Vectors

Vectors are arthropods or other invertebrate hosts e.g. mosquitoes, flies, fleas, ticks, mites and lice. Transmission by vector may be either mechanical or biological (Refer Chapter 81).

(vi) Transplacental

Some pathogens can cross the placental barrier and infect the foetus *in utero*. This is known as *vertical transmission*. This may lead to abortion, miscarriage or stillbirth. In case of congenital syphilis, live infants may be born with manifestations of the disease. Infection with rubella virus, especially in the first trimester of pregnancy, may lead to congenital malformations. Such infections are known as *teratogenic infections*. Other organisms which may cause congenital infections are toxoplasma, cytomegalovirus and herpes simplex virus.

(vii) Iatrogenic and laboratory infections

If meticulous care in asepsis is lacking, certain infections like AIDS and hepatitis B may sometimes be transmitted

during procedures such as injections, lumbar puncture, blood transfusion, dialysis and surgery. These are known as *iatrogenic infections.* Laboratory personnel handling infectious material are particularly at risk. Special care should be taken to prevent laboratory infection.

3. Susceptible Host

The infectious agent enters the body by one of the following routes: *oral, respiratory, genitourinary, conjunctival or cutaneous.* This is known as *portal of entry*.

Incubation period: It is the time interval between the entry of infective agent and the onset of clinical features of the disease. The infective agent, after reaching the selective tissue, undergoes multiplication during this period.

The outcome of an infection depends on interaction between microbial factors which predispose to pathogenicity and various host factors which contribute to resistance.

C. Factors Predisposing to Microbial Pathogenicity

Pathogenicity refers to the ability of a microbial species to produce disease. Virulence is the ability of a strain of a microorganism to cause disease. The pathogenic species *M. tuberculosis* contains strains of varying degrees of virulence including those which are avirulent, such as the vaccine strains. The virulence of a strain is not constant and may undergo variation.

Exaltation: Enhancement of virulence of a strain is known as exaltation. This can be induced by serial passage in an experimental animal.

Attenuation: Reduction of virulence of a strain is known as attenuation. This can be achieved by repeated passages through unfavourable hosts, repeated cultures in artificial media, growth under high temperature or in the presence of weak antiseptics.

Determinants of Virulence

(i) Adhesion

The initial event in the pathogenesis is the attachment of the bacteria to body surfaces. The attachment is a specific reaction between surface receptors on the epithelial cells and adhesive structures on the surface of bacteria. These adhesive structures are named *adhesins*. Adhesins usually occur as fimbriae or fibrillae and pili or as colonisation factors.

Advantages of adherence are as follows:

(*a*) It prevents the microbes from being flushed away in mucus secretions, urine and by peristalsis in the gut.

(*b*) Close adherence of the bacterium assures that the toxin will be delivered in high concentrations directly to the host cells.

(*c*) If bacterium is invasive in nature, adherence helps in penetrating host cells.

Besides above mentioned adhesins, other structures which play role in adherence are outer membrane protein, flagella, lipopolysaccharide (LPS) and glycocalyx. Most of the bacteria make use of not just one but several of these factors.

Loss of adhesins often renders the strain avirulent. Adhesins are antigenic in nature. Specific immunisation with adhesins has been attempted for prevention of gonorrhoea in human beings.

(ii) Invasiveness

This refers to the ability of an organism to spread within the host tissues after establishing infection. Highly invasive pathogens produce, spreading or generalised lesions (e.g. streptococcal infections), while less invasive pathogens cause localised lesions (e.g. staphylococcal abscess). Some pathogens though capable of causing fatal diseases lack invasiveness but remain confined to site of entry and produce the disease by elaborating a potent toxin e.g. tetanus bacillus.

(iii) Antiphagocytic factors

Macrophages and polymorphs play role in phagocytosis of microbes. There are certain factors which oppose phagocytosis for survival of bacteria. These factors are as follows:

(*a*) *Capsule*—Cell wall in many bacteria is enclosed by gelatinous layer known as capsule. Most capsules are polysaccharide in nature except that of Bacillus which is polypeptide. Capsule contributes to the virulence of bacteria by preventing phagocytosis. Non-capsulated variants usually exhibit low virulence.

(*b*) *Streptococcal M protein*—The M protein present on the surface of group A streptococci binds both fibrinogen and fibrin to the bacterial cell wall thus masking the bacterial receptors from complement.

(*c*) *Cytotoxin*—Cytotoxins produced by certain bacteria interfere with chemotaxis or kill the phagocyte. *Staph. aureus* produces haemolysins (can damage both RBCs and WBCs) and leukocidin (only WBCs are damaged).

(*d*) *Bacterial surface antigens*—Vi antigen of *S. typhi* and K antigen of *Esch. coli* enable these bacteria to resist phagocytosis and lytic activity of complement.

(iv) Survival within the phagocyte

Ingestion of a microbe by a phagocyte results in the formation of a phagolysosome by the fusion of phagosome with the lysosome. Any organism which interferes with the formation of phagolysosome can survive intracellularly.

(*a*) *Interference with the oxidative burst*—Superoxide and H_2O_2 produced are lethal to bacteria. *Staph. aureus* produces an enzyme catalase which breaks down H_2O_2 and enables the bacteria to survive. *Listeria monocytogens* produces another enzyme superoxide dismutase which neutralises the oxygen radicals leading to survival of the bacteria.

(*b*) *Prevention of fusion and degranulation*—Cell walls of some organisms modify the phagosomal membrane in such a manner that fusion of lysosome with phagosome is prevented e.g. Chlamydia.
B. pertussis produces adenylate cyclase which is inhibitor of degranulation and thus enhances intracellular survival.

(*c*) *Resistance to lysosomal enzymes*—This may be due to the presence of capsular polysaccharide (*M. lepraemurium*) and mycoside (*M. tuberculosis*).

(*d*) *Escape from phagosomes*—Some microbes escape from the phagosome into the cytoplasm of the host cell before the fusion of phagosome with the lysosome e.g. Rickettsiae are believed to escape by an effect of phospholipase A on the phagosomal membrane.

(v) Bacterial toxins

Bacteria produce two types of toxins, endotoxins and exotoxins (Table 10.1).

(*a*) *Endotoxins*—They are lipopolysaccharide (LPS) in nature and form an integral part of the cell wall of Gram negative bacteria. They are released from the bacterial surface by natural lysis of the bacteria or by the disintegration of the cell wall. They are heat stable. The toxicity of endotoxin depends on the lipid component (lipid A). Lipopolysaccharide binds to neutrophils via lipid A and alter neutrophil functions as follows:

- it decreases chemotactic activity.
- random migration of neutrophils is decreased.
- alters the metabolic and bactericidal properties of neutrophils.

The endotoxins are not destroyed by autoclaving, hence infusion of a sterile solution containing endotoxin can cause serious illness. They cannot be toxoided. They are poor antigens and the toxicity is not completely neutralised by the homologous antibodies. Massive Gram negative septicaemia may cause the syndrome of endotoxic shock characterised by fever, leucopenia, thrombocytopenia, profound fall of blood pressure and circulatory collapse leading to death.

(*b*) *Exotoxins*—They are heat labile proteins which are secreted by certain species of bacteria. They diffuse readily into the surrounding medium. They are highly potent even in minute amounts and constitute some of the most poisonous substances known. Botulinum toxin is the most poisonous, it has been estimated that 3.0 kg of Botulinum toxin can kill all the inhabitants of the world. Exotoxins

Table 10.1 Differences between Exotoxins and Endotoxins

	Exotoxins	Endotoxins
1.	Protein (polypeptides) M.W. 10,000 to 900,000.	Lipopolysaccharide in nature.
2.	Heat labile (>60°C).	Heat stable.
3.	Actively secreted by living cells into medium.	Form integral part of the cell wall; released only on disruption of bacterial cell.
4.	Highly antigenic; stimulates formation of antitoxin which neutralises toxin.	Weakly antigenic; antitoxin is not formed but antibodies against polysaccharide are raised.
5.	Converted into toxoid by formaldehyde.	Cannot be toxoided.
6.	Enzymic in action.	No enzymic action.
7.	Specific pharmacological effect for each exotoxin.	Non-specific action of all endotoxins.
8.	Very high potency.	Low potency.
9.	Highly specific for particular tissue e.g. tetanus toxin for CNS.	Non-specific in action.
10.	Don't produce fever in host.	Usually produce fever.
11.	Produced mainly by Gram-positive bacteria and also by some Gram-negative bacteria.	Produced by Gram-negative bacteria.

can be converted into toxoids by treatment with formaldehyde. Toxoids lack toxicity but retain antigenicity and thus induce protective immunity when used as vaccines.

Exotoxins are highly antigenic and stimulate formation of antitoxin which neutralises toxin. They are highly specific for a particular tissue e.g. tetanus toxin for CNS. They have specific pharmacological activities and do not produce fever in host. They are mainly produced by Gram positive bacteria but may also be produced by certain Gram negative bacteria such as *Vibrio cholerae* and enterotoxigenic *Esch. coli*.

(vi) Enzymes

Some bacteria produce enzymes which directly damage host tissue.

(*a*) *Proteases*—These enzymes cleave immuno-globulin IgA which protects at mucosal surfaces.

(*b*) *Kinase*—It enhances the spread of bacteria by dissolving fibrin clots e.g. streptococcus and staphylococcus.

(*c*) *Hyaluronidase*—It breaks down hyaluronic acid and helps in spread of infection e.g. streptococcus.

(*d*) *Coagulase*—It causes deposition of fibrin around the bacteria and thus prevents phagocytosis e.g. staphylococcus.

(*e*) *Collagenase*—It breaks down collagen in connective tissue and contributes in spread of infection e.g. *Clostridium perfringens*.

(vii) Siderophores and iron acquisition

Bacteria require iron for their metabolism. Many bacteria produce low molecular weight compounds called siderophores that can acquire iron from the host's iron binding proteins. This property enhances the virulence.

(viii) Genetic factors

Plasmids are extrachromosomal DNA segments that carry genes for antibiotic resistance known as R-factors. These factors are readily transferable by conjugation to the various bacteria and confer resistance to many antibiotics. This also indirectly contributes in survival of bacteria due to escape from action of antibiotics.

Plasmids may also code for enterotoxin production, colonisation factors and siderophore synthesis and thus enhances the virulence of the organism. All the strains of *C. diphtheriae* produce exotoxin only when they are lysogenised with a bacteriophage called betaphage. The elimination of this phage abolishes the toxigenicity of the bacillus.

(ix) Infecting dose

Adequate number of bacteria is required for successful infection. The dose of infection depends on virulence of bacteria. The dosage may be estimated as the *minimum infecting dose* (MID) or *minimum lethal dose* (MLD). Minimum number of bacteria required to produce clinical evidence of infection in a susceptible animal under standard conditions is called minimum infective dose (MID). MLD is a minimum number of bacteria that produce death in the animal under standard conditions. These doses are more correctly estimated as ID 50 and LD 50 as the dose required to infect or kill 50 per cent of the animals tested under standard conditions.

(x) Route of infection

Certain bacteria are infective when introduced through optimal route, for example, cholera vibrios can produce lesion only when administered by oral route, but unable to cause infection when introduced subcutaneously. However, *Staphylococcus aureus* can cause lesion whatever may be the portal of entry.

(xi) Communicability

The ability of a microbe to spread from one host to another is known as communicability. This property determines the survival and distribution of a microbe in a community and not necessarily produce a disease in an individual host. A correlation need not exist between virulence and communicability. Infections in which the pathogen is shed in secretions such as in respiratory or intestinal diseases, are highly communicable. In other instances, as in hydrophobia, human infection represents a dead end. Even a highly virulent microbe may not exhibit a high degree of communicability due to its rapidly lethal effect on host.

Occurrence of epidemic and pandemic diseases requires that the pathogen should possess high degree of virulence and communicability.

III. TYPES OF INFECTIOUS DISEASES

Infectious diseases may be *localised* or *generalised*.

A. Localised

There is an acute inflammatory reaction at the site of invasion to arrest infection. When this process fails, the organisms spread via the lymph channels (lymphangitis) to regional lymph nodes (lymphadenitis) and then to blood stream (bacteraemia) which leads to dissemination of organisms.

B. Generalised

1. *Bacteraemia*: It is the circulation of bacteria in blood. Transient bacteraemia is frequent even in healthy persons and may occur during chewing, brushing of teeth and straining at stools. The bacteria are immediately killed by phagocytes and are unable to initiate infection. Bacteriaemia of a longer duration is seen during generalised infections as in typhoid fever.
2. *Septicaemia*: It is the condition where bacteria circulate and multiply in the blood, form toxic products and cause high fever.
3. *Pyaemia*: It is condition where pyogenic bacteria produce septicaemia with multiple abscesses in internal organs e.g. liver, spleen, kidney.

Depending on the spread of infectious diseases in community, they may be classified as endemic, epidemic, and pandemic.

Endemic

The disease which is constantly present in a particular area, e.g. enteric fever.

Epidemic

The disease that spreads rapidly, involving many persons in a particular area at the same time, is called epidemic disease e.g. meningococcal meningitis.

Pandemic

It is an epidemic that spreads through many areas of the world involving very large number of persons within a short period e.g. cholera, influenza and enteroviral conjunctivitis.

KEY POINTS

1. The *host-parasite relationship* is determined by the interaction between host factors and the infecting microorganism. Health or disease depends on the equilibrium between the two.
2. A *parasite* is a microorganism which lives on a living host and derives nutrition from the host, without any benefit to the host.
3. *Commensals* live in complete harmony with the host without causing any harm to it.
4. Cross infection acquired in hospitals is called *nosocomial infection*.
5. *Bacterial toxins* contribute in virulence of bacteria.
6. *Endotoxins* are lipopolysaccharide (LPS) in nature while *exotoxins* are heat labile proteins.
7. Exotoxins can be converted into *toxoids* e.g. tetanus toxoid.

YOU MUST KNOW

1. Definitions of important terms such as saprophytes, parasite, commensals, pathogen, pathogenicity, virulence, infection.
2. Types of infection and their definitions.
3. Types of carriers and their definitions.
4. Modes of transmission and their examples.
5. Differences between exotoxins and endotoxins.

STUDY QUESTIONS

1. Describe the factors predisposing to microbial pathogenicity.
2. Tabulate the differences between exotoxins and endotoxins.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Which of the following may cause teratogenic infections?
   (a) Toxoplasma (b) Cytomegalovirus (c) Rubella virus (d) All of the above

2. Which toxin can be converted into toxoid by formaldehyde?
   (a) Exotoxin (b) Endotoxin (c) Both of the above (c) None of the above
3. Chemical nature of endotoxin is:
   (a) Lipopolysaccharide (b) Protein (c) Carbohydrate (d) None of the above
4. Chemical nature of exotoxin is:
   (a) Lipopolysaccharide (b) Protein (c) Carbohydrate (d) None of the above

Answers (MCQs):

1. (d) 2. (a) 3. (a) 4. (b)

UNIT II

IMMUNOLOGY

Chapter 11

IMMUNITY

The term 'immunity' is defined as resistance exhibited by the host against any foreign antigen including microorganisms. This resistance plays a major role in prevention of infectious diseases. Immunity may be *innate or acquired.*

I. INNATE IMMUNITY

It is the resistance which individual possesses by birth. It is by virtue of his genetic and constitutional make-up. It does not depend on prior contact with foreign antigen. It may be nonspecific, when there is resistance to infections in general, or specific when resistance to a particular pathogen is concerned.

A. Types of Innate Immunity

1. *Species Immunity*

It refers to the resistance to a pathogen, shown by all members of a particular species e.g. *B. anthracis* infects human beings but not chickens. The mechanism of such type of immunity is not clearly understood. The physiological and biochemical differences between tissues of different host species may be responsible for species specific resistance.

2. *Racial Immunity*

Within one species, different races may exhibit differences in susceptibility or resistance to infections. This is termed as *racial immunity*. Algerian sheep is highly resistant to anthrax which is a common disease of other races of sheep. In human beings, it is reported that American negroes are more susceptible than the white race to tuberculosis. Such differences in humans could be due to socioeconomic status. However, in a few instances genetic basis of racial immunity has been proved.

Races with sickle cell anaemia prevalent in Mediterranean coast are immune to *P. falciparum* infection. A genetic abnormality of erythrocytes (sickling) confers immunity to *P. falciparum* because such red blood cells cannot be parasitized by these parasites. Persons with hereditary deficiency of glucose-6-phosphate dehydrogenase (G-6-PD) are less susceptible to *P. falciparum*.

3. *Individual Immunity*

Resistance to infection varies with different individuals of same race and species. This is known as *individual* immunity. The genetic basis of individual immunity is

evident from the observation that homozygous twins exhibit similar degrees of susceptibility or resistance to tuberculosis. In contrast, such correlation is not seen in heterozygous twins.

B. Factors Influencing Innate Immunity

1. *Age*

The two extremes of life (foetus and old persons) carry higher susceptibility to various infections. In foetus, the immune system is immature whereas in old age there is gradual waning of immune responses. The foetus in-utero is generally protected from maternal infections through the placental barrier. However, some pathogens (rubella, cytomegalovirus and *Toxoplasma gondii*) cross this barrier and lead to congenital malformations.

In some diseases such as chickenpox and poliomyelitis, the clinical illness is more severe in adults than in young children. This may be due to more active immune response which causes greater tissue damage.

2. *Hormones*

Certain hormonal disorders such as diabetes mellitus, adrenal dysfunction and hypothyroidism enhance susceptibility to infections. Staphylococcal sepsis is more common in diabetes which may be associated with increased level of carbohydrates in tissues. Corticosteroids depress the host resistance by its antiinflammatory, antiphagocytic effects and by inhibiting antibody formation. The elevated steroid level during pregnancy may be related to higher susceptibility of pregnant women to many infections.

3. *Nutrition*

Malnutrition predisposes to bacterial infections. Both humoral and cell mediated immune responses are reduced in malnutrition.

C. Mechanisms of Innate Immunity

1. *Epithelial Surfaces*

(*i*) *Skin*

It not only acts as a mechanical barrier to microorganisms but also provides bactericidal secretions. The high concentration of salt in drying sweat, the sebaceous secretions and long chain fatty acids contribute to bactericidal activity. The skin may be freed of bacteria deposited on it (transient flora) but the bacterial flora normally present on skin (resident flora) is not easily removed even by washing and application of disinfectants. The resident bacterial flora of skin and mucous surfaces help to prevent colonisation by pathogens. Alteration of normal resident flora may lead to invasion by extraneous microbes and thus causing serious diseases. Clostridial enterocolitis following oral antibiotics is one such example.

(*ii*) *Respiratory tract*

The inhaled particles are arrested in the nasal passages on the moist mucous membrane surfaces. The mucous secretions of respiratory tract act as a trapping mechanism and hair like cilia propels the particles towards the pharynx where it is swallowed or coughed out. The cough reflex acts as an important defence mechanism. Some particles which manage to reach alveoli are ingested by phagocytes present there.

(*iii*) *Intestinal tract*

The mouth possesses saliva which has an inhibitory effect on many microorganisms. Some bacteria may be swallowed and are destroyed by acidic pH of gastric juices. The normal bacterial flora of intestine further exert a protective effect by preventing colonisation of pathogenic bacteria.

(*iv*) *Conjunctiva*

Tears have a major role by flushing away bacteria and other dust particles. In addition, lysozyme present in tears has a bactericidal action.

(*v*) *The Genitourinary tract*

The flushing action of urine eliminates bacteria from the urethra. The acidic pH of vaginal secretions in female, due to the fermentation of glycogen by lactobacillus (normal flora), renders vagina free of many pathogens. In males, semen is believed to contain some antibacterial substances.

2. *Antibacterial Substances*

Besides specific antibody formation, there are number of nonspecific antibacterial substances present in blood and tissues. These substances are properdin, complement, lysozyme, betalysin, basic polypeptides (leukins from leucocytes, plakins from platelets) and interferons which possess antiviral activity. The complement system plays an important role in the destruction of pathogenic microorganisms that invade the blood and tissues (see Chapter 15).

3. *Cellular Factors*

Once the infective agent has crossed the barrier of epithelial surfaces, the tissue factors come into play for defence.

When an infective agent invades tissue, an exudative inflammatory reaction occurs by accumulation of phagocytes at the site of infection and deposition of fibrin which entangles the organisms, to act as a barrier to spread of infection. Phagocytic cells ingest these organisms and destroy them. These phagocytic cells are classified as

(*i*) *Microphages* e.g. polymorphonuclear leucocytes (neutrophils)

(*ii*) *Macrophages* e.g. Mononuclear phagocytic cells

Phagocytic action can be divided into four stages:

(*i*) *Chemotaxis*

Phagocytes reach the site of infection attracted by chemotactic substances.

(*ii*) *Attachment*

The infective agent gets attached to phagocytic membrane.

(*iii*) *Ingestion*

Phagocyte engulfs the infective material into a vacuole (phagosome). The membrane of phagosome fuses with a lysosome to form a phagolysosome.

(*iv*) *Intracellular killing*

Most bacteria are destroyed in the phagolysosome by hydrolytic enzymes of lysosomes. However, some bacteria (*M. tuberculosis, M. leprae*) resist this type of killing and can multiply within phagolysosome and thus produce disease. Phagocytosis in such instances may actually help to disseminate infection to other parts of the body.

A class of lymphocytes called Natural Killer (NK) cells play an important role in non-specific defence against viral infections and tumour.

4. *Inflammation*

Inflammation occurs as a result of tissue injury or irritation, initiated by the entry of pathogens or other irritants. It is an important non-specific defence mechanism. Inflammation leads to vasodilation, increased vascular permeability and cellular infiltration. Microorganisms are phagocytosed and destroyed. Due to increased vascular permeability, there is an outpouring of plasma which helps to dilute the toxic products present. A fibrin barrier is laid to wall off the site of infection.

5. *Fever*

A rise in temperature following infection is a natural defence mechanism. It destroys the infecting organisms. Fever also stimulates the production of interferon which helps in recovery from viral infections.

6. *Acute phase proteins*

Following infection or injury, there is a sudden increase in plasma concentrations of certain proteins, collectively called *acute phase proteins*. These include C reactive protein (CRP), mannose binding proteins and many others. CRP and some other acute phase proteins activate the alternative pathway of complement. They are believed to prevent tissue injury and promote repair of inflammatory lesions.

II. ACQUIRED IMMUNITY

The resistance acquired by an individual during life is known as acquired immunity. It is of two types, *active* and *passive*.

A. Active Immunity

Active immunity is subdivided into two types : Natural and Artificial.

Natural—Through clinical or subclinical infection

Artificial—Induced by vaccination

It is the resistance developed by an individual as a result of contact with an antigen. This contact may be in the form of natural infection or by vaccination. It leads to stimulate the immune apparatus to form antibodies and/or the production of immunologically active cells. Active immunity develops after a latent period which is required for immune system to act but once developed, the active immunity is long lasting.

Mechanism

Active immune response stimulates both humoral and cell mediated immunity usually in parallel.

(*i*) *Humoral immunity*

It is antibody mediated immunity. It depends on the synthesis of antibodies by plasma cells. These cells produce specific circulating antibody which combines specifically with the antigens and modify their activity. This modified activity may be in the form of lysis of antigen molecules; their toxin may be neutralised, or in the form of removal of antigen by phagocytosis.

(*ii*) *Cell mediated immunity (CMI)*

It depends on T-lymphocytes developed against certain antigens. Antibody synthesis also occurs in response to these antigens, but their role is limited. The cell mediated immunity by sensitised T-lymphocytes is important in resistance to chronic bacterial infections. In these

chronic infections, organisms can multiply and survive in phagolysosome i.e. intracellular parasitism (tuberculosis, leprosy) and in viral infections (*Herpes simplex*).

Types

(i) Natural active immunity

It is acquired by natural subclinical or clinical infections. Such immunity is long lasting. Persons recovering from smallpox infection develop natural active immunity.

(ii) Artificial active immunity

It is produced by vaccination. The vaccines are prepared from live, attenuated or killed microorganisms, or their antigens or toxoids. In killed vaccines the organisms are killed by heat, formalin, phenol and alcohol. These are preserved in phenol, N-merthiolate and alcohol. Toxoids are prepared from bacterial exotoxins inactivated by formalin (formol toxoid) or by alum (alum precipitated toxoid–APT). Toxoids are immunogenic but not toxigenic.

VACCINES

(a) *Live vaccines*
- BCG for tuberculosis
- Ty 21a for typhoid
- Sabin vaccine for poliomyelitis
- MMR vaccine for measles, mumps, rubella
- 17D vaccine for yellow fever

(b) *Killed vaccines*
- TAB for enteric fever
- Killed cholera vaccine
- Salk vaccine for poliomyelitis
- Neural and non-neural vaccines for rabies
- Hepatitis B vaccine

(c) *Bacterial products*
- Tetanus toxoid for tetanus
- Diphtheria toxoid for diphtheria

B. Passive Immunity

Passive immunity is subdivided into two types : Natural and Artificial.

Natural—Through transplacental maternal IgG antibodies.

Artificial—Through antiserum injection.

Passive immunity is induced in an individual by preformed antibodies (generally in the form of antiserum) against infective agent or toxin. This antiserum is prepared by injecting infective agent or toxin in another host. The immune system has no active role in passive immunity. Protection starts immediately after transfer of immune serum. There is no latent period as present in active immunity (Table 11.1). Passive immunity is short lasting but is useful when immunity is required immediately.

Natural

It is transferred from the mother to foetus or infant. Transfer of maternal antibodies to foetus transplacentally and to infant through milk (colostrum) protects them till their own immune system matures to function.

Artificial

It is through parenteral administration of antibodies. The agents used for artificial passive immunity are hyperimmune sera of animal or human origin, convalescent sera and pooled human gammaglobulin. The oldest method is to employ horse hyperimmune sera. Antitetanus serum (ATS) is prepared by injecting a

Table 11.1 Differentiating Features of Active and Passive Immunity

	Active Immunity	Passive Immunity
1.	Produced actively by the immune system.	Received passively by the host. The host's immune system does not participate.
2.	Induced by infection or by contact with immunogens.	Conferred by administration of ready-made antibodies.
3.	Long-lasting and effective protection.	Protection short lived and less effective.
4.	Immunity is effective only after a lag period i.e. time required for generation of antibodies.	Immunity is effective immediately.
5.	Immunological memory present, therefore, subsequent challenge is more effective (booster effect).	No immunological memory. Subsequent administration of antibody is less effective due to immune elimination.
6.	Negative phase may occur. This is due to antigen combining with already existing antibody and lowering its level.	No negative phase.
7.	Not applicable in immunodeficient persons.	Applicable in immunodeficient persons.
8.	Used for prophylaxis to increase body resistance e.g. BCG vaccine.	Used for treatment of acute infections.

series of doses of tetanus toxoid to horses, and bleeding them to separate the serum. As ATS is a foreign protein, it is liable to cause hypersensitivity reactions. To eliminate these complications, human ATS is employed. This is prepared by hyperimmunisation of human volunteers with tetanus toxoid. Protection with human ATS lasts longer as there is no immune elimination of the human globulins.

Convalescent sera (sera of patients recovering from infectious diseases) contain high levels of specific antibody and therefore is employed for passive immunisation against measles and rubella. Sera of healthy adults can be pooled and is used for passive immunisation against common infectious diseases prevalent in the region. Pooled human gammaglobulin are used for passive immunisation against some viral infections e.g. hepatitis A. It has to be ensured that all preparations from human sera should be free from the risk of infections with hepatitis B, hepatitis C, hepatitis D, HIV and other viruses.

Uses of Passive Immunisation

(*i*) To provide immediate short term protection in a nonimmune host, faced with the threat of a serious infection.

(*ii*) For suppression of active immunity which may be injurious. Example is to use Rh immunoglobulins during delivery to prevent immune response to Rh factor in Rh negative mothers with Rh positive babies.

(*iii*) For treatment of serious infections.

III. MISCELLANEOUS

A. Combined Immunisation

A combination of active and passive immunisation is employed simultaneously which is known as combined immunisation. Passive immunity provides the protection necessary till the active immunity becomes effective.

B. Adoptive Immunity

Injection of immunologically competent lymphocytes is known as adoptive immunity. Instead of whole lymphocytes, an extract of immunologically competent lymphocytes can be used. This is being attempted in therapy of lepromatous leprosy. Such lymphocytes are collectively known as *transfer factor*.

C. Local Immunity

Natural infection or the live viral vaccine administered orally or intranasally provides local immunity at the site of entry such as gut mucosa and nasal mucosa respectively. A special class of immunoglobulins (IgA) plays an important role in local immunity. One type of IgA called secretory IgA is produced locally by plasma cells.

D. Herd Immunity

It refers to the overall resistance in a community. When herd immunity is low, chances of epidemics increase on introduction of a suitable pathogen. Eradication of any communicable disease depends on development of a high level of herd immunity rather than of immunity in individuals.

KEY POINTS

1. The *Immunity* is defined as resistance exhibited by the host against any foreign antigen including microorganisms.
2. Immunity may be *innate* or *acquired*.
3. Acquired immunity is of two types, *active* and *passive*.
4. Active immunity is subdivided into two types, *natural* and *artificial*.
5. Artificial active immunity may be produced by vaccination.
6. Passive immunity is also subdivided into two types, *natural* and *artificial*.
7. Artificial passive immunity is through parenteral administration of *antibodies* (antiserum).
8. *Herd immunity* refers to the overall resistance in the community.

YOU MUST KNOW

1. Innate immunity, acquired immunity, herd immunity and their role.
2. Differences between active and passive immunity.

STUDY QUESTIONS

1. Discuss the mechanisms of innate immunity.
2. Tabulate the differences between active and passive immunity.
3. Write short notes on:
   (a) Innate immunity (b) Active immunity (c) Passive immunity (d) Herd immunity.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Clinical or subclinical infections lead to
   (a) Active natural immunity (b) Passive natural immunity
   (c) Active artificial immunity (d) Passive artificial immunity
2. Vaccination induces:
   (a) Active natural immunity (b) Passive natural immunity
   (c) Active artificial immunity (d) Passive artificial immunity
3. Artificial passive immunity may be induced by:
   (a) Antiserum injection (b) Vaccination
   (c) Maternal antibodies (d) All of the above
4. All of the following are live vaccines except:
   (a) BCG (b) Sabin vaccine
   (c) MMR (d) TAB vaccine
5. All of the following are killed vaccines except:
   (a) Salk vaccine (b) Hepatitis B vaccine
   (c) Non-neural vaccines for rabies (d) MMR

Answers (MCQs):

1. (a) 2. (c) 3. (a) 4. (d) 5. (d)

Chapter 12

ANTIGEN

An antigen is a substance which, when introduced into a body evokes immune response to produce a specific antibody with which it reacts in an observable manner.

I. TYPES OF ANTIGEN

They may be classified as

A. Complete Antigen
B. Haptens/Incomplete Antigen: Complex haptens; Simple haptens.

A. Complete Antigen

These are substances which can induce antibody formation by themselves and can react specifically with these antibodies.

B. Haptens

Haptens are substances unable to induce antibody formation on its own but can become immunogenic (capable of inducing antibodies) when covalently linked to proteins, called *carrier proteins.* However, haptens can react specifically with induced antibodies. These antibodies are produced not only against the hapten but also against the carrier protein.

1. *Complex haptens* can combine with specific antibodies to form precipitates e.g. capsular polysaccharide of pneumococci.
2. *Simple haptens* combine with specific antibodies but no precipitate is produced. This is due to univalent character of simple haptens, whereas complex haptens are polyvalent. It is assumed that precipitation occurs when antigen has two or more antibody combining sites.

Proantigens are low molecular weight substances which do not induce antibody formation but can cause delayed hyper-sensitivity reaction e.g. dinitrochlorobenzene (DNCB).

Antigenic determinant (epitope) is the smallest unit of antigenicity. Antigen possesses a number of these determinants. Each type of determinant induces a specific antibody formation. The size of antigenic determinant is around 25-34A° and a molecular weight of about 400-1000. The combining site on the antibody molecule, corresponding to the epitope, is called the *paratope*.

II. FACTORS OF ANTIGENICITY

The exact basis of antigenicity is not clear but a number of factors have been implicated which make a substance antigenic. These factors are:

1. Foreignness
2. Size
3. Chemical nature
4. Susceptibility to tissue enzymes
5. Antigenic specificity
6. Species specificity
7. Isospecificity

8. Autospecificity
9. Organ specificity
10. Heterophile specificity.

1. *Foreignness*

An antigen must be foreign to the individual (non-self) to induce an immune response. The immune system does not normally mount an immune response against his own antigens (self). This tolerance to self antigens is due to continuous contact with them during the developmental stages of immune system. However, breakdown of this mechanism results in autoimmune disease.

The antigenicity of a substance is related to degree of its nonself nature. The more foreign a substance, it is likely to be more powerful antigen. Antigens from other individuals of the same species are less antigenic as compared to those from other species.

2. *Size*

Larger molecules are highly antigenic whereas low molecular weight (less than 10,000) are either nonantigenic or weakly antigenic. Substance with low molecular weight may be rendered antigenic by adsorbing these on inert particles like bentonite or kaolin.

3. *Chemical Nature*

Most naturally occurring antigens are either proteins or polysaccharides. Proteins are more effective antigens. An exception is gelatin which is non-antigenic protein due to its low tyrosine (aromatic radical) content. The presence of aromatic radical is suggested as essential for antigenicity. Lipids and nucleic acids are less antigenic on their own but do so when combined with proteins.

4. *Susceptibility to Tissue Enzymes*

Substances which can be metabolised and are susceptible to the action of tissue enzymes behave as antigens. The antigen introduced into the body is degraded by the enzymes of the phagocytic cells into fragments of appropriate size containing antigenic determinants. The hidden antigenic determinants can be identified only when molecule is broken down. Bovine serum albumin (BSA) has more than 18 determinants although only six of these are exposed in the intact (unbroken) molecule.

Substances unsusceptible to tissue enzymes such as polystyrene latex are not antigenic. Substances which cannot be metabolised in body are not antigenic. Synthetic polypeptides composed of D-aminoacids is one example.

5. *Antigenic Specificity*

Antigenic specificity depends on antigenic determinants. The position of antigenic determinant group in the antigen molecule is important for specificity. The differences in specificity are determined in compounds with the group attached at *ortho*, *meta* or *para* positions. Another factor is spatial configuration of the determinant group which makes differences in antigenic specificity of *dextro*, *laevo* and *meso* isomers of substances.

Antigenic specificity is not absolute. Cross reactions may occur between antigens which bear stereochemical similarities.

6. *Species Specificity*

Tissues of all individuals in a species possess species specific antigens. Some degree of cross reaction exists between antigens from related species. The species specificity has got phylogenetic relationship and has been of assistance in (i) tracing of evolutionary relationship (ii) forensic applications in the identification of species of blood and of seminal fluid.

7. *Isospecificity*

Isospecificity depends on isoantigens which may be found in some but not all members of a species. A species may be grouped according to the presence of different isoantigens in its members. These are genetically determined. Blood grouping is one example depending on human erythrocyte antigens (isoantigens). Blood groups are clinically very important in blood transfusion, in isoimmunisation during pregnancy and to provide valuable evidence in disputed paternity cases.

Histocompatibility antigens are associated with the plasma membrane of tissue cells. These are specific for each individual of a species. These are very important when some tissue is transplanted from one individual to another. Human leucocyte antigen (HLA) is the major histocompatibility antigen determining homograft rejection.

8. *Autospecificity*

Self antigens are generally nonantigenic, but there are exceptions. These exceptions are lens protein and sperm. These are not recognised as self antigens. Lens protein normally confined within its capsule doesn't come in contact with immune system, therefore, it is not recognised. Similarly, antigens (sperm) that are absent during the embryonic life and develop later, are also not recognised by immune system. When these antigens are released into tissues following injury, antibodies are produced against them. Mechanism in pathogenesis

of autoimmune diseases is also similar. The antigenic specificity of self antigens may be modified as a result of infection or irradiation and may thus act as nonself. The immune response against these modified antigens may lead to autoimmune disease.

9. *Organ Specificity*

Organ specific antigens are confined to a particular organ. Some organs like brain, kidney and lens protein of one species share specificity with that of another species. As brain specific antigens are shared by man and sheep, antirabies vaccines (sheep brain vaccines) may cause neuroparalytic complications in man. Antirabies vaccine (developed in sheep brain) contains large amount of partially denatured brain tissue. In man, the sheep brain tissue induces immune response causing damage to the recipient's nervous tissue.

10. *Heterophile Specificity*

The same or closely related antigens present in different tissues of more than one species are known as heterophile antigens. Antibodies to these antigens produced by one species cross react with antigens of other species. Examples include the following:

(i) Forssman Antigen

It is a lipoprotein-polysaccharide complex widely present in man, animals, birds and bacteria.

(ii) Weil-Felix Reaction

Serum of patient suffering from typhus fever (containing heterophile antibody) agglutinates strains of Proteus species (OX 19, OX 2 and OX K). This is due to presence of a hetrophile antigen on *Rickettsiae* causing typhus fever, shared by these proteus strains.

(iii) Paul-Bunnell Test

In infectious mononucleosis, heterophile antibodies appear in the serum of the patient. These antibodies agglutinate sheep erythrocytes because *Epstein Barr* virus (causative agent of infectious mononucleosis) shares antigen with sheep erythrocytes.

SUPERANTIGENS

Superantigens are molecules that can interact with antigen-presenting cells (APCs) and T-lymphocytes in a non-specific manner. These antigens do not involve the endocytic processing as required in typical antigen presentation. Interaction of superantigens with MHC class II molecules of the APC and the Vβ domain of the T-lymphocytes receptor activates a large number of T-lymphocytes than conventional antigens. This leads to massive cytokine expression and immuno-modulation. Staphylococcal enterotoxins, exfoliative toxin and some viral proteins are examples of superantigens.

KEY POINTS

1. An *antigen* is a substance which, when introduced into a body evokes immune response to produce a specific antibody with which it reacts in an observable manner.
2. Antigen may be classified as *complete antigen* and *hapten/incomplete antigen*.
3. *Heterophile antigens* are closely related antigens present in different tissues of more than one species. Antibodies to these antigens produced by one species cross react with antigens of other species.
4. *Weil-Felix reaction* and *Paul-Bunnell test* are based on the presence of heterophile antibodies.

YOU MUST KNOW

1. Types of antigen.
2. Forssmam antigen.
3. Superantigens.

STUDY QUESTIONS

1. What is an antigen? Discuss briefly about various factors of antigenicity.
2. Write short notes on: (a) Haptens (b) Heterophile antigens.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Weil-Felix reaction is a serological test based on:
   (a) Heterophile antigen
   (b) Sheep brain antigen
   (c) Specific antigen
   (d) None of the above
2. Paul-Bunnell test is based on sharing of antigens between:
   (a) Sheep erythrocytes and Epstein-Barr virus
   (b) Mycoplasma and human erythrocytes
   (c) Rickettsial antigens and Proteus antigens
   (d) None of the above

Answers (MCQs):

1. (a) 2. (a)

Chapter 13

ANTIBODIES—IMMUNOGLOBULINS

I. ANTIBODIES

Antibodies are substances which are formed in the serum and tissue fluids in response to an antigen and react with that antigen specifically and in some observable manner.

A. Properties of Antibodies

1. Chemical nature of antibodies is globulin and they are named as immunoglobulins. Serum globulins could be separated into pseudoglobulins (water soluble) and euglobulins (water insoluble). Most antibodies are found to be euglobulins. Immunoglobulins constitute about 20 to 25 per cent of the total serum proteins.
2. Based on sedimentation studies, most antibodies are sedimented at 7S (M.W. 150,000—180,000). Some heavier antibodies—19S globulins (M.W. about 900,000) were designated as M or macroglobulins.
3. Tiselius and Kabat (1938) showed that most serum antibodies on electrophoretic mobility, belong to gammaglobulins which is used now synonymously with antibody. Later, many antibodies were found to migrate as beta or alpha globulins.

The term 'Immunoglobulin' was proposed by expert committee of WHO in 1964. The name immunoglobulin (Ig) for antibody has been accepted internationally. Immunoglobulins are mainly synthesised by plasma cells. Immunoglobulin includes, besides antibody globulins, the abnormal myeloma proteins, the cryoglobulin and the macroglobulin. Thus, all immunoglobulins may not be antibodies but all antibodies are immunoglobulins.

B. Structure of Immunoglobulin

Porter, Edelman and Nisonoff (1959-64) developed a technique for cleavage of immunoglobulin molecule which has led to detailed structure of immunoglobulin.

An antibody molecule consists of two identical heavy and two identical light chains. The 'heavy' (H) chains are longer and 'light' (L) chains are shorter. Both types of chains are polypeptide in nature. The two heavy chains are held together by disulphide (S-S) bonds. Each light chain is also attached to heavy chain by disulphide bond (Fig. 13.1).

The H chains are structurally and antigenically distinct in different classes of immunoglobulins. Five different classes namely IgG, IgM, IgA, IgD, and IgE are designated depending on presence of heavy chain, gamma (γ), mu (μ), alpha (α), delta (δ) and epsilon (ε) respectively.

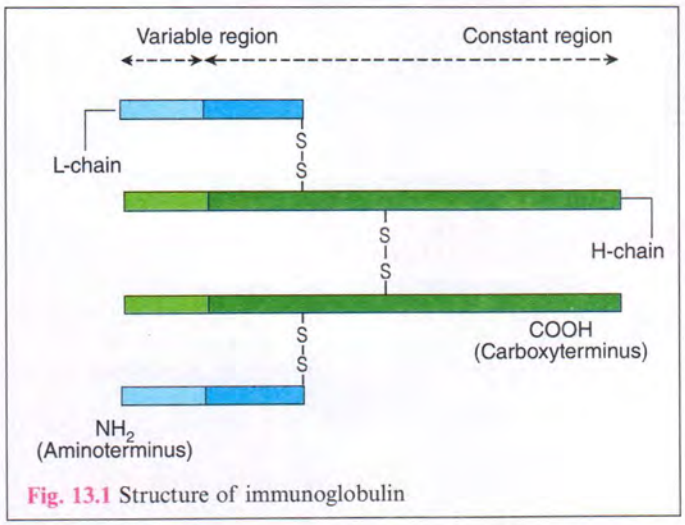


Fig. 13.1 Structure of immunoglobulin

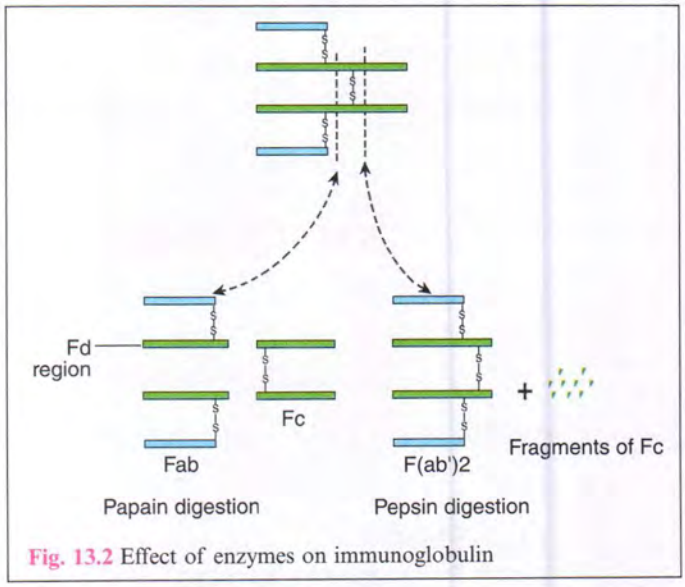


Fig. 13.2 Effect of enzymes on immunoglobulin

Class of Immunoglobulin	Heavy Chain
IgG	gamma (G)
IgM	mu (M)
IgA	alpha (A)
IgD	delta (D)
IgE	epsilon (E)

The L chains are similar in all classes of immunoglobulins. They are present in two forms kappa (K) and lambda (L). Each immunoglobulin has either two kappa or two lambda light chains but both (K & L) are never found together in a molecule. The light chains are named *kappa* and *lambda* after the investigators Korngold and Lapari. *Kappa* and *Lambda* chains are present in a ratio of 2:1 in human sera.

1. *Effect of Enzymes*

(*i*) *Papain Digestion (Fig. 13.2)*

Porter and colleagues split rabbit IgG antibody to egg albumin, by a proteolytic enzyme papain in the presence of cysteine. Papain can digest immunoglobulin molecule into three fragments one Fc fragment (Fc crystallisable) and two identical Fab (fragment antigen binding) fragments. Two Fab fragments possess the antigen binding sites but the Fc fragment lacks the ability to bind antigen. As the Fc fragment can be crystallized, it was so named.

Each Fab fragment consists of a light chain and a part of H chain. The portion of the heavy chain in Fab fragment is named Fd region. Fc fragment consists of parts of both H chains. It is composed of the carboxyterminal portion of the H chains. It determines the biological properties of the immunoglobulin molecule such as complement fixation, placental transfer, skin fixation and catabolic rate.

(*ii*) *Pepsin Digestion (Fig. 13.2)*

Pepsin cleaves at a different point of immunoglobulin molecule and gives rise to Fc portion and two Fab fragments held together in position. This Fab fragment is bivalent and can still precipitate with antigen. It is called $F(ab')_2$. Pepsin also degrades the Fc portion into smaller fragments.

2. *Aminoacid Sequences*

Each light chain contains 210-230 aminoacids whereas heavy chain has 420-460 aminoacids. Molecular weight of light chain is 25,000 and that of heavy chain is 50,000.

Both L and H chains consist of two portions each, a variable (V) region and a constant (C) region. In the L chain the two regions are of equal length while in the H chains the variable region constitutes approximately only a fifth of the chain. Variable (V) regions are present at aminoterminus (NH_2) and constant (C) region at carboxyterminus (COOH). Antigen combining site is at its aminoterminus which consists of both H and L chains (Fig. 13.1). The aminoacid sequence in the amino terminal half of the chain is highly variable, variability determines the immunological specificity of the antibody molecule. The infinite range of the antibody specificity of immunoglobulins is due to the 'variable regions' of the *H* and *L* chains. There are many hypervariable regions present in variable portions of both H and L chains and are involved with the formation of the antigen binding sites. The sites on the hypervariable regions which make actual contact with the epitope are called 'complementarity determining regions' or CDRs. There are three hypervariable regions in the L and four in the H chains.

3. *Immunoglobulin Domains (Fig. 13.3)*

Immunoglobulins are folded to form globular variable and constant domains. There are four domains in each heavy chain, one in variable region (VH) and three in constant region (CH1, CH2, CH3). There is one additional fourth domain on heavy chain (CH4) in IgM and IgE molecule. Light chain has one domain in variable region (VL) and one in constant region (CL) in all classes of immunoglobulin (IgG, IgA, IgD, IgM and IgE). Each domain has a separate function.

The variable region domains (VL and VH) are responsible for the formation of a specific antigen binding site. The constant region domains mediate the secondary biological functions. The area between CH1 and CH2 domain is hinge region where enzyme papain acts. The CH2 region in IgG binds Clq in the classical complement pathway, and the CH3 mediates adherence to the monocyte surface.

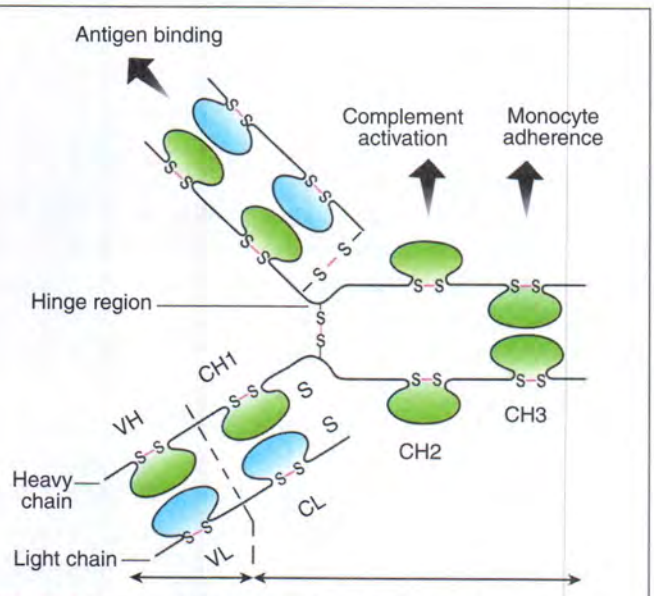


Fig. 13.3 Immunoglobulin domains (IgG molecule)

C. Immunoglobulin Antigen Determinants

Immunoglobulins are glycoproteins and can act as immunogens when inoculated into a foreign species. Differences in aminoacid sequences between different immunoglobulin classes, subclasses and types determine their antigenic specificity. There are three major types of immunoglobulin antigen determinants.

1. Isotypes
2. Allotypes
3. Idiotypes

1. *Isotypes*

These determinants are shared by all members of the same species. On the basis of isotypic markers on H chains, different classes of immunoglobulins are differentiated. Various H chain markers are gamma, mu, alpha, delta and epsilon in immunoglobulins IgG, IgM, IgA, IgD and IgE respectively. The light chains are also distinguished through isotypic markers into kappa and lambda.

2. *Allotypes*

These are individual specific determinants within a species. Allotype markers are also present on the constant regions of heavy and light chains. These markers are genetically determined. The allotype markers in humans are Gm on gamma heavy chains, Am on alpha heavy chains, and Km (originally designated as Inv) on kappa light chains. More than 25 Gm types, 3 Km allotypes and 2 Am on IgA have been identified. No allotypic markers have been found for lambda light chains or μ, δ or ε heavy chains. Allotype markers are useful in testing paternity.

3. *Idiotypes*

Idiotype markers are located in hypervariable regions of the immunoglobulin molecule. Idiotypes are specific for each antibody molecule. By immunisation with Fab fragments, antiidiotypic antibodies can be produced.

D. Immunoglobulin Classes

Characteristics of five immunoglobulins present in human sera are shown in Table 13.1.

1. *Immunoglobulin G (IgG) (Fig. 13.4)*

(*i*) IgG is the major serum immunoglobulin (about 80% of the total amount). The normal serum concentration is about 8–16 mg/ml.
(*ii*) Molecular weight is 150,000 (7S).
(*iii*) Half life is about 23 days (longest amongst all the immunoglobulins).
(*iv*) It is the only immunoglobulin that is transported through placenta and provides natural passive immunity to newborn.

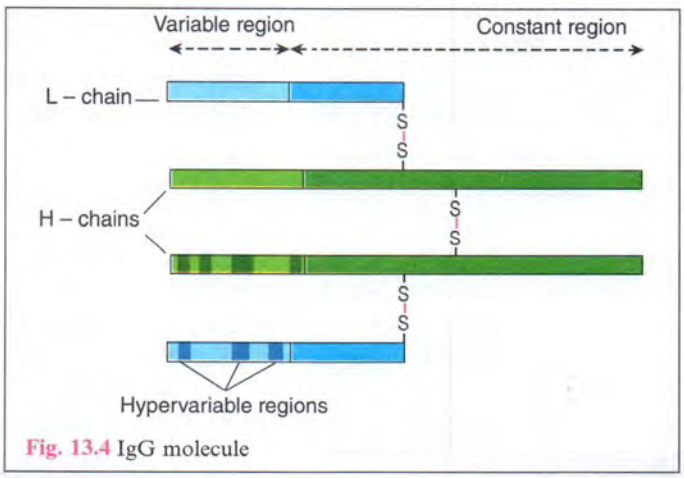


Fig. 13.4 IgG molecule

Table 13.1 Properties of Immunoglobulin Classes

Property	IgG	IgA	IgM	IgD	IgE
Molecular weight	150,000	160,000	900,000	180,000	190,000
Sedimentation coefficient(S)	7	7	19	7	8
Heavy chain	Gamma	Alpha	Mu	Delta	Epsilon
Light chain	K or L	K or L	K or L	K or L	K or L
Serum concentration (mg/ml)	12	2	1.2	0.03	0.00004
Placental transport	+	–	–	–	–
Half life	23 days	6–8 days	5 days	3 days	2–3 days
Intravascular distribution(%)	45	42	80	75	50
Present in milk	+	+	–	–	–
Complement fixation Classical Alternative	 ++ –	 – +	 +++ –	 – –	 – –
Selective secretion by seromucous glands	–	+	–	–	–
Heat stability (56°C)	+	+	+	+	–

(*v*) It is distributed equally between the intravascular and extravascular compartments.

(*vi*) IgG appears late but persists for longer period. It appears after the initial immune response which is IgM in nature.

(*vii*) It participates in precipitation, complement fixation and neutralisation of toxin and viruses.

(*viii*) IgG binds to microorganisms and enhances the process of phagocytosis.

(*ix*) Extracellular killing of target cells coated with IgG is due to recognition of Fc receptor of IgG by killer cells bearing the appropriate receptors.

(*x*) The catabolism of IgG is unique in that it depends on the serum IgG concentration. When level of IgG is raised, as in myeloma or kala-azar, the synthesis of IgG against that particular antigen is catabolised rapidly and may result in deficiency of that particular antibody. Conversely, in hypogammaglobulinaemia, IgG antibody given for therapeutic purpose will be catabolised slowly.

(*xi*) Passively administered IgG suppresses the homologous antibody synthesis by feed back mechanism. Based on this property, isoimmunisation of women is done by administration of anti-RhD IgG during delivery.

(*xii*) Four subclasses of IgG (IgG1, IgG2, IgG3, IgG4) have been recognised. These can be identified with specific antisera. Differences exist in the Fc fragment of the heavy chains of various subclasses. In human serum, these subclasses are distributed as IgG1 (65%), IgG2 (23%), IgG3 (8%) and IgG4 (4%).

(*xiii*) It is protective against those microorganisms which are active in the blood and tissues.

2. *Immunoglobulin A (IgA) (Fig. 13.5)*

(*i*) IgA is the second major serum immunoglobulin (about 10-13% of serum immunoglobulins). The normal serum concentration is 0.6 – 4.2 mg/ml.

(*ii*) Half life is about 6 – 8 days.

(*iii*) IgA occurs in two forms, serum IgA and secretory IgA.

(*iv*) Serum IgA is a monomeric 7S molecule (MW 160,000), while IgA found on mucosal surfaces and in secretions (secretory IgA, MW 400,000) is a dimer formed by two monomer units joined together by a glycoprotein named the *J chain* (J for joining). The J chain joins the two monomer units at their carboxyterminals. Secretory IgA and J chain are produced by the plasma cells situated near the mucosal or glandular epithelium. J chains are also present in other polymeric immunoglobulins such as IgM.

(*v*) Secretory IgA contains another glycine rich polypeptide called the secretory piece or secretory

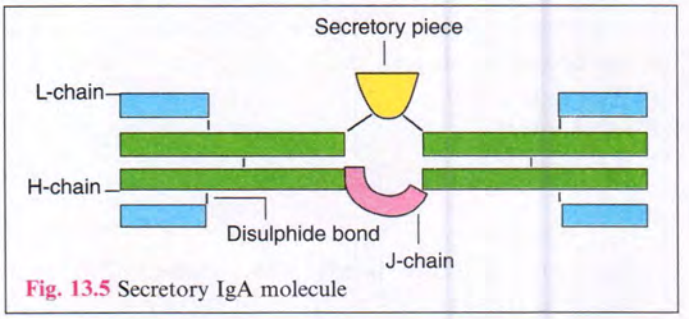


Fig. 13.5 Secretory IgA molecule

component. The S piece is not synthesised by lymphoid cells but by mucosal or glandular epithelial cells. It attaches two IgA molecules at their Fc portion during transport across the cells. The S piece is believed to protect IgA from denaturation by bacterial proteases in sites such as the intestinal mucosa which is rich in bacterial flora.

(*vi*) IgA is the principal immunoglobulin present in secretions such as milk, saliva, tears, sweat, nasal fluids, colostrum and in secretions of respiratory, intestinal and genital systems. It protects the mucous membranes against microorganisms. It forms antibody paste on mucosa. IgA covers the microorganisms to inhibit their adherence to mucosal surfaces.

(*vii*) IgA does not fix complement but can activate alternative complement pathway.

(*viii*) IgA is mainly synthesised locally by plasma cells and little is derived from serum.

(*ix*) Two subclasses of IgA (IgA1 and IgA2) are known. IgA2 is predominant (60%) in the secretions but constitutes a minor part of serum IgA. IgA2 is devoid of interchain disulphide bonds between H and L chains.

3. *Immunoglobulin M (IgM) (Fig. 13.6)*

(*i*) IgM is a pentamer consisting of 5 immunoglobulin subunits and one molecule of J chain, which joins the Fc region of the basic subunits.

(*ii*) Each H chain of IgM has four CH domains rather than three as seen in H chain of IgG molecule.

(*iii*) It constitutes about 5–8 percent of total serum immunoglobulins. The normal level in serum is 0.5–2 mg/ml.

(*iv*) Half life is about five days.

(*v*) It is heavy molecule (19S) with a molecular weight 900,000 to 1,000,000 hence also called the 'millionaire molecule'.

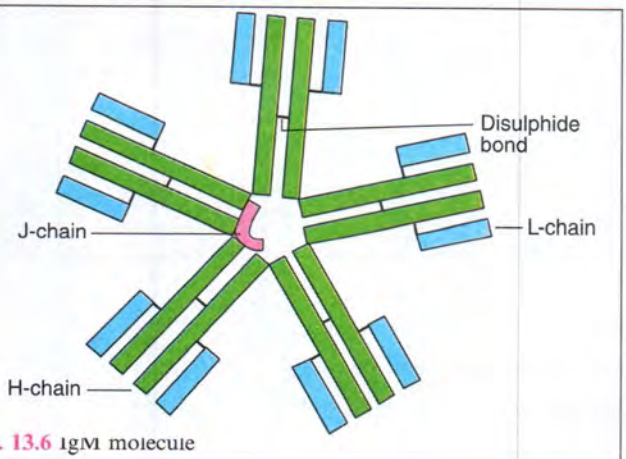


Fig. 13.6 IgM molecule

(*vi*) IgM is mainly distributed intravascularly (80%).

(*vii*) It is the earliest synthesised immunoglobulin by foetus in about 20 weeks of age.

(*viii*) It appears early in response to infection before IgG. IgM antibodies are short lived, and disappear earlier than IgG. Hence, its presence in serum indicates recent infection.

(*ix*) It can not cross the placenta, presence of IgM antibody in serum of newborn indicates congenital infection. Its detection is, therefore, useful for the diagnosis of congenital syphilis, HIV infection, toxoplasmosis and rubella.

(*x*) The valency of IgM is 10 which is observed on interactions with small haptens only. With larger antigens, the effective valency is 5.

(*xi*) It is very effective antibody in agglutination, complement fixation, opsonisation and immune haemolysis. It is more efficient than IgG in these reactions. The greater efficacy is due to the binding of complement to several Fc regions of pentameric IgM simultaneously, thus initiating complement cascade and target cell lysis with a single molecule.

(*xii*) IgM provides protection against blood invasion by microorganisms. Its deficiency is often associated with septicaemia.

(*xiii*) The isohaemagglutinins (Anti-A, Anti-B), antibodies to typhoid 'O' antigen (endotoxin), rheumatoid factor and W.R. antibodies in syphilis belong to IgM.

(*xiv*) IgM (monomeric) appears on the surface of unstimulated B lymphocytes and acts as recognition receptors for antigens.

(*xv*) Treatment of serum with 2-mercaptoethanol selectively destroys IgM without affecting IgG antibodies. This method is utilised for the differential estimation of IgG and IgM antibodies.

(*xvi*) Two subclasses (IgM1 and IgM2) of IgM are described. These are differentiated by characteristic H chains i.e. μ_1 and μ_2 H chains.

4. *Immunoglobulin D (IgD)*

(*i*) IgD resembles IgG structurally.

(*ii*) IgD is present in a concentration of 3 mg per 100 ml in serum. It is mostly intravascular in distribution.

(*iii*) Molecular weight is 1,80,000 (7 S monomer).

(*iv*) Half life is about three days.

(*v*) Like IgM, it is also present on the surface of unstimulated B lymphocytes in blood and acts as recognition receptors for antigens. Combination of cell membrane bound IgD with the corresponding

antigen leads to specific stimulation of these B lymphocytes—either activation and cloning to form antibody, or suppression.

(*vi*) Two subclasses (IgD1 and IgD2) of IgD are known.

5. *Immunoglobulin E (IgE)*

(*i*) IgE is mainly produced in the linings of respiratory and intestinal tracts. Serum contains only traces (a few nanograms per ml). It is mostly distributed extravascularly.

(*ii*) It is also referred to as reagins.

(*iii*) Molecular weight is 1,90,000 (8S molecule).

(*iv*) Half life is 2-3 days.

(*v*) It resembles IgG in structure.

(*vi*) It is heat labile (inactivated at 56°C in one hour) whereas other immunoglobulins are heat stable.

(*vii*) It has affinity for surface of tissue cells, particularly mast cells of the same species (homocytotropism).

(*viii*) IgE mediates type I hypersensitivity (atopic) reaction. This is responsible for asthma, hay fever, eczema and Prausnitz–Kustner (PK) reaction.

(*ix*) High level of IgE in serum is also seen in children with a high load of intestinal parasitism.

(*x*) It cannot cross the placental barrier or fix the complement.

(*xi*) IgE is responsible for anaphylactic type of reaction.

(*xii*) It may play some unidentified role in defence against intestinal parasitic infections.

Role of Different Immunoglobulin Classes

IgG – protects the body fluids
IgA – protects the body surfaces
IgM – protects the blood stream
IgE – mediates reaginic hypersensitivity
IgD – recognition molecule on the surface of B lymphocytes

II. ABNORMAL IMMUNOGLOBULINS

Apart from antibodies, other structurally similar proteins may be found in serum in following pathological conditions.

A. Multiple myeloma
B. Heavy chain disease
C. Cryoglobulinaemia

A. Multiple Myeloma

It is a plasma cell dyscrasia in which unchecked proliferation of one clone of plasma cells occur, resulting in the excessive production of the particular immunoglobulin synthesised by the clone. Such immunoglobulins synthesised from one clone of cells are called monoclonal. Multiple myeloma may involve plasma cells synthesising any of the five classes of immunoglobulins, IgG, IgA, IgD, IgM or IgE. About 50–60% cases are of IgG type. Myeloma involving IgM producing plasma cells is named as Waldenstrom's macroglobulinaemia.

Apart from excessive production of respective myeloma proteins (M proteins), light chains of immunoglobulins may occur as kappa or lambda chains in the urine of 50% of myeloma patients. In any one particular patient, light chain is either kappa or lambda only and never both. These light chains are called Bence-Jones (BJ) proteins, described by Bence-Jones in 1847. BJ proteins in urine have a peculiar property of coagulating when heated to 60°C but redissolves at 80°C.

B. Heavy Chain Disease

Abnormal heavy chains are produced in excess. This is due to lymphoid neoplasia.

C. Cryoglobulinaemia

It is a condition in which there is a formation of precipitate on cooling the serum, the precipitate redissolves on warming. This is due to presence of cryoglobulins in blood. Cryoglobulinaemia is often found in macroglobulinaemia, systemic lupus erythematosus (SLE) and myelomas. Most cryoglobulins consist of either IgG, IgM or their mixed precipitates.

KEY POINTS

1. *Antibodies* are substances which are formed in the serum and tissue fluids in response to an antigen and react with that antigen specifically and in some observable manner.
2. *Chemical nature* of antibodies is globulin and they are named as *immunoglobulins*.
3. An antibody molecule consists of two identical *heavy* and two identical *light* chains.
4. There are five different classes of immunoglobulins namely *IgG*, *IgM*, *IgA*, *IgD* and *IgE*.

5. IgG is the major serum immunoglobulin (about 80% of the total amount). It is the only immunoglobulin that is *transported through placenta.*
6. IgA is the second major serum immunoglobulin (about 10-13% of serum immunoglobulins). It occurs in two forms, *serum IgA* and *secretory IgA.*
7. Serum IgA is a *monomeric* while secretory IgA (found in mucosal surfaces and in secretions) is a *dimer* formed by two monomer units joined together by a glycoprotein named the *J chain* (J for joining).
8. IgA is the principal immunoglobulin *present in secretions* such as milk, saliva, tears, sweat, nasal fluids, colostrum and in secretions of respiratory, intestinal and genital systems.
9. IgM is a pentamer consisting of 5 immunoglobilin subunits and one molecule of J chain, which joins the basic subunits. It constitutes about 5-8 per cent of total serum immunoglobulins. It is heavy molecule with a molecular weight 900,000 to 1,000,000 hence aslo called the '*millionaire molecule*'.
10. IgM is mainly distributed *intravascularly* (80%). It appears early in response to infection before IgG. It can not cross the placenta, presence of IgM antibody in serum of newborn indicates *congenital infection.*
11. IgE is mainly produced in the linings of respirtory and intestinal tracts. Serum contains only traces (a few nanograms per ml). It is mostly distributed *extravascularly*. It is *heat labile* whereas other immunoglobulins are heat stable.
12. IgE mediates *type I hypersensitivity reactions.*

YOU MUST KNOW

1. Definition of antibody and structure of immunoglobulin.
2. Properties of immunoglobulin classes, i.e., IgG, IgA, IgM, IgD, IgE.
3. Diagrams of structure of IgM molecule and secretory IgA molecule.

STUDY QUESTIONS

1. Name the various classes of immunoglobulins. Describe the structure and properties of IgG, IgA and IgM.
2. Write short notes on:
   (a) IgM (b) Secretory IgA (c) IgE

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Which immunoglobulin class can pass through placenta?
   (a) IgG (b) IgM (c) IgA (d) IgE
2. Which of the following immunoglobulins is heat labile?
   (a) IgG (b) IgM (c) IgA (d) IgE
3. Which immunoglobulin class is distributed maximum intravascularly?
   (a) IgG (b) IgM (c) IgA (d) IgE
4. J chain is present in:
   (a) IgG (b) IgM (c) IgD (d) IgE
5. Which is the first immunoglobulin to appear in response to an antigen?
   (a) IgG (b) IgM (c) IgA (d) IgE
6. Which immunoglobulin class has maximum concentration in the human body?
   (a) IgG (b) IgM (c) IgA (d) IgE
7. Presence of which immunoglobulin class in serum is a useful indicator for diagnosis of congenital infections?
   (a) IgG (b) IgM (c) IgA (d) IgE
8. Which is the earliest immunoglobulin to be synthesised by the foetus?
   (a) IgG (b) IgM (c) IgA (d) IgE

9. Which immunoglobulin class is involved in type I hypesensitivity reaction?
   (a) IgG (b) IgM (c) IgA (d) IgE
10. Which immunoglobulin class can bind to mast cells?
   (a) IgG (b) IgM (c) IgA (d) IgE

Answers (MCQs):

1. (a)	2. (d)	3. (b)	4. (b)	5. (b)
6. (a)	7. (b)	8. (b)	9. (d)	10. (d)

Chapter 14

ANTIGEN-ANTIBODY REACTIONS

I. Antigen-Antibody Reactions—General Features

A. Uses
B. Characteristics
C. Stages
D. Measurement

II. Types of Antigen-Antibody Reactions

A. Precipitation Reactions
B. Agglutination
C. Complement-Fixation Test (CFT)
D. Conglutination
E. Neutralisation Test
F. Opsonisation
G. Immunofluorescence
H. Radioimmunoassay (RIA)
I. Enzyme Linked Immunosorbent Assay (ELISA)
J. Immunochromatography
K. Chemiluminescence Assay
L. Immunoelectronmicroscopic Tests
M. Immunoblotting

I. ANTIGEN-ANTIBODY REACTIONS —GENERAL FEATURES

Antigen combines with its specific antibody in an observable manner and the reaction between antigen and antibody is specific. These antigen-antibody reactions *in vitro* are known as serological tests.

A. Uses

1. In the Body or in Vivo

(*i*) It forms the basis of immunity against infectious diseases.

(*ii*) It may lead to tissue injury in some hypersensitivity reactions and autoimmune diseases.

2. In the Laboratory or in Vitro

(*i*) For diagnosis of infections

(*ii*) Helpful in epidemiological studies

(*iii*) For identification of non-infectious agents such as enzymes

(*iv*) Detection and quantitation of either antigens or antibodies.

B. Characteristics

1. Reaction is specific; an antigen combines only with its homologous antibody and vice-versa. However, cross reactions may occur due to antigenic similarity.
2. Entire molecules of antigen and antibody react and not the fragments.
3. Only the surface antigens participate in the antigen-antibody reaction.
4. The reaction is firm, but reversible. The firmness of combination depends on the affinity and avidity. Affinity is defined as the intensity of attraction between antigen and antibody molecules. Avidity is the binding strength after the formation of antigen-antibody complexes.
5. Antigens and antibodies can combine in varying proportions. Antibodies are generally bivalent,

although IgM may have five or more combining sites. Antigens may have valencies upto hundreds.

C. Stages

The antigen-antibody reaction occurs in two stages:

1. The Primary Stage

The initial interaction between antigen-antibody is rapid but without any visible effect. This reaction is reversible. The binding between antigen and antibody occurs by the weaker intermolecular forces such as Vander Waal's forces, hydrogen bonds and ionic bonds, rather than by firmer covalent binding. This primary interaction can be detected by estimating free and bound antigen or antibody separately in the reaction by a number of methods.

2. The Secondary Stage

The primary stage in most of the instances, but not all, is followed by secondary stage which has visible effects such as precipitation, agglutination, complement fixation, neutralisation and immobilisation of motile organisms. When these reactions were discovered, it was believed that different type of antibody was involved for each type of reaction. On this basis, the antibody involved in agglutination was called agglutinin and those involved in precipitation as precipitin and the corresponding antigens were called agglutinogen and precipitinogen respectively. This concept was replaced by Zinsser's unitarian hypothesis (1920) which held that an antigen would induce synthesis of only one antibody which produces different types of visible (observable) effects. Both of these extreme views are not true. It is now an established fact that a single antibody can cause different types of antigen-antibody reactions and a single antigen can stimulate production of different classes of immunoglobulins which differ in their reaction capacities as well as in other properties (Table 14.1).

Table 14.1 Role of Immunoglobulin Classes in Serological Reactions

Serological reaction	Immunoglobulin class		
	IgG	IgM	IgA
Precipitation	*Strong*	Weak	Variable
Agglutination	Weak	*Strong*	Moderate
Complement fixation	*Strong*	Weak	Negative

D. Measurement

Measurement may be in terms of mass (e.g. mg Nitrogen) or more commonly as titre of units. The antibody titre is the highest dilution of the serum which shows an observable reaction in the particular antigen-antibody reaction. Antigens may also be titrated against sera.

II. TYPES OF ANTIGEN-ANTIBODY REACTIONS

A. Precipitation Reactions

Precipitation

When a soluble antigen reacts with its antibody in the presence of electrolytes (NaCl) at an optimal temperature and pH, the antigen-antibody complex forms an insoluble precipitate and it is called *precipitation*. The precipitate usually sediments at the bottom of the tube. Precipitation may occur in liquid media or in gels such as agar, agarose or polyacrylamide.

Flocculation

When instead of sedimenting, the precipitate is suspended as floccules, the reaction is called flocculation. This is a modified form of precipitation. A serological test (e.g. VDRL) routinely used for diagnosis of syphilis is one example of flocculation test.VDRL slide is used to perform 'Venereal disease research laboratory' (VDRL) test (Fig. 14.1).

Fig. 14.1 VDRL slide

Prozone Phenomenon

When same amount of antiserum is mixed with increasing quantities of antigen in different tubes, the precipitation takes place in one of the middle tubes, in which antigen and the antibody are present in optimal or equivalent proportion (Zone of equivalence). In the preceding tubes, antibody is in excess and in later tubes, in which antigen is in excess, the precipitation is weak or even absent (Fig. 14.2). Absence of precipitation (false negative) in the presence of excess antibodies is known as prozone phenomenon. This is relevant clinically as these sera should be diluted further to get the proper results of precipitation.

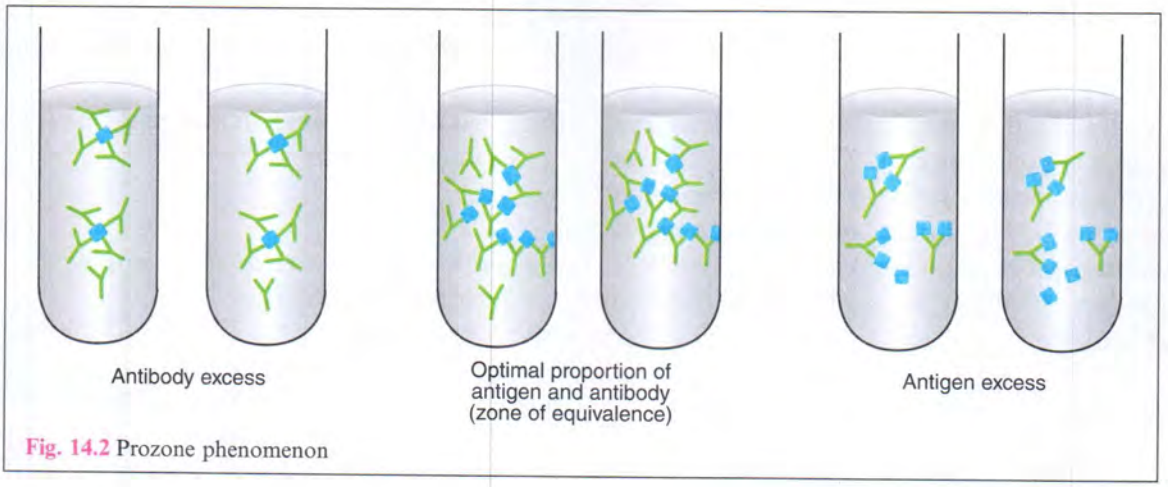


Fig. 14.2 Prozone phenomenon

(i) Mechanism of precipitation

Marrack (1934) proposed the lattice hypothesis which is widely accepted. According to this concept, multivalent antigens combine with bivalent antibodies, precipitation occurs only when a large lattice is formed. This occurs in the zone of equivalence. In the zone of antigen excess, the valencies of the antibody are fully satisfied which results in failure to form a large lattice. Similarly, in the zone of antibody excess, the valencies of the antigen are taken up with antibody and results in failure to form a large lattice (Fig. 14.3). This lattice hypothesis holds true for agglutination also.

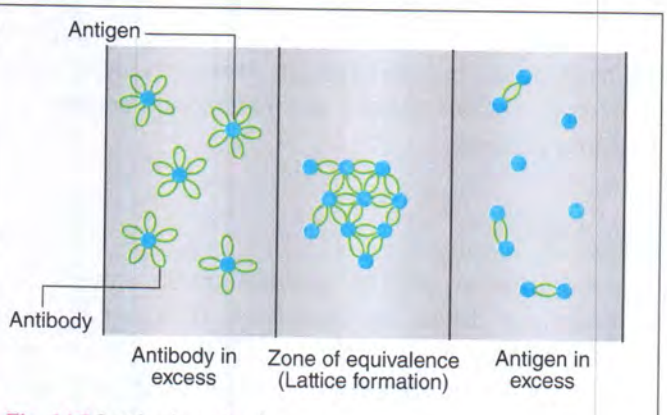


Fig. 14.3 Lattice hypothesis

(ii) Applications of precipitation reaction

Precipitation reaction may be used either as qualitative or quantitative test. It is very sensitive test and can detect as little as 1 µg of protein antigen. Precipitation test has a considerable value in:

1. Identification of bacteria e.g. Lancefield's grouping of *Streptococcus*.
2. Detection of antibody for diagnostic purposes e.g. VDRL in syphilis.
3. Forensic application in identification of human blood and seminal stains.
4. Testing for food adulterants.
5. To standardise toxins and antitoxins.

(iii) Types of precipitation reaction

(a) Ring test

Antigen solution is layered over an antiserum in a narrow tube. A precipitate ring appears at the junction of the two liquids. Examples of ring tests are C-reactive protein (CRP) test and streptococcal grouping by the Lancefield technique.

(b) Flocculation test

It can be done in slide and in tube.

Slide test: A drop of antigen solution is added to a drop of inactivated patient's serum on a slide and mixed by shaking, floccules appear. The VDRL test for detection of antibodies against syphilis is an example of slide flocculation test.

Tube test: The Kahn test (tube flocculation) was done previously for diagnosis of syphilis. Standardisation of toxins and toxoids is also done by tube flocculation test.

(c) Immunodiffusion test

These are precipitation tests in gel. Immunodiffusion is generally done in 1% agar gel. There are following advantages of precipitation in gel rather than in a liquid medium.

- The reacting band is easily visible and can be stained for preservation. This band is stable.
- The number of different antigens in the reaction can be observed. As each antigen-antibody reaction gives rise to a line of precipitation, therefore, it helps to identify different antigens.
- Identity, cross reaction and non identity between different antigens can also be observed by immunodiffusion.

Various Immunodiffusion Tests

1. Single diffusion in one dimension (Oudin procedure)

The antibody is mixed in agar gel in a test tube and antigen solution is poured over it. The antigen diffuses downward through the agar gel and a line of precipitation is formed (Fig. 14.4). The number of precipitate bands will indicate the number of different antigens present. It is single diffusion of antigen only and in one dimension i.e. towards antibody in agar gel.

2. Double diffusion in one dimension (Oakley-Fulthorpe procedure)

The antibody is incorporated in agar gel, above which is placed a column of plain agar. The antigen is poured on top of this plain agar. The antigen and antibody move towards each other through intervening column of plain agar and a precipitate band is formed where they meet in optimum concentration (Fig. 14.4).

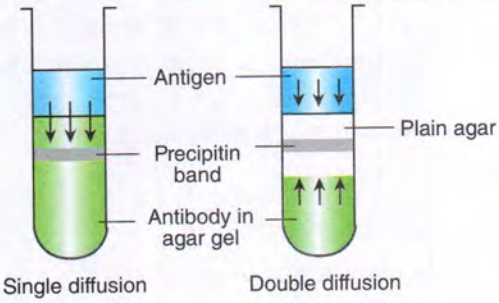


Fig. 14.4 Single and double diffusion in one dimension

3. Single diffusion in two dimensions (Radial immunodiffusion)

The antiserum (antibody) is incorporated in agar gel on a slide or petridish. The wells are cut on the surface of gel. Antigen is added to the wells. The antigen diffuses radially and ring-shaped bands of precipitation (halos) are formed around the well (Fig. 14.5a). The diameter of the ring is directly proportional to the concentration of the antigen.

Uses:

(*a*) It has been widely employed for estimation of immunoglobulin classes i.e. IgG, IgM, IgA, in sera.

(*b*) It has also been used for screening sera for antibodies to influenza viruses.

4. Double diffusion in two dimensions (Ouchterlony procedure)

Agar gel is poured on a slide. Wells are cut using a template. The antiserum (antibody) is placed in the central well and surrounding wells are filled with different antigens. If two adjacent antigens are identical, the lines of precipitate formed by them will fuse with each other. If they are unrelated, the lines cross each other. Spur formation indicates cross reaction or partial identity (Fig. 14.5b). A special variety of double diffusion in two dimensions is the Elek's test for toxigenicity in *C. diphtheriae*.

5. Immunoelectrophoresis

Immunoelectrophoresis combines electrophoresis and immunodiffusion. This is done on a glass slide layered with semisolid agar. A well is cut and antigen is filled. The first step is electrophoresis of antigen for about an hour. Rectangular trough is cut in the agar parallel to the direction of migration of antigen and filled with antibody (antiserum). Diffusion is allowed to proceed for 18-24 hours. Precipitation lines develop with each separated component of the antigen. By this technique, a number of antigens can be identified in human serum. It is particularly useful for detection of normal and abnormal serum proteins like myeloma proteins (Fig. 14.6).

6. Electroimmunodiffusion

Immunodiffusion can be speeded up if antigen and antibody are driven by electricity. It is combination of electrophoresis and diffusion. Of these methods, counterimmunoelectrophoresis (CIE or CIEP) and rocket electrophoresis are used frequently.

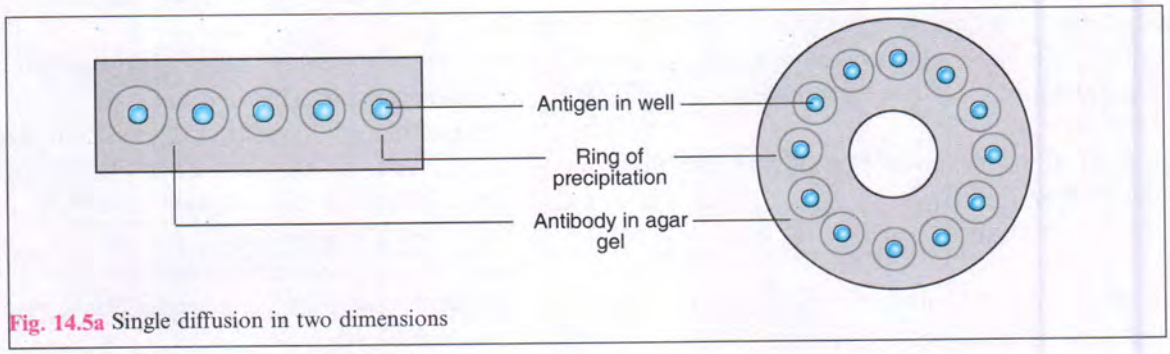


Fig. 14.5a Single diffusion in two dimensions

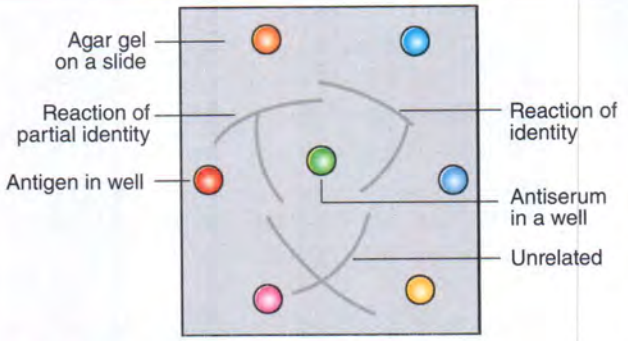


Fig. 14.5b Double diffusion in two dimensions

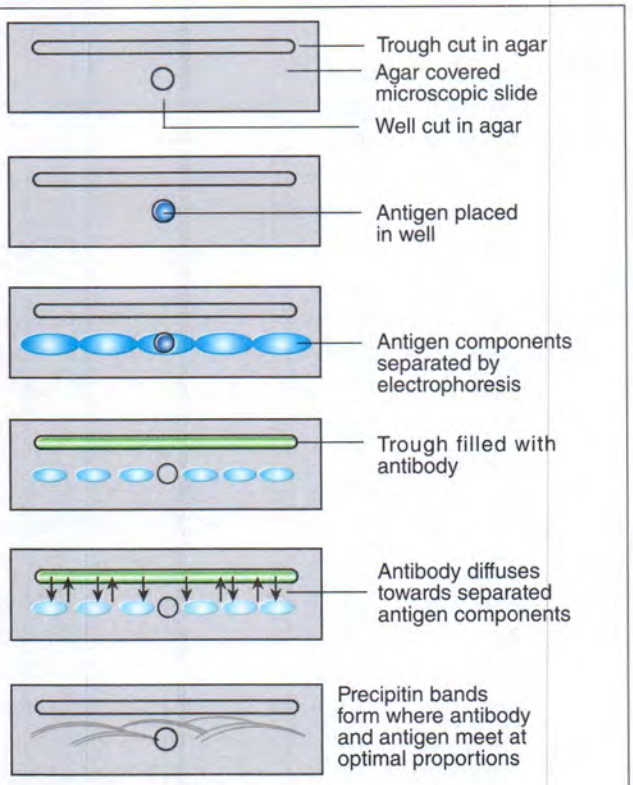


Fig. 14.6 Immunoelectrophoresis

(i) Counterimmunoelectrophoresis (Counter-current-immunoelectrophoresis, CIE or CIEP)

It is one dimensional double electro-immunodiffusion. This test is based on movement of antigen and antibody in opposite direction. This is performed on a glass slide layered with agarose and wells are cut on the surface. One well is filled with antigen and other with antibody. Electric current is passed through the gel. The antigen and antibody move towards each other resulting in a precipitation line at a point between them (Fig. 14.7). It takes about thirty minutes and is more sensitive than standard immunodiffusion technique. It is applied clinically for detecting hepatitis B antigens and antibodies, antigens of cryptococcus in cerebrospinal fluid and in number of other diseases.

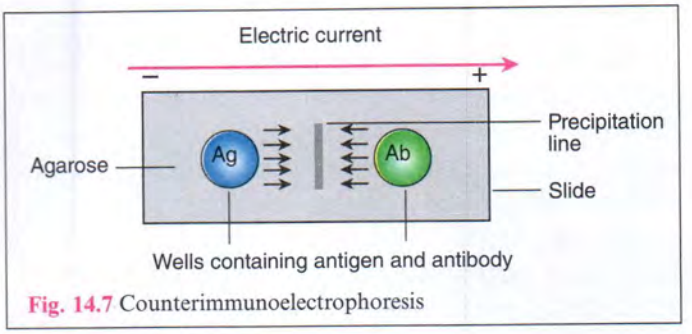


Fig. 14.7 Counterimmunoelectrophoresis

(ii) Rocket electrophoresis

It is one dimensional single electro-immunodiffusion. This technique is mainly applied for quantitation of antigens. The antiserum to the antigen to be quantitated is mixed in agarose and gelled on the glass slide. The wells are punched in the set gel and filled with increasing concentrations of the antigen. It is then electrophoresed. Precipitation is formed in the shape of cone like structures (appearance of a rocket). The length of these rocket like structures corresponds to the concentration of the antigen (Fig. 14.8).

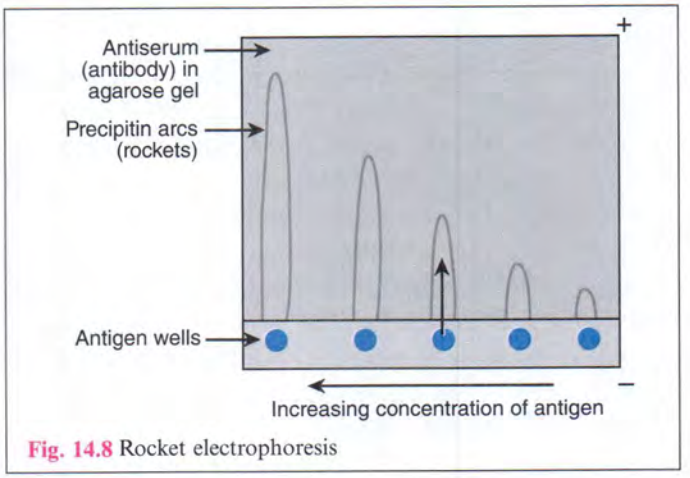


Fig. 14.8 Rocket electrophoresis

(iii) Laurell's two dimensional electrophoresis

It is a variant of rocket electrophoresis. By this method, several antigens in a mixture can be quantitated. In the first stage of the technique, the antigen mixture is electrophoretically separated and in second stage, electrophoresis is done perpendicular to that of first stage to get rocket like precipitation (Fig. 14.9).

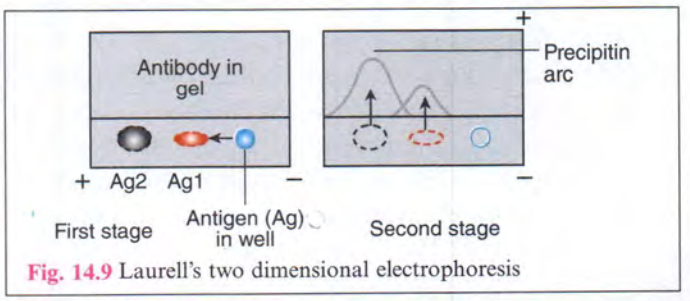


Fig. 14.9 Laurell's two dimensional electrophoresis

B. Agglutination

It is an antigen-antibody reaction, in which a particulate antigen combines with its antibody in the presence of electrolytes at an optimal temperature and pH, resulting in visible clumping of particles. It differs from precipitation in which soluble antigen is present in contrast to particulate antigen of agglutination. The agglutination is more sensitive than precipitation for the detection of antibodies. The agglutination reaction takes place better with IgM antibody than with IgG antibody. Principles governing agglutination are the same as that of precipitation. Agglutination occurs when antigen and antibody are present in optimal proportions. Lattice formation hypothesis holds good for agglutination too. The zone phenomenon may occur when either an antigen or an antibody is in excess. Occasionally incomplete antibodies (e.g. anti-Rh and anti-*Brucella*) are formed that combine with the antigen but do not cause agglutination. They act as 'blocking' antibodies, inhibiting the agglutination by the complete antibody added subsequently.

TYPES OF AGGLUTINATION REACTION

1. Slide Agglutination Test

A uniform suspension of antigen is made in a drop of saline on a slide or tile and a drop of the appropriate antiserum is added. The agglutination reaction is facilitated by mixing the antigen and the antiserum with a wire loop or by gently rocking the slide. Clumping occurs instantly or within seconds when agglutination test is positive. Clumping after a minute may be due to drying of the fluid and should be disregarded. A control consisting of antigen suspension in saline, without adding antiserum must be included on the same slide. It is to ensure that antigen is not autoagglutinable.

Uses:

(*i*) It is a routine procedure to identify the bacterial strains isolated from clinical specimens. One example is to identify Salmonella species.

(*ii*) It is also used for blood grouping and cross matching.

2. Tube Agglutination Test

This is a standard quantitative method for determination of antibodies. Serum is diluted serially by doubling dilution in test tubes. An equal volume of a particulate antigen is added to all tubes. The highest dilution of serum at which agglutination occurs is antibody titre. Tube agglutination is routinely employed for antibody detection in diagnosis of typhoid (Widal test), brucellosis and typhus fevers (Weil-Felix reaction).

Uses of tube agglutination test

(*i*) Serological diagnosis of
- (*a*) enteric fever (Widal test)
- (*b*) typhus fever (Weil-Felix reaction)
- (*c*) infectious mononucleosis (Paul-Bunnel test)
- (*d*) brucellosis

(*ii*) For diagnosis of primary atypical pneumonia (Streptococcus MG agglutination test)

Complications related to tube agglutination

Two main complications may be

(*i*) Prozone phenomenon

(*ii*) Blocking antibody

(i) Prozone phenomenon

Serum with high concentration of antibody may not be able to react with antigen and gives a false negative result. Several dilutions of the serum should be tested to prevent such false negative results due to prozone.

(ii) Blocking antibody

Blocking or incomplete antibodies may be detected by performing the test in hypertonic (5%) saline or albumin saline. Antiglobulin (Coombs) test is more reliable for detecting these antibodies.

3. The Antiglobulin (Coombs) Test

This test was devised originally by Coombs, Mourant and Race (1945) for the detection of incomplete anti-Rh antibodies. When sera containing incomplete anti-Rh antibodies are mixed with corresponding Rh-positive erythrocytes, the incomplete antibody globulin coats the erythrocytes but no agglutination occurs. When such coated erythrocytes are treated with antiglobulin or Coombs serum (rabbit antiserum against human gamma globulin), the cells are agglutinated. This is the principle of Coombs test (Fig. 14.10).

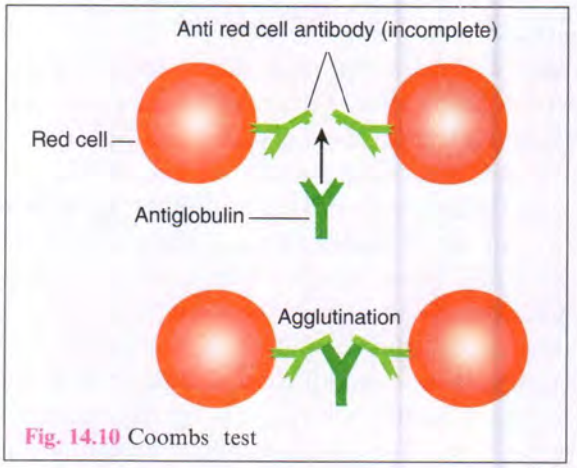


Fig. 14.10 Coombs test

There are two types of Coombs test:

(*i*) Direct Coombs test

(*ii*) Indirect Coombs test

The only difference between the two is that the sensitisation of the erythrocytes with incomplete antibodies takes place in vivo in direct type whereas it occurs in vitro in indirect type.

Uses of Coombs test

(*a*) For detection of anti Rh antibodies

(*b*) For demonstration of any type of incomplete antibody eg. brucellosis

4. Heterophile Agglutination Test

Heterophile antibodies have a property to react with microorganisms or cells of unrelated species due to common antigenic sharing.

(i) Weil-Felix reaction

Some Proteus (OX19, OX2, OXK) strains are agglutinated by sera of patients with rickettsial infections. This is due to antigenic sharing between these Proteus strains and Rickettsial species.

(ii) Paul-Bunnel test

Sheep erythrocytes are agglutinated by sera of infectious mononucleosis.

(iii) Streptococcus MG agglutination test

It is positive in primary atypical pneumonia.

5. Passive Agglutination Test

A precipitation reaction can be converted into agglutination test by attaching soluble antigens to the surface of carrier particles such as latex particles, bentonite and red blood cells. Such tests are called *passive agglutination* tests. This conversion is done because agglutination tests are more sensitive for detection of antibodies. Passive agglutination tests are very sensitive. When instead of antigen, the antibody is adsorbed on the carrier particles for estimation of antigens, it is known as *reversed passive agglutination*.

(i) Latex agglutination test

Polystyrene latex particles (0.8 – 1 μm in diameter) are widely employed to adsorb several types of antigens. Latex particles can also be coated with antibody for detection of antigen (Fig. 14.11). These tests are very convenient, rapid and specific. These are used for detection of hepatitis B antigen, ASO, CRP, RA factor, HCG, bacterial typing (*N. meningitidis*) and many other antigens. Latex agglutination tile is used to perform latex agglutination test (Fig. 14.12).

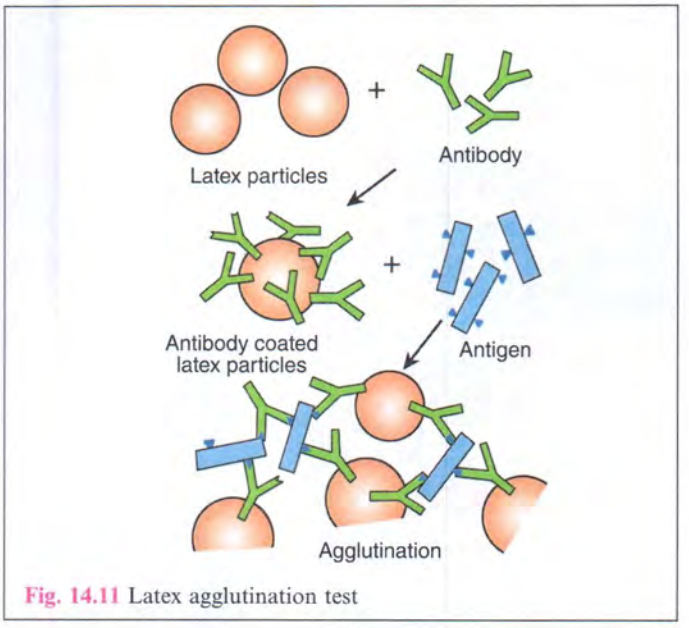


Fig. 14.11 Latex agglutination test

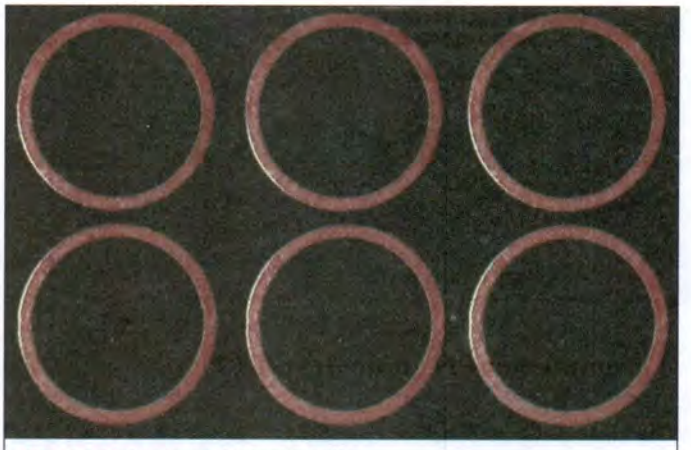


Fig. 14.12 Latex agglutination tile

(ii) Haemagglutination test

Erythrocytes sensitised with antigen are used for detection of antibodies. In rheumatoid arthritis, an autoantibody (RA factor) appears in the serum. This RA factor acts as an antibody to gammaglobulin. Thus, RA factor can agglutinate red cells coated with gammaglobulins. The antigen used for the test is sheep red blood cells sensitised with rabbit antisheep erythrocyte antibody (amboceptor). The amboceptor is antibody, therefore, gammaglobulin in nature. This is the principle of Rose-Waaler test employed for detection of RA factor in cases of rheumatoid arthritis.

(iii) Coagglutination

Principle

It is based on the presence of protein A on the surface of some strains of *Staph. aureus* (especially Cowan 1 strain). Specific IgG immunoglobulin is coated on these Cowan

1 strains of *Staphylococcus aureus*. Fc portion of IgG molecule binds to protein A whereas antigen combining Fab terminal remains free. When the corresponding antigen is mixed with these coated cells, Fab terminal binds to antigen resulting in agglutination (Fig. 14.13). This test is used for detection of bacterial antigens in blood, urine and CSF. *N. gonorrhoeae*, *Streptococcus pyogenes* and *H. influenzae* antigens can be detected by this method.

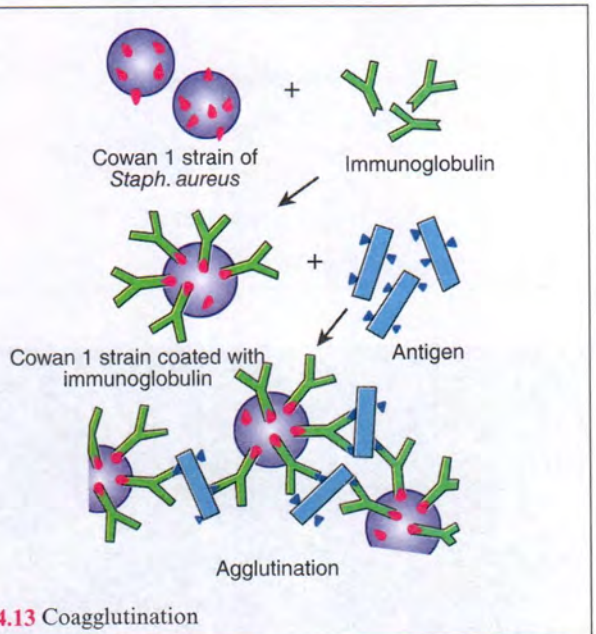


Fig. 14.13 Coagglutination

C. Complement Fixation Test (CFT)

1. Principle (Fig. 14.14)

The antigen-antibody complexes have ability to 'fix' complement. This reaction has no visible effect. To detect the fixation of complement, an indicator system consisting of sheep erythrocytes coated with amboceptor (rabbit antibody to sheep erythrocytes) is used. Complement can lyse these erythrocytes coated with antibodies. If complement is fixed and utilized in the antigen-antibody reaction, there is no free complement to act on the indicator system and hence no lysis of erythrocytes, which indicates the positive complement fixation test. Lysis of erythrocytes indicates that complement was not fixed in the first step and therefore, the serum is negative for antibodies (negative CFT).

2. Procedure

The serum (to be tested) should be inactivated by heating at 56°C for 30 minutes to destroy any complement activity the serum may have and to remove some non-specific inhibitors of complement. The antigen may be used as soluble or particulate. Fresh guinea pig serum is used as source of complement. Complement activity is heat labile so it is used as fresh. Alternatively, the serum is preserved either in the lyophilised or frozen state or with addition of special preservatives.

Controls should be included in the test. Antigen and serum controls are included to ensure that they are not anti-complementary. Complement control is used to ensure that the desired amount has been added, and cell control to make sure that sensitised erythrocytes (erythrocytes coated with amboceptor) do not undergo lysis in the absence of complement.

3. Standardisation of Complement

The guinea pig serum is titrated for complement activity. One unit of minimum haemolytic dose (MHD) of

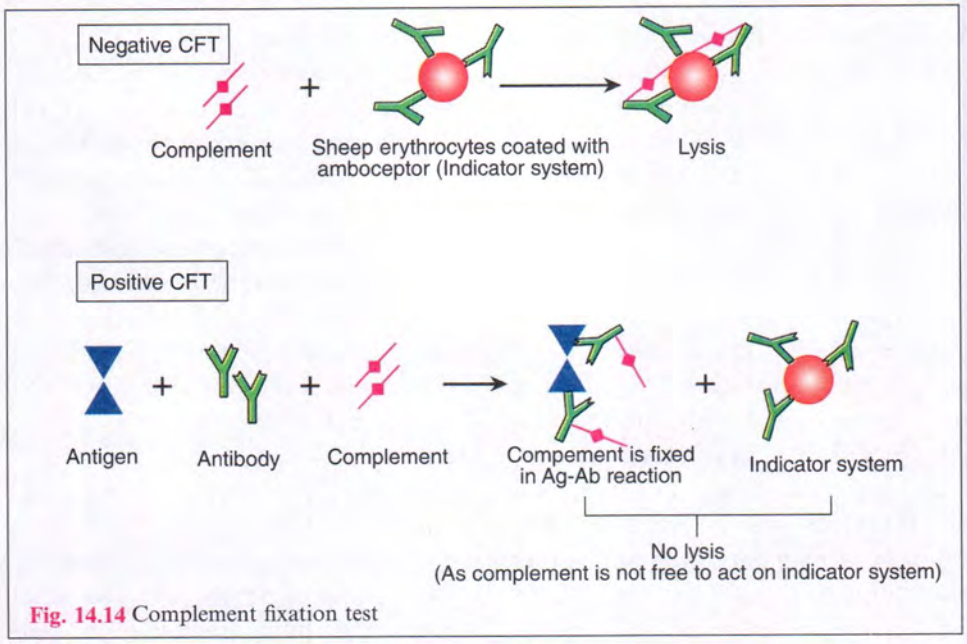


Fig. 14.14 Complement fixation test

complement is defined as the highest dilution (smallest quantity) of guineapig serum which lyses one unit volume of washed sheep red blood cells in the presence of excess haemolysin (amboceptor) within a fixed time (30 to 60 minutes) and at a fixed temperature (37°C).

4. Titration of Amboceptor

The amboceptor is titrated for haemolysin activity. One unit of minimum haemolytic dose (MHD) of haemolysin is defined as the highest dilution (smallest quantity) of the inactivated amboceptor which lyses one unit volume of washed sheep red blood cells in the presence of excess complement within a fixed time (30 to 60 minutes) and at a fixed temperature (37°C). Normal saline with added calcium and magnesium is used as diluent for titrations of complement, amboceptor and for CFT.

INDIRECT COMPLEMENT FIXATION TEST

Certain avian (e.g. duck, parrot) and mammalian (e.g. horse, cat) sera cannot fix guinea pig complement. Indirect complement fixation test is used for testing such sera. Test is done in duplicate and after the first step, the standard antiserum known to fix complement is added in one set.

First step: Antigen + test serum (negative for antibody) + guinea pig complement

Second step: Standard antiserum will react with antigen and fix the free complement.

Indicator system: No haemolysis because complement is not free to act on indicator system.

In a positive test, antigen would have been used up by serum in the first step and standard antiserum would not be able to fix the complement which remains free to act on indicator system resulting in haemolysis (positive result).

First step: Antigen + test serum (positive for antibody) + Guinea pig complement

Second step: Standard antiserum cannot react with antigen because antigen has been used up by antibody in the first step but complement is free as it is not fixed.

Indicator system: Haemolysis occurs because complement is free to act on the system.

D. Conglutination

This is an alternative method for systems which do not fix guinea pig complement. Horse complement (non-haemolytic) is used. The indicator system is sheep erythrocytes sensitised with bovine serum. Bovine serum contains a beta globulin component named conglutinin, which acts as antibody to complement. Conglutinin can cause agglutination of sensitised sheep erythrocytes if these are combined with complement, this process is called *conglutination*.

First step : Antigen + Antiserum (positive) + Horse complement → complement is fixed

Second step : Sheep erythrocytes with conglutinin → No agglutination occurs because complement is not free to act with sensitised erythrocytes.

No agglutination—Positive result

Agglutination—Negative result

OTHER COMPLEMENT DEPENDENT SEROLOGICAL TESTS

1. Immobilisation Test

When live motile *Treponema pallidum* is mixed with patient's serum in the presence of complement, the organism becomes non-motile. This is the principle of Treponema pallidum immobilisation test.

2. Immune Adherence

The antigen-antibody complexes in some bacteria (*V. cholerae, T. pallidum*) adhere to particulate material such as erythrocytes or platelets. This bacterial adherence to cells is known as *immune adherence*.

3. Cytolytic or Cytocidal Tests

When a live *Vibrio cholerae* is mixed with its antibody in the presence of complement, the bacterium is lysed. This forms the basis for measurement of anticholera antibodies.

E. Neutralisation Test

Bacterial exotoxins are capable of producing neutralising antibodies (antitoxins) which play a role in protection against diseases such as diphtheria and tetanus. The toxicity of bacterial endotoxins is not neutralised by antisera. Viruses may also be neutralised by their antibodies and these are named as virus neutralisation tests.

Toxin-antitoxin neutralisation can be measured *in vivo* and *in vitro*.

IN VIVO TESTS

1. Toxigenicity Test

Toxigenicity test is done for detection of toxin of *C. diphtheriae*. It is an intradermal test to inject bacterial toxin in animal previously protected by antitoxin of *C. diphtheriae* and a test animal without antitoxin. No biological effects of toxin are observed in the control animal but test animal (unprotected) dies.

2. Shick Test

This is similar kind of test in humans. Diphtheria toxin is injected intradermally in man, there is no reaction at the site of injection if person is immune to diphtheria ie. antitoxin is present in blood. Injected toxin is neutralised by circulating antitoxin.

IN VITRO TESTS

1. Antistreptolysin 'O' (ASO) Test

Serum of patients suffering from *Streptococcus pyogenes* infection contains antistreptolysin 'O' (antitoxin) which neutralizes the haemolytic activity of streptococcal 'O' haemolysin (toxin).

2. Virus Neutralisation Tests

Neutralisation of viruses can be demonstrated in cell cultures, eggs and animals It is used mostly in typing viral isolates.

3. Nagler Reaction

Clostridium welchii toxin (alphatoxin) is neutralised by antitoxin when the bacteria is grown in egg yolk medium containing antitoxin. It is useful for rapid detection of *C. welchii* in clinical specimens.

F. Opsonisation

Opsonisation is the process by which a particulate antigen becomes more susceptible to phagocytosis. This occurs by help of opsonin which combines with an antigen and facilitates phagocytosis. Opsonin may be an antibody-like substance or other component present in serum. *Opsonic index* is defined as ratio of phagocytic activity of the patient's blood for a given bacterium, to that of a normal individual. *Phagocytic index* is the average number of phagocytosed bacteria per polymorphonuclear leucocyte from stained blood films.Phagocytic index denotes the phagocytic activity of the blood and thus helps in measuring opsonic index.

G. Immunofluorescence

Fluorescence is the property of certain dyes which absorb rays of one particular wavelength (ultraviolet light) and emit rays with a different wavelength (visible light). Coons and his colleagues (1942) showed that fluorescent dyes can be conjugated to antibodies and these 'labelled' antibodies can be used to detect antigens in tissues. The commonly used fluorescent dyes are *fluorescin isothiocyanate* and *lissamine rhodamine*, exhibiting blue green and orange red fluorescence respectively. Immunofluorescence test is of two types:

1. Direct immunofluorescence test
2. Indirect immunofluorescence test

1. DIRECT IMMUNOFLUORESCENCE TEST

Principle

The specific antibodies tagged with fluorescent dye (i.e. labelled antibodies) are used for detection of unknown antigen in a specimen. If antigen is present, it reacts with labelled antibodies and fluorescence can be observed under ultraviolet light of fluorescent microscope (Fig. 14.15). This is depicted in flow diagram as follows:

Specimen (Positive for antigen)
+
Labelled antibodies
↓
Fluorescence observed
↓
Antigen is present in specimen

Uses

1. It is commonly employed for detection of bacteria, viruses or other antigens in blood, CSF, urine, faeces, tissues and other specimens.
2. It is a sensitive method to diagnose rabies, by detection of the rabies virus antigens in brain smears.

Disadvantage

Separate specific fluorescent labelled antibody has to be prepared against each antigen to be tested.

2. INDIRECT IMMUNOFLUORESCENCE TEST

The indirect method is employed for detection of antibodies in serum or other body fluids.

Principle

A known antigen is fixed on a slide. The unknown antibody (serum) is applied to the slide. If antibody (globulin) is present in the serum, it attaches to known antigen on the slide. For detection of this antigen-antibody reaction, fluorescin-tagged antibody to human globulin is added. In positive test, fluorescence occurs under ultraviolet light (Fig. 14.15). One specific example of detecting antibodies in serum of syphilis patient is also included.

Advantages

A single antihuman globulin fluorescent conjugate can be employed for detection of antibody to any antigen. All antibodies are globulin in nature, therefore, antihuman globulin attaches to all antibodies. This has overcome the disadvantage of direct immunofluorescence test.

SANDWICH' TECHNIQUE OF IMMUNOFLUORESCENCE

Antigen being in the middle with labelled and unlabelled antibody on either side, forms a sandwich. This is used

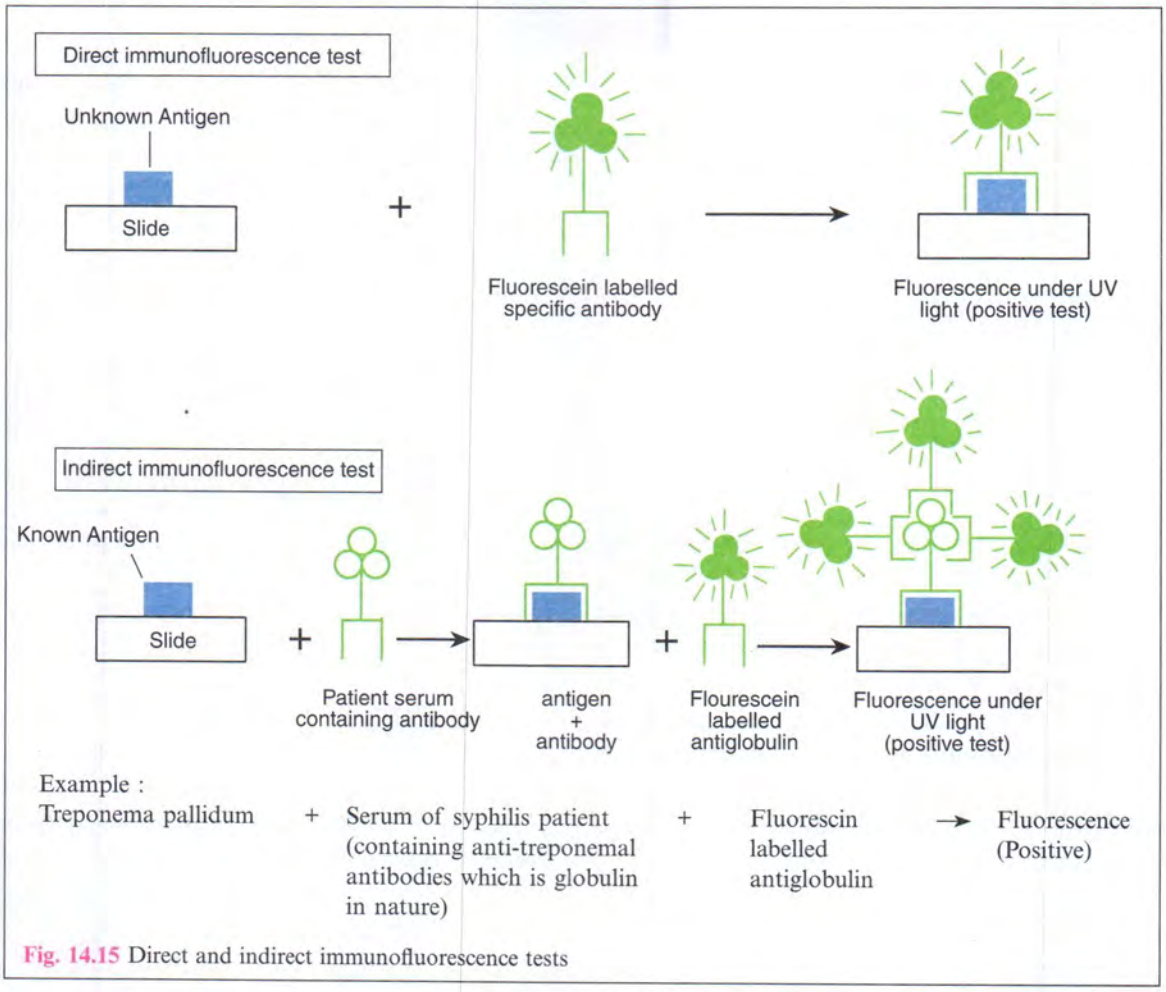


Fig. 14.15 Direct and indirect immunofluorescence tests

for detection of antibodies.

Antibody

+

Antigen

+

Labelled antibody

H. Radioimmunoassay (RIA)

Berson and Yallow (1959) first described the test radioimmunoassay (RIA) and since then it has been utilised for quantitation of hormones, drugs, hepatitis B surface antigen, IgE and viral antigens. In 1977, Yallow was awarded the Nobel Prize for the discovery of RIA test. This test can detect antigens up to picogram (10^{-12}g) quantities.

RIA is based on competition for a fixed amounts of specific antibody between a known radiolabelled antigen and unknown unlabelled (test) antigen (Fig. 14.16). This competition is determined by the level of the test antigen present in the reacting system. After antigen-antibody reaction, the antigen is separated into the 'free' and 'bound' fractions and their radioactivity is measured. The concentration of test (unlabelled) antigen is calculated from the ratio of the bound and total antigen labels, using a reference curve (Fig. 14.17).

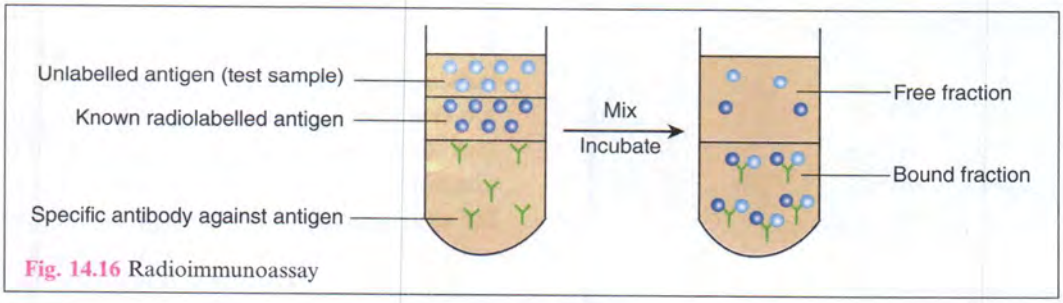


Fig. 14.16 Radioimmunoassay

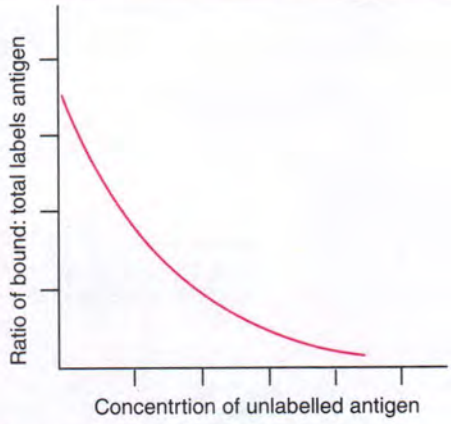


Fig. 14.17 Reference curve

The standard dose response or reference curve has to be prepared for calculations. This is done by running the reaction with fixed amounts of antibody and labelled antigen but with varying known amounts of unlabelled antigen. The ratio of bound: total labels (B:T ratio) plotted against the unlabelled antigen concentrations gives the standard reference curve. The concentration of antigen in the test sample is calculated with the help of B:T ratio of the test by using standard dose response or reference curve.

I. Enzyme Linked Immunosorbent Assay (ELISA)

ELISA has been applied widely for detection of a variety of antibodies and antigens. It is simple and nearly as sensitive as radioimmunoassay. It requires only microlitre quantities of test reagents. The principle of ELISA is almost same as that of immunofluorescence, the only difference being, an enzyme is used instead of fluorescent dye. The enzyme acts on substrate to produce a colour in a positive test. ELISA can be used for detection of antigen or antibody. Tests for specific immunoglobulin classes (e.g. IgM ELISA) are also available. It is done on a solid phase. The test can be done in polystyrene tubes (macro-ELISA) or polyvinyl microtitre plates (micro-ELISA) (Fig. 14.18).

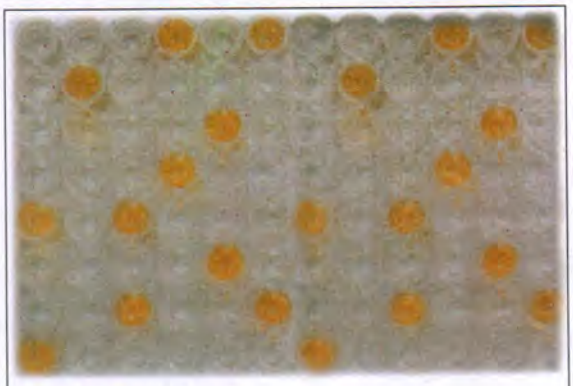


Fig. 14.18 ELISA test

SANDWICH ELISA

For antigen detection in a specimen, the wells of microtitre plate are coated with specific antibody against the antigen to be detected. Specimens to be tested are added in coated wells. If antigen is present in specimen, it binds to coated antibody. To detect this antigen-antibody reaction, antiserum (antibody) conjugated with an enzyme is added. This conjugated antiserum binds to an antigen already attached to coated antibody. A substrate is added to know the binding of conjugated antiserum to antigen-antibody complex. In case of binding (positive result), an enzyme acts on substrate to produce colour, intensity of which can be read by spectrophotometer or ELISA reader. Colour detection can also be seen by naked eye. This type of ELISA test is also known as sandwich ELISA (Fig. 14.19). Positive and negative controls should always be included in the test. At every step of ELISA test, incubation and washing is done to wash off unbound reagents.

INDIRECT ELISA

For antibody detection, the wells of microtitre plate are coated with antigen. Sera to be tested are added in these coated wells. If antibody is present in specimen, it binds to coated antigen. To detect this antigen-antibody reaction, a goat antihuman immunoglobulin antibody conjugated with an enzyme is added. Enzyme conjugated antihuman immunoglobulin binds to antibody (immunoglobulin in nature). To detect this binding, a substrate is added and enzyme acts on substrate to produce colour in a positive reaction. This procedure is also named as indirect ELISA (Fig. 14.19). Reading of the test is same as described in sandwich ELISA. Positive and negative controls are always put up alongwith test sera. Incubation and washing is done at every step to wash off unbound reagents. Substrates are specific for each enzyme. The enzyme (horseradish peroxidase, alkaline phosphatase) gives rise to a colour change by adding specific substrate (o-phenyl-diamine dihydrochloride for peroxidase, p-nitrophenyl phosphate for alkaline phosphatase). Alkaline phosphatase with this substrate produces a yellow colour.

COMPETITIVE ELISA

It has been used for detection of HIV antibodies. Positive result shows no colour whereas appearance of colour indicates a negative test. Like radioimmunoassay, there are two specific antibodies, one conjugated with enzyme and other present in serum (if serum is positive for antibodies). Competition occurs between two antibodies for same antigen. The microtitre plate wells are coated with HIV antigen. Sera to be tested is added to these wells and incubated at 37°C and then washed. If antibodies

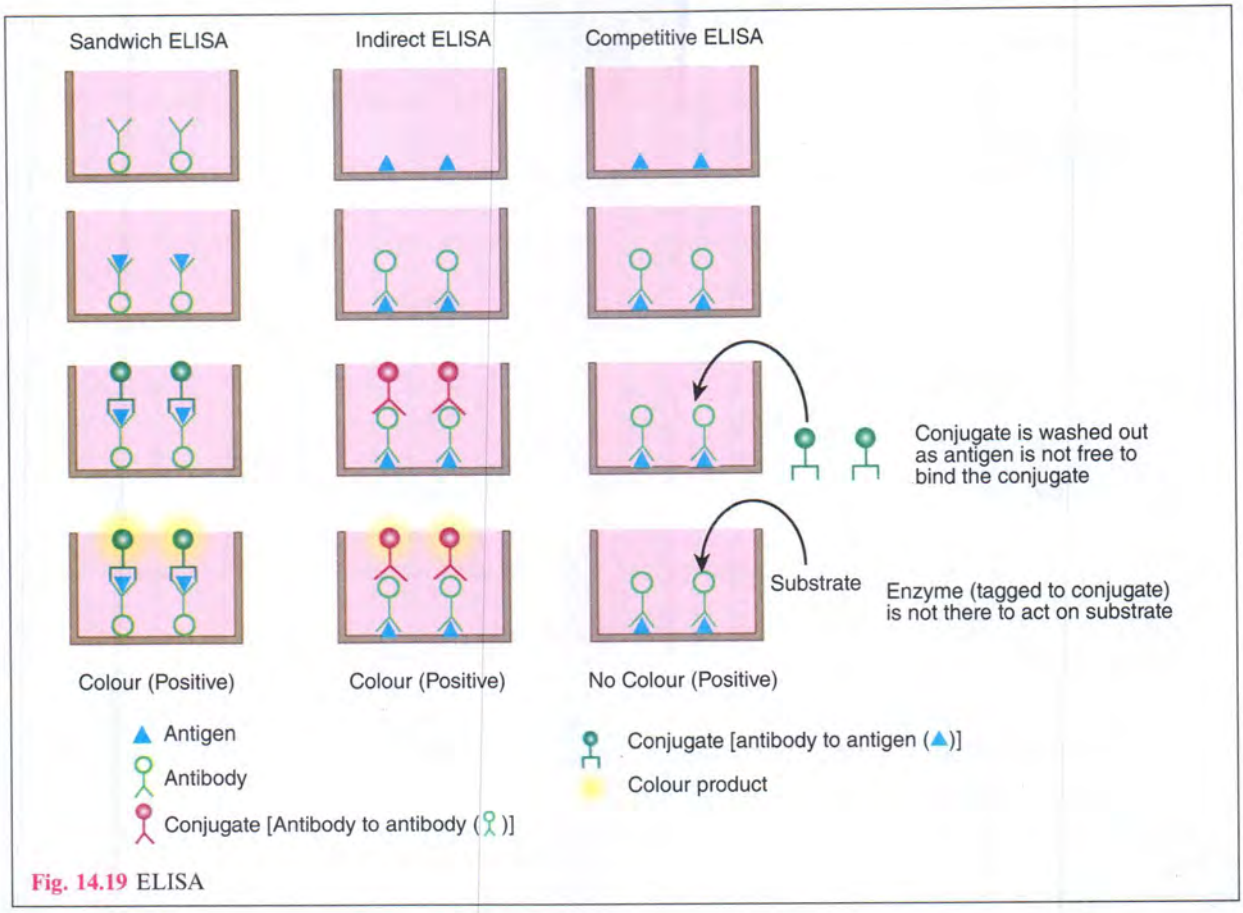


Fig. 14.19 ELISA

are present, antigen-antibody reaction occurs. To detect this reaction, enzyme labelled specific HIV antibodies are added. There is no antigen left for these antibodies to act. These antibodies remain free and washed off during washing. Substrate is added but there is no enzyme to act on it. Therefore, positive results show no colour (Fig. 14.19). If serum to be tested is negative for antibodies, antigen is there to combine with enzyme conjugated antibodies and enzyme acts on substrate to produce colour.

CASSETTE OR CYLINDER ELISA

It is a simple modification of ELISA for testing one or few samples of sera at a time. The test is rapid (about 10 minutes) as compared with the 2-4 hours taken for micro-ELISA. The result is read visually.

Cassette ELISA is being used for detection of HIV type 1 and 2 antibodies. Specific type 1 and 2 antigens are immobilised on the nitrocellulose membrane in the cassette. Test serum is added on the membrane. In positive serum, antibody will bind to the appropriate antigen. After washing to remove unbound antibody, conjugate (enzyme labelled antihuman immunoglobulin antibody) is added. It is washed again to remove unbound conjugate and a substrate is added. A positive result shows a coloured spot. Human immunoglobulin immobilised at a spot on the membrane serves as a control, as shown by development of colour at that site.

Uses of ELISA

It has been used for detection of antigens and antibodies of various microorganisms. Some examples are

(*i*) Detection of HIV antibodies in serum
(*ii*) Detection of mycobacterial antibodies in tuberculosis
(*iii*) Detection of rotavirus in faeces
(*iv*) Detection of hepatitis B markers in serum.
(*v*) Detection of enterotoxin of *Escherichia coli* in faeces.

J. Immunochromatography

Immunochromatography or lateral flow immunoassay is one of the most popular form of rapid immunoassays. It can detect both antigens and antibodies. It has advantage of being a one step test. It can be completed within 30 minute. It is a strip based test. The strip contains a chromatographic pad with three zones: sample pad, conjugate pad and capture line (Fig 14.20). The conjugate pad may be having colloidal gold, dye, or latex beads as conjugate which produces signal. The specimen is

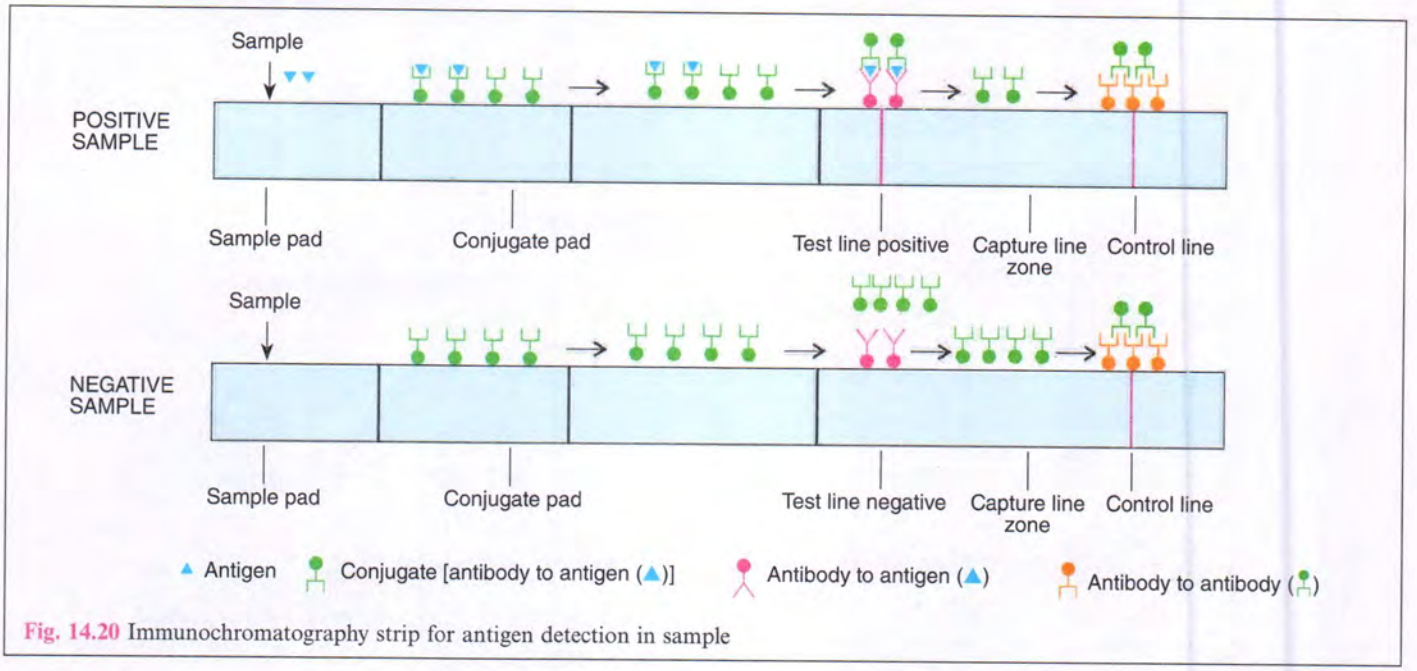


Fig. 14.20 Immunochromatography strip for antigen detection in sample

applied to the sample pad and flows laterally by capillary action. Upon reaching the conjugate pad, it may bind to conjugate if antigen or antibody is present in the specimen and forms antigen antibody complex. This complex then flow laterally to reach capture line. Here it is captured by antigen or second antibody present in the capture line. The presence of colour line is a positive test. There is a positive control line also to check that the test was properly performed.

K. Chemiluminescence Assay

Chemiluminescence refers to a chemical reaction in which energy is emitted in the form of light. On the basis of this property chemiluminescence compounds are used in this method to provide the signal (i.e. light) during the antigen-antibody reaction. The emitted light can be measured and the concentration of the analyte calculated. The method has been fully automated. This property of chemiluminescence has also been applied for drug sensitivity testing of *M. tuberculosis* (refer chapter 42).

L. Immunoelectronmicroscopic Tests

1. *Immunoferritin Test*

Ferritin (electron dense substance) conjugated antibody is used to react with an antigen. This antigen-antibody reaction can be visualised under electron microscope.

2. *Immunoelectronmicroscopy*

Viral particles are mixed with specific antisera and are observed under the electron microscope. These are seen as clumps. This method is applied in some viruses such as hepatitis A virus and viruses causing diarrhoea.

3 *Immunoenzyme Test*

Some enzymes such as peroxidase can be conjugated with antibodies. Tissue sections are treated with peroxidase labelled antisera to detect corresponding antigen. The peroxidase bound to the antigen is visualised under the electron microscope.

M. Immunoblotting

In immunoblots, antibodies can detect proteins (antigens) in mixtures. The mixture of proteins (antigens) is electrophoretically separated in a gel. The separated proteins are then transferred from gel to a nitrocellulose paper. These nitrocellulose paper strips are reacted with test sera and subsequently with enzyme-conjugated anti-human immunoglobulin. A suitable substrate is added, colour is produced by enzyme where specific antibody in test sera has reacted with separated proteins on strip. This test has been widely used to confirm the ELISA positive HIV antibody cases. This is known as Western Blot test. It detects antibodies against various protein (antigen) fractions in test sera.

The above procedure may also be applied to analyse DNA or RNA. When DNA is transferred on nitrocellulose strips from gel, this test is referred to as Southern Blot test. Similarly, if RNA is transferred, it is named as Northern Blot test.

KEY POINTS

1. Antigen combines with its specific antibody in an observable manner and the reaction between antigen and antibody is specific. These antigen-antibody reactions *in vitro* are known as *serological tests*.
2. There are various types of antigen-antibody reactions. Some of these include *precipitation*, *agglutination*, *complement fixation test*, *immunofluorescence* and *enzyme linked immunosorbent assay* (*ELISA*).
3. When a soluble antigen reacts with its antibody in the presence of electrolytes (NaCl) at an optimal temperature and pH, the antigen-antibody complex forms an insoluble precipitate and it is called *precipitation*. When instead of sedimenting, the precipitate is suspended as floccules, the reaction is called *flocculation*. This is a modified form of precipitation.
4. *Flocculation test*, *radial immunodiffusion*, *immunoelectrophoresis*, *counterimmunoelectrophoresis* and *rocket electrophoresis* are different types of precipitation reactions.
5. *Agglutination* is an antigen-antibody reaction, in which a particulate antigen combines with its antibody in the presence of electrolytes at an optimal temperature and pH, resulting in visible clumping of particles. It differs from precipitation in which soluble antigen is present in contrast to particulate antigen of agglutination.
6. *Slide agglutination test*, *tube agglutination test*, *heterophile agglutination test* and *passive agglutination test* are different types of agglutination reaction.
7. *Passive agglutination reaction* is a precipitation reaction which has been converted into agglutination test by attaching soluble antigens to the surface of carrier particles such as *latex particles*, *bentonite* and *red blood cells*.
8. *Latex agglutination test*, *haemagglutination test* and *coagglutination* are examples of passive agglutination reaction.
9. Immunofluorescence test is of two types: *direct immunofluorescence test* and *indirect immunofluorescence test*. The antibodies tagged with flourescent dye (i.e., labelled antibodies) are used in these tests.
10. Enzyme linked immunosorbent assay (ELISA) has been applied widely for detection of a variety of antibodies and antigens.
11. Different types of ELISA include *sandwich ELISA*, *indirect ELISA* and *competitive ELISA*.
12. Detection of HIV antibodies, mycobacterial antibodies, rotavirus in faeces and hepatitis B markers in serum are some examples where ELISA is commonly used.

YOU MUST KNOW

1. Difference between precipitation and agglutination.
2. Prozone phenomenon.
3. Mechanism and applications of precipitation reaction.
4. Types of precipitation reaction.
5. Principles and applications of radial immunodiffusion, immunoelectrophoresis, counter current immunoelectropheresis (CIEP).
6. Types of agglutination reactions and their uses.
7. Principles and uses of latex agglutination test and coagglutination.
8. Principle of complement fixation test.
9. Principles and uses of immunofluorescence tests and enzyme linked immunosorbent assay (ELISA).

STUDY QUESTIONS

1. Name various antigen-antibody reactions and describe the principle and application of precipitation reactions.
2. Define agglutination reaction? Discuss the principle and application of agglutination reactions.

3. Write short notes on:
   (a) Immunoelectrophoresis (b) CIEP (c) Rocket electrophoresis
   (d) Prozone phenomenon (e) Latex agglutination test (f) Coagglutination
   (g) CFT (h) Conglutination (i) Neutralisation test.
4. Discuss briefly about:
   (a) Immunofluorescence tests (b) RIA
   (c) ELISA (d) Chemiluminescence assay.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Which immunoglobulin class is the most efficient to produce agglutination reaction?
   (a) IgG (b) IgM (c) IgA (d) IgE
2. Which immunoglobulin class is the most efficient to produce precipitation reaction?
   (a) IgG (b) IgM (c) IgA (d) IgE
3. Ring test is used for:
   (a) C-reactive protein test (b) Streptococcal grouping by Lancefield technique
   (c) Both of the above (d) None of the above
4. VDRL test is an example of:
   (a) Agglutination test (b) Flocculation test (c) Immunofluorescence (d) All of the above
5. Radial immunodiffusion can be used to estimate the following immunoglobulin classes:
   (a) IgG (b) IgM (c) IgA (d) All of the above
6. Counterimmunoelectrophoresis is used for detecting:
   (a) Hepatitis B antigens (b) Cryptococcal antigens (c) *Neisseria meningitidis* (d) All of the above
7. Tube agglutination test is used for serological diagnosis of:
   (a) Enteric fever (b) Infectious mononucleosis
   (c) Typhus fever (d) All of the above
8. Which of the following is/are example/s of heterophile agglutination test?
   (a) Weil-Felix reaction (b) Paul-Bunnel test
   (c) Streptococcus MG agglutination test (d) All of the above
9. Which of the following is/are example/s of passive agglutination test?
   (a) Latex agglutination test (b) Haemagglutination test
   (c) Coagglutination (d) All of the above
10. Which of the following is/are example/s of neutralisation test?
    (a) Schick test (b) Antistreptolysin 'O' test
    (c) Nagler reaction (d) All of the above
11. Direct immunofluorescence test may be used for detection of:
    (a) Rabies virus antigens (b) Antibodies in syphilis
    (c) Both of the above (d) None of the above
12. Indirect immunofluorescence test may be used for detection of:
    (a) Rabies virus antigens (b) Antibodies in syphilis
    (c) Both of the above (d) None of the above
13. ELISA can be used for detection of antigens and/or antibodies in:
    (a) HIV (b) Rotavirus (c) Hepatitis B virus (d) All of the above
14. The technique of immunoblotting to analyse RNA is named:
    (a) Southern blot (b) Northern blot (c) Western blot (d) None of the above

Answers (MCQs):

1. (b)	2. (a)	3. (c)	4. (b)	5. (d)
6. (d)	7. (d)	8. (d)	9. (d)	10. (d)
11. (a)	12. (b)	13. (d)	14. (b)	

Chapter 15

COMPLEMENT SYSTEM

I. COMPLEMENT

The 'complement' (C) refers to a system of some non-specific proteins present in normal human and animal serum, which are activated characteristically by antigen-antibody reaction and subsequently lead to a number of biologically significant consequences. The term complement was coined by Paul Ehrlich, because it augmented (complemented) the action of antibody. The complement constitutes 10-15 percent of total human serum globulins. C as a whole is heat labile (inactivated at 56°C for 30 minutes) though some of its components are heat stable. C ordinarily does not combine with either free antigen or antibody, but only with antigen-antibody complex. Only IgM, IgG 3, IgG 1 and IgG 2 in that order fix complement. This property is due to the presence of C binding site on the Fc portion of these immunoglobulins. The complement system consists of about 20 proteins which include the complement components, the properdin system and the control proteins.

A. Components of Complement

There are nine components of complement called C1 to C9. The component C1 is made up of three protein subunits named C1q, C1r and C1s. In normal serum C3 is present in the highest concentration (1.2 mg/ml) whereas C2 in the lowest concentration (0.015 mg/ml).

Complement is normally present in circulation in inactive form, but when its activity is induced by antigen-antibody reaction or other stimuli, complement components react in a specific sequence as a cascade either through the classical or alternative pathway. Both the pathways have same result i.e. lysis or damage of target cell. Classical pathway is triggered by specific antigen-antibody complex; the alternative pathway can be initiated by endotoxin, lipopolysaccharides or zymosan (yeast cell wall).

B. Classical Pathway

Complement components react in a specific sequence, following activation by antigen-antibody complex, and results in immune cytolysis. This is known as the classical pathway of complement (Fig. 15.1).

The traditional model used to explain C activity for immune cytolysis is the lysis of erythrocytes sensitised by its antibodies. The erythrocyte (E)-antibody (A) complex is named EA and later on, when C components are attached to EA, it is called EAC.

Immune cytolysis is initiated by the binding of component C1 to EA. C1q is the recognition unit of C1, hence, C1q reacts with the Fc piece of bound antibody molecule (IgM or IgG) in EA. The binding of C1q in the presence of calcium ions leads to activation of C1r

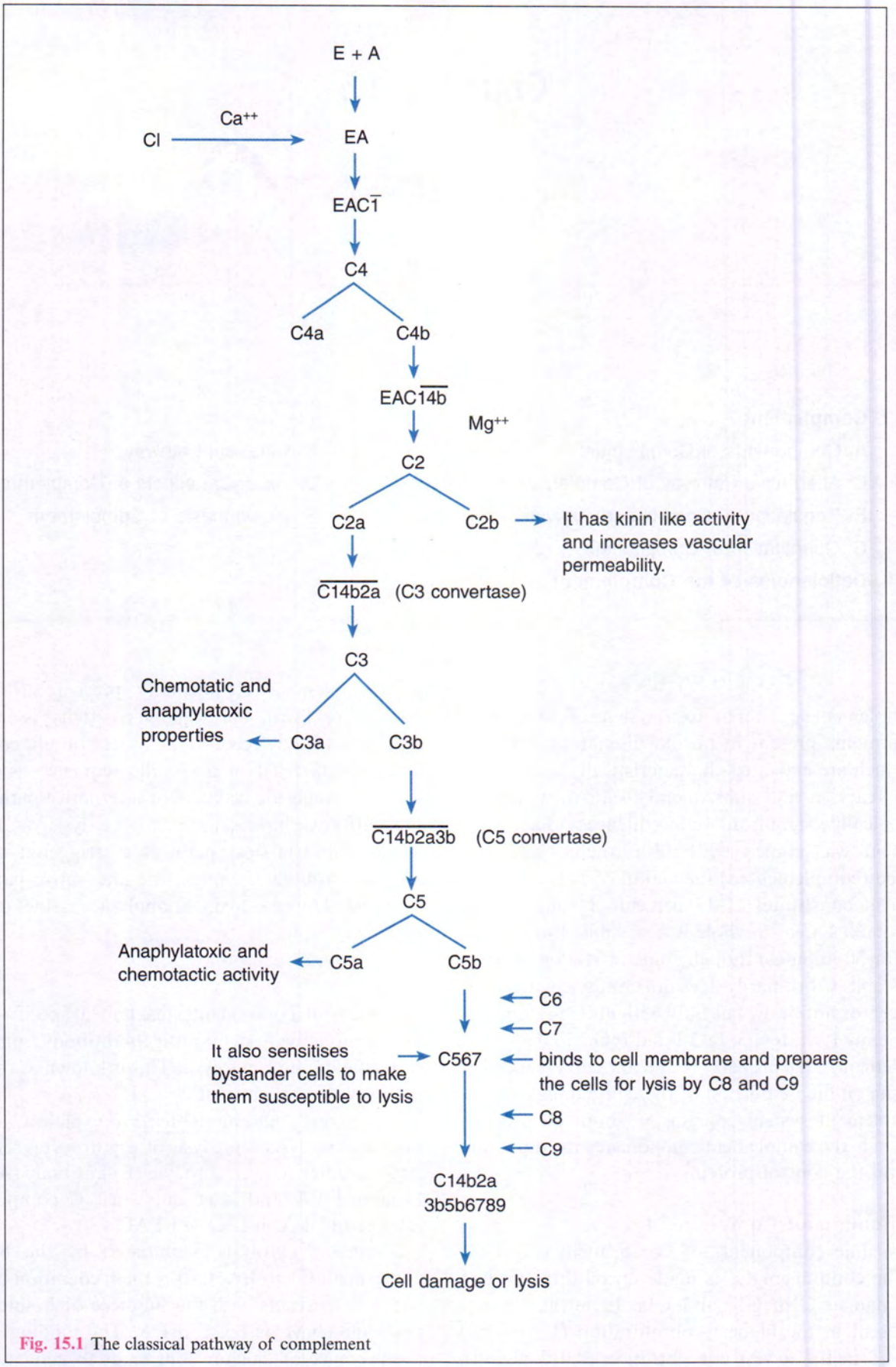


Fig. 15.1 The classical pathway of complement

and C1s. The activated C1s is an esterase which splits C4 into C4a and C4b, of which C4b joins the cascade. C14b in presence of magnesium ions act on C2 and forms C2a and C2b. The larger fragment C2a attaches to C4 to form $\overline{C42}$ which has enzymatic activity and is called *C3 convertase*. The other C2 fragments possess kinin like activity and increase vascular permeability. $\overline{C42}$ splits C3 into C3a and C3b, of which, C3b joins the cascade. C3a has chemotactic and anaphylatoxic properties. $\overline{C14b2a3b}$ has enzymatic activity and is referred to as *C5 convertase*. It acts on C5, to split it into C5a (anaphylatoxic and chemotactic) and C5b which joins the cascade. C6 and C7 join to form $\overline{C567}$, some of which binds to cell membrane and prepares the cell for lysis by C8 and C9. Most of $\overline{C567}$ absorb to unsensitised 'bystander' cells making them susceptible to lysis by C8 and C9. In this way, $\overline{C567}$ serves to amplify the reaction. The unbound $\overline{C567}$ complex has chemotactic and leucocyte activating properties. The lysis is done by producing 'holes' approximately 100°A in diameter on cell membrane. This disrupts the osmotic integrity of the cell membrane which results into release of the contents of the cell.

C. Alternative Pathway of Complement Activation

The activation of C3 has the major role in the complement cascade. In the classical pathway, C3 activation occurs by classical C3 convertase ($C\overline{142}$). This activation of C3 without help of C142, is known as the 'alternative pathway'.

The alternative pathway contributes to antimicrobial defence without requiring specific antibodies. A wide range of substances (activators) are known to activate alternative pathway. These include bacterial endotoxins, yeast cell walls, IgA and D, the cobra venom factor and the nephritic factor (a protein present in the serum of glomerulonephritis patient). The binding of C3b to an activator is the first step in the alternative pathway. Although C3b is present in the circulation but in the free state it is rapidly inactivated by the serum protein factors H and I. However, bound C3b is protected from such inactivation.

The bound C3b, in the presence of Mg^{++}, interacts with plasma protein factor B forming C3bB. The factor B portion of C3bB complex is split by factor D into Ba and Bb. Bb fragment binds to C3b forming C3bBb. This C3bBb is C3 convertase of alternative pathway (Fig. 15.2). This enzyme is extremely labile. The properdin or factor P helps to stabilise the enzyme C3 convertase. C3 convertase splits more C3 to C3a and C3b. The newly formed C3b binds more factor B. The alternative pathway then proceeds from C3 to C9 in the similar way as that occurs in the classical pathway.

D. Biological Effects of Complement

1. Bacteriolysis and Cytolysis

Complement mediates immunological membrane damage. This results in bacteriolysis and cytolysis. Neutralisation of certain viruses requires the participation of complement.

2. Amplification of Inflammatory Response

C3a and C5a are anaphylatoxic by degranulation of mast cells to release histamine and other mediators.

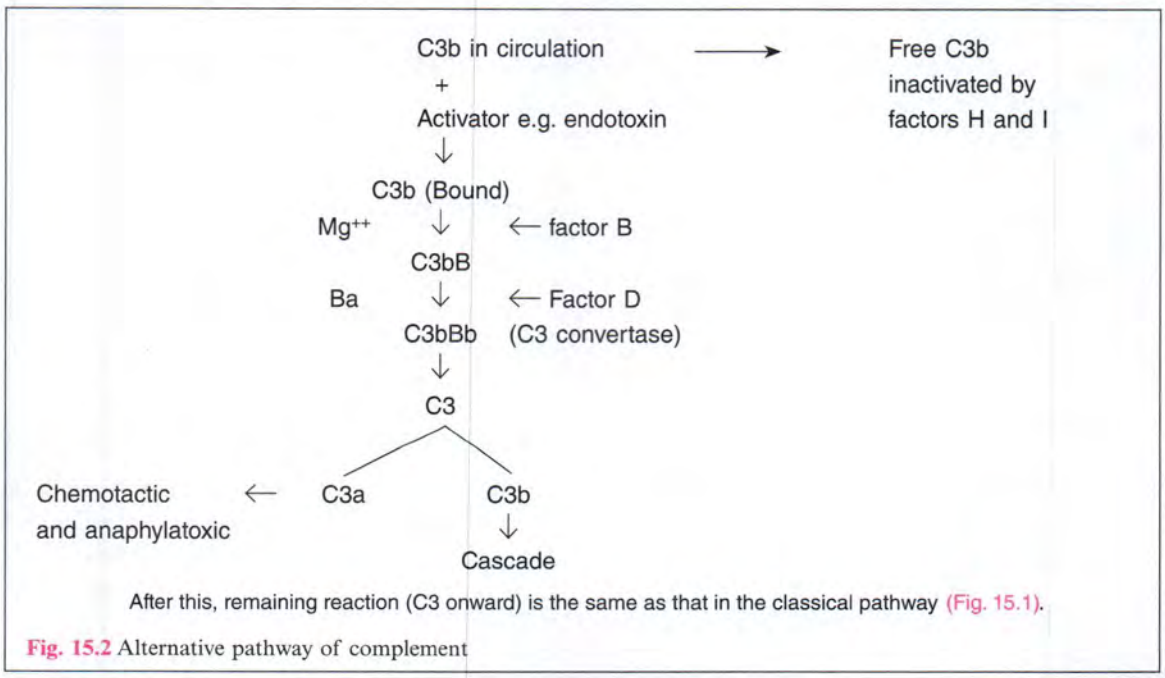


Fig. 15.2 Alternative pathway of complement

They cause increased vascular permeability and are also chemotactic. C567 is chemotactic and also brings about reactive lysis.

3. Hypersensitivity Reactions

Complement participates in Type II (cytotoxic) and Type III (immune complex) hypersensitivity reactions.

4. Endotoxic Shock

Endotoxin can activate the alternative pathway of the complement cascade. In endotoxic shock there is excessive C3 activation and platelet adherence. Platelet lysis and release of large amount of platelet factor lead to disseminated intravascular coagulation (DIC) and thrombocytopenia. Gram negative septicaemias and dengue haemorrhagic syndrome may have a similar pathogenesis. Schwartzman reaction is a good model of excessive C3 activation and depletion of complement protects against this reaction.

5. Immune Adherence

Complement bound to antigen-antibody complexes adheres to erythrocytes or to platelets. This reaction is called immune adherence. Adherent particles are rapidly phagocytosed and thus help in eliminating the pathogenic microorganisms. C3 and C4 are necessary for immune adherence.

6. Opsonisation

An important function of complement is to facilitate the destruction of pathogens by phagocytic cells. Phagocytic cells (macrophages, monocytes, neutrophils and others) possess surface receptors for C3b. If immune complexes have activated the complement system, the C3b bound to them stimulate phagocytosis and removal of immune complexes. This facilitated phagocytosis is referred to as opsonisation.

7. Autoimmune diseases

Serum complement levels are decreased in many autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis. They may, therefore, be involved in a pathogenesis of autoimmune diseases.

E. Regulation of Complement Activation

The destruction of tissues by excessive activity of complement is prevented by regulation of complement activation. Apart from damage to tissues, unchecked complement activity can also cause exhaustion of the complement system. Control of the complement cascade is exerted by two types of regulatory proteins: inhibitors which bind to complement components and halt their further function, and inactivators which destroy complement proteins.

1. Inhibitors

Normal serum contains natural inhibitors of complement which regulate the complement activity. A heat labile alpha neuraminoglycoprotein in serum inhibits C1 esterase. The S protein binds to $\overline{C567}$ and modulates the cytolytic action of the membrane attack complex.

2. Inactivators

A serum betaglobulin, factor I provides control of C3 activation. It cleaves C3b and C4b. Another betaglobulin factor H regulates alternative pathway by binding to C3b. C4 binding protein is a normal serum protein, which controls the activity of cell bound C4b. An enzyme named anaphylatoxin inactivator degrades C3a, C4a and C5a which are anaphylatoxins released during the complement cascade.

F. Biosynthesis of Complement

Complement components are synthesised in various sites as follows:

Site	*Complement Component*
Intestinal epithelium	C1
Macrophages	C2, C4
Spleen	C5, C8
Liver	C3, C6, C9
Not known	C7

Rise in complement levels (C4, C3, C5 and C6) occurs in response to acute inflammation. Complement alongwith some other plasma proteins (CRP) are collectively known as '*acute phase substances*' which show a rise in acute inflammation.

G. Quantitation of Complement

Complement activity of serum is measured by estimating the highest dilution of the serum that lyses sheep erythrocytes sensitised by antierythrocytic antibody.

Complement components can be measured by radial immunodiffusion method but this does not differentiate between active and inactive fractions.

II. DEFICIENCIES OF THE COMPLEMENT

Deficiency of complement leads to poor host resistance against infections and results in recurrent bacterial and fungal infections and collagen disorders. Some deficiency states of various complement components are listed in Table 15.1.

Table 15.1 Complement Deficiency and Associated Syndromes

Deficiency	Syndrome
C1 inhibitor	Hereditary angioneurotic oedema
C1, C2, C4 components	Systemic lupus erythematosus (SLE) and other collagen vascular diseases
C3 and its regulatory protein C3b inactivator	Recurrent pyogenic infections
C5, C6, C7, C8, C9 components	Bacteraemia, mainly with Gram negative diplococci; toxoplasmosis
C9	No particular disease

KEY POINTS

1. The *complement* refers to a system of some non-specific proteins present in normal human and animal serum, which are activated characteristically by antigen-antibody reaction and subsequently lead to a number of biologically significant consequences.
2. There are *nine components* of complement C1 to C9.
3. Complement components react in a specific sequence as a cascade either through the *classical* or *alternative pathway*.
4. Classical pathway is triggered by specific antigen-antibody complex; the alternative pathway can be initiated by *endotoxin*, *lipopolysaccharides* or *zymosan* (yeast cell wall).
5. Deficiency of complement leads to poor host resistance against bacterial and fungal infections and collagen disorders.

YOU MUST KNOW

1. Components of complement.
2. Steps in activation of classical pathway and alternative pathway of complement.
3. Biological effects of complement.
4. Complement deficiency and associated syndromes.

STUDY QUESTIONS

1. Discuss briefly the classical pathway of complement activation.
2. Write short notes on:
   (a) Alternative pathway of complement
   (b) Biological effects of complement.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Classical pathway of the complement is activated by:
   (a) Antigen
   (b) Antibody
   (c) Antigen-Antibody complex
   (d) None of the above
2. The alternative pathway of the complement is initiated by:
   (a) Endotoxins
   (b) Lipopolysaccharides
   (c) Yeast cell walls
   (d) All of the above
3. What is the chemical nature of components of complement?
   (a) Protein
   (b) Lipopolysaccharide
   (c) Lipid
   (d) None of the above

4. First component of complement which binds to antigen-antibody complex in classical pathway is:
   (a) C1q (b) C1r
   (c) C1s (d) C3
5. Which component of complement is present in the highest concentration in the serum?
   (a) C1 (b) C2
   (c) C3 (d) C5
6. Factor B and Factor D are important components of:
   (a) Classical pathway of complement (b) Alternative pathway of complement
   (c) Both of the above (d) None of the above

Answers (MCQs):

1. (c) 2. (d) 3. (a) 4. (a) 5. (c) 6. (b)

Chapter 16

STRUCTURE AND FUNCTIONS OF IMMUNE SYSTEM

The lymphoreticular system is responsible for immune response and it consists of lymphoid and reticuloendothelial components. The lymphoid cells (lymphocytes and plasma cells) are responsible for specific immunity. The phagocytic cells (polymorphonuclear leucocytes and macrophages) which are part of reticuloendothelial system, are mainly concerned with scavenger functions, of eliminating microorganisms and other foreign particles from blood and tissue, thus contributing to non-specific immunity.

I. THE LYMPHOID SYSTEM

Lymphoid cells
e.g. Lymphocytes
Plasma cells
Lymphoid organs
A. Central (Primary) lymphoid organs
e.g. Thymus
Bursa of Fabricius in birds
Bone marrow in mammals
B. Peripheral (Secondary) lymphoid organs
e.g. Spleen
Lymph nodes
Mucosa associated lymphoid tissue (MALT)
Lymphoid tissues in gut, lungs, liver, bone marrow

Types of Immune Response

The specific immune response to an antigen can be of two types:

1. The humoral or antibody mediated immunity (HMI or AMI)
   It is mediated by antibodies which are produced by plasma cells.
2. The cell-mediated immunity (CMI)
   It is mediated by sensitised lymphocytes.

The central lymphoid organs are structures in which precursor lymphocytes proliferate, develop and become immunologically competent. After acquiring immunological capabilities, the lymphocytes migrate to peripheral lymphoid organs, where appropriate immune response occurs when exposed to an antigen.

A. Central Lymphoid Organs

1. Thymus

The primary function of the thymus is to produce thymic lymphocytes. It is a major site for lymphocyte proliferation in the body. During maturation, the lymphocytes acquire new surface antigens (*Thy antigens*) and are called T-lymphocytes or T-cells (thymus dependent). Unlike in the peripheral lymphoid organs, lymphocyte proliferation in the thymus is not dependent on antigenic stimulation. The thymus confers immunological competence on these

cells during their stay in the organ. Majority of these proliferative lymphocytes die in the thymus and only about one percent migrate to the secondary lymphoid organs. These T-cells circulate through blood and lymphatics in man. The T-lymphocytes are responsible for cell mediated immunity (CMI). The importance of thymus in CMI is evident from, the lymphopaenia, deficient graft rejection and so called 'runting disease' seen in neonatally thymectomised mice. Congenital aplasia of the thymus in man (Di-George syndrome) and in mice ('nude mice') are other examples of deficient CMI.

2. Bone Marrow

Some lymphoid cells develop and mature within the bone marrow and are called B-cells (B for Bursa or Bone marrow). In birds, these B lymphocytes mature in Bursa of Fabricius. Bone marrow in mammals is equivalent to bursa. Bone marrow is the site for stem cell proliferation, the origin of pre-B cells and their maturation into functional B-lymphocytes. Competence for IgM production by B-cells is acquired early (14th day of embryonation) and for IgG late (21st day). Mature B-lymphocytes also acquire C3 and Fc receptor on their surface. B-lymphocytes bear either surface IgM alone or in combination with IgG or IgA depending upon the production of a particular immunoglobulin class. Following appropriate antigenic stimulation, B-lymphocytes transform into plasma cells and secrete antibodies.

B. Peripheral Lymphoid Organs

1. Lymph Nodes

The lymph nodes are round bodies and lie along the course of lymphatic vessels. The mature node has an outer cortex and an inner medulla. A paracortical area lies between cortical follicles and base of medullary cords. The cortex contains primary lymphoid follicles (accumulation of lymphocytes) within which secondary follicles (germinal centres) develop during antigenic stimulation. Besides these lymphocytes, the lymphoid follicles also contain dendritic macrophages which capture and process the antigen.The medulla is predominantly composed of medullary cords which are elongated branching bands of the lymphocytes, plasma cells and macrophages. The cortical follicles and medullary cords contain B-lymphocytes whereas paracortical area (a zone between the cortical follicles and medullary cords) contains T-lymphocytes.

Lymph nodes are filter for the lymph. They phagocytose foreign particles including microorganisms. They help in the proliferation and circulation of T and B lymphocytes. They enlarge following antigenic stimulation.

2. Spleen

It is the largest lymphoid organ. It contains two distinct areas—*White* and *red pulps*. Spleen consists of a cortex containing densly packed T and B lymphocytes and a loosely structured medulla. The periarterial lymphoid collections in the white pulp are called Malphigian corpuscles or follicles. Germinal centres develop within white pulp following antigenic stimulation. Surrounding the germinal centre is a mantle layer of lymphocytes. Like in lymph node, T and B lymphocyte areas are segregated.

(*i*) B-lymphocyte area: Perifollicular region, germinal centre and mantle layer.

(*ii*) T-lymphocyte area: The lymphatic sheath immediately surrounding the central arteriole.

The spleen serves as the graveyard for blood cells and as a systemic filter for trapping circulating blood borne foreign particles.

3. Mucosa Associated Lymphoid Tissue (MALT)

The subepithelial accumulation of lymphoid tissue protects the mucosa lining the alimentary, respiratory and genitourinary tracts which is exposed to numerous antigens. The lymphoid tissue may be present as diffuse collection of lymphocytes or as aggregates with well formed follicles (pharyngeal tonsils; small intestinal Peyer's patches). These are collectively named as *mucosa-associated lymphoid tissues* (MALT).

Such lymphoid tissues in the gut are called the gut associated lymphoid tissue (GALT) and those in the respiratory tract, the bronchus associated lymphoid tissue (BALT). MALT contains both lymphoid cells (B and T cells) as well as phagocytes. The predominant immunoglobulin in the mucosa is secretory IgA, other immunoglobulin classes are also formed locally.

C. Cells of Lymphoreticular System

These consist of structural cells (reticulum cells, endothelial cells and fibroblasts) and immunologically competent cells (lymphocytes, plasma cells and macrophages). In the circulating blood, about 70% lymphocytes are T-lymphocytes, 20% B-lymphocytes and 10% Null Cells.

1. Lymphocytes

These are small, round cells present in blood, lymph, lymphoid organs and many other tissues. Lymphocytes constitute 20–45% of leucocyte population in peripheral blood, while they are predominant cell types in lymph and lymphoid organs. Lymphocytes may be small (5–8 μm), medium (8–12 μm) and large (12–15 μm). Among these, the small lymphocytes are the most numerous. According to

their life span, the lymphocytes are classified as short lived (10–12 days) and long lived (three years or more, or even for life). The short lived lymphocytes act as effector cells in immune response, while the long-lived cells are store houses of immunological memory and act as memory cells. The long-lived lymphocytes are mostly thymus derived.

LYMPHOCYTE RECIRCULATION

Lymphopoiesis occurs at three places—the bone marrow, central lymphoid organs and the peripheral lymphoid organs. The lymphocytes of these three sites mix together in the process known as *lymphocyte recirculation*. There is a constant circulation of lymphocytes through the blood, lymph, lymphatic organs and tissues. This recirculation of lymphocytes from one site to another ensures that lymphocytes of appropriate specificity would reach the site during their careless wandering, following introduction of antigen. Most of the recirculating lymphocytes are T-cells. B-lymphocytes tend to be more sessile.

Lymphocytes which are 'educated' by the central lymphoid organs become *immunologically competent cells* (ICCs). They are fully equipped to deal against an antigen. They serve the following functions:

1. recognition of antigens ('R' for recognition).
2. storage of immunological memory ('R' for rememberance or memory).
3. immune response to antigens ('R' for response).

The nature of immune response depends on the type of cells stimulated by an antigen. Stimulated T-cells produce certain activation products (lymphokines) and induce CMI, while stimulated B-cells divide and transform into plasma cells which synthesise antibodies and induce HMI.

DIFFERENCES BETWEEN T AND B CELLS

1. T cells bind to sheep erythrocytes to form rosettes (E rosette or SRBC rosette) while B cells do not. T cells bind to sheep erythrocytes by CD2 antigen.
2. B cells also form rosette (EAC rosette or Erythrocyte amboceptor complement rosette) but with sheep erythrocytes coated with antibody (amboceptor) and complement. T cells are unable to form EAC rosette. B-cell surface has C3 receptor which binds to complement of EAC.
3. T cells have thymus specific antigens on surface, which are lacking on B-cells.
4. All T cells have CD3 receptor on their surface.
5. B-cells have immunoglobulins on surface and surface receptors for the Fc fragement of IgG. These are absent on T-cells. Instead T cells have T cell receptors (TCR) composed of two chains of polypeptides, linked to CD3.
6. Blast transformation of T-cells occur with mitogens such as phytohaemagglutinin (PHA) or concanavalin A (ConA) while B cells have similar transformation with bacterial endotoxins.
7. Viewed under the scanning microscope, B-cells possess numerous microvilli on their surface while T-cells are free of these projections (Table 16.1).

Table 16.1 Characteristic Features of T Cells and B Cells

	Feature	T Cell	B Cell
A.	Location		
	1. Peripheral blood	65-85%	15-25%
	2. Lymph node	60-75%	30-35%
	3. Spleen	25-45%	55-60%
	4. Thoracic duct	80-90%	10-20%
	5. Thymus	96%	Negligible
B.	Thymus specific antigens	+	–
C.	CD3 receptor	+	–
D.	Surface immunoglobulins	–	+
E.	Receptor for Fc fragment of IgG	–	+
F.	SRBC rosette (E-rosette)	+	–
G.	EAC rosette (C3 receptor)	–	+
H.	Numerous microvilli on surface	–	+
I.	Blast transformation with		
	1. anti-CD3	+	–
	2. anti-Ig	–	+
	3. Phytohaemagglutinin (PHA)	+	–
	4. Concanavalin A	+	–
	5. Endotoxins	–	+

(i) T-lymphocytes

T-lymphocytes can be classified into different *subpopulations* or *subsets* based on functional capacity and on surface antigenic determinants.

(a) ON THE BASIS OF FUNCTIONS

Various subsets are as follows:

REGULATORY T CELLS

T helper (CD4) cells

There are two subsets of T helper (Th) cells, Th1 and Th2. Th1 produce mainly the cytokines such as interferon gamma (IFN-γ) and interleukin-2 (IL-2) which activate macrophages and T cells to promote cell mediated immunity (CMI). Th2 are the helper T cells which interact with B-lymphocytes to develop them into plasma cells that produce immunoglobulins. This interaction is mediated by lymphokines (such as IL4, IL5, IL6) secreted by Th2 cells. Thus T helper cells facilitate B cell response to produce immunoglobulins.

Balanced activity of helper and suppressor T-cells produces optimum immune response. Overactivity of helper cells or decreased suppressor cell activity leads to autoimmunity, while decreased helper cell function or increased suppressor cell activity results in immunodeficiency state. Helper cells are about 65 percent and suppressor cells about 35 percent of circulating T-lymphocytes.

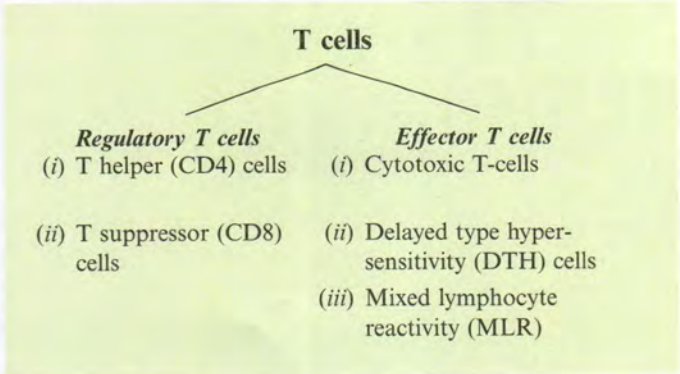
T suppressor (CD8) cells

Suppressor T-cells block immune response by their action on T–helper or B cells.

EFFECTOR CELLS

Cytotoxic T (Tc) cells

These cells can lyse specific target cells whose surface antigens are recognised by them e.g. virally infected cells, tumour cells. Cytotoxic T cells possess membrane glycoprotein CD8.



Delayed type hypersensitivity (DTH) cells

These cells are responsible for delayed (Type IV) hypersensitivity. These cells are not distinguishable from Th cells on the basis of surface markers. They possess CD4 markers as those in Th cells. It is believed that these cells may be one type of T helper cells (Th1).

These cells secrete different lymphokines (e.g. gamma interferon) responsible for delayed type of hypersensitivity.

Mixed lymphocyte reactivity (MLR) cells

These are cells which undergo rapid proliferation in mixed lymphocytic reactivity.

(b) ON THE BASIS OF SURFACE ANTIGENIC DETERMINANTS (Table 16.2)

Table 16.2 Subsets of Cells in Man

Type of Cell	Old Term	New Term
Pan T marker (present on all T-cells)	T3 T11	CD3 CD2
T helper/delayed hypersensitivity	T4	CD4
T cytotoxic/suppressor	T8	CD8
B cells	B4	CD19

The surface antigens on the T-cells can be detected by monoclonal antibodies. These thymic antigens or markers were previously designated by the letters T1 to T11. These markers are now designated as CD (*clusters of differentiation*). More than fifty T-cell antigens are identified, relevant CD molecules are given below.

CD2 (T11)

It is an early antigen which acts as a receptor for sheep red blood cells. It is present in all mature T-lymphocytes.

CD3 (T3)

It is present on all T-lymphocytes. It is closely associated with T cell receptor (TCR) on cell membrane. It is supposed to be involved in transmitting signal to the cell following antigen binding.

CD4 (T4)

It is present on all T-helper cells. It is also present on macrophages and monocytes. CD4 also acts as a receptor for HIV (human immunodeficiency virus). CD4 cells recognise MHC class II antigens.

CD5 (T1)

Its function is not known but it is found in majority of T-cells from the stage of maturation.

CD8 (T8)

It is present on suppressor T cells and cytotoxic T-cells. CD8 cells recognise MHC class I antigens.

(ii) Null Cells

The small proportion of lymphocytes which are neither T-cells nor B-cells are known as *null cells*. Among the null cells, killer cells (K cells), natural killer (NK) cells and lymphokine activator killer (LAK) cells are recognised. K cells are now classified with NK cells.

(a) Killer Cells (K Cells)

These lymphocytes possess surface receptor for Fc portion of IgG. These cells are capable of killing target cells sensitised with IgG antibodies. They do not require complement for such lysis or killing. These are responsible for antibody dependent cell mediated cytotoxicity (ADCC). This antibody dependent cell mediated cytotoxicity differs from the action of cytotoxic T lymphocytes, which is independent of antibody.

(b) Natural Killer Cells (NK Cells)

These are large lymphocytes which contain azurophilic granules in the cytoplasm and for which, they are called *large granular lymphocyte* (LGL). NK cells are cytolytic for virally transformed target cells, certain tumor lines and are involved in allograft rejection. NK cells are found in spleen and peripheral blood.

Action of NK cells is independent of antibody in contrast to K cells which are dependent on antibody. Their activity is 'natural' or 'nonimmune' as it does not require sensitisation by prior antigenic contact. They release several cytolytic factors. One of these, *perforin*, which resembles complement component C9, causes transmembrane pores through which cytotoxic factors enter the cell and destroy it by *apoptosis* (programmed cell death). NK cell activity is enhanced by interferon. It is believed that NK cells play an important role in antiviral and antitumour immunity.

(c) Lymphokine Activated Killer Cells (LAK Cells)

These are NK cells treated with interleukin-2 (IL-2), which are cytotoxic to tumour cells without affecting normal cells. IL-2 also acts as a growth factor for NK cells.

(iii) B-Lymphocytes and Plasma Cells

Antigenically stimulated B-lymphocytes undergo blast transformation to become plasmablasts, intermediate transitional cells and then plasma cells. A plasma cell can synthesise an antibody of single specificity, of a single immunoglobulin class (IgM or IgG or IgA). An exception is seen in primary immune response, when a plasma cell synthesising IgM initially, may later switch over to IgG production. Mature plasma cells survive only a few weeks. However, myeloma plasma cells are capable of an unlimited number of cell divisions. Plasma cells are present in germinal centres of lymph nodes, spleen and diffuse lymphoid tissue of respiratory and alimentary tracts. Although plasma cell is the principle antibody producing cell, but other cells like lymphocytes, lymphoblasts and transitional cells may also synthesise antibody to a certain extent.

B-lymphocytes bind to sheep RBC (SRBC) coated with antibody and complement (EAC) forming EAC rosette. B-lymphocytes also have immunoglobulin on their surface. They possess receptor for Fc fragment of IgG. There are numerous microvilli on the surface of B-lymphocytes when these cells are viewed under scanning electron microscope.

Interaction of T-lymphocytes and B-lymphocytes in cell mediated immunity (CMI) and humoral or antibody mediated immunity (AMI) is shown in Fig. 16.1.

2. Phagocytic Cells

The phagocytic cells are the mononuclear macrophages (of blood and tissues) and the polymorphonuclear microphages.

(i) Macrophages

The blood macrophages (monocytes) are the largest of the lymphoid cells present in peripheral blood. Tissue macrophages (histiocytes) are named as alveolar macrophages in the lungs and Kupffer cells in the liver. Tissue macrophages proliferate locally. Macrophages express many surface receptors such as Ia proteins, those for the Fc part of IgG, activated complement components and various lymphokines.

Functions

(1) Phagocytosis

The primary function of macrophages is phagocytosis. Macrophage envelops the foreign particle by small pseudopodia. The membranes of pseudopods fuse to form phagosome. Lysosomes containing hydrolytic enzymes fuse with the membrane of phagosome to form phagolysosome. Most bacteria are killed intracellularly by lysosomal enzymes, however, some (bacilli of brucellosis and tuberculosis) may resist and multiply intracellularly for transportation to other locations.

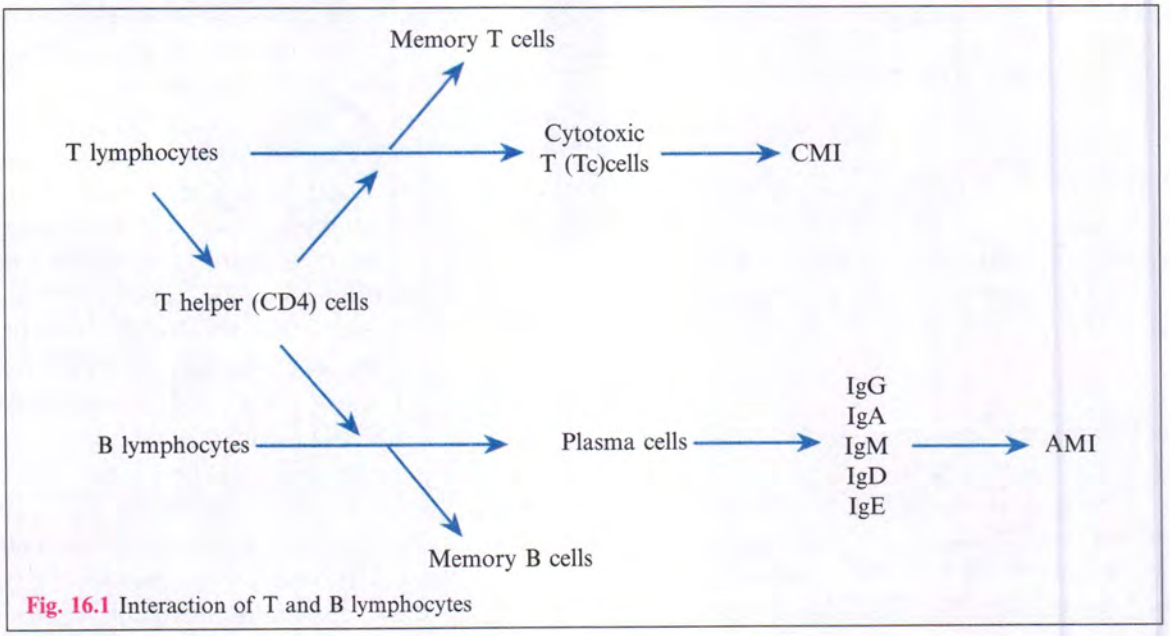


Fig. 16.1 Interaction of T and B lymphocytes

(2) Specific immune response

(*i*) They trap and process bacterial antigens and present them in optimal concentration to the lymphocytes for induction of specific immune response. Too high a concentration of antigen may be tolerogenic and too low a concentration may not be able to induce a immune response. It is an essential prerequisite for some antigens. The T cells accept the processed antigen on macrophage only when both the cells (T cell and macrophage) possess surface determinants coded for by the same major histocompatibility complex (MHC) genes. This is known as MHC *restriction*.

(*ii*) Activated macrophages secrete IL-1 which induces T-cells to synthesise IL-2. IL-2 facilitates T-cell activation and thus initiate CMI. Activated T-cells also help B-cells to form antibodies.

(3) Antitumour activity and graft rejection

The functional activity of macrophages may be increased by lymphokines, complement components or interferon. Activated macrophages are not antigen specific. They secrete a number of biologically active substances, including interleukin-1, tumour necrosis factor and colony stimulating factor (CSF). When stimulated by cytophilic antibodies and certain lymphokines, macrophages become 'armed'. These armed macrophages are capable of antigen specific cytotoxicity and are important in antitumour activity and graft rejection.

(ii) Microphages

The microphages contain polymorphonuclear leucocytes (neutrophils, eosinophils and basophils) of blood.

(a) Neutrophils

They have non-specific phagocytic property. They play a prominent role of phagocytosis in acute inflammation by locating a foreign particle.

(b) Eosinophils

Eosinophils are less phagocytic than neutrophils. They are found in large numbers in allergic inflammation, parasitic infections and diseases associated with antigen-antibody complexes, but their function is not clear.

(c) Basophils

They are present in blood and tissues (mast cells). Their cytoplasm contain basophilic granules which contain heparin, histamine, serotonin and other hydrolytic enzymes. Degranulation of mast cells release these pharmacologically active agents resulting in anaphylaxis and atopic allergy.

3. Dendritic Cells

Although macrophages are the major antigen presenting cells (APC) but the dendritic cells also perform this function. Dendritic cells are bone marrow derived cells. They possess MHC Class II antigens. They have little or no phagocytic activity. They are present in peripheral blood and in the peripheral lymphoid organs. These cells

are specially involved in the presentation of the antigens to T cells during primary immune response.

II. MAJOR HISTOCOMPATIBILITY COMPLEX (MHC)

Transplants from one individual to another member of the same species ('allografts') are recognised as foreign and rejected. Gorer (1930) identified the antigens responsible for allograft rejection in inbred mice that led to the discovery of the major histocompatibility complex (MHC). He identified two blood group antigens in mice. Antigen 1 was common to all strains of mice while antigen 2 was present in certain strains only and was responsible for allograft rejection. This was named H2 antigen. The H2 antigen was found to be the major histocompatibility antigen and to be coded for by a closely linked multiallelic clusters of genes called the major histocompatibility complex named as H-2 complex. The MHC in humans is known as the human leucocyte antigen (HLA) complex. The major transplantation antigens of man include carbohydrate antigens of erythrocytes (blood groups) and glycoprotein antigens of cell membranes (HLA system). In 1980, Snell, Dausset and Benacerraf were awarded the Nobel Prize for their work on MHC and the genetic control of immune response.

A. The HLA Complex

Histocompatibility antigens mean cell surface antigens that evoke immune response to an incompatible host resulting in allograft rejection. These alloantigens are present on surface of leucocytes in man and are called human leucocyte antigens (HLA) and the set of genes coding for them is named the *HLA Complex*.

The HLA complex of genes is located on short arm of chromosome 6 and is grouped in three classes (Fig. 16.2).

Class I — HLA-A, HLA-B and HLA-C
Class II — HLA-DR, HLA-DQ and HLA-DP (All of these are present within HLA-D region of HLA complex.)
Class III — Complement loci encode for C2, C4 and Factor B of complement system and tumour necrosis factors (TNF) alpha and beta.

A locus is the position where a particular gene is located on the chromosome. HLA loci are multiallelic i.e. the gene present on the locus can be any one of several alternative forms (alleles). Each allele determines a distinct antigen. There are 24 alleles at HLA-A locus and 50 at HLA-B. HLA system is very pleomorphic. Every individual inherits one set of HLA-genes from each parent.

The nomenclature of the HLA-system is regulated by official committee of WHO. They have officially recognised alleles and their corresponding antigens by the locus and a number e.g. HLA-A1, HLA-DR7 etc.

1. Class I MHC Antigens (A, B, C)

The MHC class I antigens are present on the surface of all nucleated cells. They are involved in graft rejection and cell mediated cytolysis. The cytotoxic T cells (CD8) recognise MHC class I antigens for their action.

2. Class II MHC Antigens (DR, DQ and DP)

They have a very limited distribution and are principally found on the surface of macrophages, monocytes, activated T-lymphocytes (CD4) and B-lymphocytes. They are primarily responsible for the graft-versus-host response and the mixed leucocyte reaction (MLR). The immune response genes (Ir) in mice are identical to MHC class II genes in man. The antigens coded for by the Ir genes are termed the Ia (I region associated) antigens.

3. Class III MHC Antigens

Class III genes encode C2, C4 complement components of the classical pathway and properdin factor B of the alternative pathway.

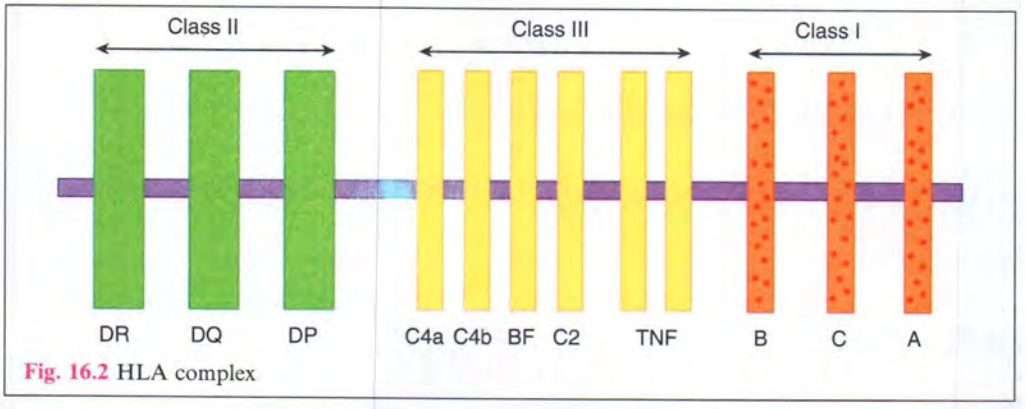


Fig. 16.2 HLA complex

B. HLA Typing

Antisera from multiparous women are collected for HLA typing. These multiparous women tend to have antibodies to HLA antigens of their husbands, due to sensitisation during pregnancy. However, monoclonal antibodies are now available. Typing is done serologically by complement dependent cytotoxic reaction. Lymphocytes of donor is typed against recipient sera in the presence of complement. However, serological typing is not possible for HLA-D and HLA-DP antigens. HLA-D is detected by mixed leucocytic reaction (MLR) and HLA-DP by primed lymphocyte typing (PLT).

Indications of HLA Typing

1. Tissue typing prior to transplantation
2. Paternity determination
3. Diseases and HLA association
   HLA-B27 – Ankylosing spondylitis
   HLA-DR4 – Rheumatoid arthritis

MHC RESTRICTION

T cells respond to processed antigens on macrophages only when they are presented along with the self-MHC antigen. This is called *MHC restriction*. Both class I and class II MHC antigens interact in this phenomenon. Cytotoxic T cells (CD8) react to an antigen in association with Class I MHC antigens while helper T cells (CD4) recognise class II MHC antigens. Cytotoxic T cells can kill or lyse virally infected target cells only when T cells and target cells are of the same MHC type.

KEY POINTS

1. The *lymphoreticular system* is responsible for immune response and it consists of *lymphoid* and *reticuloendothelial components*.
2. The lymphoid cells (*lymphocytes* and *plasma cells*) are responsible for specific immunity.
3. Lymphocytes which are 'educated' by the central lymphoid organs become *immunologically competent* cells (ICCs). They are fully equipped to deal against an antigen.
4. The nature of immune response depends on the type of cells stimulated by an antigen. Stimulated *T-cells* produce certain activation products (lymphokines) and induce cell mediated immunity (CMI), while stimulated B-cells divide and transform into plasma cells which synthesise antibodies and induce humoral immunity.
5. The surface antigens on the T-cells can be detected by monoclonal antibodies. These thymic antigens are designated as CD (*clusters of differentiation*).
6. On the basis of functions, T cells can be classified into *T helper (CD4) cells* and *T suppressor (CD8) cells*.
7. *Histocompatibility antigens* mean cell surface antigens that evoke immune response to an incompatible host resulting in allograft rejection. These alloantigens are present on surface of leucocytes in man and are called *human leucocyte antigens (HLA)* and the set of genes coding for them is named the *HLA complex*.

YOU MUST KNOW

1. Differences between T and B cells.
2. Clusters of differentiation (CD).
3. Killer cells and Natural killer cells.
4. Human leucocyte antigen (HLA) and the HLA complex.

STUDY QUESTIONS

1. Differentiate between T and B cells in a tabulated form.
2. Write short notes on:
   (a) Subsets of T lymphocytes
   (b) B-lymphocytes
   (c) Null cells
   (d) Mucosa associated lymphoid tissue.

3. Describe briefly about:
   (a) Major histocompatibility complex
   (b) HLA typing
   (c) MHC restriction.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. E-rosettes are formed by
   (a) T cells (b) B cells
   (c) Macrophages (d) Monocytes
2. EAC rosettes are formed by
   (a) T cells (b) B cells
   (c) Macrophages (d) None of the above
3. CD3 receptor is present on:
   (a) T cells (b) B cells
   (c) Macrophages (d) Monocytes
4. CD4 antigen is present on:
   (a) T-helper cells (b) Macrophages
   (c) Monocytes (d) All of the above
5. CD8 antigen is present on:
   (a) T helper cells (b) T suppressor cells
   (c) Both of the above (d) None of the above
6. Natural killer cells play an important role in:
   (a) Antiviral activity (b) Antitumour activity
   (c) Allograft rejection (d) All of the above
7. CD8 cells can recognise:
   (a) MHC class I antigens (b) MHC class II antigens
   (c) MHC class III antigens (d) All of the above
8. Class II MHC antigens are present on:
   (a) Macrophages (b) Monocytes
   (c) Activated T lymphocytes (CD4) (d) All of the above
9. Which of the following HLA types is associated with ankylosing spondylitis?
   (a) HLA-B27 (b) HLA-DR4
   (c) HLA-DP (d) None of the above
10. Which of the following HLA types is associated with rheumatoid arthritis?
    (a) HLA-B27 (b) HLA-DR4
    (c) HLA-A1 (d) None of the above

Answers (MCQs):

1. (a)	2. (b)	3. (a)	4. (d)	5. (b)
6. (d)	7. (a)	8. (d)	9. (a)	10. (b)

Chapter 17

IMMUNE RESPONSE

I. INTRODUCTION

The specific reactivity induced in a host following an antigen stimulus is known as the immune response. It is of two types:

1. *Humoral or antibody mediated immunity*
2. *Cell-mediated Immunity*

Antibody mediated immunity (AMI) provides primary defence against most extracellular bacteria and helps in defence against viruses those infect through respiratory or intestinal tracts. AMI also participates in the pathogenesis of immediate (type I, II and III) hypersensitivity and certain autoimmune diseases. Cell mediated immunity (CMI) protects against fungi, viruses and intracellular bacteria like *M. tuberculosis*, *M. leprae* and parasites such as *Leishmania* and trypanosomes. It plays an important role in allograft rejection, graft-versus-host reaction (GVH), and mediates the pathogenesis of delayed (type IV) hypersensitivity and certain autoimmune diseases. It also provides immunological surveillance and immunity against cancer. Both AMI and CMI usually develop together, though at times one or other may be the predominant type.

II. HUMORAL IMMUNE RESPONSE

Antibody production follows a characteristic pattern (Fig. 17.1) which consists of:

1. *a lag phase*—The immediate stage following antigenic stimulation when no antibody is detectable in circulation.
2. *a log phase*—There is steady rise in titre of antibodies.
3. *a plateau*—There is an equilibrium between antibody synthesis and catabolism.

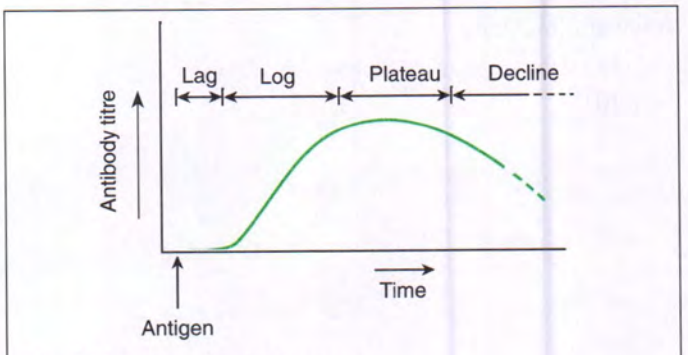


Fig. 17.1 Primary humoral response

4. the *phase of decline*—Catabolism exceeds the production and the titre falls.

A. Primary and Secondary Humoral Responses

The primary humoral response has a long lag phase and low titre of antibodies that do not persist for long. In contrast, the secondary humoral response has a short or negligible lag phase and a much higher level of antibodies that last for long periods. (Fig. 17.2). The antibody formed in the primary humoral response is predominantly IgM and in secondary humoral response IgG. The duration of the lag phase depends upon the nature of antigen, its amount, route of administration and species of animal. With some antigens such as diphtheria toxoid, the lag phase in primary humoral response may be 2-3 weeks, while with pneumococcal polysaccharide, it may be as short as a few hours.

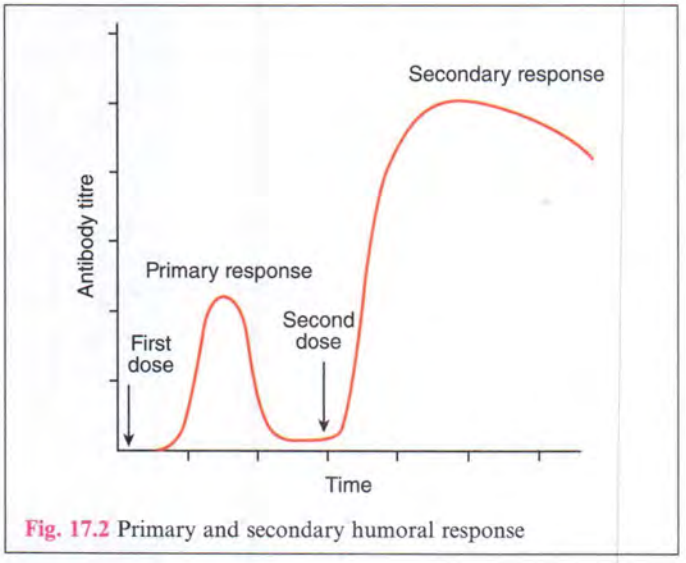


Fig. 17.2 Primary and secondary humoral response

A single injection of an antigen helps more in priming the immunocompetent cells to produce the particular antibody rather than in actual elaboration of high levels of antibody. Effective levels of antibody are usually induced by subsequent exposures to the same antigen. Due to this reason, the killed vaccines are given in multiple doses for immunisation. The first injection is known as the 'priming' dose and the rest as 'booster' doses. A single dose of live vaccine is sufficient as multiplication of the organism in the body provides a continuous antigenic stimulus that acts both as priming and booster doses.

When the same animal is subsequently exposed to the same antigen, a temporary fall in the level of circulating antibody occurs due to the combination of antigen with pre-existing antibody (antibody formed during the first antigen exposure). This has been called the 'negative phase'. It is followed by an increase in antibody level exceeding the initial level. These effects of repeated antigenic stimulus forms the basis of secondary humoral response.

B. Fate of Antigen in Tissues

Antigens introduced intravenously are rapidly localised in the spleen, liver, bone-marrow, kidneys and lungs. About 70-80% of these antigens are broken down by reticuloendothelial cells and excreted in the urine. On the other hand, antigens introduced subcutaneously are mainly localised in the draining lymph nodes, only small amount being found in the spleen.

Antigens are presented to immunocompetent cells in two ways—by macrophages and by the dendritic cells in the lymph node follicles. With many antigens, processing by macrophages appears to be a necessary prerequisite for antibody formation. Both macrophages and dendritic cells present the antigen, native or processed, at the cell surface. Macrophages modulate the dose of antigen presented to lymphocytes so that it may be optimum to induce immune response.

C. Production of Antibodies

Antigens are presented to immunocompetent cells by antigen presenting cells (APC) (macrophages and dendritic cells). With many antigens (T cell dependent antigens such as proteins and erythrocytes), processing by macrophages is essential pre-requisite for antibody formation. In case of T cell independent antigens, such as polysaccharides, antibody production does not require T cell participation.

Only when the processed antigen is presented on the surface of APC, in association with MHC molecules, to the T cell carrying antigen recognition receptors (TCRs), is the T cell able to recognise it. In the case of helper T cells, the antigen has to be presented with MHC Class II and for cytotoxic T cells with MHC class I molecules.

The TH cell requires two signals for activation. The first is a combination of TCR with MHC class II-complexed antigen. The second is interleukin-1 (IL-1) produced by APC. The activated TH cell produces interleukin-2 (IL-2) and other cytokines required for B-cell stimulation. These include IL-4, IL-5 and IL-6 which act as B-cell growth factor (BCGF) and the B cell differentiation factor (BCDF) for activation of B cells which have combined with their respective antigen. These B cells undergo clonal proliferation and differentiate into plasma cells that synthesise and secrete antibodies. B cells carry surface receptors which consist of IgM or other immunoglobulin classes. Depending upon these

receptors, a plasma cell secretes an antibody of a single specificity of a single antibody class (IgM, IgG or any other single class). However, in primary humoral response, plasma cells initially secrete IgM and later switching over to form IgG. Following antigenic stimulus, not all B-lymphocytes get converted into plasma cells. A small proportion of them develop into 'memory cells' which have a long lifespan and can recognise the same antigen on subsequent exposure. The increased antibody response during secondary antigenic stimulus is due to the memory cells induced by the primary contact with the antigen.

Antibody production by B lymphocytes is regulated by T cells. Helper T cells (TH) stimulate and suppressor T cells (TS) inhibit antibody production. Optimum antibody response depends on the balanced activity of these TS and TH cells.

D. Theories of Antibody Formation

There are two broad groups:

1. Instructive theories
2. Selective theories

The instructive theories postulate that an immunocompetent cell (ICC) is capable of synthesising antibodies of all specificity. The antigen instructs ICC to produce the complementary antibody. Selective theories, on the contrary, postulate that ICCs have only a restricted immunological range. The antigen selects the appropriate ICC to synthesise an antibody.

1. Instructive Theories

(i) Direct template theory

According to this theory, the antigen (or the antigenic determinant) enters the antibody forming cell and serves as a 'template' against which antibodies are synthesised so that they have combining sites complementary to the antigenic determinant.

(ii) Indirect template theory

This was proposed by *Burnet* and *Fenner* (1949). According to this theory, the antigenic determinant enters into the ICC so that a 'genocopy' of the antigenic determinant is incorporated in its genome and transmitted to the progeny cells (indirect template).

2. Selective Theories

(i) Side chain theory

According to side chain theory, ICCs have surface receptors capable of reacting with antigens which have complementary side chains. When foreign antigens are introduced into the body, they combine with those cell receptors which have a complementary fit. This inactivates the receptors. As a compensatory mechanism, there is an over production of the same type of receptors which circulate as antibodies.

(ii) Natural selection theory

This theory was proposed by Jerne (1955). He postulated that about a million globulin (antibody) molecules were formed in embryonic life, which covered the full range of antigens. When an antigen is introduced, it combines selectively with the globulin molecule that has the nearest complementary 'fit'. The globulin, with the combined antigen, stimulates antibody forming cells to produce same kind of antibody.

(iii) Clonal selection theory

This theory was proposed by Burnet (1957). The theory states that during foetal development a large number of clones of immunological competent cells (ICCs) bearing specific antibody patterns are produced by a process of somatic mutation of ICCs against all possible antigens. Clones of cells with immunological reactivity with self antigens are eliminated during embryonic life. Such clones are known as 'forbidden clones'. Persistence of forbidden clones or their development in later life by somatic mutation lead to autoimmune processes. Each ICC is capable of reacting with one antigen or a small number of antigens. Contact with specific antigen leads to cellular proliferation to form clones synthesising the antibody. The clonal selection theory is widely accepted nowadays.

E. Monoclonal Antibodies

1. Principle

Antibodies that are usually produced in response to a single antigen are heterogenous as they are synthesised by several different clones of cells i.e. polyclonal. A single antibody forming cell or clone produces antibodies directed against a single antigen or antigenic determinant only and such antibodies are called monoclonal antibodies. In multiple myeloma, antibodies are produced by a single clone of plasma cells against a single antigenic determinant and hence the antibodies are monoclonal.

The method for production of monoclonal antibodies against any desired antigen was first described by Kohler and Milstein in 1975. They were awarded Nobel Prize for Medicine in 1984.

2. Technique

Antibody forming spleen cells are fused with myeloma cells to produce hybrid cells (hybridomas). The resultant hybridoma retains the antibody producing capacity of the

spleen cells and the ability of myeloma cells to multiply indefinitely (Fig. 17.3). The details of technique are as follows:

(*i*) Animal (usually mouse) is immunised with the desired antigen and lymphocytes are harvested from the spleen.

(*ii*) Spleen cells (lymphocytes) are then fused with mouse myeloma cells, grown in culture, which are deficient in the enzyme hypoxanthine phosphoribosyl transferase (HPRT). Fusion is done by incubating these cells in the presence of polyethylene glycol (PEG).

(*iii*) The fused cells (hybrid cells) are grown in basal culture medium containing hypoxanthine, aminopterin and thymidine (HAT medium).

(*iv*) Only hybrid cells having properties of both the splenic lymphocytes (HPRT+) and myeloma cells (HPRT–) can grow in culture. The enzyme HPRT is necessary for nucleic acid synthesis and is provided by the splenic lymphocytes in hybrid cells. Splenic lymphocytes alone (unfused) cannot replicate indefinitely while unfused myeloma cells are killed by aminopterin in HAT medium.

(*v*) Clones that secrete the desired antibody are selected for continuous cultivation. These hybridomas can be maintained indefinitely and will continue to form monoclonal antibodies. They can also be grown as tumours in the peritoneal cavity of mice by intraperitoneal inoculation and monoclonal antibodies are obtained by harvesting the ascitic fluid produced. Hybridomas may be frozen for prolonged storage.

Mouse monoclonal antibodies, however, proved unsuitable for human therapeutic use because of strong antimouse immune response. Human monoclonal antibodies have been developed by modification of the original technique. Genes for particular antibody fragments have been fused to bacteriophage genes. Large quantities of the desired antibody can be obtained by infecting bacteria with the appropriate bacteriophage. Such antibodies hold great promise for immunotherapy.

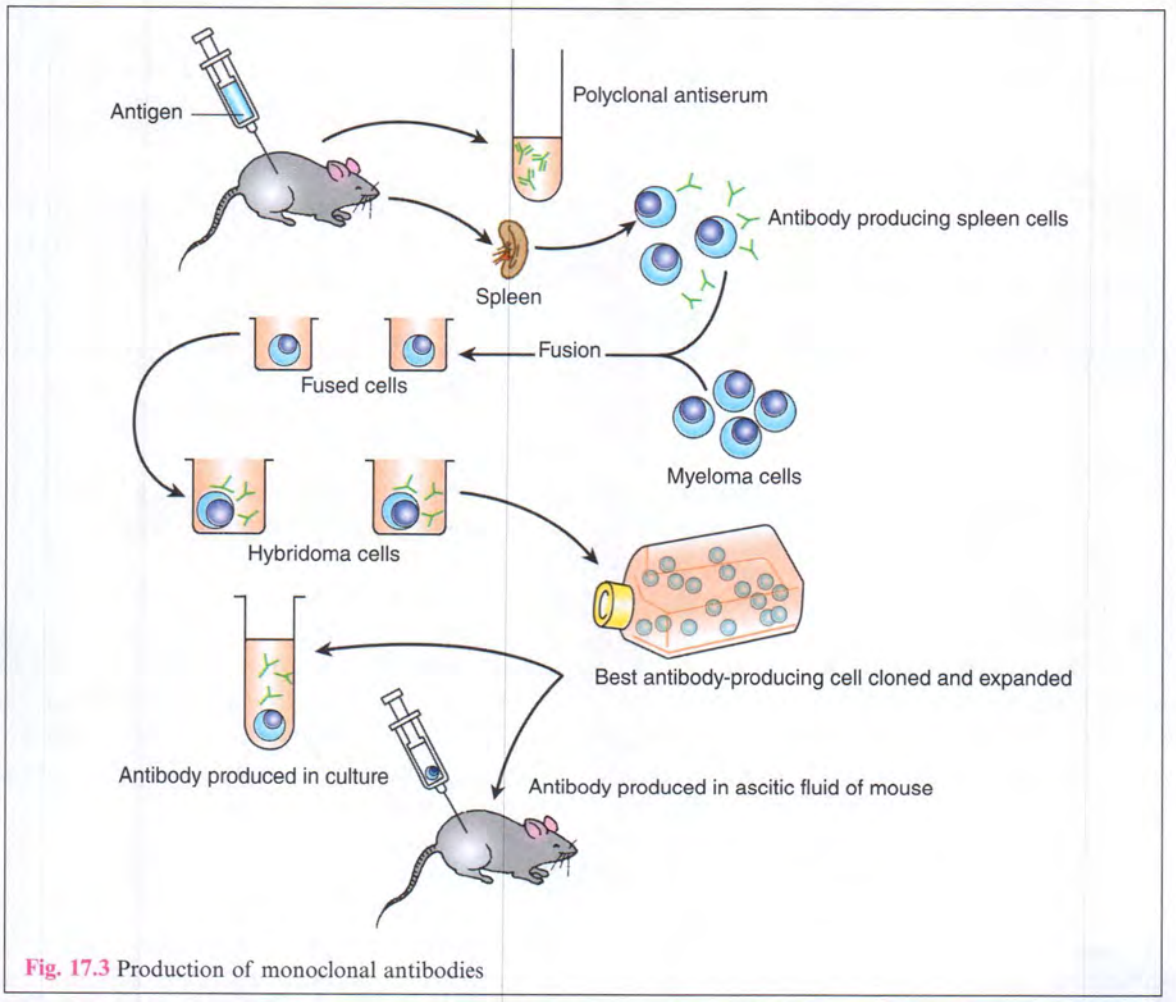


Fig. 17.3 Production of monoclonal antibodies

3. Applications

(*i*) *Diagnostic Use*: Many commercial diagnostic systems use monoclonal antibodies for identification of bacterial, viral and other antigens. Direct fluorescence and enzyme-linked assays utilise monoclonal antibody conjugates.

(*ii*) *Pure antibody*: Large amount of pure antibody of defined class can be prepared.

F. Factors Influencing Antibody Production

1. Age

The embryo is immunologically immature. Production of antibodies starts after the development and differentiation of lymphoid organs. During embryonic life clones of cells that have specificity towards self-antigens are eliminated.

Immunocompetence is not complete at birth. However, full competence is acquired only by the age of 5-7 years for IgG and 10-15 years for IgA.

2. Genetic Factors

The immune response in different individuals to same antigen varies due to genetic factors. Persons capable of responding to a particular antigen are called *responder* and those who do not respond are termed *nonresponder*. The Ir (immune response) genes control this property.

3. Nutritional Status

Protein calorie malnutrition suppresses both humoral and cellular immunity.

4. Route of Administration

There is better immune response following parenteral administration of antigen than through oral or nasal routes.

5. Dose of Antigen

An antigen is immunogenic only above a minimum critical dose. Very small dose does not stimulate immune system to produce antibodies while very large dose may inhibit the antibody producing system and paralyse it. This phenomenon is known as *immunological paralysis*.

6. Multiple Antigens

When two or more antigens are administered simultaneously, the effects may vary. Antibodies may be produced against all the antigens, or antibody response to one or the other of the antigens may be enhanced as in triple vaccine (diphtheria, pertussis, tetanus), or the response to one or more of them may be diminished (antigenic competition). For optimal effect, the nature and relative proportions of the different antigens should be carefully adjusted.

7. Adjuvants

Any substance that enhances the immunogenicity of an antigen is called *adjuvant*. A number of substances such as aluminium hydroxide or phosphate and incorporation of protein antigens in the water phase of a water-in-oil emulsion (Freund's incomplete adjuvant), delay the release of antigen and prolong the antigenic stimulus.

(i) Types of adjuvants

(a) *Depot*: Aluminium hydroxide or phosphate, alum and Freund's incomplete adjuvant (water in archis oil).

(b) *Bacterial*: Freund's complete adjuvant is the Freund's incomplete adjuvant along with a suspension of killed tubercle bacilli.

(c) *Chemical*: Bentonite, calcium alginate and silica particles.

(ii) Action of adjuvants

(a) Sustained release of antigen from depot

(b) Stimulate lymphocytes non-specifically

(c) Activate macrophages

(d) Stimulate CMI

Freund's complete adjuvant develops delayed hypersensitivity in addition to enhanced antibody formation. This adjuvant cannot be used in humans because it produces local granuloma.

8. Immunosuppressive Agents

These agents inhibit the immune response. They are useful in certain situations such as transplantation when it becomes necessary to prevent graft rejection. Immunosuppressive agents include X-irradiation, radiomimetic drugs, corticosteroids, antimetabolites and antilymphocytic serum.

(i) X- irradiation

It is more cytotoxic to replicating cells and has been used to prolong transplant survival.

(ii) Radiomimetic drugs

They belong to the class of alkylating agents e.g. cyclophosphamide, nitrogen mustard. Cyclophosphamide given for three days after the antigen, completely suppresses the antibody response. It is much less effective when given before the antigen. It selectively prevents B cell replication.

(iii) Corticosteroids

They cause depletion of lymphocytes from the blood and lymphoid tissues. They are antiinflammatory drugs that diminish the responsiveness of both B and T cells. They impair maturation of activated cells by suppressing the

production of interleukins. However, therapeutic doses have little effect on immune response.

(iv) Antimetabolites

These are substances that interfere with the synthesis of DNA, RNA or both and thus inhibit cell division and differentiation necessary for humoral and cellular immune responses. They are used clinically in the prevention of graft rejection. Some examples of antimetabolites are folic acid antagonists (methotrexate), analogues of purine (6-mercaptopurine, azathioprine), cytosine (cytosine arabinoside) and uracil (5-Fluorouracil).

(v) Antilymphocytic serum (ALS)

Antilymphocytic serum is a heterologous antiserum raised against lymphocytes. Antiserum prepared against thymus cells is called antithymocyte serum (ATS). The corresponding globulin preparations are called antilymphyocyte globulin (ALG) or antithymocyte globulin (ATG). ALS is raised in horses. It is devoid of undesirable effects and its action is only on circulating lymphocytes and not on lymphocytes in lymphoid organs. It is mainly used to prevent graft rejection. Antilymphocytic serum is effective primarily against T-lymphocytes. Humoral response to thymus independent antigens is unaffected but the response to thymus dependent antigens may be inhibited. As ALS is a foreign protein, it may lead to hypersensitivity reactions.

(vi) Cyclosporine

It has been widely employed as an immunosuppressant in organ transplantation. It does not have any cytotoxic effect on lymphocytes and has no antimitotic activity. It selectively inhibits helper T-cell activity.

III. CELL MEDIATED IMMUNE RESPONSES

The term cell mediated immunity refers to specific acquired immune responses mediated by sensitised T cells. This form of immunity can be transferred from donor to recipient with intact lymphocytes, but not with antisera, hence it is called *cell mediated immune reaction*. Cell mediated immunity (CMI) plays an important role in the following immunological functions:

1. delayed hypersensitivity (type IV hypersensitivity)
2. immunity in infectious diseases caused by intracellular organisms
3. transplantation immunity and graft-versus-host (GVH) reaction
4. immunological surveillance and immunity against cancer
5. pathogenesis of certain autoimmune diseases e.g. thyroiditis.

A. Induction of CMI

Foreign antigen is presented by antigen presenting cells (APCs) to T-lymphocytes. T-lymphocytes possess antigen recognition receptors known as T cell receptors (TCRs) that recognise foreign antigen and a self MHC molecule on the surface of the APC. These sensitised T-lymphocytes undergo blast transformation, clonal proliferation and differentiation into memory cells and effector cells (Th, Tc, Td and Ts). The activated lymphocytes release biologically active products (lymphokines) which are responsible for various manifestations of CMI.

T cells recognise antigens only when presented with MHC molecules. CD8 + cells can recognise the combination of foreign antigen and Class I MHC antigen and differentiate into Tc and Ts lymphocytes whereas CD4 + cells can recognise the combination of foreign antigen with Class II MHC antigen and differentiate into Th and Td cells. Tc lymphocytes recognise foreign antigen and Class I MHC antigen and gets attached to the target cell. This stimulates Tc lymphocytes to release cytolysins which leads to lysis of the target cell. Subsequently, the Tc cell may detach from the target cell and repeat the same process with another. Tc lymphocytes also synthesise and secrete interferon-γ and thus they probably also contribute to some extent to macrophage activation.

B. Cytokines

These are biologically active substances secreted by monocytes, lymphocytes and other cells. They are named lymphokines if they are derived from lymphocytes and monokines if they are derived from monocytes and macrophages. Interleukins are chemical substances that function primarily as growth and differentiating factors. They exert a regulatory influence on other cells. All these biologically active substances (lymphokines, monokines, interleukins) are collectively known as cytokines. They are not specific for antigens. Various cytokines are shown in Table 17.1.

Some of the important cytokines are described below:

1. *Interleukin–1*: Interleukin-1(IL-1) is principally secreted by macrophages and monocytes. It occurs in two forms IL-1 alpha and beta. Its production is stimulated by antigens, toxins, inflammatory processes and inhibited by corticosteroids and cyclosporin A. It is a stable polypeptide.

Immunological effects of IL-1

1. Stimulation of T cells for the production of IL-2 and other lymphokines
2. B cell proliferation and antibody synthesis
3. Neutrophil chemotaxis and phagocytosis

Table 17.1 Cytokines and their Functions

	Cytokine	Major sources	Main functions
1.	Interleukins:		
	IL-1	Macrophages and monocytes	Stimulation of T cells for the production of IL-2 and other lymphokines; B cell proliferation; neutrophil chemotaxis.
	IL-2	T cells	Major activator of T and B cells, cytotoxicity of T and NK cells, helps in destruction of tumour cells.
	IL-3	T cells	Acts as a growth factor for bone marrow stem cells.
	IL-4	TH cells	Proliferation of B and cytotoxic T cells, augments IgE synthesis.
	IL-5	TH cells	Proliferation of activated B cells.
	IL-6	T, B cells, macrophages, fibroblasts	Induces immunoglobulin synthesis by B cells; formation of IL-2 receptors on T cells.
	IL-7	Spleen, bone marrow stromal cells	B and T cell growth factor.
	IL-8	Macrophages	Neutrophil chemotactic factor.
	IL-9	T cells	Proliferation of T cells.
	IL-10	T, B cells, macrophages	Inhibit interferon production.
	IL-11	Bone marrow stromal cells	Induce acute phase proteins.
	IL-12	T cells	Activate natural killer (NK) cells.
	IL-13	T cells	Inhibit the functions of mononuclear cells.
	IL-14	T cells	Stimulates proliferation of activated B cells, inhibits Ig secretions.
	IL-15	Monocytes	Proliferation of T cells and activated B cells.
	IL-16	Eosinophils, CD8+ T cells	Chemoattraction of CD4+ cells.
	IL-17	CD4+ T cells	Release of IL-6, IL-8, GCF and PGE2.
	IL-18	Hepatocytes	Induces interferon-γ production, enhances NK cells activity.
2.	Colony stimulating factors (CSF):		
	G-CSF	Fibroblasts, endothelium	Granulocyte growth stimulation.
	M-CSF	Fibroblasts, endothelium	Mononuclear growth stimulation.
3.	Tumour necrosis factors (TNF):		
	TNF-α	Activated macrophages and monocytes	Tumour cytotoxicity, antiviral effects, endotoxic shock.
	TNF-β	TH cells	Induce other cytokines.
4.	Interferons (IFN):		
	IFN-α	Leucocytes	Antiviral activity.
	IFN-β	Fibroblasts	Antiviral activity.
	IFN-γ	Activated T cells	Antiviral, macrophage activation, increases the cytotoxicity of NK cells.

Together with the tumour necrosis factor (TNF), it is responsible for many of the hematological changes in septic shock.

2. *Interleukin-2*: Interleukin-2 (IL-2) is the major activator of T and B cells and stimulates cytotoxic T (T_C) cells and NK cells. It converts certain null cells (LGL) into lymphokine activated killer (LAK) cells which help to destroy NK resistant tumour cells. This property has been used in the treatment of cancers. IL-2 was previously named as T cell growth factor (TCGF). It is produced by T cells.
3. *Interleukin-3*: IL-3 is a growth factor for bone marrow stem cells. It is also known as multicolony stimulating factor (multi-CSF).

4. *Interleukin-4*: IL-4 acts as B cell differentiating factor. It activates resting B cells. It also acts as growth factor for T cells and mast cells. It augments IgE synthesis and therefore may have a role in atopic hypersensitivity. It enhances the activity of cytotoxic T cells. It was formerly known as B cell growth factor-I (BCGF-I).
5. *Interleukin-5*: IL-5 causes proliferation of activated B cells. It was formerly known as B cell growth factor-II (BCGF-II).
6. *Interleukin-6*: IL-6 is produced by stimulated T and B cells. It is also produced by macrophages and fibroblasts. It induces immunoglobulin synthesis by B cells and formation of IL-2 receptors on T cells.
7. *Colony stimulating factors* (*CSF*): They stimulate the growth and differentiation of pluripotent stem cells in the bone marrow. They are named after the types of cells they induce—for example, granulocyte (G), or mononuclear (M) CSF. IL-3 induces growth of all types of haematopoietic cells and thus known as multi-CSF. They are responsible for adjusting the rate of production of blood cells according to requirements e.g. massive granulocyte response is seen in pyogenic infections.
8. *Tumour necrosis factors* (*TNF*): The tumour necrosis factor occurs as two types namely alpha and beta. TNF-alpha is produced principally by activated macrophages and monocytes. It resembles IL-1 in having a very wide spectrum of biological activities such as its role in the manifestations of endotoxic shock. It also has immunomodulatory influences on other cytokines. TNF-beta is formed principally by T helper cells. Its actions are similar to those of TNF-alpha. TNF-beta was formerly known as lymphotoxin.
9. *Interferons* (*IFN*): There are three classes of IFNs, alpha, beta and gamma. IFN-alpha is produced by leucocytes, beta produced by fibroblasts and gamma by activated T cells. IFN-gamma increases the cytotoxicity of NK cells and macrophages. It has inhibitory effect on malignant cells. Interferons also have antiviral activity.

Cytokine production is regulated by exogenous factors such as antigens, as well as by endogenous factors. They also regulate each other by positive and negative feedbacks. IL-1, 2, 3, CSF and IFNs have already found therapeutic application.

Lymphokines

1. *Migration inhibiting factor* (MIF): It inhibits the migration of normal macrophages.
2. *Macrophage activating factor* (MAF): It restricts macrophage movement and increases phagocytic activity.
3. *Macrophage chemotactic factor* (MCF): It stimulates chemotaxis of macrophages.
4. *Macrophage stimulating factor* (MSF): It stimulates macrophage migration to the site of action.

C. Detection of CMI

CMI can be detected by following methods:

1. Skin tests for delayed hypersensitivity
2. Lymphocyte transformation test
3. Migration inhibiting factor (MIF) test
4. Rosette formation
5. Detection of T-cells by immunofluorescence technique.

1. Skin Tests for Delayed Hypersensitivity

It is useful to detect delayed hypersensitivity to commonly encountered antigens. Tuberculin skin test is commonly used. Other antigens used for skin testings are 1% solution of 1-Chloro-2, 4 dinitrochlorobenzene (DNCB) or dinitrofluorobenzene (DNFB).

2. Lymphocyte Transformation Test

This is in vitro test which detects transformation of cultured sensitised T lymphocytes on contact with specific antigen.

3. Migration Inhibiting Factor (MIF) Test

Human peripheral leucocytes are incubated in capillary tubes in culture chambers containing culture fluid. In the absence of antigen, the leucocytes migrate out to the open end of the tube to form a fan like pattern. When an antigen to which the individual has CMI is introduced into the culture fluid, the leucocytes are prevented from migrating. By comparison with the control (without antigen), it is possible to make a semiquantitative assessment of the migration inhibition.

4. Rosette Formation

Most T cells form rosettes when incubated with sheep erythrocytes. Rosette is a lymphocyte to which three or more sheep erythrocytes are attached. T cell rosette is called as E-rosette. T cells can be estimated by counting E-rosettes and thus help in detection of CMI status.

5. Detection of T cells by Immunofluorescence Technique

T cells can be detected by immunofluorescence technique using monoclonal antibodies.

D. Transfer Factor

Lawrence (1954) reported transfer of CMI in man by injecting the extracts from the leucocytes. The extract contains a soluble factor known as 'transfer factor' (TF). The transferred immunity is specific as CMI can be transferred only to those antigens to which the donor is sensitive.

TF is a low molecular weight substance (MW 2000 to 4000), resistant to trypsin but gets inactivated at 56°C in 30 minutes. It remains stable for several years at –20°C and in the lyophilised form at 4°C. It is not antigenic. Chemically, it appears to be polypeptide-polynucleotide. It is highly potent. The transferred CMI is systemic and not local. Humoral immunity is not transferred by transfer factor. The mechanism of action of TF is not known. It probably stimulates the release of lymphokines from sensitised T lymphocytes. TF has been used in:

1. T cell deficiency (Wiskott-Aldrich syndrome) patients
2. treatment of disseminated infections associated with deficient CMI (lepromatous leprosy and tuberculosis).
3. treatment of malignant melanoma and other types of cancer.

IV. IMMUNOLOGICAL TOLERANCE

Immunological tolerance is defined as a state in which contact with an antigen specifically abolishes the capacity to mount an immune response against that particular antigen when it is administered subsequently, the immune reactivity to other antigens being unaffected. Immune tolerance may be of two types, natural or acquired.

Natural tolerance is non-responsiveness to self antigens. Any antigen that comes in contact with the immunological system during its embryonic life would be recognised as self antigen and would not provoke an immune response in the mature animal. Burnet and Fenner (1949) postulated that tolerance could also be induced against foreign antigens if they were administered during embryonic life.

Acquired tolerance arises when a potential antigen induces a state of unresponsiveness to itself. For acquired tolerance to be maintained, the antigen must persist or be repeatedly administered.

The induction of tolerance depends on the species and immunocompetence of the host, physical nature, dose and route of administration of antigen. Rabbits and mice can be rendered tolerant more rapidly than guinea pigs and chickens. It is difficult to induce tolerance when the degree of immunocompetence of the host is higher. For this reason, embryos and newborns are particularly susceptible for induction of tolerance. Tolerance can be induced in adults when immunocompetence is temporarily interrupted by immunosuppressive agents.

Soluble antigens and haptens are more tolerogenic than particulate antigens. For example, when human gamma globulin is heat aggregated, it is immunogenic in mice, but when de-aggregated, it is tolerogenic. The induction of tolerance is dose dependent. High doses of antigen induce B cell tolerance whereas repeated minute doses induce T cell tolerance. A moderate dose of same antigen may be immunogenic. The route of administration is also important for induction of tolerance. Certain haptens are immunogenic in guinea pigs by the intradermal route but are tolerogenic orally or intravenously.

Tolerance to humoral and cellular immunity is usually induced simultaneously. When unresponsiveness is established for one parameter (humoral or cellular) of the immune response, it is called 'split tolerance'. In guinea pigs, delayed hypersensitivity to tuberculin can be inhibited, without affecting the production of a circulating antibody, by injection of tuberculoprotein prior to vaccination with BCG.

Tolerance can be overcome spontaneously or by an injection of cross reacting immunogens. Tolerance to bovine serum albumin in rabbits can be abolished by immunisation with cross-reacting human serum albumin.

Mechanisms of Tolerance

Tolerance can arise through following mechanisms:

1. Clonal Deletion

In embryonic life clones of B and T lymphocytes that recognise self antigens are selectively deleted and are no longer available to respond upon subsequent exposure to that antigen. This is called clonal deletion.

2. Clonal Anergy

Clones of B and T lymphocytes that recognise self antigens might be present but they cannot be activated. This is known as clonal anergy.

3. Suppression

Clones of B and T lymphocytes expressing receptors that recognise self antigens are preserved. Antigen recognition might be capable of causing activation, however, expression of immune response might be inhibited through active suppression.

4. Other Mechanisms

Access of the antigen to immunocompetent cells is interfered with (afferent block), or antibody synthesised is neutralised or destroyed (efferent block).

KEY POINTS

1. The specific reactivity induced in a host following an antigen stimulus is known as the *immune response*.
2. Immune response is of two types: *humoral immunity* and *cell mediated immunity*.
3. Humoral immunity is due to *antibody* production while cell immunity is because of sensitised T cells.
4. There are many theories of antibody formation but the *clonal selection theory* is widely accepted.
5. Antibodies that are usually produced in response to a single antigen are *polyclonal*, i.e., synthesised by several different clone of cells.
6. A single antibody forming cell or clone produces antibodies directed against a single antigen or antigenic determinant only and such antibodies are called *monoclonal antibodies*.
7. *Hybridoma* technique is used to produce monoclonal antibodies.
8. Many commercial diagnostic systems use monoclonal antibodies for identification of bacterial, viral and other antigens. *Direct fluorescence* and *enzyme-linked assays* utilise monoclonal antibody conjugates.
9. Cell mediated immunity (CMI) plays an important role in *delayed hypersensitivity* (*type IV hypersensitivity*), *transplantation immunity*, immunity in infectious diseases caused by *intracellular organisms*, *immunity against cancer* and pathogenesis of certain *autoimmune diseases*.
10. The biologically active substances are responsible for various manifestation of CMI. These are named *cytokines*. These include *lymphokines*, *monokines* and *interleukins*.
11. *Immunological tolerance* is defined as a state in which contact with an antigen specifically abolishes the capacity to mount an immune response against that particular antigen when it is administered subsequently, the immune reactivity to other antigens being unaffected.

YOU MUST KNOW

1. Primary and secondary humoral immune responses.
2. Theories of antibody formation.
3. Principle, technique and applications related to monoclonal antibodies.
4. Cytokines and their functions.
5. Immunological tolerance.

STUDY QUESTIONS

1. Discuss primary and secondary humoral responses.
2. Discuss briefly about:
   (a) Theories of antibody formation
   (b) Production of monoclonal antibodies and their applications.
3. Write short notes on:
   (a) Cytokines (b) Adjuvants (c) Transfer factor
   (d) Immunological tolerance (e) Clonal selection theory.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Cell mediated immunity (CMI) plays an important role in:
   (a) Allograft rejection (b) Type IV hypersensitivity reactions
   (c) Graft-versus-host reaction (d) All of the above
2. B-lymphocytes which have a long lifespan and can recognise the same antigen on subsequent exposure are named:
   (a) Memory cells (b) Killer cells
   (c) Natural killer cells (d) None of the above

3. The widely accepted theory of antibody formation is:
   (a) Direct template theory (b) Indirect template theory
   (c) Natural selection theory (d) Clonal selection theory
4. The animal used for monoclonal antibodies production is:
   (a) Mouse (b) Guinea pig
   (c) Rabbit (d) None of the above
5. The technique used for monoclonal antibodies production is called:
   (a) Hybridoma (b) Rosette formation
   (c) Opsonisation (d) Immunofluorescence
6. Interleukin-1 (IL-1) is principally secreted by:
   (a) Macrophages (b) Monocytes
   (c) Both of the above (d) None of the above
7. Main functions of interleukin-1 (IL-1) are:
   (a) Stimulation of T cells for production of IL-2 (b) B cell proliferation
   (c) Neutrophil chemotaxis (d) All of the above
8. Which of the following methods can be used to detect cell mediated immunity?
   (a) Skin tests for delayed hypersensitivity (b) Lymphocyte transformation test
   (c) Migration inhibiting factor test (d) All of the above
9. Which of the following mechanisms can be used to induce immunological tolerance?
   (a) Clonal anergy (b) Clonal deletion
   (c) Suppression (d) All of the above

Answers (MCQs):

1. (d)	2. (a)	3. (d)	4. (a)	5. (a)
6. (c)	7. (d)	8. (d)	9. (d)	

Chapter 18

IMMUNODEFICIENCY DISEASES

I. Primary Immunodeficiencies

- A. Humoral Immunodeficiencies (B Cell Defects)
- B. Cellular Immunodeficiencies (T Cell Defects)
- C. Combined Immunodeficiencies (Both B and T Cell Defects)
- D. Disorders of Complement
- E. Disorders of Phagocytosis

II. Secondary Immunodeficiencies

- A. Depression of Humoral Immune Responses
- B. Depression of Cell-Mediated Immunity

Immunodeficiency diseases are conditions where the defence mechanisms of the host are impaired, leading to repeated microbial infections and sometimes enhanced susceptibility to malignancies. Such defects may be primary or secondary. Primary immunodeficiencies result from abnormalities in the development of immune mechanisms. Secondary immunodeficiencies are due to consequences of some other disease, malnutrition, drugs and other processes that affect the normal functioning of the mature immune system. These immunodeficiency diseases may involve specific immune functions—humoral immunity, cell mediated immunity or both–or nonspecific mechanisms such as phagocytosis and complement.

I. PRIMARY IMMUNODEFICIENCIES

The defects may be in T cell, B cell or both, complement system or phagocytes. Some primary immunodeficiency syndromes are listed in Table 18.1.

It must be realised that there is considerable overlapping due to intimate interaction between the B cell and the T-cell. For instance, T cell deficiencies of helper or suppressor T cells will also have a profound effect on antibody response.

A. Humoral Immunodeficiencies (B Cell Defects)

1. X-Linked Agammaglobulinaemia

This syndrome is the first immunodeficiency disease to have been recognised. It was first described by Bruton in 1952 and is also called *Bruton's disease*. It is seen only in male infants. Manifestations are not apparent till about six months of age due to passive protection by maternal immunoglobulins. Patients suffer from recurrent infections with pyogenic bacteria, particularly with pneumococci, streptococci, meningococci, *Pseudomonas* and *Haemophilus influenzae*.

The basic defect is a failure of pre-B cells to differentiate into mature B cells. All classes of immunoglobulins are grossly depleted in the serum. Tonsils and adenoids are atrophic. Lymph node reveals a depletion of cells of the bursa-dependent areas. Plasma cells and germinal centres are absent even after stimulation with antigen. Affected individuals have a marked decrease in the proportion of B-cells in circulation but pre-B cells are present in normal numbers in bone marrow. T cell functions are normal in these patients and can normally handle viral, fungal, protozoal diseases. Allograft rejection is

Table 18.1 Primary Immunodeficiency Syndromes and their Classification

A.	**Humoral Immunodeficiencies (B cell Defects)**
	1. X-linked agammaglobulinaemia
	2. Transient hypogammaglobulinaemia of infancy
	3. Common variable immunodeficiency
	4. Selective immunoglobulin deficiencies
	5. Immunodeficiencies with hyper-IgM
	6. Transcobalamin II deficiency
B.	**Cellular Immunodeficiencies (T Cell Defects)**
	1. Thymic hypoplasia (DiGeorge's syndrome)
	2. Purine nucleoside phosphorylase (PNP) deficiency
C.	**Combined Immunodeficiencies (Both B and T Cell Defects)**
	1. Cellular immunodeficiency with abnormal immunoglobulin synthesis (Nezelof's syndrome)
	2. Ataxia telangiectasia
	3. Wiskott–Aldrich syndrome
	4. Immunodeficiency with thymoma
	5. Severe combined immunodeficiency diseases
	6. MHC Class II deficiency
D.	**Disorders of Complement**
	1. Complement component deficiencies
	2. Complement inhibitor deficiencies
E.	**Disorders of Phagocytosis**
	1. Chronic granulomatous disease (CGD)
	2. Myeloperoxidase deficiency
	3. Chediak–Higashi syndrome
	4. Leucocyte G-6-PD deficiency

normal and delayed hypersensitivity to tuberculin can be demonstrated. Treatment consists of routine injections of normal immunoglobulins.

2. *Transient Hypogammaglobulinaemia of Infancy*

This is due to an abnormal delay in the initiation of IgG synthesis in some infants. It may occur in infants of both sexes. Maternal IgG is slowly catabolised and reaches a very low level by the second month. Ordinarily, the infants start producing their own IgG by this age. When there is a delay, immunodeficiency occurs. Treatment with gammaglobulin is not recommended in this condition, as it may contribute to prolongation of immunodeficiency by a negative feedback inhibition of IgG synthesis.

3. *Common Variable Immunodeficiency*

This is also known as *late onset hypogammaglobulinaemia* because it usually manifests only by 15-35 years of age. It is characterised by recurrent pyogenic infections. This may also be associated with an increased incidence of autoimmune disease. B cells may be present in circulation in normal numbers, but they appear defective and are unable to differentiate into plasma cells and secrete immunoglobulins. Increased suppressor T cell and decreased helper T cell activity have been proposed as a cause of this disorder. In this condition, the total immunoglobulin level is usually less than 300 mg per 100 ml, with IgG level less than 250 mg per 100 ml. Treatment is by administration of gammaglobulin preparations intramuscularly or intradermally.

4. *Selective Immunoglobulin Deficiencies*

In these conditions, there occurs selective deficiency of one or more immunoglobulin classes, whereas the other immunoglobulin classes remain normal or elevated.

(a) Selective IgA deficiency

There is absence or near absence of serum and secretory IgA in this most common well-defined immunodeficiency disorder. These patients exhibit increased susceptibility to respiratory and gastrointestinal infections. Atopic disorders among IgA deficient individuals are high. Anti-IgA antibodies are present in many of these patients.

(b) Selective IgM deficiency

Selective IgM deficiency has been found to be associated with septicaemia due to meningococci and other Gram-negative bacteria.

5. *Immunodeficiencies with Hyper-IgM*

There is low levels of IgA and IgG with elevated IgM. Some of these immunodeficiencies are X-linked and some inherited as autosomal recessive. Patients are vulnerable to infections and autoimmune disorders such as thrombocytopaenia, neutropaenia and haemolytic anaemia. Some patients develop malignant infiltration with IgM producing cells.

6. *Transcobalamin II Deficiency*

It is an autosomal recessive disorder. These individuals show metabolic effects of vitamin B12 deficiency including megaloblastic anaemia and intestinal villous atrophy. Immunological defects include plasma cells depletion, diminished immunoglobulin levels and impaired phagocytosis. Treatment with vitamin B_{12} may restore haematopoietic, gastrointestinal and B cell functions but not phagocytic activity.

B. Cellular Immunodeficiencies (T Cell Defects)

Abnormalities of T cell development results in viral, intracellular bacterial, fungal and protozoal infections rather than acute bacterial infections.

1. Thymic Hypoplasia (DiGeorge's Syndrome)

This is a developmental defect affecting the third and fourth pharyngeal pouches, which leads to aplasia or hypoplasia of the thymus and parathyroid glands. T cells are deficient or absent in the circulation. The thymus dependent areas of spleen and lymph nodes are depleted of T lymphocytes. Delayed hypersensitivity and graft rejection are depressed. The humoral immune response is largely unaffected.

Patients show enhanced susceptibility to viral, fungal and bacterial infections. Most of these infants usually show other associated developmental anomalies such as cardiac defects particularly involving great vessels. Neonatal tetany is present. Transplantation of foetal thymus tissue has been reported to restore the immunological functions.

2. Purine Nucleoside Phosphorylase (PNP) Deficiency

Inherited deficiency of enzyme PNP occurs due to a gene defect in chromosome 14 which results in impaired metabolism of cytosine and inosine to purines. These patients show decreased T cell proliferation leading to decreased cell mediated immunity and recurrent or chronic infections.

C. Combined Immunodeficiencies (Both B and T Cell Defects)

1. Cellular Immunodeficiency with Abnormal Immunoglobulin Synthesis (Nezelof's Syndrome)

There is depressed cell mediated immunity associated with selectively elevated, decreased or normal levels of immunoglobulin. Patients are susceptible to recurrent viral, fungal, bacterial and protozoal diseases. The thymus is small and peripheral lymphoid tissues are hypoplastic. Autoimmune processes such as haemolytic anaemia are common. Bone marrow transplantation, transfer factor and thymus transplantation have been used for treatment, with success in some cases. Adequate antimicrobial therapy is essential for the treatment of microbial infections.

2. Ataxia Telangiectasia

This is an autosomal recessive genetic disorder, where combined immunodeficiency is associated with cerebellar ataxia, telangiectasia (dilated capillaries), ovarian dysgenesis and chromosomal abnormalities. Death occurs due to sinopulmonary infection early in life, or malignancy in second or third decade. The majority of patients lack IgA and IgE but some patients possess antibody to IgA. Cell mediated immunity is also defective which results in an impairment of delayed hypersensitivity and graft rejection. Transfer factor and foetal thymus transplants have been tried with limited benefit.

3. Wiskott-Aldrich Syndrome

It is an X-linked recessive disease characterised by eczema, bleeding and recurrent infections. Most of the affected children die due to complications such as bleeding, infection or lymphoreticular malignancy during childhood. The bleeding occurs due to thrombocytopenia, eczema from elevated IgE level and recurrent infections from abnormalities in cell-mediated (thymic hypoplasia) and antibody mediated immunity. B cells are unable to respond to polysaccharide antigens. Treatment with transfer factor and bone-marrow transplantation have been found to be beneficial.

4. Immunodeficiency with Thymoma

This syndrome consists of a benign thymic tumour, impaired cell mediated immunity and agammaglobulinaemia. It occurs usually in adults. Antibody formation is poor and progressive lymphopenia develops. Patients have been shown to have excessive suppressor T cell activity.

5. Severe Combined Immunodeficiency Diseases

These include many syndromes with severe combined deficiency of both humoral and cell mediated immunity. They are inherited in the autosomal recessive mode. Many distinct patterns of this immunodeficiency have been described.

(a) Swiss type agammaglobulinaemia

Such persons are born with lymphoid stem-cell defects and lack both humoral and cell-mediated immunity. They have agammaglobulinaemia and lymphocytopaenia. The basic defect is presumed to be at the level of the lymphoid stem cell.

(b) Reticular dysgenesis

This is the most serious form of combined immunodeficiency. Here the defect is at the level of multipotent bone marrow stem cell. There is a total failure of myelopoiesis. The individual is born with lymphopenia, neutropaenia, thrombocytopaenia, anaemia and bone marrow aplasia. The condition is invariably fatal in the first week of life.

(c) Adenosine deaminase deficiency

Adenosine deaminase (ADA) is an enzyme which catalyses the conversion of adenosine to inosine in purine metabolic pathway. In these patients, ADA levels are low in all the tissues, including red blood cells. The mechanism by which

this deficiency causes immunological impairment is not clear. It is, however, believed that deficiency of ADA leads to accumulation of adenosine and deoxyadenosine triphosphate, which are toxic to lymphocytes, particularly T-lymphocyte lineage. There is greater loss of T-cell immunity than that of B-cell. The immunodeficiency varies from complete absence to mild abnormalities of B and T cell functions. Infants with these severe immune disorders are vulnerable to all forms of infections and most die during the first year of life.

6. MHC Class II Deficiency

It is an autosomal hereditary recessive disorder in which failure to express MHC II molecules on the surface of antigen presenting cells (macrophages and B cells) results in a deficiency of CD4+ T-cells. This is due to the fact that T-cell (CD4+) development is dependent on positive selection by MHC class II molecules in thymus. Thus MHC II deficient infants exhibit the deficiency of T cells (CD4+). The lack of these helper T-cells results in deficient antibodies.

D. Disorders of Complement

1. Complement Component Deficiencies

Genetic deficiencies of almost all complement components have been detected in man. Deficiency of C1r and C4 is associated with systemic lupus erythematosus. The most severe abnormalities of host defences occur with C3 deficiencies which result in increased susceptibility to pyogenic infections. Deficiency of C6, C7, C8 is associated with neisserial infections.

The defects of all these complement components are transmitted as autosomal recessive traits, except that of properdin which is X-linked recessive disease.

2. Complement Inhibitor Deficiencies

Hereditary angioneurotic oedema is due to a genetic deficiency of C1 inhibitor and this defect is transmitted as an autosomal dominant. Normally, C1 inhibitor is involved in inactivation of complement system and other pathways, like clotting, fibrinolytic and kinin. In C1 inhibitor deficiency, these systems may be activated. Activation of factor XII (Hageman factor) leads to formation of *bradykinin* and C2 *kinin*. These kinins act on the post-capillary venules causing contraction of endothelial cells and formation of gaps that allow the plasma leakage and production of oedema.

E. Disorders of Phagocytosis

Phagocytosis may be impaired either by intrinsic or extrinsic defects. Intrinsic defects are within phagocytic cells such as enzyme deficiency and extrinsic defects may be due to deficiency of opsonin antibody, complement or other factors promoting phagocytosis. Phagocytic dysfunction leads to increased susceptibility to infections.

1. Chronic Granulomatous Disease (CGD)

It is a fatal genetic disorder in which there is a deficiency of NADPH oxidase. In these patients, polymorphonuclear leucocytes phagocytose invading bacteria normally but are unable to kill ingested microorganisms because engulfment of bacteria is not followed by activation of oxygen-dependent killing mechanisms. The bacteria involved in the recurrent infections are catalase positive organisms such as staphylococci and coliforms. Catalase negative bacteria are handled normally. Leucocytes from these patients fail to reduce nitroblue tetrazolium (NBT) during phagocytosis. This property has been used (NBT test) for the diagnosis of chronic granulomatous disease.

2. Myeloperoxidase Deficiency

In this rare disease, leucocytes are deficient in myeloperoxidase. Patients are liable to develop recurrent *Candida albicans* infection.

3. Chediak-Higashi Syndrome

It is an autosomal recessive disorder. Polymorphonuclear leucocytes in these patients possess large lysosomes. These abnormal lysosomes do not fuse readily with phagosome. Thus leucocytes possess diminished phagocytic activity. Patients suffer from severe pyogenic infections.

4. Leucocyte G-6-PD Deficiency

Leucocytes are deficient in glucose-6-phosphate dehydrogenase and show diminished bactericidal activity after phagocytosis. Such patients are vulnerable to microbial infections.

II. SECONDARY IMMUNODEFICIENCIES

These can occur secondary to a number of disease states such as metabolic disorders, malnutrition, malignancy or as a consequence of certain therapeutic measures which depress the immune system. Secondary immunodeficiencies are more common than primary immunodeficiencies.

A. Depression of Humoral Immune Responses

This results when B-cells are depleted as in lymphoid malignancy, particularly in chronic lymphatic leukaemia; when excessive loss of serum proteins occurs as in exfoliative skin disease and in protein losing enteropathies. In multiple myeloma, there is excessive production of abnormal immunoglobulins but normal immunoglobulin level is decreased.

B. Depression of Cell-Mediated Immunity

Cell mediated immunity is depressed in acquired immunodeficiency syndrome (AIDS), Hodgkin's lymphoma, obstruction to lymph circulation, lepromatous leprosy and some cases of sarcoidosis. Acquired immunodeficiency syndrome (AIDS) is the most important of secondary immunodeficiency diseases (see Chapter 63).

KEY POINTS

1. *Immunodeficiency* diseases are conditions where the defence mechanisms of the host are impaired leading to repeated microbial infections and sometimes enhanced susceptibility to malignancies.
2. *Primary immunodeficiencies* result from abnormalities in the development of immune mechanisms.
3. *Secondary immunodeficiencies* are due to consequences of some other disease, malnutrition, drugs and other processes that affect the normal functioning of the mature immune system.
4. These immunodeficiency diseases may involve specific immune functions—*humoral immunity*, *cell mediated immunity* or *both*—or *nonspecific* mechanisms such as *phagocytosis* and *complement*.

YOU MUST KNOW

1. List of primary immunodeficiency diseases.
2. Secondary immunodeficiency diseases.

STUDY QUESTIONS

1. What are immunodeficiency diseases? Classify primary immunodeficiency diseases and describe briefly about DiGeorge's syndrome.
2. Write short notes on:
   (a) B cell defects (b) T cell defects (c) Disorders of complement.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. DiGeorge's syndrome is an immunodeficiency disease due to:
   (a) T cell defects (b) B cell defects (c) Both of the above (d) None of the above
2. Which of the following defects occur in Nezelof's syndrome.
   (a) T cell defects (b) B cell defects (c) Both of the above (d) None of the above
3. Wiskott-Aldrich syndrome is an immunodeficiency disease which occurs due to:
   (a) T cell defects (b) B cell defects (c) Both of the above (d) None of the above
4. Chediak-Higashi syndrome is an immunodeficiency disease which occurs due to:
   (a) T cell defects (b) B cell defects
   (c) Complement disorder (d) Diminished phagocytic activity
5. Chronic granulomatous disease occurs due to deficiency of:
   (a) NADPH Oxidase (b) G6PD enzyme (c) Both of the above (d) None of the above

Answers (MCQs):

1. (a) 2. (c) 3. (c) 4. (d) 5. (a)

Chapter 19

HYPERSENSITIVITY

I. Classification

A. Type I (Anaphylactic) Reaction
B. Type II (Cytotoxic) Reaction
C. Type III (Immune Complex) Reaction
D. Type IV (Delayed or Cell Mediated) Reaction
E. Type V (Stimulatory Type) Reaction

II. Shwartzman Reaction

Immune response is generally a protective process but it may sometimes be injurious to the host. Hypersensitivity refers to a condition in which immune response results in excessive reactions leading to tissue damage, disease or even death in the sensitised host.

Hypersensitivity occurs in individuals who have had previous contact with the antigen (allergen). The initial contact sensitises the immune system by priming appropriate B or T lymphocytes. It is known as *'priming'* or *'sensitising'* dose. Subsequent contact with the same antigen causes hypersensitivity. It is known as *'shocking'* dose. These allergens may be complete antigens or haptens.

Allergy is most commonly used as a synonym for hypersensitivity. The term 'allergy' means an altered state of reactivity to an antigen, it may include both protective as well as injurious immune response. This term allergy was originally coined by *von Pirquet* (1905).

I. CLASSIFICATION

Hypersensitivity reactions are classified into two main types, 'immediate' and 'delayed' types based on the time required by sensitised host to develop clinical reactions upon exposure to the shocking dose of the antigen. The major differences between the two hypersensitivity reactions are shown in Table 19.1.

Coomb and Gel (1963) classified hypersensitivity reactions into four major types, types I to IV.

Table 19.1 Distinguishing Features of Immediate and Delayed Type of Hypersensitivity

	Feature	Immediate type	Delayed type
1.	Onset and duration	Appears and recedes rapidly	Appears slowly in 24-72 hours and lasts longer
2.	Immune response	Antibody mediated	Cell mediated (T-lymphocytes)
3.	Passive transfer	Possible with serum	Cannot be transferred with serum but possible with lymphocytes or transfer factor
4.	Desensitisation	Easy but short lived	Difficult but long lasting
5.	Induction	Antigens or haptens, by any route	By antigen injected intradermally or with Freund's adjuvant or by skin contact

Type I (Anaphylactic)
Type II (Cytotoxic)
Type III (Immune complex)
Type IV (Delayed or cell mediated)

Type I, II and III depend on the interaction of antigen with humoral antibodies and are known as immediate type reactions. Type IV reaction is mediated by T-lymphocytes. Reaction develops in 24 to 72 hours and is called delayed hypersensitivity reaction. Later on type V hypersensitivity reaction was also described.

A. Type I (Anaphylactic)

Antibodies (IgE type) are fixed on the surface of tissue cells (mast cells and basophils) in sensitised individuals. The antigen combines with the cell fixed antibody, leading to release of pharmacologically active substances which produce the hypersensitivity reaction.

B. Type II (Cytotoxic)

It is a cytotoxic reaction mediated by antibodies that react with antigens present on the surface of cell or other tissue components resulting in damage of the cell. Combination with antibody may sometimes cause stimulation instead of cell damage. An example is 'long acting thyroid stimulator' (LATS), an antibody against thyroid cells, which stimulates the excessive secretion of thyroid hormone.

C. Type III (Immune Complex)

The damage is caused by antigen-antibody complexes. These complexes may be deposited in tissues causing immune complex disorders.

D. Type IV (Delayed or Cell Mediated)

The tissue damage is mediated by T-lymphocytes and not by antibody, hence, is also called cell mediated hypersensitivity. The antigen activates specifically sensitised T-lymphocytes which lead to the secretion of lymphokines, with fluid and phagocyte accumulation.

E. Type V (Stimulatory Type)

It is a modification of Type II hypersensitivity reaction. Antibodies interact with antigens on cell surface that leads to cell proliferation and differentiation instead of inhibition or killing. Antigen-antibody reaction enhances the activity of affected cell.

TYPE I (ANAPHYLACTIC) REACTION

Anaphylaxis is the classical example of type I hypersensitivity reaction. It is IgE mediated reaction. The term anaphylaxis (ana-without, phylaxis-protection) was described by Richet (1902) who observed that dogs which had survived a sublethal dose of toxic extracts of sea anemones, were rendered highly susceptible to minute doses of the same extract given days or weeks later, instead of becoming immune to it.

Type I reactions occur in two forms—the acute, potentially fatal, systemic form called *anaphylaxis* and the recurrent non-fatal localised form called *atopy*.

1. Mechanism of Anaphylaxis

Anaphylaxis occurs when a sensitised individual comes in contact with a shocking dose of antigen. Sensitisation may occur by any route such as parenteral, inhalation or ingestion but the most effective route is parenteral. Antigens as well as haptens may induce anaphylaxis. An interval of 2-3 weeks is required between sensitising and shocking dose, during which cytotropic antibody IgE (previously known as reagenic antibody) produced against the antigen attaches to surface receptors of mast cells and basophils. IgE molecules bind to these receptors by their *Fc* end. Once sensitised, the individual remains so for a long period. When a shocking dose of the same or immunologically related antigen is given, the antigen combines with cell bound IgE antibody on mast cells rapidly. The antigen-antibody complex stimulates mast cells and basophils to release mediators that cause clinical manifestations of anaphylaxis (Fig. 19.1). Shocking dose is most effective when given intravenously, less effective subcutaneously, and least effective intradermally.

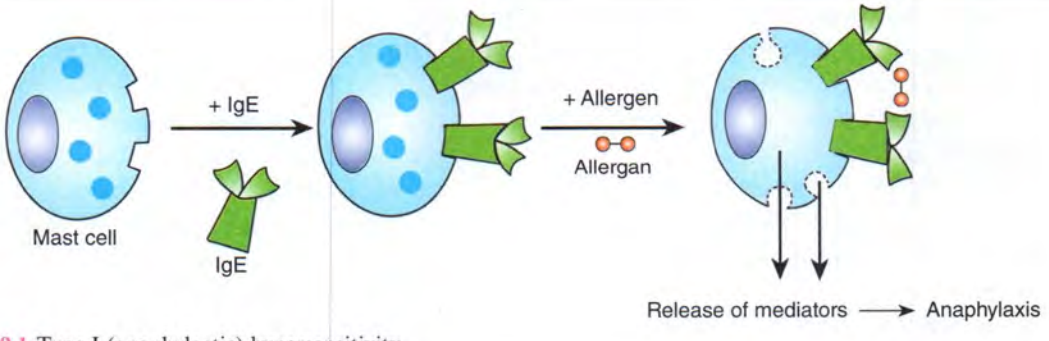


Fig. 19.1 Type I (anaphylactic) hypersensitivity

2. Chemical Mediators

The chemical mediators are of two types—*primary* mediators which are the preformed contents of mast cell and basophil granules and the *secondary* mediators which are newly formed upon stimulation of mast cells, basophils and other leucocytes.

(*i*) *Primary mediators:* e.g. Histamine, serotonin, eosinophil chemotactic factor of anaphylaxis, neutrophil chemotactic factor of anaphylaxis, various proteolytic enzymes.

(*a*) *Histamine*
This is the most important in human anaphylaxis. It is formed by the decarboxylation of histidine present in the granules of mast cells, basophils and in platelets. It causes vasodilatation, increased capillary permeability and contraction of smooth muscle.

(*b*) *Serotonin*
It causes vasoconstriction, increased capillary permeability and smooth muscle contraction. It is found in the brain tissue, intestinal mucosa and platelets.

(*c*) *Eosinophil chemotactic factor of anaphylaxis (ECF-A)*
These are released from mast cell granules and are strongly chemotactic for eosinophils. These probably contribute to the eosinophilia associated with many hypersensitivity conditions. Another chemotactic factor which attracts neutrophils is known as neutrophil chemotactic factor of anaphylaxis.

(*d*) *Proteolytic enzymes*
Proteases and hydrolases are also released from mast cell granules.

(*ii*) *Secondary mediators:* e.g. Slow reacting substances (SRS), prostaglandins and thromboxane, platelet activating factor (PAF), cytokines such as IL3 to IL6, GM-CSF, IL1, INF-γ, INF-α.

(*a*) *Slow reacting substance of anaphylaxis (SRS-A)*
It is produced by leucocytes. These are much more potent bronchoconstrictors than histamine. These are principal mediators in bronchoconstriction of asthma. They cause sustained contraction of smooth muscles. These are not inhibited by antihistaminics.

(*b*) *Prostaglandins and thromboxane*
Both of these are derived from arachidonic acid which is formed from disrupted cell membranes of mast cells and leucocytes. Prostaglandin F2a is a bronchoconstrictor. Thromboxane A2 is also a powerful, but transient, bronchoconstrictor.

(*c*) *Platelet activating factor (PAF)*
It is released from basophils which causes aggregation of platelets and release of their vasoactive amines.

(*d*) *Other mediators of anaphylaxis*
These are the anaphylatoxins released by complement activation and bradykinin and other kinins from plasma kininogens.

3. Features of Anaphylaxis

(*i*) Anaphylaxis occurs within a few seconds to few minutes following shocking dose of antigen.
(*ii*) Cytotropic IgE antibody is responsible.
(*iii*) Tissues or organs which are affected in anaphylaxis are called 'target tissues' or 'shock organs'. Lung is the principal shock organ in humans. Bronchospasm, laryngeal oedema, respiratory distress, shock and death may occur.
(*iv*) It can be induced artificially by serum of sensitised individual.
(*v*) It is not related to heredity.

4. Types of Anaphylaxis

Apart from systemic anaphylaxis, there are other types of anaphylaxis which are as follows:

(i) Anaphylaxis in vitro (Schultz-Dale phenomenon)

Isolated tissues (uterus or ileum) from sensitised guinea pigs when kept in a bath of Ringer's solution, the organ contracts vigorously on addition of the specific antigen to the bath. This is named as *Schultz-Dale phenomenon.*

(ii) Cutaneous anaphylaxis

Cutaneous anaphylaxis may be induced when a small shocking dose of an antigen is administered by intradermal route to a sensitized host. It is manifested by appearance of local 'wheal and flare' response. Cutaneous anaphylaxis is useful in testing for hypersensitivity and in identifying the allergen responsible in atopy.

(iii) Passive cutaneous anaphylaxis (PCA)

This is a sensitive *in vivo* method for detection of antibodies. A small volume of the antibody is inoculated intradermally into a normal animal. If the antigen along with a dye Evans blue is injected intravenously 4-24 hours afterwards, there will be an immediate blueing at the site of intradermal injection due to vasodilatation and increased capillary permeability (wheal and flare reaction). PCA can be used to detect human IgG antibody which is heterocytotropic (antibody is capable of fixing to cells

of other species) but not IgE which is homocytotropic (can fix to cells of homologous species only).

5. Atopy

The term 'atopy' (atopy meaning out of place or strangeness) was first introduced by *Coca* (1923) to refer to familial hypersensitivities which occur spontaneously in man. It is typified by hay fever and asthma. The antigens commonly involved in atopy are pollens, house dust and foods. These atopens induce IgE antibodies, formerly termed as 'reagin' antibodies. Atopic sensitisation occurs spontaneously following natural contact with atopens. Predisposition to atopy is genetically determined, probably linked to MHC genotypes.

(i) Features of atopy

1. Atopy runs in families. These individuals have tendency to produce reagin antibody in unusually large amounts.
2. Reactions occur at the site of entry of the antigen, inhalation of pollens affect lungs (bronchial asthma), ingestion of fish, milk, eggs, drugs etc. (gastrointestinal disorders or cutaneous eruptions) and contact leads to local allergy (conjunctivitis).
3. It is IgE mediated hypersensitivity reaction. IgE is homocytotropic i.e. species specific. Only human IgE can fix to the surface of human cells.
4. Induction of atopy is difficult artificially because atopens are poor antigens.

(ii) Mechanism of atopy

The atopen combines with the cell bound IgE antibodies fixed on the surfaces of mast cells and the basophils and this antigen-antibody complex stimulates these cells to release the mediators resulting in clinical features of atopy.

Examples

1. Food allergy e.g. egg, mushroom, prawn, shell fish.
2. Dust allergy e.g. pollens of ragweeds, grasses or trees, house dust.
3. Drug allergy e.g. penicillin, sulphonamides.

(iii) Prausnitz-Kustner (PK) reaction

This was the original method for detecting atopic antibody. Prausnitz and Kustner (1921) reported that if serum collected from Kustner, who had a gastrointestinal allergy to certain cooked fish, was injected intracutaneously into Prausnitz, followed 24 hours later by injecting (intracutaneously) small amount of cooked fish extract into the same site, a wheal and flare reaction occurred at the site of injection within minutes. The special affinity of IgE (reaginic antibody) for skin cells forms the basis of PK reaction. As reaginic IgE is homocytotropic, the test has to be performed on human skin. This test is not done nowadays due to risk of transmission of serum hepatitis.

In-vitro methods for detection of IgE include radioallergosorbent test (RAST), ELISA and passive agglutination.

6. Anaphylactoid Reaction

This reaction resembles anaphylactic shock clinically and is provoked by intravenous injection of peptone, trypsin and some others substances. It has a nonspecific mechanism involving the activation of complement pathway with release of anaphylatoxins. It has no immunological basis. The clinical resemblance is due to action of same chemical mediators participating in both reactions.

TYPE II (CYTOTOXIC) REACTION

Cytotoxic reaction is mediated by IgG (or rarely IgM) antibodies directed against antigens on the surface of cells resulting in cell damage. Antibodies bind to an antigen on the cell surface and cause (*i*) phagocytosis of the cell through opsonic or immune adherence (*ii*) cytotoxicity by natural killer (NK) cells (*iii*) lysis through activation of complement system (Fig. 19.2).

Examples

1. *Autoimmune anaemias and haemolytic disease of the newborn*

   Lysis of red cells occur due to formation of antierythrocyte antibodies.
2. *Drug reactions*

   Sedormid purpura is a classical example. A sedative drug sedormid (not used nowadays) combines with platelets and antibodies are formed against these sedormid coated platelets. On subsequent exposure to drug, antibodies attack the platelets leading to thrombocytopenic purpura. Other drugs such as sulphonamide, thiazide diuretics and quinidine cause similar type of purpura. Many drugs attach to the cell membranes of erythrocytes, neutrophils or platelets and lead to antibody formation. On subsequent exposure to drug, antigen-antibody reaction produces cytotoxic or cytolytic (haemolysis) reactions.

Demonstration of type II reaction

Coombs test (direct antiglobulin test) is usually positive.

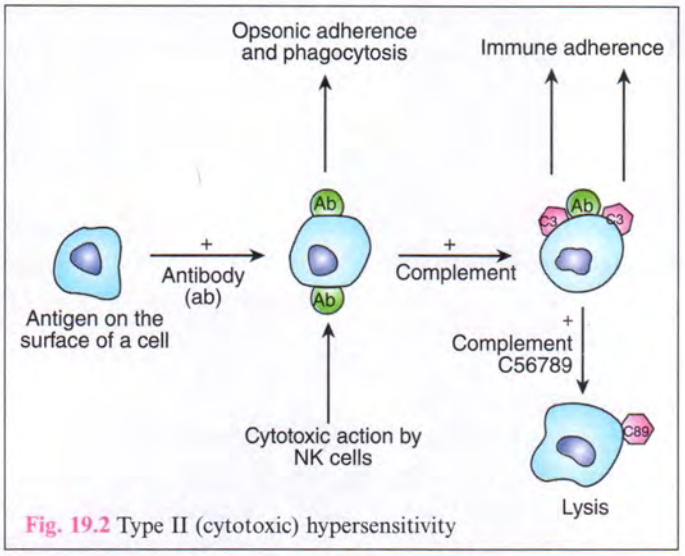


Fig. 19.2 Type II (cytotoxic) hypersensitivity

TYPE III (IMMUNE COMPLEX) REACTION

It is characterised by deposition of antigen-antibody complexes in tissues, activation of complement and infiltration of polymorphonuclear leuococytes leading to tissue damage. Type III reactions differ from type II reactions because its antigens are not attached to the surface of a cell. The interaction of these antigens with antibodies produce free floating complexes which can be deposited in the tissues leading to an *immune complex reaction*.

Two typical type III reactions include *Arthus reaction* (localised) due to relative antibody excess and *serum sickness* (generalised) because of relative antigen excess.

1. Arthus Reaction

Arthus (1903) observed that with repeated subcutaneous injections of normal horse serum (antigen) into rabbits, the initial injections were without any local effect, but with later injections, intense local oedema and haemorrhagic necrosis develop. This type of reaction is called Arthus reaction. The tissue damage is due to antigen-antibody complexes formed at equivalence or slight antibody excess. The antigen antibody complexes are deposited on the walls of blood vessels. These complexes activate complement and attract neutrophils at the local site. Leucocyte-platelet thrombi are formed which reduce the blood supply and cause tissue necrosis. The Arthus reaction can be passively transferred with sera containing high titre of antibodies (IgG, IgM).

2. Serum Sickness

It is a systemic form of type III hypersensitivity reaction. It appears following a single injection of high concentration of foreign serum. Antibodies to foreign serum reach high enough titres by 7-12 days but still some excess antigen remains in the circulating blood. Immune complexes get deposited on the endothelial lining of blood vessels in various parts of the body, causing inflammatory infiltration. Massive complement activation and fixation by antigen-antibody complexes leads to fall in complement concentration. Serum sickness differs from other hypersensitivity reactions in that a single injection serves both as the sensitising and shocking dose. Immune complexes damage the tissue in the same way as that in Arthus reaction. Serum sickness is characterised by fever, urticaria, arthralgia, lymphadenopathy and splenomegaly. Some important immune complex diseases are poststreptococcal glomerulonephritis, endocarditis, hepatitis B, dengue haemorrhagic fever and malaria.

TYPE IV (DELAYED OR CELL MEDIATED) REACTION

The reaction is mediated by sensitised T-lymphocytes which, on contact with specific antigen, release lymphokines that cause biological effects on macrophages, leucocytes and tissue cells (Fig. 19.3). In contrast, all others hypersensitivity reactions are induced by circulating antibodies. Type IV or delayed type of hypersensitivity occurs within 48-72 hours of antigen challenge. As it is not antibody mediated, it cannot be passively transferred by serum, but can be transferred by lymphocytes or the transfer factor. Two types of delayed hypersensitivity reactions are well recognised, the tuberculin (infection) type and the contact dermatitis type.

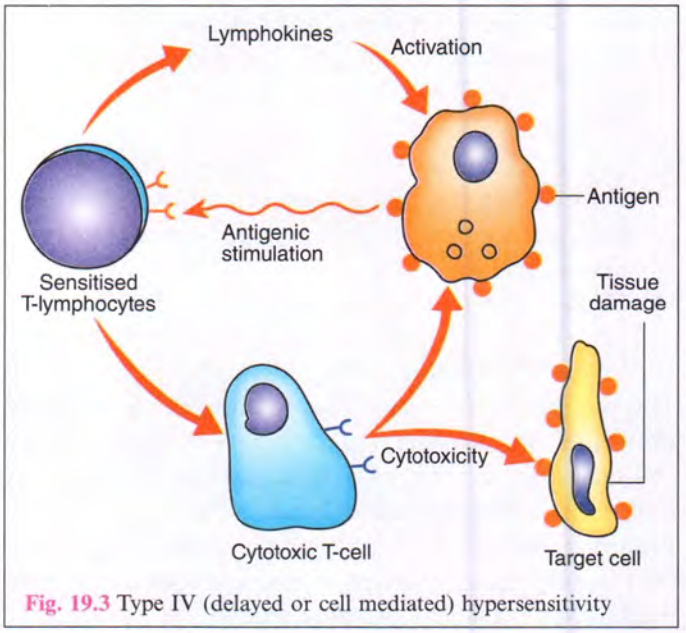


Fig. 19.3 Type IV (delayed or cell mediated) hypersensitivity

1. Tuberculin (Infection) Type

When a small dose of tuberculin is injected intradermally in an individual sensitised to tuberculoprotein by prior infection or immunisation, an erythema and swelling (induration) occurs at the site of injection within 48-72 hours. The injection site is infiltrated by large number of lymphocytes and about 10-12% macrophages. In unsensitised individuals, the tuberculin injection provokes no response. Purified protein derivative (PPD) which is the active material of tubercle bacilli, is used in tuberculin test.

The tuberculin test is a useful indicator for delayed hypersensitivity (cell mediated immunity) to the bacillus. Cell mediated hypersensitivity reaction develops in many infections with bacteria (*M. tuberculosis*, *M. leprae*), fungi and parasites. It occurs when infection is subacute or chronic and the pathogen is intracellular.

Various skin tests are performed to detect delayed type of hypersensitivity. Positive skin test does not indicate present infection but implies that the person has been infected or immunised by the microorganism in the past. Some of these skin tests include:

(*i*) Lepromin test: It is positive in tuberculoid leprosy but negative in lepromatous type of leprosy.

(*ii*) Frei test: This test is positive in lymphogranuloma venereum (LGV).

(*iii*) Histoplasmin test: It is positive in histoplasmosis (a fungal infection caused by *Histoplasma capsulatum*).

2. Contact Dermatitis Type

Delayed hypersensitivity may sometimes develop as a result of skin contact with a range of sensitising materials—metals such as nickel and chromium, drugs such as penicillin or other antibiotics in ointments, simple chemicals like hair dyes, picryl chloride, dinitrochlorobenzene, cosmetics and soaps.

These substances can act as haptens. After absorption through skin, these molecules combine with skin protein to become antigenic. Cell mediated immunity is induced in skin. As most of the antigens involved are fat soluble, their likely portal of entry is along the sebaceous glands. Sensitisation is particularly liable to occur when the chemical is applied in an oily base (ointment or cream) on an inflammed area of the skin. The Langerhan's cells of skin carry these antigens to regional lymph nodes where T-lymphocytes are sensitised. On subsequent exposure to the offending agent, sensitised lymphocytes release lymphokines which cause superficial inflammation of skin characterised by redness, induration, vesiculation within 24-48 hours. The dermis is infiltrated predominantly by lymphocytes and few macrophages.

Detection

Patch test

The suspected allergen is applied to a small area of skin under an adherent dressing. Sensitivity is indicated by itching appearing in 4-5 hours, and local reaction (erythema, vesicle or blister formation) after 24-48 hours.

TYPE V (STIMULATORY TYPE) REACTION

It is a modification of Type II hypersensitivity reaction. Antibodies interact with antigens on cell surface that leads to cell proliferation and differentiation instead of inhibition or killing. Antigen-antibody reaction enhances the activity of affected cell.

The typical example is *Grave's disease* in which thyroid hormones are produced in excess quantity. Long acting thyroid stimulating (LATS) antibody is an autoantibody to thyroid membrane antigen. It is presumed that LATS combines with a TSH receptor on thyroid cell surface and produces the same effect as TSH resulting in excessive secretion of thyroid hormone.

II. SHWARTZMAN REACTION

It is not an immune response but it has superficial resemblance to hypersensitivity reaction. It is probably a specialised type of intravascular coagulation precipitated by endotoxin.

Shwartzman (1928) injected a culture filtrate of *S. typhi* intradermally in a rabbit and same filtrate was injected intravenously after 24 hours. A haemorrhagic necrotic lesion developed at the site of intradermal inoculation. The initial intradermal (preparatory) injection causes accumulation of leucocytes which by release of lysosomal enzymes damage capillary walls. Following the intravenous (provocative) injection, there occurs intravascular clotting, the thrombi leading to necrosis of vessel walls and haemorrhage. This is known as *Shwartzman reaction*.

When both preparatory and provocative doses are given intravenously, the animal dies 12-24 hours after the second dose. Autopsy reveals bilateral cortical necrosis of the kidneys and patchy haemorrhagic necrosis in other organs such as liver, spleen etc. The reaction is also called the *Sanarelli-Shwartzman reaction* or the generalised Shwartzman reaction.

Mechanisms similar to the Shwartzman reaction may operate in certain clinical conditions, such as fulminating meningococcal septicaemia (Waterhouse–Friderichsen syndrome).

KEY POINTS

1. *Hypersensitivity* refers to a condition in which immune response results in excessive reactions leading to tissue damage, disease or even death in the sensitised host.
2. Hypersensitivity reactions are mainly of four types: *type I* (*anaphylactic*), *type II* (*cytotoxic*), *type III* (*immune complex*) and *type IV* (*delayed* or *cell mediated*).
3. Type I, II and III depends on the interaction of antigen with *humoral antibodies* and are known as *immediate* type reactions. Type IV reaction is mediated by *T-lymphocytes*.
4. *IgE* is involved in type I hypersensitivity reaction. Type I reactions occur in two forms, the systemic form called *anaphylaxis* and the localised form called *atopy*.
5. Type II reaction is mediated by antibodies that react with antigens present on the surface of cell or other tissue components resulting in damage of the cell. Examples of this reaction are *autoimmune anaemias* and *haemolytic disease of the new born*.
6. Type III reaction is caused by *antigen-antibody complexes*. These complexes may be deposited in tissues causing immune complex disorders. Two typical type III reactions include *Arthus reaction* (localised) and *serum sickness* (*generalised*).
7. Type IV reaction is mediated by T-lymphocytes. The antigen activates specifically *sensitised T-lymphocytes* which lead to the secretion of *lymphokines*, with fluid and phagocyte accumulation. Two types of delayed hypersensitivity reactions include the *tuberculin* (*infection*) *type* and the *contact dermatitis type*.

YOU MUST KNOW

1. Differences between immediate and delayed type of hypersensitivity reactions.
2. Mechanisms and examples of type I, type II, type III and type IV hypersensitivity reactions

STUDY QUESTIONS

1. Classify hypersensitivity reactions. Describe in detail about type I hypersensitivity reactions.
2. Write short notes on:
   (a) Arthus reaction (b) Serum sickness (c) Delayed hypersensitivity reaction
   (d) Atopy (e) Anaphylaxis (f) Type II hypersensitivity reactions.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Which of the following hypersensitivity reactions has cell mediated immune response?
   (a) Type I (b) Type II
   (c) Type III (d) Type IV
2. Which class of immunoglobulin mediates type I hypersensitivity reactions?
   (a) IgG (b) IgM
   (c) IgA (d) IgE
3. Chemical mediators released during type I hypersensitivity reactions may be:
   (a) Histamine (b) Serotonin
   (c) Eosinophil chemotactic factors of anaphylaxis (d) All of the above
4. Schultz-Dale phenomenon is an example of:
   (a) Type I hypersensitivity reaction (b) Type II hypersensitivity reaction
   (c) Type III hypersensitivity reaction (d) Type IV hypersensitivity reaction
5. Arthus reaction is an example of:
   (a) Type I hypersensitivity reaction (b) Type II hypersensitivity reaction
   (c) Type III hypersensitivity reaction (d) Type IV hypersensitivity reaction

6. Delayed hypersensitivity reaction is mediated by:
   (a) T lymphocytes
   (b) B lymphocytes
   (c) Macrophages
   (d) All of the above
7. Lepromin test is an example of:
   (a) Type I hypersensitivity reaction
   (b) Type II hypersensitivity reaction
   (c) Type III hypersensitivity reaction
   (d) Type IV hypersensitivity reaction
8. Excess production of thyroid hormones in Grave's disease is due to:
   (a) Type I hypersensitivity reaction
   (b) Type II hypersensitivity reaction
   (c) Type III hypersensitivity reaction
   (d) Type IV hypersensitivity reaction
9. Shwartzman reaction is an example of:
   (a) Type I hypersensitivity reaction
   (b) Type II hypersensitivity reaction
   (c) Type III hypersensitivity reaction
   (d) None of the above

Answers (MCQs):

1. (d)	2. (d)	3. (d)	4. (a)	5. (c)
6. (a)	7. (d)	8. (d)	9. (d)	

Chapter 20

AUTOIMMUNITY

I. INTRODUCTION

Normally an animal's immune system recognises its own tissue antigens as 'self' and therefore it doesn't produce antibodies against these self antigens. Autoimmunity is a condition when the body produces autoantibodies and immunologically competent T-lymphocytes against its own tissues. This leads to structural or functional damage of tissues. Autoimmunity literally means 'protection against self', while it leads to development of 'injury to self'.

Self tolerance was originally described by Ehrlich (1901) as *'horror autotoxicus'*. He observed that after immunisation of a goat with erythrocytes of other goats, antibodies were produced against erythrocytes of other goats but not against their own.

II. MECHANISMS OF AUTOIMMUNITY

Autoimmunisation can occur under the following conditions:

A. Hidden or Sequestered Antigens

Certain hidden or 'sequestered' antigens are not recognised as self antigens. When these antigens are released into circulation, they may evoke an immune response. Examples are lens antigen of eye, sperm and thyroglobulin. These self antigens are present in closed system and have no access to the immune apparatus. The lens protein is enclosed in its capsule and has no contact with blood circulation. Hence immunological tolerance against lens protein is not developed during foetal life. When this antigen leaks out, following cataract surgery or injury, it may elicit an immune response which results in damage to the lens of the other eye. As spermatozoa develop only during puberty, the tolerance against this antigen is not induced during foetal life. Following mumps, the virus damages the basement membrane of seminiferous tubules which leads to leakage of sperms and therefore induce an immune response resulting in orchitis.

B. Antigen Alteration

Tissue antigens may be altered by physical, chemical or biological factors and these new cell surface antigens are called 'neoantigens'. These neoantigens are no longer recognised as 'self' and may elicit an immune response.

Viruses and other intracellular pathogens may induce alteration of cell surface antigens leading to autoimmunity.

C. Cross Reacting Foreign Antigens

Sharing of antigens by different organisms is the basis of 'cross reacting antigen' theory. Streptococcus (M proteins) and heart muscle share antigenic characteristics. Repeated streptococcal infection can therefore induce the immune response which damages the heart. Nephritogenic strains of streptococci may lead to glomerulonephritis due to the antigenic sharing.

Injection of Semple rabies vaccine may elicit an immune response against sheep brain antigens. This may lead to encephalitis due to cross reaction between human and sheep brain antigens.

D. Forbidden Clones

According to clonal selection theory an innumerable number of clones of immunologically competent cells (ICCs) bearing specific antibody patterns are produced against all possible antigens. Any clone of cells carrying a pattern reactive against self antigens is destroyed during embryonic life. Such clones are known as 'forbidden clones'. At the time of birth only cells against nonself persist. Persistence of forbidden clones or development in later life by somatic mutation could lead to autoimmune diseases.

E. T and B Cell Defects

T helper cells facilitate B cell response to antigens while T suppressor cells inhibit antibody production by B cells. Optimal antibody response depends on the balanced activity of T helper and T suppressor cells. Enhanced function of T helper cell and decreased T suppressor cell function are suggested as causes of autoimmunity.

Another hypothesis is polyclonal B cell activation. An antigen generally activates only its corresponding B cell but certain microorganisms and their products can cause polyclonal (i.e. antigen nonspecific) activation of B-lymphocytes to produce antibodies.

Nonspecific antibodies are formed in some infectious diseases, such as antihuman erythrocyte cold antibodies in mycoplasma pneumonia.

III. CLASSIFICATION OF AUTOIMMUNE DISEASES

Autoimmune diseases may be classified as

A. Haemocytolytic diseases
B. Localised or organ specific diseases
C. Systemic or non-organ-specific diseases.

A. Haemocytolytic Diseases

These conditions involve various cells present in blood circulation. Haemocytolytic diseases include autoimmune haemolytic anaemias, leucopenia and thrombocytopenia.

B. Localised or Organ-Specific Diseases

In these diseases, specific organs are targets for autoimmune reactions.

C. Systemic or Non-Organ-Specific Diseases

In systemic diseases, immune response occurs against a variety of self antigens and involves damage to several organs and tissue system (Table 20.1).

Table 20.1 Autoimmune Diseases

	Type	Disease	Autoantibody
A.	Haemocytolytic diseases	1. Autoimmune haemolytic anaemia 2. Idiopathic thrombocytopenic purpura	1. Anti-red blood cell antibodies. 2. Antiplatelet antibodies.
B.	Localised or Organ specific diseases	1. Grave's disease 2. Hoshimoto's thyroiditis 3. Myasthenia gravis 4. Pernicious anaemia 5. Addison's disease 6. Chronic active hepatitis 7. Sympathetic ophthalmia	1. Anti-TSH receptor autoantibody 2. Antibodies to thyroglobulin and to microsomal antigens 3. Antiacetylcholine antibodies 4. Antibody to gastric parietal cells and to vitamin B-12 binding site of intrinsic factor 5. Antibodies to adrenal cells 6. Antinuclear antibodies; antihepatocyte antibodies 7. Antibody to uveal or retinal tissue
C.	Systemic diseases (non-organ specific)	1. Rheumatoid arthritis 2. Systemic lupus erythematosus antibodies 3. Goodpasture's syndrome	1. Antigammaglobulin antibodies 2. Antinuclear (anti DNA) 3. Antibasement membrane

IV. PATHOGENESIS OF AUTOIMMUNE DISEASES

Autoimmune diseases may occur either by humoral response or by cellular immune response against self antigens. Autoantibodies are more easily detectable than T-cell assessment for cellular response. Antibodies may cause damage by type II (cytotoxic) or type III (immune complex) hypersensitivity reactions. Type II reaction is important in autoimmune haemolytic anaemias in which autoantibodies alongwith complement destroy erythrocytes. Type III reaction has a role in diseases like SLE, myasthenia gravis and Grave's disease. In SLE, deposition of circulating immune complexes in glomerular basement membrane results in tissue damage. A third mechanism in autoimmune diseases is by type IV reaction (delayed type of hypersensitivity). Humoral and cellular immune responses may act together in some autoimmune diseases. Experimental orchitis can be induced only when both type of immune responses are in action.

Progress of autoimmune diseases can be arrested by immunosuppressive therapy.

KEY POINTS

1. *Autoimmunity* is a condition when the body produces autoantibodies and immunologically competent T-lymphocytes against its own tissues.
2. Autoimmunity can occur in certain conditions such as *hidden or sequestered antigens, antigen alteration, cross reacting foreign antigens, forbidden clones, T and B cell defects.*

YOU MUST KNOW

1. Mechanisms of autoimmunity.
2. List of autoimmune diseases.

STUDY QUESTIONS

1. What is autoimmunity? Discuss the various mechanisms of autoimmunity.
2. Classify autoimmune diseases and describe their pathogenesis.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Lens protein of eye is an example of:
   (a) Sequestered antigen (b) Neoantigen
   (c) Cross-reacting foreign antigen (d) None of the above
2. Which antibody is responsible for systemic lupus erythematosus?
   (a) Antiplatelet antibodies (b) Antinuclear antibodies
   (c) Antibasement membrane antibodies (d) None of the above

Answers (MCQs):

1. (a) 2. (b)

◆◆◆

Chapter 21

TRANSPLANTATION AND TUMOUR IMMUNITY

I. TRANSPLANTATION

It is a useful procedure for replacement of diseased tissues or organs which are irrepairably damaged. Transplantation is necessary for restoration of function. The tissue or organ transplanted is called transplant or graft. It has been known for a long time that transplants survive only when the tissue or organ is taken from the recipient himself, while grafts from another individual of the same species or from a different species would be rejected. The immune response induced by the *transplantation* (HLA) *antigens* is the reason for rejection of these exogenous grafts.

A. Types of Transplants

1. *Autograft*: It is a tissue or organ taken from one site and engrafted to another site in the same individual.
2. *Isograft*: It is a graft taken from an individual and placed on another individual of the same genetic constitution e.g. grafts made between identical twins.
3. *Allograft* (formerly called homografts): It is graft transfer between two genetically nonidentical members of the same species.
4. *Xenograft* (formerly called heterografts): These are grafts between members of different species and are called xenografts.

   Autografts and isografts are usually accepted and survive. Allografts and xenografts are usually rejected due to genetic and antigenic incompatibility.

B. The Allograft Reaction

Rejection of the graft by the recipient is called the allograft reaction. The graft becomes vascularised during the first two or three days and appears to be accepted initially. By the fourth day, inflammation occurs and the graft is invaded by lymphocytes and macrophages. Vascularity diminishes due to thrombosis and the graft undergoes ischaemic necrosis. By the tenth day, graft sloughs off. These events resulting in the rejection of allograft is known as the 'first set response'. Cell mediated reaction is responsible for the reaction. Rejection is primarily by helper T lymphocytes, which activate cytotoxic T lymphocytes, macrophages and B lymphocytes.

When a *second allograft* from the same donor is applied on a sensitised recipient (in which previous graft has been rejected by the first set response), it will be rejected in an accelerated fashion. Necrosis sets in early

and the graft sloughs off by the sixth day. This type of rejection is known as *'second set response'*. In this, antibodies play a dominant role along with cell mediated immunity. 'Second set response' is seen only if the second graft is from the same donor as the first. Application of the graft from another donor will evoke only the first set response.

C. Histocompatibility Antigens

Histocompatibility antigens are specific for each individual and are under genetic control. When the recipient possesses all the antigens present in the graft, there will be no immune response and the transplantation is successful. Rejection of graft depends upon the antigenic differences between graft and recipient. The term 'major histocompatibility complex' (MHC) is a system of cell antigens that are responsible for allograft rejection or acceptance. The MHC in man is the 'human leucocyte antigen (HLA) system'. A description of the HLA system is presented in Chapter 16.

D. Histocompatibility Testing

Procedures used for matching donor and recipient for transplantation are described below.

1. ABO Grouping

ABO compatibility is essential in all tissue transplantation because blood group antigens are strong histocompatibility antigens.

2. Tissue Typing (Detection of MHC Antigens)

The HLA antigens of class I type on leucocytes are identified by means of antisera obtained from multiparous women, from persons who have received multiple blood transfusions, from individuals who are repeatedly skin grafted and from volunteers who have been immunised with cells from another individual with a different HLA haplotype. Monoclonal antibodies are now being used instead of these antisera. Following methods are used:

(i) Cytotoxic test

Lymphocytes are incubated with a panel of standard sera for HLA antigens (class I MHC antigens) in presence of complement. Cells carrying antigens corresponding to the HLA antiserum are killed. These can be detected by the addition of trypan blue which stains only dead cells.

(ii) Mixed lymphocytes culture (MLC)

MHC class II antigens are identified by this method. Lymphocytes from both donor and recipient are cultured together. Donor cells are irradiated to prevent DNA synthesis and proliferation. A radioactive DNA precursor is also added to the mixture of donor and recipient lymphocytes. If the class II antigens are foreign, the recipient cells will be stimulated to divide. As these stimulated recipient lymphocytes replicate their DNA, they incorporate the radioactive precursor. The amount of radioactivity incorporated can be easily measured.

E. Prevention of Graft Rejection

1. Immunosuppression

Immunosuppression is produced in the recipient so that the transplanted tissue survives for a longer period. Irradiation, corticosteroids and antilymphocytic serum (ALS) are employed for immunosuppression in clinical transplantation. Fungal metabolites such as cyclosporin A and repamycin are also used. They show specific T cell inhibitory activity.

2. Transplantation in anatomically protected sites

There are certain privileged sites where allografts are permitted to survive. These include cornea, cartilage and testicle grafting. Lack of vascularity prevents graft rejection in case of corneal transplants.

The foetus can be one example of an intrauterine allograft as it contains antigens which are foreign to the mother. The reason why the foetus is exempt from rejection is not clear. Various explanations are as follows:

(*i*) The placenta produces certain hormones which are locally immunosuppressive.

(*ii*) The placenta produces mucoproteins which coat foetal cells, thus masking HLA-antigens and prevent recognition.

(*iii*) The mother produces specific blocking antibodies to foetal antigens and thus blocking immune recognition.

(*iv*) The high concentration of alpha-fetoprotein may protect the foetus against immunological damage from any maternal leucocytes entering foetal circulation. This is due to immunosuppressive properties of alpha-fetoprotein.

(*v*) β_1-glycoprotein, of foetal origin, has been shown to inhibit maternal cellular immunity.

These factors depress the immune system of the mother and its response is localised in the uterus.

F. Graft-Versus Host Reaction

Graft rejection is generally due to the reaction of the host to the grafted tissue (*host-versus-graft response*). Contrary to that, the graft may mount an immune response against

the antigens of the host this is known as the 'graft-versus-host (GVH) reaction'.

The GVH reaction occurs when the following conditions are present:

1. The graft contains immunocompetent T-lymphocytes. MHC antigens of recipient activate transplanted immunocompetent T-lymphocytes which lead to the production of lymphokines, Tc cells, antibodies etc. They attack the recipient cells leading to manifestations of GVH reaction.
2. The recipient possesses HLA antigens that are absent in the graft.
3. The recipient's immunological responsiveness is either destroyed or so impaired (following whole body irradiation) that he cannot reject a graft.

The GVH reactions are predominantly cell mediated. The manifestations of GVH reaction consist of splenomegaly, fever, rash, anaemia, weight loss and sometimes death. Neonatally thymectomised animal receiving an allograft of spleen or blood lymphocytes do not grow normally but develop a fatal wasting syndrome known as *runting disease*.

II. TUMOUR IMMUNITY

When a cell undergoes malignant transformation, it expresses new surface antigens and may also lose some normal antigens. The tumour associated antigens are immunologically distinct from normal tissue antigens. The host recognises the tumour associated antigens as "nonself," therefore, tumour can be considered as an allograft and is expected to induce an immune response.

A. Tumour Antigens

They can be classified into two groups:

1. Tumour specific antigens (TSAs)
2. Tumour associated antigens (TAAs).

1. Tumour Specific Antigens (TSAs)

Tumour specific antigens are present on the membrane of cancer cells and not on the normal cells. They induce an immune response when the tumour is transplanted in syngenic animals. Such tumour specific antigens reject tumour transplants in immunised hosts, these are termed as 'tumour specific transplantation antigens' (TSTA) or 'tumour associated transplantation antigens' (TATA).

In chemically induced tumours, these antigens are tumour specific. Different tumours possess different antigens, even if they are induced by the same carcinogen. In contrast, the TSAs of virus induced tumours are virus specific. All tumours produced by one virus will possess the same antigens, even if the tumours are in different animal strains or species.

2. Tumour Associated Antigens (TAAs)

These are present on tumour cells and also on some normal cells. These include:

(*i*) *Tumour associated carbohydrate Antigens* (TACAs): They represent abnormal forms of glycoproteins and glycolipids such as mucin-associated antigen detected in pancreatic and breast cancers.

(*ii*) *Oncofetal antigens*: These are foetal antigens which are present in embryonic and malignant cells, but not in normal adult cells. The best known examples are alpha-fetoprotein in hepatomas and carcinoembryonic antigen (CEA) in colonic cancers.

(*iii*) *Differentiation antigens*: CD10, an antigen expressed in early B-lymphocytes, is present in B-cell leukaemias. Similarly, prostate specific antigen (PSA) has been used as a diagnostic indicator in prostatic cancer.

B. Immune Response in Malignancy

Both cell-mediated and humoral responses can be demonstrated in malignancy. The sensitised T-cells attack the 'foreign' tumour cells and tend to limit its growth. Of all the T-cell subsets, delayed-type hypersensitivity T (Td) cells play a significant role in tumour killing by means of lymphokines that they release. The natural killer (NK) cells can also kill the tumour cells in absence of antibody. In addition to direct lysis of tumour cells, NK cells also participate in antibody dependent cytotoxicity (ADCC). Thus NK cells may provide the first line of defence against many tumours.

Humoral responses may also participate in tumour cell killing by activation of complement and induction of ADCC by NK cells.

C. Immunosurveillance

It is believed that malignant cells arise by mutation of somatic cells that occur frequently. It is postulated that the immune system keeps a constant vigilance on these malignant mutation of somatic cells and destroy them on the spot. Inefficiency of the immunosurveillance, either as a result of ageing or in congenital or iatrogenic immunodeficiencies, leads to an increased incidence of cancer. The development of tumours represents an escape from this surveillance. The mechanisms of such escape are not clear but various possibilities have been suggested. These are as follows:

1. *Modulation of Surface Antigens*: Certain tumour cells may shed or stop expressing the surface antigens thus

making the tumour cells immunologically invisible.
2. *Masking Tumour Antigens*: Some cancers produce a mucoprotein called sialomucin. It binds to the surface of the tumour cells. Since sialomucin is a normal component, the tumour cells are not recognised as foreign by the immune system.
3. *Production of Blocking Antibodies*: Certain tumour cells invoke immune system to produce blocking antibodies which can not fix and activate complement resulting in prevention of tumour cell lysis.
4. *Fast Rate of Proliferation of Malignant Cells*: Due to the fast rate of proliferation of malignant cells, tumour cells may be able to 'sneak through' before the development of an effective immune response and once they reach a certain mass, the tumour load may be too great for the host's immune system to control.
5. *Suppression of Cell Mediated Immunity* (CMI): Some tumours may form cytokines like Transforming Growth Factor β (TGF-β) which suppresses CMI.

D. Immunotherapy of Cancer

Immunotherapy of cancer is of two types:

1. Non-Specific Active Immunotherapy

This activates the immune system in a generalised manner which destroys the tumour cells. BCG vaccine when injected directly into certain tumours may lead to tumour regression. Antitumour effect of BCG is believed to be due to activation of macrophages and NK cells. BCG has been reported to be useful in malignant melanomas, bladder cancer, lung cancer and certain leukaemias. *Corynebacterium parvum* has been reported to be beneficial in various types of lung cancers and metastatic breast cancer. Dinitrochlorobenzene (DNCB) has been tried in squamous and basal cell carcinoma of the skin. Levamisole, originally introduced as an antihelminthic, has been tried for stimulating cell mediated immunity and macrophage functions.

Other nonspecific immune modulators include thymic hormones to restore T-cell function, interferon to stimulate NK cell function and IL-2 to stimulate killing of cancers by Tc cells, NK cells and macrophages.

2. Specific Immunotherapy

This has been attempted by vaccination with tumour antigens, treatment with 'immune RNA' and transfer factor. Monoclonal antibody to tumour antigens may be administered, either alone or tagged with a cytotoxic drug, will bind to and specifically destroy only cancer cells. Lymphokine activated killer (LAK) cells obtained by treatment of natural killer (NK) cells with interleukin-2 (IL-2) have been tried in the treatment of certain malignancies, such as renal carcinomas.

KEY POINTS

1. *Transplantation* is a useful procedure for replacement of diseased tissues or organs which are irrepairably damaged. The tissue or organ transplanted is called *transplant* or *graft*.
2. There are four types of transplant namely *autograft*, *isograft*, *allograft* and *xenograft*.
3. Rejection of graft depends upon the antigenic differences between graft and recipient. The *transplantation* (*HLA*) *antigens* are responsible for graft rejection or acceptance.
4. Graft rejection is generally due to the reaction of the host to the grafted tissue (*host-versus-graft response*). Contrary to that, the graft may mount an immune response against the antigens of the host this is known as the '*graft-versus-host* (*GVH*) *reaction*'.
5. It is believed that malignant cells arise by mutation of somatic cells that occur frequently. It is postulated that the immune system keeps a constant vigilance on these malignant mutation of somatic cells and destroy them on the spot. This is known as *immunosurveillance*.
6. The development of tumour represents an escape from this immunosurveillance.

YOU MUST KNOW

1. Types of transplants.
2. Histocompatibility antigens.
3. Graft-versus-host (GVH) reaction.
4. Immunosurveillance.

STUDY QUESTIONS

1. Define various types of transplants. Describe briefly about the allograft reaction.
2. Write short notes on:
   (a) Histocompatibility antigens
   (b) Graft-versus-host (GVH) reaction.
3. Discuss briefly about role of immunity in tumours.
4. Write short notes on:
   (a) Tumour antigens
   (b) Immunosurveillance.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Grafts between two genetically non-identical members of the same species are known as:
   (a) Autografts
   (b) Isografts
   (c) Allografts
   (d) Xenografts
2. Which of the following cells may provide the first line of defence against many tumours?
   (a) Eosinophils
   (b) Natural killer cells
   (c) Monocytes
   (d) None of the above

Answers (MCQs):

1. (c) 2. (b)

Chapter 22

IMMUNOHAEMATOLOGY

The ABO system is the most important of all the blood group systems known and its discovery made blood transfusion possible. The other blood group systems include Rh, MN, P, Lutheran, Lewis, Kell, Duffy, Kidd, Diego, Yt, Kg, Dombrock and Colton. The ABO and Rh systems are the major blood group antigens.

I. ABO BLOOD GROUP SYSTEM

The ABO blood group system was originally described by Landsteiner (1900) and now contains four blood groups. In 1930, Landsteiner was awarded the Nobel Prize for the discovery of human blood groups. The blood group is determined by presence or absence of two antigens A and B on the surface of the red cell membrane. Red cells of blood group A carry antigen A, cells of blood group B antigen B and cells of blood group AB have both A and B antigens, while blood group O cells have neither A nor B antigens. These groups are also distinguished by the presence or absence of two distinct isoantibodies in the serum. The serum contains the isoantibodies for the antigen that is absent on the red cell. The serum of blood group A individual possesses anti-B antibodies, blood group B has anti-A antibodies, and blood group O both anti-A and anti-B, while in blood group AB both anti-A and anti-B antibodies are absent (Table 22.1).

Anti-A and anti-B isoantibodies are called natural antibodies because they are seen to arise without any apparent antigenic stimulation.

Table 22.1 Distribution of ABO Antigens on the Red Cells and Isoantibodies in the Serum.

Blood group	Antigen on red cells	Isoantibodies in serum
A	A	Anti-B
B	B	Anti-A
AB	AB	None
O	None	Anti-A and Anti-B

H Antigen

The H antigen (H substance) is present on red cells of all ABO blood groups. It is the precursor substance on which A-B-O genes operate to produce A and B antigens. Due to universal distribution of H antigen, it is not ordinarily important in blood grouping or blood transfusion. However, Bhende et al (1952) from Bombay reported a rare instance in which A and B antigens as well as H antigens were absent from red cells. This is called as OH blood group or Bombay type. Sera of these persons contain anti-A, anti-B and anti-H antibodies, therefore, they can accept the blood only from their own group (OH or Bombay type).

A, B and H antigens are glycoproteins. H antigen is structurally L-fucose, A and B antigens are formed by addition of N-acetylgalactosamine and galactose respectively to H antigen (L-fucose). In addition to erythrocytes, these antigens are also present in almost all tissues and fluids of the body.

II. RH BLOOD GROUP SYSTEM

Landsteiner and Wiener produced an anti-Rhesus serum by injecting rabbits with red cells from Rhesus monkey. Anti-Rhesus serum agglutinated about 83% human red cells. This type of antigen was then named Rhesus antigen or Rh factor.

Of all the Rh antigens, antigen D (Rho) is the most potent antigen. Rh positive or Rh-negative blood depends on the presence or absence of D-antigen on the surface of red cells respectively. Among Indians, approximately 93% are Rh positive and about 7% negative. The Rh factor can be detected by testing the blood with anti-D (anti-Rh) serum.

In contrast to ABO system, there are no natural antibodies against Rh antigen. Rh antibodies arise in serum only in consequence of Rh incompatible pregnancy or transfusion. Most of these antibodies belong to the IgG class, and being 'incomplete antibodies', they do not agglutinate Rh positive red cells in saline. A minority are complete (saline agglutinating) antibodies of IgM class. These are not relevant in the pathogenesis of haemolytic disease of newborn as they do not cross the placenta. The IgG class incomplete antibodies can be detected in the maternal blood by indirect Coombs test and in cord blood of newborn by direct Coombs test.

III. HAEMOLYTIC DISEASE IN NEWBORN

When an Rh negative person gets transfusion with Rh positive blood or when an Rh negative mother carries an Rh-positive foetus, antibodies to Rh are produced.

The haemolytic disease of the newborn occurs most commonly when the mother is Rh negative while the father and foetus are Rh positive. The mother is usually immunised at first delivery and consequently the first child escapes damage (except when the woman has already been transfused earlier with Rh positive blood). During first delivery, foetal cells enter the maternal circulation in large numbers and induce Rh antibodies. During subsequent pregnancy, the Rh antibodies (IgG class) pass from the mother to the foetus and damage its erythrocytes. The clinical features of haemolytic disease of newborn may vary from erythroblastosis foetalis to intrauterine death due to hydrops foetalis.

However, the incidence of haemolytic disease of newborn is much less than the expected figures which is probably due to the following:

1. *Immunological unresponsiveness to Rh antigens*: Some Rh-negative individuals do not form Rh antibodies even after repeated injection of Rh-positive cells. They are called 'nonresponders'. The reason for this unresponsiveness is not known.
2. *ABO incompatibility between foetus and mother*: In faeto-maternal ABO incompatibility, the foetal cells entering the maternal circulation are destroyed rapidly by the mother's incompatible anti-A or anti-B antibodies before they can induce Rh antibodies.
3. *Number of pregnancies*: The risk increases with each successive pregnancy of mother with Rh-positive foetuses. The first child usually escapes damage because sensitisation occurs only during its delivery.

Rh isoimmunisation can be prevented by administration of anti-Rh IgG immediately after delivery. To be effective, this should be employed from first delivery onwards. The passively administered IgG antibody may prevent isoimmunisation by a negative feedback mechanism.

ABO Haemolytic Disease

Due to maternofoetal ABO incompatibility haemolytic disease may occur in the newborn. Persons of blood group A or B possess IgM natural antibodies in blood, which cannot cross the placenta to harm the foetus. But persons with blood group O contain predominantly IgG antibodies in blood. Hence ABO haemolytic disease is seen more frequently in O group mothers having A or B group foetus. As ABO haemolytic disease is because of naturally occurring maternal isoantibodies, it may occur even in first birth, without prior immunisation. ABO haemolytic disease is more common than Rh haemolytic disease but is much milder. The *direct Coombs test* with foetal erythrocytes is often negative but *indirect Coombs test* with neonatal serum (cord blood) is more commonly positive.

IV. COMPLICATIONS OF BLOOD TRANSFUSION

The complications of blood transfusion may be of two types, immunological and non-immunological.

1. Immunological Complications

Following an incompatible blood transfusion, the red cells undergo intravascular haemolysis or they may be coated by antibodies and engulfed by phagocytes, removed from circulation and subjected to extravascular lysis.

Some transfusion reactions may be due to processes other than blood group incompatibility. These are due to the recipient being hypersensitive to some allergen present in the donor blood. Patient develops rigor, urticaria and other manifestations.

2. Non-immunological Complications

The most important is transmission of infectious agents during blood transfusion. These may include viruses, bacteria and protozoa. HIV and hepatitis viruses are of

great concern among all the infectious agents associated with blood transfusion. List of various infectious agents transmitted via blood transfusion is as follows:

1. Viruses

Human immunodeficiency viruses (HIV-1 and HIV-2)
Hepatitis B virus (HBV)
Hepatitis C and D viruses
Cytomegalovirus (CMV)

2. Bacteria

Treponema pallidum
Leptospira interrogans

3. Protozoa

Plasmodia
Leishmania donovani
Toxoplasma gondii

KEY POINTS

1. The *ABO system* is the most important of all the blood group systems known. Another important system is *Rh blood group system.*
2. Transmission of infectious agents is an important complication of blood transfusion. *HIV* and *hepatitis viruses* are of great concern among all the infectious agents associated with blood transfusion.

YOU MUST KNOW

1. Rh blood group system.
2. List of infectious agents transmitted via blood transfusion.

STUDY QUESTIONS

1. Name various blood group systems and describe Rh blood group system.
2. Write short notes on:
   (a) Haemolytic disease in newborn (b) Complications of blood transfusion.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Red cells of individual with group A carry:
   (a) Antigen A (b) Antigen B (c) Both of the above (d) None of the above
2. Erythrocytes of individual with blood group B carry:
   (a) Antigen A (b) Antigen B (c) Both of the above (d) None of the above
3. Red cells of individual with blood group AB carry:
   (a) Antigen A (b) Antigen B (c) Both of the above (d) None of the above
4. Erythrocytes of individual with blood group O carry:
   (a) Antigen A (b) Antigen B (c) Both of the above (d) None of the above
5. Haemolytic disease in newborn may occur when:
   (a) An Rh negative mother carries an Rh positive foetus
   (b) An Rh positive mother carries an Rh negative foetus
   (c) Both of the above
   (d) None of the above
6. The following infectious agents may be transmitted via blood transfusion:
   (a) Human immunodeficiency virus (b) Hepatitis C virus
   (c) *Treponema pallidum* (d) All of the above

Answers (MCQs):

1. (a) 2. (b) 3. (c) 4. (d) 5. (a) 6. (d)

UNIT III

SYSTEMIC BACTERIOLOGY

Chapter 23

STAPHYLOCOCCUS

Staphylococci are Gram positive cocci arranged in grape-like clusters. They are the commonest cause of suppuration. *Sir Alexander Ogston* (1880) a Scottish surgeon, established the pathogenic role of coccus in abscesses and other suppurative lesions. He gave the name *Staphylococcus* (*Staphyle*, meaning a bunch of grapes, *kokos*, meaning a berry) from the typical arrangement of the cocci in grape like clusters. He also observed that staphylococcal strains from pyogenic lesions produce golden yellow colonies while those from the normal skin produce white colonies on solid culture media. *Rosenbach* (1884) named them *Staph. aureus* and *Staph. albus* respectively. *Passet* (1885) described a third strain of Staphylococcus, that produces lemon yellow colour and named it as *Staph. citreus*. Since pigment production is not a constant character, this classification is now obsolete.

I. SPECIES

The genus *Staphylococcus* contains various species but the medically important species are:

A. Staphylococcus aureus

Staphylococcus aureus is a pathogenic staphylococcus that causes pyogenic infections in man.

B. Staphylococcus epidermidis

Staphylococcus epidermidis is a skin commensal.

C. Staphylococcus saprophyticus

Staphylococcus saprophyticus acts as an opportunistic pathogen.

Pathogenic strains (e.g. *Staph. aureus*) have certain characteristics like production of enzymes coagulase, phosphatase, deoxyribonuclease and their ability to ferment mannitol. Based on production of enzyme coagulase, staphylococci are classified as *coagulase positive* and *coagulase negative*. Most of the *coagulase positive* strains produce golden yellow colonies and are known as *Staph. aureus* (also named as *Staph. pyogenes*). These strains are toxigenic. The *coagulase negative* strains are generally non-pathogenic, non-toxigenic and form white colonies, these are called *Staph. epidermidis* (formerly known as *Staph. albus*).

II. STAPHYLOCOCCUS AUREUS

A. Morphology

They are Gram positive cocci arranged in grape-like clusters, non-motile, non-sporing, approximately 1 μm in diameter (Fig. 23.1). Cluster formation is due to sequential division of bacteria in three perpendicular planes with daughter cells remaining in close proximity. They may also be present singly, in pairs, tetrads or short chains of three or four cells especially when examined from liquid culture. A few strains may possess microscopically visible capsules, especially in young cultures. Many apparently non-capsulated strains also have some amount of capsular material on the surface.

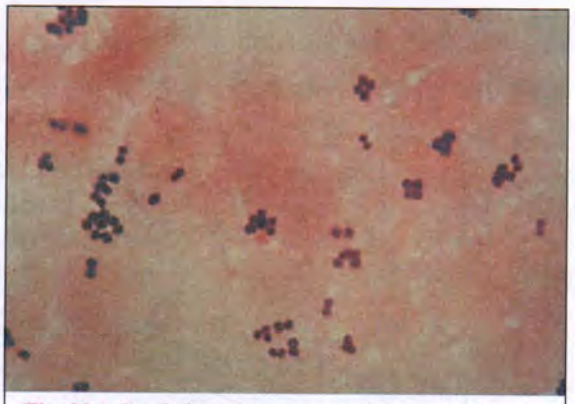


Fig. 23.1 *Staphylococcus aureus* on Gram staining

B. Culture

They grow readily on ordinary culture media within a temperature range of 10–42°C, the optimal temperature being 37°C and pH 7.4–7.6. They are aerobes and facultative anaerobes.

1. Nutrient Agar

After overnight or 24 hours incubation, the colonies are 2–4 mm in diameter, circular, smooth, convex, opaque and easily emulsifiable. Most of the strains produce golden yellow pigment. The pigment is not diffusable into the medium. Pigment production is best seen at 22–25°C and only in aerobic cultures. Pigment production is enhanced by incorporation of 1% glycerol monoacetate or milk in the medium. The pigment is believed to be a **carotenoid**.

2. Blood Agar

Colonies are similar to those on nutrient agar and in addition a beta type of haemolysis is seen. Haemolysis is best observed with sheep or rabbit blood.

3. MacConkey's Agar

Colonies are very small and pink due to lactose fermentation.

4. Selective Media

These media are used for isolating *Staph. aureus* from samples, such as faeces containing other bacteria. Staphylococci can tolerate 8–10% NaCl, lithium chloride, tellurite and polymyxin. Incorporation of these reagents into media make them selective. Examples of selective media include **salt milk agar** (containing 8-10% Nacl) and **Ludlam's medium** (containing lithium chloride and tellurite).

5. Mannitol Salt Agar

This is both a selective and an indicator medium. It contains nutrient agar with 1% mannitol, 7.5% sodium chloride and phenol red as indicator. Yellow coloured colonies are seen on this medium due to fermentation of mannitol by most strains of *Staph. aureus*. Mannitol fermentation leads to production of acid and lowers the pH of medium (phenol red produces yellow colour in acidic pH).

6. Milk Agar

This medium is prepared by mixing sterile nutrient agar and sterilised milk. Colonies of *Staph. aureus* are larger than those on nutrient agar and pigment production is enhanced.

7. Liquid Medium

Uniform turbidity is produced in peptone water or nutrient broth.

C. Biochemical Reactions

Staph. aureus is catalase positive (unlike streptococci) and oxidase negative. It breaks down carbohydrates by

fermentation whereas micrococci break down sugars aerobically. This property can be tested on Hugh-Leifson (O/F) medium.

It ferments a number of sugars producing acid without gas. These fermentation reactions are of no diagnostic value except mannitol fermentation which may be useful to differentiate *Staph. aureus* (mannitol positive) from *Staph. epidermidis* (mannitol negative).

The following characteristics help to distinguish a pathogenic strain of staphylococcus (*Staph. aureus*) from other non-pathogenic strains (e.g. *Staph. epidermidis*).

1. Beta type of haemolysis on blood agar
2. Production of a golden yellow pigment
3. Coagulase production
4. Mannitol fermentation
5. Gelatin liquefaction
6. Phosphatase production
7. Production of enzyme deoxyribonuclease
8. Tellurite reduction

Phosphatase test

Most strains of *Staph. aureus* produce enzyme phosphatase. For detection of this enzyme, *Staph. aureus* is grown on nutrient agar containing phenolphthalein diphosphate. Enzyme phosphatase acts on phenolphthalein salt to release free phenolphthalein. The colonies turn pink when exposed to ammonia vapour due to the presence of free phenolphthalein.

Deoxyribonuclease test

Staph. aureus produces enzyme deoxyribonuclease which hydrolyses DNA.

Tellurite reduction

Staph. aureus reduces tellurite to tellurium producing black colonies, when grown in a medium containing potassium tellurite (e.g. Potassium tellurite blood agar used for *C. diphtheriae*).

Other biochemical tests

Staphylococci hydrolyse urea, reduce nitrates to nitrites, are indole negative and are MR and VP positive. Most strains are lipolytic and produce a dense opacity when grown on egg yolk medium. These tests are of lesser importance.

D. Resistance

Staphylococci are more resistant among the non-sporing bacteria. They survive in dried pus for 2–3 months. Most of the staphylococci are killed at 62°C for 30 minutes but some may require 80°C for one hour. Heat resistant strains may even grow at a higher temperature of 45°C. Most strains grow well in the presence of 10% NaCl. These features are significant in food preservation.

They are killed by crystal violet at a concentration of one in five hundred thousand and by brilliant green, at a concentration of one in ten million. This is the reason that crystal violet agar is used as selective medium for isolation of streptococci. Staphylococci are resistant to 1% phenol for 15 minutes while mercury perchloride (1%) solution kills them in 10 minutes. They are resistant to lysozyme but some micrococci are sensitive to it. Staphylococci are generally sensitive to lysostaphin, which is a mixture of enzymes produced by a particular strain of *Staph. epidermidis*.

E. Antigenic Structure (Fig. 23.2)

1. Capsule

Some strains of *Staph. aureus* possess capsule and inhibit phagocytosis. The capsule is composed of polysaccharide. Capsulated strains tend to be more virulent.

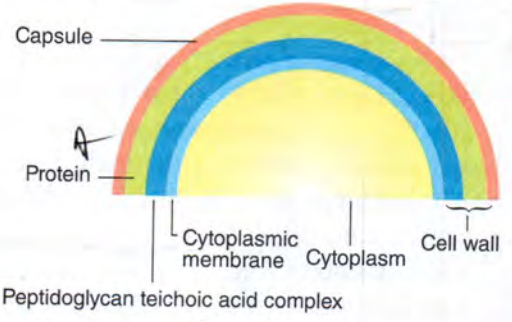


Fig. 23.2 Antigenic structure of *Staphylococcus aureus*

2. Peptidoglycan

Peptidoglycan is a polysaccharide polymer that provides rigidity to the cell wall. It activates complement and evokes production of inflammatory cytokines.

3. Teichoic Acid

It is a major antigenic determinant of all strains of *Staph. aureus*. It is the group specific ribitol teichoic acid of the cell wall. It facilitates adhesion of the cocci to the host cell surface and protects them from complement mediated opsonisation. It is associated with the peptidoglycan in an insoluble form. It is absent in *Staph. epidermidis*. The latter contains glycerol teichoic acid.

4. Protein A

It is a cell wall component of most strains of *Staph. aureus* (especially Cowan I strain). It is chemotactic, antiphagocytic, anticomplementary and elicits platelet injury. Protein A has ability to bind the Fc portion of immunoglobulin G (IgG). Binding IgG in this manner

can block phagocytosis. Cowan I strains coated with any IgG antiserum will be agglutinated if mixed with its corresponding antigen. This procedure is known as *Coagglutination* (refer Chapter 14).

F. Toxins and Enzymes

Staph. aureus forms a number of toxins and enzymes. They are important virulence factors of the organism for producing a disease in the host.

1. Toxins

(i) Haemolysins

Four antigenically distinct types called alpha, beta, gamma and delta haemolysins are produced by staphylococci. These are exotoxins.

(a) Alpha lysin (α–lysin)

Alpha lysin (also known as *alpha* toxin) is the most important in pathogenicity. In cultures, it is produced only under aerobic conditions. It is a protein and is inactivated at 60°C. The toxin regains activity if it is further heated to 80°C–100°C. This is due to toxin combining with a inhibitor at 60°C. At higher temperature, the inhibitor is inactivated resulting in setting the toxin free. Alpha-lysin is lytic to rabbit erythrocytes but less active against sheep or human red cells. It is also cytotoxic (platelet), leucocidal and dermonecrotic. It causes injury to the circulatory system and muscle tissue.

(b) Beta lysin (β–lysin)

Beta lysin is haemolytic for sheep cells, but not for human or rabbit erythrocytes. Lysis is initiated at 37°C but it is evident only on cold temperature. It is named as *hot-cold phenomenon*. It is produced both aerobically as well as anaerobically. It has been identified as sphingomyelinase C. It acts on sphingomyelin in the plasma membrane of erythrocytes and is also called the *hot-cold lysis*.

(c) Gamma lysin (γ–lysin)

It acts on human, sheep and rabbit erythrocytes.

(d) Delta lysin (δ–lysin)

The delta lysin is lytic to human, sheep and rabbit red blood cells.

(ii) Leucocidin

It is composed of two components, S (slow) and F (fast). These components damage polymorphonuclear leucocytes and macrophages. This toxin is also called the *Panton-Valentine toxin* after its discoverers.

(iii) Enterotoxin

The toxin is responsible for staphylococcal food poisoning—nausea, vomiting and diarrhoea occurring within 2–6 hours of consuming contaminated food. Nine (A, B, C1, C2, C3, D, E, H and I) antigenically distinct enterotoxins have been identified. Some strains may form toxin of more than one type. Type A toxin is responsible for the most cases. The enterotoxin is resistant to gut enzymes. It also resists boiling for 30 minutes, a temperature that kills *Staph. aureus*. Toxin is produced when *Staph. aureus* grows in carbohydrate and protein foods, usually cooked food. Milk and milk products have also been implicated. The food may contain preformed toxin but not the bacteria. The source of infection is usually a staphylococcal lesion in skin of fingers of food handler. Ingestion of micrograms of toxin can lead to food poisoning. The toxin is believed to act directly on the autonomic nervous system to cause the illness. Enterotoxin is also a **superantigen** and thus stimulates T-lymphocytes to release interleukins and tumour necrosis factor. These factors contribute in production of clinical illness. Enterotoxins have also been implicated in some cases of post-antibiotic diarrhoea. The toxin can be detected by serological tests such as ELISA and latex agglutination.

(iv) Toxic shock syndrome toxin (TSST)

Toxic shock syndrome (TSS) is characterised by fever, hypotension, vomiting, diarrhoea and an erythematous rash with subsequent desquamation and hyperaemia of mucous membranes. Most TSST producing strains belong to bacteriophage group-I. TSST type-1 (formerly also known as enterotoxin F) is most often responsible. The TSST-1 toxin producing strains colonise vagina more frequently. TSST is a **superantigen** and thus a potent activator of T-lymphocytes resulting in release of interleukins and tumour necrosis factor. This results in clinical condition of TSS.

(v) Exfoliative (epidermolytic) toxin

Two types of exfoliative toxin, A and B have been described. Toxin A is heat stable while toxin B is heat labile. Production of toxin A is under chromosomal control whereas toxin B is plasmid mediated. These toxins are produced by some strains of *Staph. aureus* (phage group II). They cause epidermal splitting resulting in blistering diseases. It may lead to generalised desquamation producing *staphylococcal scalded skin syndrome (SSSS)*. The severe form of SSSS is known as Ritter's disease in the new born. Milder forms are pemphigus neonatorum and bullous impetigo.

2. Enzymes

Staph. aureus produces a number of enzymes, coagulase, phosphatase and deoxyribonuclease which are related to virulence of the bacteria.

Staph. aureus produces an enzyme coagulase. There are eight (A–H) antigenic types of coagulase. Most human strains produce coagulase type A. It has a property to clot human or rabbit plasma. Coagulase is secreted free into the culture medium. It is heat labile. It requires a plasma factor (**coagulase reacting factor**, *CRF*) for its clotting action. CRF is present in rabbit and human plasma. Clotting does not take place with guinea pig plasma because it lacks CRF. Coagulase converts fibrinogen into fibrin.

Clumping factor (also called **bound coagulase**) is heat stable constituent of the cell wall which reacts directly with the fibrinogen and causes clumping of cocci due to the precipitation of fibrin on the cell surface.

The test for coagulase is done by the slide and the tube method. It is done to differentiate pathogenic (*Staph. aureus*) strain from non-pathogenic strains.

(i) Slide coagulase test

Slide coagulase test detects *bound coagulase*. A few colonies of bacteria are emulsified in a drop of normal saline on a clean glass slide and mixed with a drop of undiluted rabbit or human plasma. Prompt clumping of the suspension occurs with coagulase positive strains. False positive results may occur in cases of citrate utilising bacteria (*Enterococcus* and *Pseudomonas*). Some strains which are negative for *bound coagulase* but positive for *free coagulase* gives false negative results by 'slide coagulase test'. Hence, these should be confirmed by 'tube coagulase test'.

(ii) Tube coagulase test

Tube coagulase test is done for detection of extracellular **free coagulase**. 0.1ml of an overnight broth culture or an agar culture suspension of the organism is mixed with 0.5 ml of a 1 in 5 dilution of human or rabbit plasma. Diluted plasma alone in a second tube serves as a control. The tubes are incubated in a water bath at 37°C for three to six hours. In case of a positive test, the plasma clots and does not flow when the tube is inverted. If clot does not appear it is left overnight at room temperature and is re-examined. On continued incubation, the clot may be lysed by production of fibrinolysin by some strains. Controls with known coagulase-positive and coagulase-negative cultures must be included with each batch of tests.

False positive

Citrated plasma should not be used because some contaminated (e.g. Pseudomonas) bacteria may utilize the citrate and produce false positive reaction. Oxalate, EDTA or heparin are more suitable anticoagulants.

G. Pathogenesis

Staph. aureus is an important pyogenic organism and lesions are localised in nature in contrast to streptococcal lesions which are spreading in nature. Coagulase enhances virulence of *Staph. aureus* by inhibiting phagocytosis. It forms a wall of fibrin clot around the lesion. Thick creamy pus is formed in staphylococcal infections.

Staphylococcal diseases may be classified as cutaneous and deep infections; food poisoning, nosocomial infections, skin exfoliative diseases and toxic shock syndrome.

1. Cutaneous Infections

Superficial infections include pustules, boils, carbuncles, abscesses, styes, impetigo, pemphigus neonatorum, wound and burn infections.

2. Deep Infections

These include osteomyelitis, tonsillitis, pharyngitis, sinusitis, pneumonitis, empyema, endocarditis, meningitis, bacteriaemia septicaemia and pyaemia.

They may cause urinary tract infections especially in association with local instrumentation or diabetes.

3. Food Poisoning

Staphylococcal food poisoning may follow 2–6 hours after the ingestion of contaminated food which contains preformed enterotoxin of *Staph. aureus*.

4. Nosocomial Infections

They are important cause of hospital acquired infections.

5. Skin Exfoliative Diseases

These diseases are produced by the strains of *Staph. aureus* that produce epidermolytic toxin. Stripping of the superficial layers of the skin from the underlying tissue occurs in the various exfoliative syndromes caused by staphylococci (bullous impetigo, pemphigus neonatorum, Ritter's disease). Staphylococcal scalded skin syndrome (SSSS) is one example of exfoliative diseases in which toxin spreads systemically. It is seen, but not exclusively, in small children.

6. Toxic Shock Syndrome (TSS)

It is caused by toxin shock syndrome toxin (TSST-1) and characterised by high fever, hypotension, vomiting,

diarrhoea and scarlatiniform rash. Although TSS became widely known in association with the use of tampons by menstruating women but it occurs in other situations also. Subsequently non-menstrual associated TSS has also been reported in both males and females as a complication of staphylococcal abscesses, osteomyelitis, post-surgical wound infections etc.

H. Typing Methods

1. Bacteriophage Typing

Strains of *Staph. aureus* may be distinguished by their susceptibility to different bacteriophages. An internationally accepted set of phages is used. A set of 23 bacteriophages is employed. The strain to be typed is grown on nutrient agar to produce a lawn culture. After drying, the phages of basic set are applied in a fixed dose (routine test dose, RTD). After overnight incubation, the culture will be observed for lysis by the phages. The phage type is designated according to the phages capable of lysing the bacterial strain (Fig. 23.3). Thus a strain of phage type 52/80/94 is the one that is lysed only by phages 52, 80 and 94. Phage typing is important in epidemiological studies of staphylococcal infections.

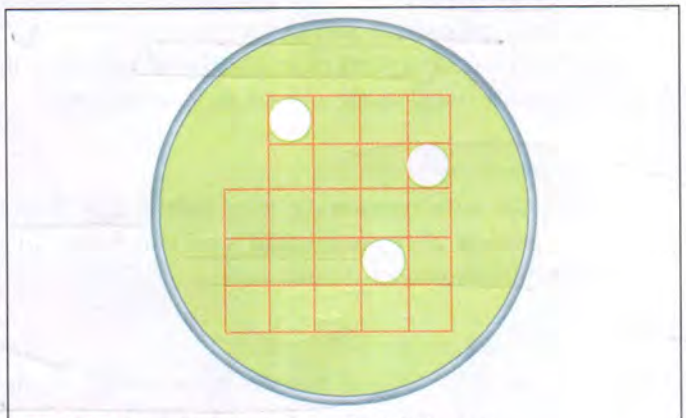


Fig. 23.3 Bacteriophage typing of staphylococci

The reference centre for staphylococcal phage typing in India is located in the Department of Microbiology, Maulana Azad Medical College, New Delhi.

Typing set of staphylococcal phages

Group I	29, 52, 52A 79, 80
Group II	3A, 3C, 55, 71
Group III	6, 42E, 47, 53, 54, 75, 77, 83A, 84, 85
Group IV	—
Group V	94, 96
Not allocated	81, 95,

2. Other Typing Methods

Molecular typing methods such as DNA fingerprinting, ribotyping and PCR are used. Pulse field gel electrophoresis (PFGE) and sequence-based typing methods are now used in investigating of an staphylococcal outbreak.

I. Antibiotic Sensitivity

Most of the strains of *Staph. aureus* were sensitive to penicillin originally. Soon after penicillin came to be used clinically, resistant strains began to emerge. Penicillin resistance in staphylococci is due to the following:

1. Production of **beta lactamase** (*penicillinase*) which is plasmid coded and transmitted by transduction or conjugation. The same plasmid may also carry genes for resistance to other antibiotics (erythromycins, tetracyclines, aminoglycosides etc.) and heavy metals. Beta lactamase inactivates penicillin by splitting the beta lactam ring. Four types (A to D) of penicillinases are produced by staphylococci.
2. **Methicillin resistant staphylococci:** There is reduction in affinity of penicillin binding proteins of the staphylococcal cell wall for β-lactam antibiotics. This change is normally chromosomal in nature. This type of resistance also occurs in beta lactamase resistant penicillins such as methicillin, nafcillin and oxacillin. They may cause outbreaks of hospital infection. These strains are called *methicillin resistant Staph. aureus (MRSA)*. These are important cause of postoperative wound infections and other hospital acquired infections. They may also cause epidemics of hospital cross infections. Vancomycin or teicoplanin is used for treatment of infections with MRSA. Methicillin resistant strains of *Staph. epidermidis* (MRSE) have also been described. As methicillin is an unstable drug, oxacillin is used for sensitivity testing. These strains are resistant to all β-lactam agents and often to other agents such as aminoglycosides and fluoroquinolones. Methicillin resistance correlates with the presence of the resistance gene *Mec A* that codes for a unique penicillin binding protein PBP_{2a} (or PBP2') not affected by beta lactamase resistant penicillins. This gene is transmitted chromosomally. Although predominantly a hospital pathogen, MRSA is becoming more common in community. These MRSA strains are named community acquired MRSA strains (CA-MRSA). MRSA strains restricted to hospital settings are named hospital acquired MRSA strains (HA-MRSA). Hospital personnel harboring MRSA have been implicated as the chief source of nosocomial infection. These strains can cause minor and systemic staphylococcal infections.

MRSE has been recognised as important agents of nosocomial infections in hospitalised patients who have undergone prosthetic heart valve surgery. Eradication of colonised sites (e.g. nose or skin) with topical agents is often indicated for reducing the risks of cross infection in hospital.

The following measures may be adopted to control staphylococcal infections in hospitals.

- To isolate the patients with open staphylococcal lesions.
- Detection of staphylococcal lesions among hospital staff and keeping them away from work till they are treated for their lesions.
- Strict aseptic techniques to be followed.
- Hand washing, the most effective method to control hospital infections.
- In case of out-break of staphylococcal sepsis, a search may be made for carriers among hospital staff. Carriers detected should be treated with local application of muprocin or chlorhexidine.

3. Bacterium may also develop tolerance to penicillin, by which it is only inhibited and not killed.

J. Epidemiology

Human patients and carriers are the commonest source of infection. About 10–30% of healthy persons carry the bacteria in the nose and less often in skin, axilla, perineum and throat. In hospital, more than 50% of nursing staff are carriers of *Staph. aureus*. Staphylococcal disease may be exogenous or endogenous. It spreads by contact, either direct or through fomites or by other vehicles. Staphylococci are the commonest cause of hospital cross infections. These bacteria are often antibiotic resistant strains. These hospital strains spread from the hospital staff or other patients. The postoperative wards, operating rooms, intensive care units, newborn nursery and cancer chemotherapy wards are at the highest risk of these infections.

K. Laboratory Diagnosis

1. Specimens

These are collected according to the nature of lesion as follows:

Specimen	Lesion
Pus	Suppurative lesions
Sputum	Respiratory infections
Blood	Septicaemia or PUO
Urine	Urinary tract infection
CSF	Meningitis
Faeces	Food poisoning
Food or vomit	Food poisoning

Nasal and perineal swabs are collected for detection of carriers

2. Collection and Transport

Specimens should be collected in sterile containers under all aseptic conditions. Sterile swabs should be used for collection of specimens from sites such as nose or perineum. In case of urine, midstream urine should be collected. Blood should be collected in blood culture bottles comprising of glucose broth and taurocholate broth.

Specimens should be transported immediately to the laboratory and processed.

3. Direct Microscopy

Direct microscopy with Gram-stained smears of pus or wound exudate is useful, where Gram positive cocci in clusters may be seen (Fig. 23.4). This is of no value for specimens where mixed bacterial flora are normally present e.g. sputum.

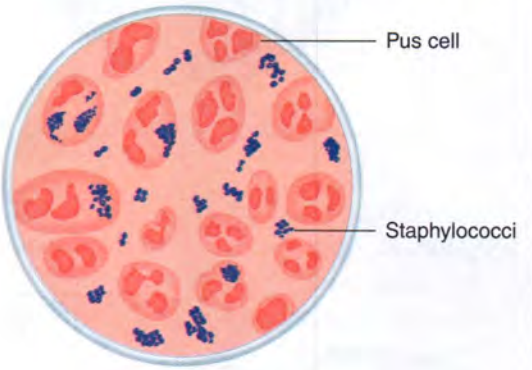


Fig. 23.4 Staphylococci in stained smear of pus

4. Culture

The specimens are inoculated on following media:

- Blood agar
- Peptone Water

Specimens where staphylococci are expected to be scanty or outnumbered by other bacteria (e.g. faeces, swabs from carriers), are inoculated on selective media such as:

- Salt agar
- Salt milk agar
- Robertson's cooked meat medium (RCM) containing 10% NaCl.

The inoculated media are incubated at 37°C for 18–24 hours. Next day, culture plates are examined for morphology of bacterial colonies and other characters. Uniform turbidity is produced in liquid medium such

as peptone water. Gram staining from colony on blood agar and hanging drop preparation from peptone water are done to further characterise the bacteria.

5. Colony Morphology and Gram Staining

On blood agar, colonies are 2–4 mm in diameter, circular, raised, opaque and produce golden yellow pigment. Beta haemolysis is seen around these colonies. The pigment is not diffusable into the medium (Fig. 23.5).

On Gram staining, they are Gram positive cocci (1 μm in diameter) arranged in grape like clusters. Non-motile cocci in clusters are seen in hanging drop preparation.

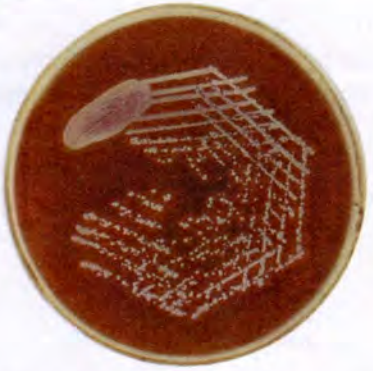


Fig. 23.5 *Staphylococcus aureus* on blood agar

6. Biochemical Reactions

(*i*) Catalase test: All staphylococci (pathogenic and non-pathogenic) are catalase positive. This test distinguishes Staphylococcus from Streptococcus which is catalase negative.

(*ii*) Coagulase test: It is positive in *Staph. aureus* but negative in other staphylococci. It helps in differentiating a pathogenic strain (*Staph. aureus*) from non-pathogenic strains. This test has been described in detail on page no. 181 of this chapter. Other tests which are positive in pathogenic strain (*Staph. aureus*) and distinguish it from non-pathogenic staphylococci are described below.

(*iii*) Mannitol fermentation: Acid production without gas

(*iv*) Gelatin liquefaction: Positive

(*v*) Tellurite reduction: Positive

(*vi*) Production of enzymes phosphatase and deoxyribonuclease: Both are positive.

Besides mannitol fermentation, other carbohydrates may also be fermented but are not helpful in diagnosis.

7. Bacteriophage Typing

It is done for epidemiological purposes to trace the source of *Staph. aureus* infections. It is especially useful in food poisoning in large number of persons affected in the community. Other typing methods include antibiogram pattern, plasmid typing, ribotyping and DNA fingerprinting.

8. Antibiotic Susceptibility

This is important as staphylococci develop resistance to drug readily. Antibiotic sensitivity can be determined by Stokes method or Kirby-Bauer method.

L. Treatment

Benzyl penicillin is the most effective antibiotic in sensitive strains. Cloxacillins are used against beta-lactamase producing strains. Vancomycin is used in treatment of infections with MRSA strains. Strains resistant to vancomycin and teicoplanin have appeared in hospitals. For mild superficial lesions, topical applications of bacitracin or chlorhexidine may be sufficient.

Some strains show drug tolerance. These strains will be found susceptible in the disc diffusion method but their minimum bactericidal concentration (MBC) will be very much higher than minimum inhibitory concentration (MIC). They are not killed by antibiotics in the routine doses.

The carriers are treated by local application of antibiotics such as bacitracin and antiseptics such as chlorhexidine. In resistant cases, rifampicin along with another oral antibiotic may be useful.

M. Control

1. Source: Isolation and treatment.
2. Detection of carriers among hospital staff, their isolation and treatment.
3. Sterilisation of instruments.
4. Detection of staphylococcal lesions and their treatment.
5. Stop misuse of antibiotics.

III. OTHER COAGULASE POSITIVE STAPHYLOCOCCI

Certain strains of coagulase positive staphylococci do not possess all the characteristics of *Staph. aureus*. These organisms are called *Staphylococcus intermedius*. They are mostly of animal origin. *Staph. hyicus* is another coagulase positive staphylococcus but does not infect humans.

IV. COAGULASE NEGATIVE STAPHYLOCOCCI (CoNS)

Coagulase negative staphylococci (CoNS) form the part of the normal flora of the skin. They are opportunistic pathogens, which cause infection in debilitated or immunocompromised patients.

A. Staphylococcus epidermidis

It is a skin commensal and acts as opportunistic pathogen in prosthetic devices e.g. prosthetic heart valves, intraperitoneal catheters, orthopaedic prostheses and vascular grafts. It may cause septicaemia and subacute bacterial endocarditis. It may produce stitch abscess. It mainly acts as pathogen in immunocompromised individual. Their aetiological role may be proved by repeated isolation.

B. Staphylococcus saprophyticus

It acts as an opportunistic pathogen. It is an important cause of urinary tract infection in young, sexually active females. When present in urine cultures, *Staph. saprophyticus* may be found in low numbers ($<10^4$/mL) and still be considered as significant. *Staph. saprophyticus* can also cause septicaemia and endocarditis in patients with cardiac surgery. The aetiological role is again by repeated isolation. *Staph. saprophyticus* is novobiocin resistant which distinguishes it from *Staph. epidermidis*. Novobiocin resistance can be determined by using novobiocin disc (5 μg) over the lawn culture of a test strain and measuring the zone of inhibition. A zone of inhibition less than 12 mm indicates resistance.

Differentiating features of *Staph. aureus, Staph. epidermidis* and *Staph. saprophyticus* are shown in Table 23.1.

Table 23.1 Differentiating Features of Three Species of Staphylococcus

Feature	*Staph. aureus*	*Staph. epidermidis*	*Staph. saprophyticus*
Coagulase test	+	–	–
Mannitol fermentation	+	–	–
Production of			
DNAase	+	–	–
Phosphatase	+	–/weak +	–
α–lysin	+	–	–
Protein A in the cell wall	+	–	–
Sensitivity to lysostaphin	+	–	–
Novobiocin resistance	–	–	+
Pathogenicity	Primary	Opportunistic	Opportunistic

C. Other coagulase negative staphylococci

Staphylococcus haemolyticus may cause wound infection, bacteraemia, urinary tract infection and endocarditis. Other coagulase negative staphylococci include *Staph. lugdunensis, Staph. schleiferi* and *Staph. simulans.*

V. MICROCOCCI

Micrococci are free living in the environment. These are Gram positive cocci, catalase positive, coagulase negative, arranged in clusters which differ from staphylococci in attacking sugars oxidatively. They may appear in irregular clusters, groups of four or of eight. They are often larger than staphylococci. Colonies are generally white in colour. Their staining is often not uniform. They are saprophytes and commensals. They may rarely cause opportunistic infection. Micrococci can be differentiated from staphylococci by Hugh-Leifson's oxidation-fermentation (O/F) test in which micrococci show oxidative and staphylococci show fermentative breakdown of carbohydrate. They are also resistant to lysostaphin while *Staphylococcus aureus* is sensitive (Table 23.2).

Table 23.2 Distinguishing Characters of Staphylococcus aureus and Micrococcus

Character	Staphylococcus aureus	Micrococcus
Gram staining	Gram positive	Gram positive, darkly stained
	Grape like clusters	Present in groups of four (tetrad) or eight
	Uniform staining	Often staining is not uniform
	1 μm in size	Larger than staphylococcus
Colony characters	Colonies are golden yellow in colour	Colonies are generally white in colour
Coagulase test	Positive	Negative
Breakdown of carbohydrates (O/F test)	Fermentatively	Oxidatively
Aerobic acid production from glycerol in the presence of erythromycin	Positive	Negative
Sensitivity to lysostaphin (200 μg disc)	Sensitive	Resistant
Furazolidine susceptibility (100 μg of furazolidone disc)	Sensitive	Resistant
Bacitracin (0.04 unit disc) susceptibility	Resistant	Sensitive

KEY POINTS

1. Staphylococci are *Gram positive coci* arranged in *grape-like clusters*. They are the commonest cause of *suppuration*.
2. Medically important species of staphylococus include *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Staphylococcus saprophyticus*.
3. *Staphylococcus aureus* is a pathogenic staphylococcus.
4. Pathogenic strains (e.g. *Staph. aureus*) have certain characteristics like production of enzymes *coagulase*, *phosphatase*, *deoxyribonuclease* and their ability to *ferment mannitol*.
5. Based on production of enzyme coagulase staphylococci are classified as *coagulase positive* (e.g. *Staph. aureus*) and *coagulase negative* (e.g. *Staph. epidermidis*, *Staph. saprophyticus*).
6. Mannitol salt agar is a selective medium for isolation of *Staph. aureus*.
7. *Haemolysins*, *leucocidin*, *enterotoxin*, *toxic shock syndrome toxin* (TSTT), *exfoliative toxin* are some toxins produced by *Staph. aureus*.
8. *Staph. aureus* also produces a number of enzymes, *coagulase*, *phosphatase* and *deoxyribonuclease*.
9. The test for coagulase is done by the *slide* and the *tube* method. It is done to differentiate pathogenic (*Staph. aureus*) strain from non-pathogenic strains.
10. *Staph. aureus* is an important *pyogenic organism* and lesions are *localised* in nature. They may also cause *food poisoning*, *nosocomial infections*, *scalded skin syndrome* and *toxic shock syndrome*.
11. Strains of *Staph. aureus* may be distinguished by *bacteriophage typing*.
12. Penicillin resistance in staphylococci may be due to production of *beta lactamase* (*penicillinase*) which is *plasmid coded* and transmitted by *transduction*.
13. Methicillin resistant *Staph. aureus* (MRSA) may cause outbreaks of hospital infection.
14. Coagulase negative staphylococci form the part of the normal flora of the skin. They are *opportunistic pathogens*, which cause infection in debilitated or immunocompromised patients.
15. *Staph. saprophyticus* is *novobiocin resistant* which distinguishes it from *Staph. epidermidis* and *Staph. aureus*.
16. Micrococci are *saprophytes* and *commensals*. They may rarely cause opportunistic infections.
17. Micrococci differ from staphylococci in attacking sugars *oxidatively*.

YOU MUST KNOW

1. Morphology and culture characteristics of *Staph. aureus*.
2. Toxins and enzymes of *Staph. aureus*.
3. Diseases caused by *Staph. aureus*.
4. Methicillin resistant *Staph. aureus* (MRSA).
5. Laboratory diagnosis of infections caused by *Staph. aureus*.
6. Differences between *Staph. aureus*, *Staph. epidermidis* and *Staph. saprophyticus*.
7. Coagulase negative staphylococci (CNS).
8. Micrococci.

STUDY QUESTIONS

1. Describe the laboratory diagnosis of infections caused by *Staph. aureus*.
2. Write short notes on:
   (a) Toxins and enzymes produced by *Staph. aureus*.
   (b) Toxic shock syndrome
   (c) Staphylococcal food poisoning

(d) Coagulase test
(e) Clumping factor
(f) Bacteriophage typing
(g) Staphylococcal scalded skin syndrome.

3. Write briefly about:
(a) MRSA
(b) Coagulase negative staphylococci
(c) Micrococci
(d) Antigenic structure of *Staph. aureus.*

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Which of the following characteristics help to distinguish *Staphylococcus aureus* from non-pathogenic strains of Staphylococcus?
(a) Beta type of haemolysis on blood agar
(b) Production of a golden yellow-pigment
(c) Coagulase production
(d) All of the above

2. Protein A is a cell wall component of:
(a) *Staphylococcus aureus*
(b) Coagulase negative staphylococci
(c) Micrococci
(d) None of the above

3. Which of the following toxins is responsible for Staphylococcal scalded skin syndrome?
(a) Enterotoxin (b) Leucocidin (c) Haemolysin (d) Exfoliative toxin

4. Slide coagulase test detects:
(a) Free coagulase (b) Bound coagulase (c) Both of the above (d) None of the above

5. Tube coagulase test detects:
(a) Free coagulase (b) Bound coagulase (c) Both of the above (d) None of the above

6. Which of the following bacteria produce coagulase enzyme?
(a) *Staphylococcus aureus* (b) *Staph. epidermidis* (c) *Staph. saprophyticus* (d) None of the above

7. Which of the following staphylococci is/are novobiocin resistant?
(a) *Staphylococcus aureus* (b) *Staph. epidermidis* (c) *Staph. saprophyticus* (d) None of the above

8. All of the following are coagulase negative staphylococci except:
(a) *Staphylococcus epidermidis*
(b) *Staph. saprophyticus*
(c) *Staph. aureus*
(d) *Staph. haemolyticus*

Answers (MCQs):

1. (d) 2. (d) 3. (d) 4. (b) 5. (a)
6. (a) 7. (c) 8. (c)

Chapter 24

STREPTOCOCCUS AND ENTEROCOCCUS

Streptococci are Gram positive cocci which are arranged in chains. They are part of the normal human flora. Some of them are important human pathogens causing pyogenic infections. They grow poorly in simple media but their growth is enhanced by the addition of fermentable carbohydrate (e.g. glucose), blood or serum.

The name streptococci (*streptos* meaning twisted or coiled) was given by *Billroth* (1874). *Rosenbach* (1884) coined the name *Streptococcus pyogenes* for the organisms which he isolated from human suppurative lesions.

I. CLASSIFICATION (FIG. 24.1)

The streptococci are divided into aerobic streptococci, obligate anaerobes and facultative anaerobes. Obligate anaerobic streptococci are described in chapter on 'Nonsporing anaerobes' (Chapter 30). Aerobic and facultative anaerobic streptococci are classified on the basis of their haemolytic properties. Three types of haemolytic reactions (α, β and γ) are observed on blood agar medium.

A. Alpha (α) Haemolytic Streptococci

They produce a greenish discolouration around the colonies. This is due to partial haemolysis. The zone of lysis is small (1 or 2mm wide) with presence of unlysed erythrocytes which are detectable microscopically. Alpha haemolysis is seen with viridans group of streptococci and pneumococcus.

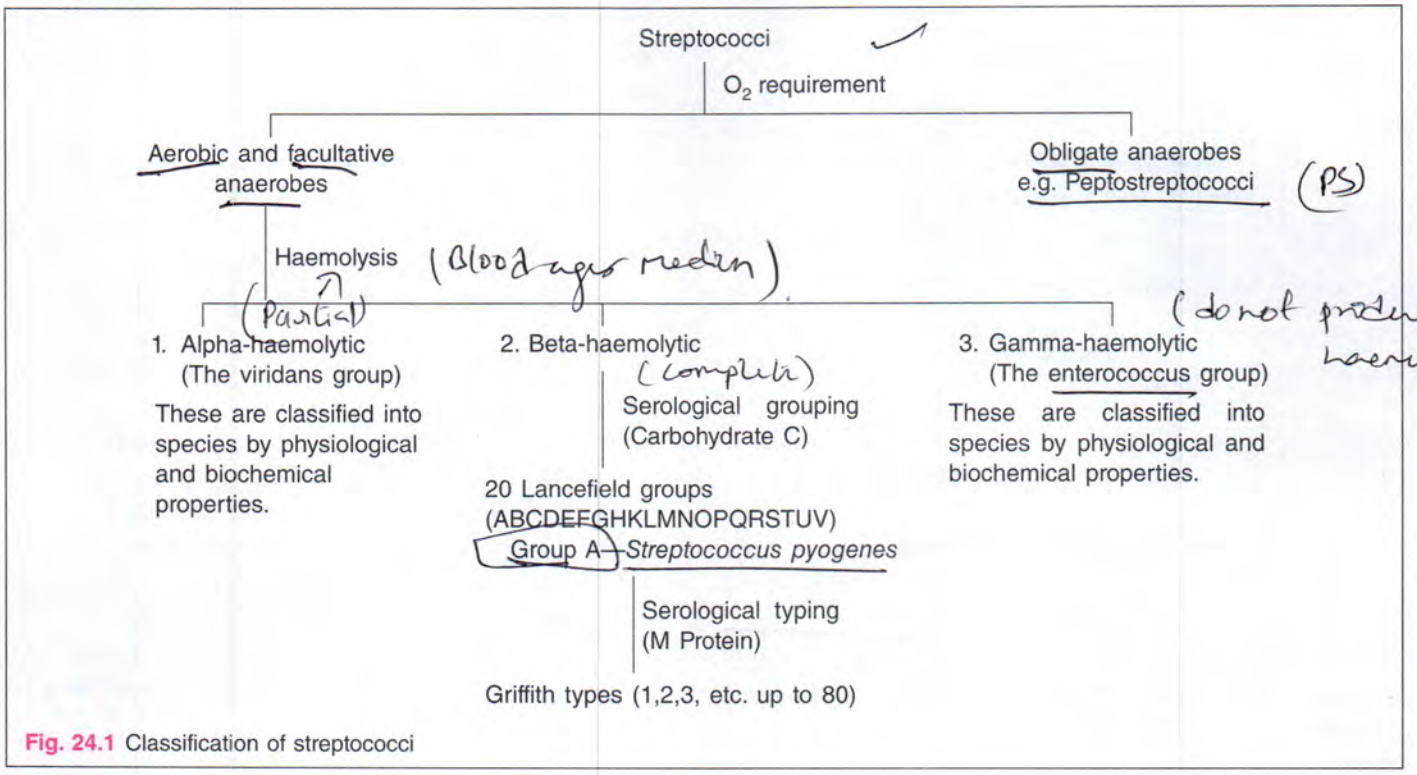


Fig. 24.1 Classification of streptococci

B. Beta (β) Haemolytic Streptococci

These streptococci produce a clear, colourless zone of complete haemolysis (2–4 mm wide) around the colonies, within which erythrocytes are completely lysed. The lysis of erythrocytes is due to the production of two types of streptolysin by the organisms: *streptolysin O and streptolysin S*. The former is destroyed by oxygen and the latter is oxygen stable (S for stable). Streptolysin S is responsible for surface colony haemolysis. Since most strains produce both types of haemolysin, they can usually be recognised by β haemolysis of surface colonies. Most of the pathogenic streptococci fall into this group. *Streptococcus pyogenes* is the most important and is responsible for many important human infections.

C. Gamma (γ) or Non-Haemolytic Streptococci

They do not produce haemolysis and *Str. faecalis* is a typical example.

The beta haemolytic streptococci were classified by *Lancefield* (1933) serologically into a number of broad groups based on the nature of a carbohydrate (C) antigen on the cell wall. These are known as *Lancefield groups*, 20 of which have been identified, named A–V (without I and J) by precipitation reaction performed with appropriate sera. Majority of streptococci that produce human infections belong to group A named as *Streptococcus pyogenes*. These are further subdivided by type specific antisera into approximately 80 Griffith serotypes (type 1, 2 etc.) based on their surface proteins (M, T and R). Serotyping of isolated strains is useful in epidemiological studies.

II. STREPTOCOCCUS PYOGENES

A. Morphology

The individual cocci are spherical or oval, 0.5 to 1.0 μm in diameter and are arranged in chains. Chain formation is due to successive cell divisions occurring in one plane only and daughter cells failing to separate completely (Figs. 24.2 and 24.3).

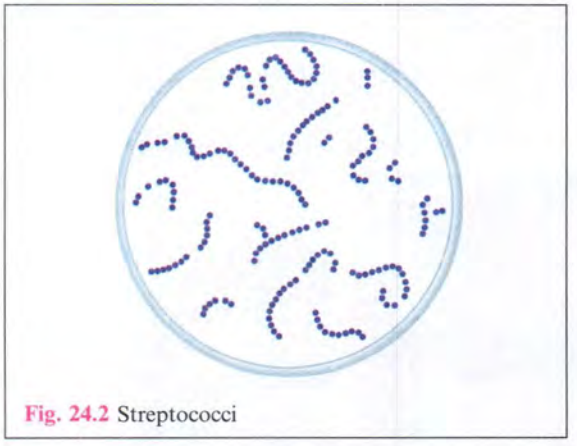


Fig. 24.2 Streptococci

Larger chains are formed in liquid than in solid media. The length of bacterial chain has no relevance to virulence or other properties. In fact, non-pathogenic

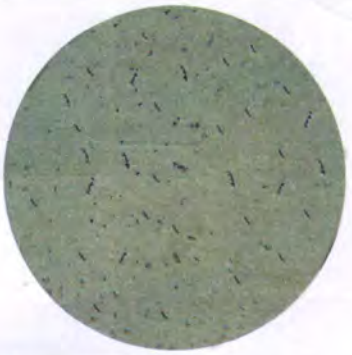


Fig. 24.3 *Streptococcus pyogenes*

strains such as *Streptococcus salivarius* forms the longest chain.

Streptococci are Gram positive, non-motile and non-sporing. Some strains of *Streptococcus pyogenes* (Group A) and group C have capsules composed of hyaluronic acid, while members of groups B and D show polysaccharide capsules. These capsules are best noticeable in very young cultures.

B. Culture

They are aerobes and facultative anaerobes, growing best at a temperature of 37°C (range 22–42°C). These are most exacting in nutritive requirements, growth occurring only in media containing blood, serum or sugars (fermentable carbohydrates). On blood agar, after overnight incubation, the colonies are small (0.5–1.0 mm, pin point), circular, semitransparent, low convex with a wide zone of β–haemolysis around them (Fig. 24.4). Growth and haemolysis are promoted by presence of 10% CO_2 in the environment. Mucoid colones are formed by strains which produce large capsules. Selective media containing 1:500,000 crystal violet (crystal violet blood agar) permit growth of streptococci but inhibit other bacteria especially staphylococci. Another selective medium is PNF medium (horse blood agar containing polymyxin B sulphate, neomycin sulphate and fusidic acid) used for isolation of streptococci.

Fig. 24.4 *Streptococcus pyogenes*

In liquid media, such as glucose broth, growth occurs as a granular turbidity with a powdery deposit. Bacterial chains being heavier settle down as deposit.

C. Biochemical Reactions

Streptococci are catalase negative, unlike staphylococci which are catalase positive. They are not soluble in 10 per cent bile, unlike *Str. pneumoniae*. Hydrolysis of pyrrolidonyl naphthylamide (PYR test) and failure to ferment ribose are useful to differentiate *Str. pyogenes* from other streptococci. *Str. pyogenes* produces the enzyme L-pyrrolidonyl-β-naphthylamidase (Pyrase). This enzyme hydrolyses L-pyrrolidonyl-β-naphthylamide to p-naphthylamine which produces red colour in the presence of β-dimethylamino cinnamaldehyde. PYR-positive bacteria produce a deep red colour whereas PYR-negative organisms produce a slight orange colour or no colour change.

Several sugars are fermented by streptococci producing acid but no gas. These are of little value in laboratory identification.

D. Resistance

Streptococcus pyogenes is a delicate organism which is inactivated by heat at 56°C for 30 minutes. It dies in a few days in cultures but can be stored in Robertson's cooked meat medium at 4°C. It is rapidly inactivated by antiseptics. It is more resistant to crystal violet than many other bacteria, therefore this dye is used to prepare selective media for the isolation of streptococci. It is susceptible to sulphonamide and many antibiotics. Unlike *Staph. aureus*, it does not develop drug resistance. Sensitivity to bacitracin is employed for differentiating *Str. pyogenes* (Group A) from other haemolytic streptococci. *Str. pyogenes* is resistant to SXT (trimethoprim-suphamethoxazole)

E. Antigenic Structure

1. Capsular Hyaluronic Acid

Capsule may be present on groups A and C streptococci. The capsule when present inhibits phagocytosis.

2. Group Specific Polysaccharide Antigen

The cell wall is composed of an outer layer of protein (fimbria containing protein) and lipoteichoic acid, a middle layer of group-specific C carbohydrate and inner layer of peptidoglycan (mucoprotein) (Fig. 24.5). The peptidoglycan is responsible for cell wall rigidity. It has also some biological activities e.g. pyrogenic and thrombolytic activity. Lancefield grouping is done on the basis of group specific C carbohydrate. *Str. pyogenes* belongs to group A.

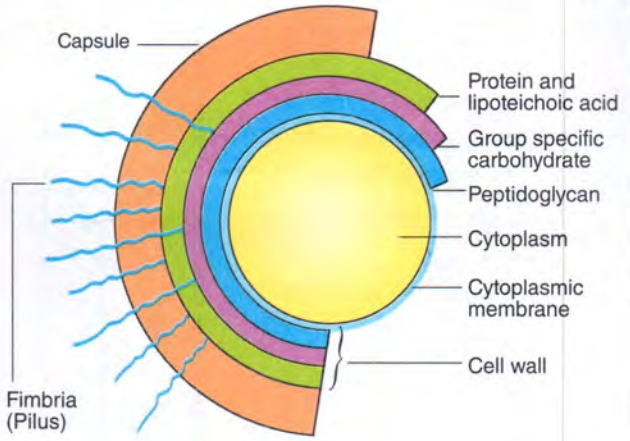


Fig. 24.5 Antigenic structure of *Streptococcus pyogenes*

3. Type Specific Antigen

The outer part of the cell wall contains protein antigens. *Str. pyogenes* is further subdivided on the basis of their surface proteins M, T and R.

(i) M protein

It is the most important antigen and acts as a virulence factor by inhibiting phagocytosis. It is antigenic in nature. The antibody to M protein is protective. The M protein is heat and acid stable but susceptible to trypsin. About 80 M-protein types have been recognised. After extraction of M protein, typing is done with type specific sera. M-protein along with other surface antigens probably have an important role in pathogenesis of rheumatic fever.

(ii) T and R proteins

The T protein is an acid labile, trypsin resistant antigen. It may be specific, but many different M types may have the same T antigen. It is usually differentiated by agglutination with specific antisera. Another surface protein R has been identified in some types of *Str. pyogenes* (type 2,3, 28 and 48) and some strains of group B, C and G. The T and R proteins have no relation to virulence of streptococci.

(iii) Other associated factors

A non-type-specific protein associated with the M protein is identified. This is known as M associated protein (MAP). Some M types of *Str. pyogenes* produce a lipoproteinase which is known as serum opacity factor (SOF). These strains produce opacity when applied to agar gel containing horse or swine serum.

Hair-like pili (fimbria) which consist partly of M protein and are covered with lipoteichoic acid are present in group A streptococci. They are important in the attachment of streptococci to epithelial cells.

F. Toxins and Enzymes

Str. pyogenes produces several exotoxins and enzymes which contribute to its virulence.

1. Toxins

(i) Haemolysins

Streptococci produce two type of haemolysins, *streptolysin 'O'* and *'S'*. Streptolysin 'O' is so named because it is oxygen labile. It is inactivated in the presence of oxygen, but may be reactivated by treatment with reducing substances. It is heat labile. It lyses red cells and is also cytotoxic for neutrophils, platelets and cardiac tissue. It is demonstrable only in deep colonies and not in surface cultures. It is antigenic and antistreptolysin 'O' (ASO) regularly appears in sera following streptococcal infection. An ASO titre in serum in excess of 200 units suggests either recent or recurrent streptococcal infection. Streptolysin 'O' and 'S' are produced by groups A, C and G.

Streptolysin 'S' is an oxygen stable haemolysin and is responsible for the haemolysis seen around colonies of streptococci on the surface of blood agar plates. In addition to haemolytic property, it has got leucocidal action. It is protein in nature but is not antigenic. Convalescent sera do not neutralise streptolysin 'S' activity.

(ii) Pyrogenic exotoxin (erythrogenic toxin)

This toxin is responsible for the rash of scarlet fever. It is only produced by lysogenic strains of group A streptococci. Three antigenically distinct forms of pyrogenic exotoxin, A, B and C have been described. Types A and C are coded for by bacteriophage genes but type B gene is chromosomal. These exotoxins are 'superantigens'—T cell mitogens which induce massive release of inflammatory cytokines. The toxin is antigenic and is neutralised by antibodies present in the convalescent sera. It causes pyrogenicity, cytotoxicity and enhancement of susceptibility to endotoxin. This toxin was renamed *Streptococcal pyrogenic exotoxin* (Spe) because induction of fever is the primary effect of the toxin. There are four distinct types of these exotoxins namely Spe A, Spe B, Spe C and Spe F. These toxins act as superantigens.

This toxin is responsible for Dick test and Schultz Charlton reaction. When the toxin is injected intradermally into the skin of a susceptible child, a localised erythematous reaction appears. This is called the *Dick test*. The test becomes negative during convalescence due to neutralisation of toxin by antibody. In scarlet fever, when homologous antitoxin is injected locally into the rash, blanching of the rash occurs, it is named as *Schultz Charlton reaction*. These tests are now only of historical importance as scarlet fever is now rarely seen.

2. Enzymes

(i) Streptokinase (fibrinolysin)

It promotes the lysis of human fibrin clot by catalysing the conversion of plasminogen (plasma precursor) into plasmin. It is protein in nature and antigenic. Neutralising antibodies (antistreptokinase) appear in convalescent sera which may be estimated for retrospective study of streptococcal infection. Streptokinase facilitates the spread of infection by breaking down the fibrin barrier around the lesions.

(ii) Deoxyribonucleases (streptodornase)

Group A streptococci elaborate four antigenically distinct deoxyribonucleases (DNAases), A,B,C, and D, of which, type B is the most antigenic in man. These enzymes liquefy the highly viscous DNA that accumulate in thick pus. DNA present in pus is derived from the nuclei of necrotic cells. This explains the thin serous character of streptococcal exudate. Enzyme preparation containing both streptokinase and streptodornase have been applied clinically in liquefying localised collections of thick exudates, as in empyema.

Antibodies against deoxyribonuclease B is useful in retrospective diagnosis of *Str. pyogenes* infections, particularly in skin infections, where ASO titre may be low. Apart from group A, deoxyribonuclease is also elaborated by group C and G streptococci.

(iii) Nicotinamide adenine dinucleotidase (NADase)

NADase acts on the coenzyme NAD and liberates nicotinamide from the molecule. It is produced by groups A, C and G streptococci and is antigenic. It is believed to be leucotoxic.

(iv) Hyaluronidase

It is produced by strains of group A,B,C, and G streptococci. It breaks down hyaluronic acid of the tissues and favours spread of streptococcal lesion along intercellular spaces. The enzyme is antigenic and specific antibodies appear in patient's sera.

Streptococci possess a hyaluronic acid capsule and also produce an enzyme hyaluronidase—a seemingly self destructive process. But it is found that those strains which form hyaluronidase in large quantities are non-capsulated. There is also evidence that hyaluronidase produced in tissues is more in amounts than in cultures.

(v) Other extracellular products

Many strains produce neuraminidase, esterases, phosphatase, amylase, lipase, N-acetyl glucosaminidase and beta-glucuronidase. Contribution of these products in pathogenesis is not known. Some M types of *Str. pyogenes* produce serum opacity factor (SOF) which has been described earlier in this chapter.

G. Pathogenesis (Table 24.1)

Str. pyogenes produces pyogenic infections with a tendency to spread locally. Non-suppurative sequelae of local infections include acute glomerulonephritis and rheumatic fever.

Table 24.1 Streptococcal Diseases

Streptococcus	Lesions
Str. pyogenes	
(i) *Pyogenic infections*	
Respiratory tract	Acute tonsillitis or pharyngitis (sore throat), scarlet fever
Skin infections	Infections of wounds, burns and skin lesions (eczema), erysipelas, impetigo, necrotizing fascitis
Genital infections	Puerperal sepsis
Streptococcal toxic shock syndrome	Bacteraemia, necrotizing fascitis
Deep infections	Bone and joint infections, lymphadenitis, septicaemia, abscess in internal organs
(ii) *Non-suppurative complications*	Rheumatic fever, acute glomerulonephritis
Group B Streptococci	Neonatal infections (septicaemia and meningitis)
Enterococcus faecalis	Urinary tract infection, endocarditis
Viridans Streptococci	Endocarditis, dental caries

1. Pyogenic Infections

(i) Respiratory infections

Sore throat (acute tonsillitis and/or pharyngitis) is the most common of streptococcal diseases. Tonsillitis is more common in older children and adults. The organisms may spread to surrounding tissues, leading to complications such as cervical adenitis, otitis media, quinsy, Ludwig's angina and mastoiditis. It may rarely lead to meningitis.

Scarlet fever

It consists of a combination of sore throat and a generalised erythematous rash. It is caused by a strain producing the erythrogenic toxin. It is now rarely seen in U.K. and other colder countries. It does not occur in India.

(ii) Skin infections

Str. pyogenes causes suppurative infections of the skin with a predilection to produce lymphangitis and cellulitis. Infection of minor abrasions may sometimes lead to fatal septicaemia. *Str. pyogenes* has been associated with necrotizing fascitis (NF), an invasive, infection characterised by inflammation and necrosis of the skin, subcutaneous fat and fascia. Although uncommon, NF is a life-threatening infection. The strains causing NF have been named is '**flesh eating bacteria**'.

The two typical streptococcal skin infections are erysipelas and impetigo. Erysipelas is an acute spreading lesion. Skin shows massive brawny oedema with erythema. Elderly persons are usually affected. Impetigo is found mainly in young children. It is caused by *Str. pyogenes* belonging to certain limited number of serotypes. These skin infections are the main cause leading to acute glomerulonephritis in children in the tropics.

In skin infections, antibody response to streptolysin O is not high, therefore ASO titre does not have as much clinical significance as in pharyngeal infections. Antibodies to DNAase B are more useful in retrospective diagnosis of skin infections antecendent to acute glomerulonephritis.

(iii) Streptococcal toxic shock syndrome

Streptococcal toxic shock syndrom (TSS) is a condition in which the entire organ system collapses, leading to death. Group A streptococci associated with streptococcal TSS produce a streptococcal pyrogenic exotoxin notably Spe A. These toxins play a major role in pathogenesis and act as superantigens leading to overstimulation of the immune response. Other virulence factors, such as SLO and various cell wall antigens, can also contribute to toxic shock. Streptococcal TSS resembles Staphylococcal TSS. Patients are often bacteraemic and have necrotizing fascitis. Young children with chickenpox (vericella), and elderly adults seem to be at greater risk.

(iv) Other pyogenic infections

(a) Puerperal sepsis: *Str. pyogenes* was an important cause of puerperal sepsis. It used to take a heavy toll of life before antibiotics became available.
(b) Sepsis: Infections of skin lesions (eczema, psoriasis, scabies), wounds and burns.
(c) Pyaemia, septicaemia, abscess in internal organs (brain, lung, liver and kidney).

2. Non-Suppurative Complications

Str. pyogenes infections are sometimes followed by two important non-suppurative sequelae, *acute rheumatic fever* and *acute glomerulonephritis*. These complications occur one to four weeks after the acute infection. *Str. pyogenes* is no longer detectable when these complications set in. The latent period suggests an immune response. Rheumatic fever is often preceded by sore throat while acute glomerulonephritis by the skin infection. These sequelae or complications are believed to be the result of hypersensitivity to some streptococcal components. Rheumatic fever may follow infection with any serotype of *Str. pyogenes* while acute glomerulonephritis is caused by only a few nephritogenic types (Table 24.2).

Table 24.2 Differentiating Features of Acute Rheumatic Fever and Acute Glomerulonephritis

Feature	Acute Rheumatic Fever	Acute Glomerulonephritis
Primary site of infection	Throat	Throat or skin
Prior sensitisation	Essential	Not necessary
Serotypes of *Str. pyogenes* responsible	Any	Pyoderma types 49, 52–55, 57–61 and throat infection types 12, 1, 25, 4 and 3
Immune response	Marked	Moderate
Complement level	Unaffected	Lowered
Repeated attacks	Common	Absent
Course	Progressive or static	Spontaneous resolution
Prognosis	Variable	Good
Hereditary tendency	Present	Not known
Penicillin prophylaxis	Essential	Not indicated

The mechanism by which streptococci produce rheumatic fever is still not clear. A common cross-reacting antigen may exist in some group A streptococci and heart, therefore, antibodies produced in response to the streptococcal infection could cross react with myocardial and heart valve tissue, causing cellular destruction.

Acute glomerulonephritis probably develops because some components of glomerular basement membrane are antigenically similar to the cell membranes of nephritogenic streptococci. Antibodies formed against streptococci cross react with glomerular basement membrane and result in damage. Alternatively, streptococcal antigen-antibody complexes may deposit in the glomeruli. In either case, the activation of the complement components C3 and C5 leads to tissue destruction.

H. Epidemiology

The major sources of infection are patients and carriers. Symptomless infection is common and maintains the

organism in the community. Transmission is either by direct contact or fomites. Streptococcal infections of respiratory tract are more frequent in children 5–8 years of age. Outbreaks of infection occur in closed communities such as army camps or boarding schools. Control measures include early detection of patients and carriers and their treatment.

I. Laboratory Diagnosis

Diagnosis of acute suppurative infections is made by culture, while in the non-suppurative complications, diagnosis is mainly based on the demonstration of antibodies.

1. Acute Suppurative Infections

(i) Specimens

Specimen is collected according to the site of lesion, such as swab, pus, blood or CSF.

(ii) Collection and Transport

Specimens should be collected in sterile containers under all aseptic conditions. These should be plated immediately or sent to the laboratory in Pike's transport medium (blood agar containing 1 in 1,000,000 crystal violet and 1 in 16,000 sodium azide).

(iii) Gram Staining of Smears

Gram positive cocci in chains is indicative of streptococcal infection. Smears are of no value where streptococci are present as part of the resident flora such as in infections of the throat and genitalia. Smear examination is important in specimens such as pus and CSF.

(iv) Culture

The specimen is inoculated on blood agar medium and incubated at 37°C for 18–24 hours. Haemolysis develops better under anaerobic conditions or in the presence of 5–10% carbon dioxide. Sheep blood agar is preferred as human blood may contain certain inhibitors.

(v) Colony Morphology and Staining

The colonies of streptococci are small 0.5–1.0 mm (size of pinpoint as compared to pinhead size of staphylococci), circular, low convex with a zone of β-haemolysis around them.

In liquid medium, such as glucose broth, growth occurs as a granular turbidity with a powdery deposit. Streptococci chains being heavier settle down as a powdery deposit.

Gram staining from colonies show Gram positive cocci in chains. Hanging drop preparation from liquid medium shows non-motile cocci.

(vi) Biochemical Reactions

Streptococci are catalase negative which is an important test to differentiate streptococci from staphylococci. They are not soluble in 10% bile unlike *Streptococcus pneumoniae*.

Streptococci ferment sugars producing acid but no gas. PYR test and failure to ferment ribose are useful in identification of *Str. pyogenes* from other streptococci.

(vii) Identification of Various Groups of Streptococci

Str. pyogenes (group A) is sensitive to bacitracin (0.04 unit/disc). Group 'A' streptococci can be rapidly identified by fluorescent antibody technique. PYR test may be done to differentiate *Streptococcus pyogenes* from other streptococci. PYR test is positive for *Streptococcus pyogenes*. Group 'B' streptococci may be identified by the CAMP reaction (Christie, Atkins and Munch-Peterson). Both group A and group B are resistant to SXT (trimethoprim-sulphamethoxazole) while all other streptococci are sensitive to it. However enterococci are also resistant to SXT. Group 'D' can be isolated by performing heat resistance test. Details of these tests are described in respective streptococci group.

(viii) Lancefield Grouping

Haemolytic streptococci are grouped serologically by the Lancefield technique. It is done for definite classification and epidemiological studies. Lancefield grouping depends on C carbohydrate, which is specific for each group. C Carbohydrate is extracted from the cell wall of streptococci and grouping done by a precipitation test with group antisera. Streptococci are grown in Todd-Hewitt broth and the extraction of C carbohydrate may be done with hydrochloric acid (Lancefield's acid extraction method), or formamide (Fuller's method) or by autoclaving (Rantz and Randall's method) or by an enzyme produced by *Streptomyces albus* (Maxted's method). The extract and the specific antisera are allowed to react in capillary tubes. Precipitation occurs within five minutes. Besides capillary precipitation, grouping may also be done by agar gel precipitation.

(ix) Antigen Detection Tests

ELISA and agglutination tests are used to demonstrate group A streptococcal antigen from throat swabs.

2. Non–Suppurative Complications

In rheumatic fever and glomerulonephritis, serological tests provide retrospective evidence of streptococcal infection. The routine test done is antistreptolysin O (ASO) titration. A titre of 200 units or more is significant

in rheumatic fever and is indicative of prior streptococcal infection.

ASO test is a neutralisation reaction where antibodies to streptolysin 'O' (ASO) are neutralised with streptolysin 'O' antigen. ASO titre is usually found in high levels in rheumatic fever but in glomerulonephritis, titres tend to be low, therefore, anti-deoxyribonuclease B (anti-DNAase B) estimation is more reliable. Titres higher than 300 or 350 are significant. This test is very useful for retrospective diagnosis of streptococcal pyoderma, for which ASO is of less value. Antihyaluronidase is another useful test for pyoderma infection of streptococci.

The streptozyme test, a passive slide hemagglutination test is a sensitive and specific screening test. It becomes positive after nearly all types of streptococcal infection, whether of the throat or the skin.

J. Treatment

Penicillin G is the drug of choice. In patients allergic to penicillin, erythromycin or cephalexin is used. Antibiotics have no effect on established glomerulonephritis and rheumatic fever.

K. Prophylaxis

Prophylaxis is indicated only in the prevention of rheumatic fever. This prevents streptococcal reinfection and further damage to the heart. Penicillin is administered for a long period in children who have developed early signs of rheumatic fever.

III. OTHER HAEMOLYTIC STREPTOCOCCI

Besides *Str. pyogenes*, haemolytic streptococci belonging to other groups like B, C, D, F, G and rarely H, K, O and R may also lead to human infections.

A. Group B Streptococci (GBS)

Str. agalactiae is an important pathogen of bovine mastitis in cattle. In recent years, it has been recognised as the single most important pathogen in neonates causing neonatal septicaemia and meningitis. It may also cause septic abortion and puerperal sepsis. *Str. agalactiae* is a commensal of female genital tract from where bacterial infection in neonates occur. Other Group B infections in neonates include osteomyelitis, arthritis, conjunctivitis, respiratory infections, endocarditis and peritonitis.

The presumptive identification depends on their ability to hydrolyse hippurate. Hippurate positive bacteria produce a deep purple colour whereas hippurate negative organisms produce a slightly yellow pink colour or fail to produce any colour. Group B streptococci possess the enzyme hippuricase (also called hippurate hydrolase), which hydrolyses sodium hippurate to form sodium benzoate and glycine. This reaction can be detected by adding ninhydrin, which reacts with glycine to form a purple colour. Another test to identify group B is the CAMP reaction (Christie, Atkins and Munch-Peterson), which can be demonstrated by the production of accentuated zone of haemolysis (as butterfly appearance) when Group B streptococci are inoculated perpendicular to a streak of *Staph. aureus* grown on blood agar (Fig. 24.6). Group B streptococci produce a CAMP factor that enhances the lysis of sheep erythrocytes by staphylococcal β-lysin. Other two methods to perform CAMP test include disc method and rapid CAMP test (or spot CAMP test). In disc method, a disc impregnated with the β-lysin is used on the plate containing growth of Group B streptococci. There is an enhanced haemolysis around the disc but it is not of butterfly appearance as disc is round. A third method, the rapid CAMP test, a drop of extracted β-lysin is placed on the growth of suspected Group B streptococci in the culture plate. It is then incubated at 35°C for at least 20 minutes, enhanced haemolysis is observed.

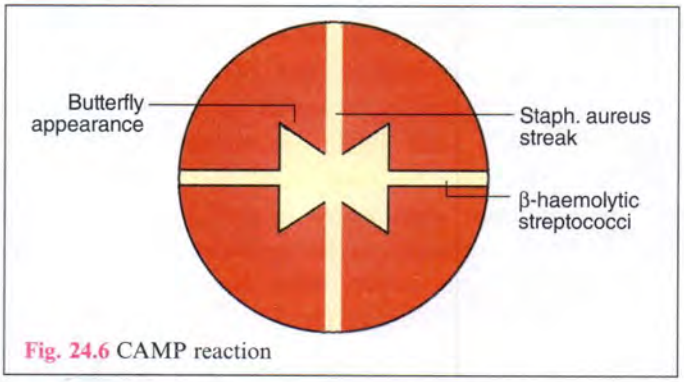


Fig. 24.6 CAMP reaction

Besides hippurate hydrolysis and CAMP test, resistance to sulphamethoxazole (SXT) is another test in identification of Group B streptococci. This test can be performed by applying SXT disc on the growth of Group B streptococci. Resistance to SXT can be observed in case of Group B streptococci.

Nine capsular serotypes of Group B streptococci have been identified.

B. Group C and G Streptococci

The species *Str. dysgalactiae* subsp. *equisimilis* is usually isolated from human sources. The clinical spectrum of disease caused by this species resembles infections caused by *Str. pyogenes*. It may cause human infections like pharyngitis, skin infections, endocarditis, septicaemia and meningitis. *Str. dysgalactiae* subsp *equisimilis* resembles *Str. pyogenes* in fermenting trehalose but differs in fermenting

ribose. Most isolates of this species possess either the Lancefield group C or group G antigen. However some strains containing Lancefield group L or A antigen have been reported. Similar to *Str. pyogenes* cases of glomerulonephritis and acute rheumatic fever have been reported following *Str. dysgalactiae* subsp. *equisimilis* infections. *Str. equi* subsp. zoo*epidemicus* may also cause human infections. It possesses Lancefield group C antigen. *Str. dysgalactiae* subsp. *dysgalactiae* and *Str. equi* subsp. *equi* are animal pathogens. Both species possess Lancefield group C antigen. Another group is *Str. anginosus group* which can harbour the Lancefield group antigen A, C, F, G or none at all.

C. Group D Streptococci

Streptococci belonging to group 'D' are classified as enterococci (faecal streptococci) and non-enterococci (non-faecal streptococci). They are usually non-haemolytic while some strains may be alpha or beta haemolytic. The enterococci group has been reclassified as a separate genus called *Enterococcus*. Non-enterococci of group D (*Str. bovis group*) are inhibited by 6.5 per cent sodium chloride. These are now named as Group D streptococci. Both Group D streptococci and enterococci are positive for bile-aesculin hydrolysis test. They can grow in 40% bile and hydrolyse aesculin to form aesculetin which combines with ferric citrate to give a black complex. Aesculin and ferric citrate are present in the bile aesculin agar slant which is used to perform this test. The test is named as *bile-aesculin hydrolysis test.*

Group D streptococci may cause genitourinary infection or endocarditis rarely. They are susceptible to penicillin.

IV. ENTROCOCCUS

Enterococci also belong to group 'D' as they contain the same antigen as that of group 'D' streptococci. They are normal inhabitants of human intestinal tract and possess some distinctive properties as follows:

1. They are positive for bile-aesculin hyrolysis test.
2. They are relatively heat resistant and can withstand heat at 60° C for 30 minutes *(heat test or heat resistance test)*.
3. Their ability to grow in the presence of 6.5 per cent sodium chloride.
4. Their ability to grow at 45°C and at pH 9.6.
5. They are PYR test positive.
6. They are resistant to SXT (trimethoprim-sulphamethoxazole).

Enterococcal species have been divided into five groups (Group I to Group V) based on acid formation from mannitol and sorbose, and arginine hydrolysis. *Enterococcus faecalis* and *E. faecium* belong to Group II.

On MacConkey's medium they grow as tiny deep pink colonies. On Gram's staining, enterococci appear as pairs of oval cocci and short chains. The identification of the species is based on biochemical reactions. *Enterococcus faecalis* is the most commonly isolated enterococcus from human sources. Other enterococci are *E. faecium* and *E. durans*. *E. faecalis* can be identified by fermentation of mannitol, sucrose, aesculin and sorbitol, and by producing black colonies when grown on tellurite blood agar.

E. faecalis is frequently isolated from cases of urinary tract infection and wound infection. They may also cause other infections like subacute bacterial endocarditis, septicaemia, peritonitis and infection of biliary tract. Strains resistant to penicillin and other antibiotics occur frequently. Vancomycin is the primary alternative drug to penicillin for treating enterococcal infections. Vancomycin resistant enterococci (VRE) have also been isolated. Resistance is most common in *E.faecalis* and *E. faecium*. Seven types of resistance to vancomycin in enterococci have been described. These seven types of resistance are encoded by seven genes namely Van A, Van B, Van C, Van D, Van E, Van G and Van L.

Enterococcus can be differentiated from Group 'D' streptococcus by a number of tests as shown in Table 24.3.

Table 24.3 Differences between enterococcus and Group D streptococcus

Test	Enterococcus	Group 'D' streptococcus
1. Bile-aesculin hydrolysis test	+	+
2. Growth in the presence of 6.5% NaCl	+	–
3. PYR test	+	–
4. Sensitivity to SXT	R	S
5. Ability to grow at 45°C	+	–

V. VIRIDANS STREPTOCOCCI

This group of streptococci produce alpha haemolysis on blood agar. They are known as viridans streptococci (from *viridis* meaning green) due to greenish discolouration (α-haemolysis) on blood agar. They are commensals of mouth and upper respiratory tract. *Str. viridans* includes *Str. mitis* group, *Str. anginosus* group, *Str. salivarius* group, *Str. mutans* group and *Str. bovis* group. Each

group contains many species. *Str. milleri* and Group F streptococci are now included in *Str. anginosus* group. This group can possess Lancefield group antigen A, C, F, G or none at all.

They are ordinarily non-pathogenic but on occasions cause disease. The viridans streptococci are associated with dental caries and subacute bacterial endocarditis. Dental caries is caused by *Str. mutans*. It breaks down dietary sucrose, producing acid and a tough adhesive dextran. The acid damages dentine while the dextran binds together food debris, mucus, epithelial cells and bacteria to form dental plaques. These plaques lead to dental caries.About 40% of subacute bacterial endocarditis is caused by viridans group of streptococci.Members of *Str. mitis* group are the predominant agent in urogenital and gastrointestinal tract. They cause endocarditis in persons with predisposing factor such as valvular disease of the heart, congenital heart disease and cardiac surgery. Following some dental procedures such as tooth extraction, they may cause transient bacteraemia and get implanted on damaged or prosthetic valves or in a congenitally diseased heart, and grow to form vegetations. Prophylactic antibiotic should be given in such persons before tooth extraction or other procedures. Viridans streptococci are generally penicillin sensitive but some strains may be resistant. Hence, antibiotic sensitivity of these bacteria must be determined for appropriate treatment of endocarditis.

Important laboratory tests for diagnosis of different groups of streptococci are shown in Table 24.4.

Table 24.4 Some characteristic features of different groups of streptococci

Lancefield group	Species or common name	Diagnostic tests	Diseases caused
A	*Str. pyogenes*	Bacitracin sensitive; PYR positive; ribose not fermented; resistant to SXT	Upper respiratory tract infections; skin infections; acute rheumatic fever; acute glomerulonephritis
B	*Str. agalactiae*	CAMP test positive; hippurate hydrolysis positive; resistant to SXT	Neonatal septicaemia and meningitis
C or G	*Str. dysgalactiae* subsp. *equisimilis*	Ribose fermented; trehalose fermented	Pharyngitis; endocarditis
D	(i) *Enterococcus sp.* (*E. faecalis* and other enterococci)	Bile aesculin test positive; heat test positive; growth in the presence of 6.5% NaCl; PYR positive; resistant to SXT	Urinary tract infections; wound infections
	(ii) Non-enterococci (*Str. bovis*)	Bile aesculin test positive; no growth in 6.5% NaCl; PYR negative; sensitive to SXT	Genitourinary infections
Not typed	Viridans streptococci (*Str. salivarius* group, *Str. mutans* group and many others)	Optochin resistant; species differentiation on biochemical tests	Endocarditis; dental caries

KEY POINTS

1. *Streptococci* are *Gram positive cocci* which are arranged in *chains*. They grow poorly in simple media but their growth is enhanced by the addition of fermentable carbohydrate (e.g. glucose), blood or serum.
2. Three types of haemolytic reactions (α, β and γ) are observed on blood agar medium.
3. The beta haemolytic streptococci were classified by *Lancefield* serologically into *20 Lancefield groups* named *A-V* (without I and J). These groups are based on the nature of a carbohydrate (C) antigen on the cell wall.
4. *Streptococcus pyogenes* belongs to Lancefield *group A*.
5. *Crystal violet blood agar* is a *selective medium* for isolation of streptococci.
6. *Haemolysins* and *pyrogenic exotoxins* are the toxins produced by *Str. pyogenes*.
7. *Streptokinase*, *deoxyribonucleases*, *nicotinamide adenine dinucleotidase* (*NADase*) and *hyaluronidase* are important enzymes produced by *Str. pyogenes*.

8. *Str. pyogenes* produces *pyogenic infections* with a tendency to *spread locally*. *Sore throat* (*acute tonsilitis* and/or *pharyngitis*) is the most common of streptococcal diseases. Non-suppurative sequelae of local infections include *acute glomerulonephritis* and *rheumatic fever*.
9. Group B streptococci are important pathogens in neonates causing *neonatal septicaemia* and *meningitis*. *CAMP reaction* is used to identify Group B streptococci.
10. *Enterococcus faecalis* is frequently isolated from cases of *urinary tract infection* and *wound infection*. Heat resistance test is positive for *E. faecalis*.
11. *Viridans streptococci* produce *alpha haemolysis* on blood agar. They are *commensals* of mouth and upper respiratory tract. They are ordinarily *non-pathogenic* but on occasions cause disease. They are associated with *dental caries* and *subacute bacterial endocarditis*.

YOU MUST KNOW

1. Classification of streptococci.
2. Morphology and culture characteristics of *Streptococcus pyogenes*.
3. Toxins and enzymes of *Str. pyogenes*.
4. Infections caused by different streptococci.
5. Non-suppurative complications of *Str. pyogenes* infections.
6. Laboratory diagnosis of infections caused by *Str. pyogenes*.
7. Group B streptococci, Group D streptococci and viridans streptococci.

STUDY QUESTIONS

1. Classify streptococci. Describe the laboratory diagnosis of streptococcal sore throat.
2. Write short notes on:
   (a) Antigenic structure of *Str. pyogenes*
   (b) Toxins and enzymes of *Streptococcus pyogenes*
   (c) Non-suppurative complications of *Str. pyogenes* infections
   (d) Lancefield grouping.
3. Write briefly about:
   (a) Group B streptococci
   (b) Group D streptococci
   (c) Enterococci
   (d) Viridans streptococci
   (e) CAMP reaction
   (f) Heat test.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. On blood agar which type of haemolysis is produced by *Streptococcus pyogenes*?
   (a) Alpha haemolysis (b) Beta haemolysis
   (c) Gamma haemolysis (d) None of the above
2. Erythrogenic toxin is responsible for:
   (a) Dick test (b) Schultz-Charlton reaction
   (c) Both of the above (c) None of the above
3. Which of the following is selective medium for *Streptococcus pyogenes*?
   (a) Blood agar (b) Crystal violet blood agar
   (c) Potassium tellurite blood agar (d) Chocolate agar

4. Sensitivity to bacitracin can be used to identify:
   (a) *Streptococcus pyogenes* (b) *Str. agalactiae*
   (c) *Str. equisimilis* (d) *Str. mitis*
5. CAMP reaction can be used to identify:
   (a) *Streptococcus pyogenes* (b) *Str. agalactiae*
   (c) *Str. equisimilis* (d) *Str. mitis*
6. The most important bacterial cause of sore throat is:
   (a) *Streptococcus pyogenes* (b) *Staphylococcus aureus*
   (c) *Staphylococcus epidermidis* (d) *Neisseria gonorrhoeae*
7. Which of the following are non-suppurative complications of *Streptococcus pyogenes* infections?
   (a) Acute rheumatic fever (b) Acute glomerulonephritis
   (c) Both of the above (d) None of the above
8. Which of the following tests may be used for diagnosis of non-suppurative complications of *Streptococcus pyogenes* infections?
   (a) ASO test (b) Anti DNAase B test
   (c) Streptozyme test (d) All of the above
9. Enterococci contain the same antigen as that of:
   (a) Group A streptococci (b) Group B streptococci
   (c) Group C streptococci (d) Group D streptococci
10. Which of the following can cause dental caries?
   (a) *Streptococcus mutans* (b) *Staphylococcus aureus*
   (c) Enterococcus (d) *Streptococcus agalactiae*

Answers (MCQs):

1. (b)	2. (c)	3. (b)	4. (a)	5. (b)
6. (a)	7. (c)	8. (d)	9. (d)	10. (a)

Chapter 25

PNEUMOCOCCUS

Pneumococcus

A. Morphology
B. Culture
C. Biochemical Reactions
D. Resistance
E. Antigenic Structure
F. Variation
G. Toxins and Other Virulence Factors
H. Pathogenesis
I. Epidemiology
J. Laboratory Diagnosis
K. Treatment
L. Prophylaxis

Pneumococci are normal commensals of the upper respiratory tract. They are important pathogens of pneumonia and otitis media in children. They are reclassified as *Streptococcus pneumoniae* because of its genetic relatedness to streptococcus. They differ from streptococci in their morphology (diplococci), bile solubility, optochin sensitivity and by a specific polysaccharide capsule.

PNEUMOCOCCUS

A. Morphology

Pneumococci are Gram positive, small (1 μm diameter), slightly elongated cocci arranged in pairs (diplococci) with the broad ends in apposition. Each coccus has one end broad or rounded and other pointed (flame shaped or lanceolate appearance) (Fig. 25.1). They are capsulated and the capsule encloses each pair. The capsule may be demonstrated as a clear halo in India ink preparation (Fig. 25.2). In old cultures, the capsule is usually lost. They are non-motile and non-sporing.

B. Culture

Pneumococci have complex nutritional requirement and therefore grow only in enriched media especially

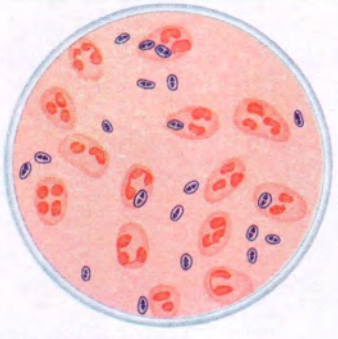


Fig. 25.1 *Str. pneumoniae* in pus

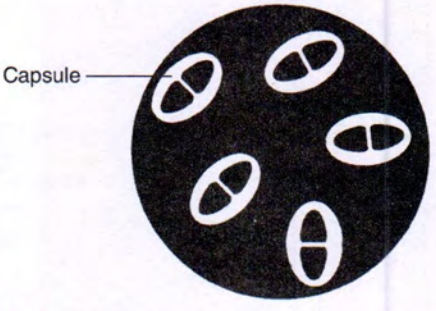


Fig. 25.2 India ink preparation to show pneumococcus capsule

supplemented with blood. They are aerobes and facultative anaerobes and their growth is improved by 5–10 per cent CO_2. The optimum temperature for growth is 37°C (range 25°C to 42°C) and pH 7.8 (range 6.5–8.3).

On blood agar, after incubation for 18 hours, the colonies are usually small (0.5–1 mm), dome shaped, with an area of greenish discolouration (alpha haemolysis) around them. On prolonged incubation, the colonies become flat, with raised edges and central umbonation (due to autolysis occuring at centre) which creates a draughtsman appearance (concentric rings are seen when viewed from above). Some strains such as those of serotype 3 and 7 form large mucoid colonies due to production of abundant capsular polysaccharides. Pneumococci are typically alpha haemolytic but under anaerobic conditions colonies show beta haemolysis due to liberation of oxygen labile pneumolysin O by these bacteria.

In liquid medium such as glucose broth, pneumococci produce uniform turbidity. These cocci readily undergo autolysis in cultures due to the action of intracellular enzymes. Autolysis is enhanced by bile salts and other surface active agents. This property is helpful to differentiate it from other streptococci.

C. Biochemical Reactions

Pneumococci ferment several sugars with production of acid only. Fermentation is tested in Hiss's serum water. Fermentation of inulin by pneumococci is of great value to differentiate them from streptococci.

Pneumococci are soluble in bile. When 2% sodium deoxycholate solution is added to a broth culture at neutral pH, the culture clears due to the lysis of the cocci. Alternatively, if a loopful of 10% sodium deoxycholate solution is placed on a pneumococcus colony, lysis of colony occurs within a few minutes. Bile solubility test is an important diagnostic test to differentiate pneumococcus from other streptococci. This test is based on the presence of an autolytic enzyme amidase in the pneumococci. This enzyme cleaves the bond between alanine and muramic acid in the peptidoglycan. The amidase is activated by bile salts, resulting in lysis of the bacteria.

Pneumococci are catalase and oxidase negative.

D. Resistance

Pneumococci are delicate organisms and are destroyed at 52°C for 15 minutes. They are more sensitive to usual antiseptics.

They are sensitive to a wide range of antibiotics including penicillins, erythromycin, tetracycline, chloramphenicol, vancomycin and clindamycin. Penicillin resistant strains first appeared in 1967. It may be due to mutation or gene transfer. The drug resistance to penicillin is not due to production of beta lactamase, but alteration in the penicillin binding proteins on the bacterial surface. These strains are also resistant to multiple drugs.

Pneumococci are sensitive to optochin (ethyl-hydrocuprein hydrochloride) in a concentration of 1/500,000. When a 6 mm optochin disc (5 µg) is applied on a blood agar plate inoculated with pneumococci, a wide zone (14 mm or more) of inhibition occurs on incubation. This is very useful test to differentiate pneumococci from other streptococci which do not show zone of inhibition by optochin disc.

E. Antigenic Structure

The most important antigen of the pneumococcus is *capsular polysaccharide.* Other antigens are *somatic M protein* and a group specific *cell wall carbohydrate.*

1. Capsular Polysaccharide

Capsular polysaccharide is type specific. Since this polysaccharide is soluble in tissue and culture fluid, it diffuses into the culture medium or tissues and infective exudates, hence, it is also called the *soluble specific substance* (SSS). Pneumococci are classified into types based on the nature of the capsular polysaccharide. More than 90 serotypes are recognised. These are named 1, 2, 3, and so on.

Serological typing of pneumococcus is carried out by three methods.

(*i*) Agglutination of organisms with type specific antiserum.

(*ii*) Precipitation of capsular polysaccharide with type specific antiserum.

(*iii*) Quellung reaction or capsule swelling reaction was described by *Neufeld* (1902). In this reaction, a suspension of pneumococci is mixed on a slide with a drop of specific antiserum and a loopful of methylene blue solution. In presence of the homologous antiserum, the capsule around pneumococci reveals an apparent swelling, sharply delineated and refractile under the microscope. The Quellung test can be done either in sputum or in culture, and used to be a routine bedside procedure in olden days.

2. M Protein

M protein is characteristic for each type of pneumococcus. It is not associated with virulence and antibody to M protein is non-protective.

3. Cell Wall Carbohydrate (C-Substance)

Pneumococci contain a species specific carbohydrate antigen which is named as C-substance. It is present in all pneumococci. The *C-substance* is precipitated by an

abnormal protein (β-globulin), that appears in the acute phase sera of cases of pneumonia but disappears during convalescence. It is also detected in blood of patients with some other illnesses. This is known as the *C-reactive protein* (CRP). It is not an antibody of C substance. It is an 'acute phase' substance, produced in hepatocytes. Its production is stimulated by bacterial infections, malignancies and tissue destruction. CRP is used as an index of activity in rheumatic fever and certain other conditions. CRP is routinely detected in serum by latex agglutination test.

F. Variation

Pneumococcus is virulent in capsulated (smooth) form but non-capsulated (rough) forms are avirulent. On repeated subculture, a smooth to rough (S→R) variation occurs. R forms arise as spontaneous mutants and outgrow the parental S forms in artificial culture. Such R mutants are eliminated in tissues by phagocytosis.

Rough pneumococci of one serotype may be made to produce capsules of the same or different serotypes, on treatment with DNA from the respective serotypes of pneumococci. This transformation was discovered by *Griffith* (1928) as the first instance of genetic exchange of information in bacteria.

G. Toxins and Other Virulence Factors

Pneumococci produce an oxygen labile intracellular haemolysin and a leucocidin. The virulence of pneumococci is dependant upon its capsule which prevents or inhibits phagocytosis. Non-capsulated strains are avirulent. The antibody to the capsular polysaccharide protects against infection. Due to the abdundance of capsular material, the virulence of type-3 pneumococcus is enhanced.

Pneumolysin, a toxin produced by penumococci, is another virulence factor. It has cytotoxic and complement activating properties. It is immunogenic.

H. Pathogenesis

Str. pneumoniae is one of the most common bacteria causing pneumonia, both lobar and bronchopneumonia. It is also responsible for acute tracheobronchitis and empyema.

1. Lobar Pneumonia

Pneumonia results only when the general resistance is lowered. Common infective types of *Str. pneumoniae* include types 1–12 in adults and types 6, 14, 19 and 23 are responsible in children.

2. Bronchopneumonia

It is almost always a secondary infection following viral infections of the respiratory tract. Any serotype of pneumococcus can produce bronchopneumonia. Other causative agents responsible for bronchopneumonia include *Staph. aureus, K. pneumoniae, Str. pyogenes, H.influenzae, Fusobacterium species and Bacteroides.*

3. Meningitis

It is the most serious of pneumococcal infections. *Str. pneumoniae* is the second most important cause of pyogenic meningitis after *N.meningitidis*. This disease is commoner in children. Pneumococcus spreads from the pharynx to the meninges via blood stream. Other bacterial agents of pyogenic meningitis include *N. meningitidis, H. influenzae, Str. agalactiae* (group-B) and *Listeria monocytogenes.*

4. Other Infections

Pneumococcus may also produce empyema, pericarditis, otitis media, sinusitis, conjunctivitis, peritonitis and suppurative arthritis, usually as complications of pneumonia.

I. Epidemiology

The source of human infection is carrier and less often patient. Pneumococci are transmitted by inhalation of contaminated dust, droplets or droplet nuclei. Infection usually leads to pharyngeal carriage. Disease results only when the host resistance is lowered by factors such as respiratory viral infections, malnutrition etc.

Lobar pneumonia is usually a sporadic disease but epidemics may occur. The incidence of bronchopneumonia is more when an epidemic of influenza or other viral infections of the respiratory tract occurs.

J. Laboratory Diagnosis

1. Specimens

Clinical samples, such as sputum, cerebrospinal fluid (CSF), pleural exudate or blood are collected according to the site of lesion. Blood culture is useful in pneumococcal septicaemia.

2. Collection and Transport

All the specimens should be collected in sterile containers under all aseptic conditions. They should be processed immediately. In case of delay, CSF specimen should never be refrigerated but kept at 37°C (*H.influenzae*, another causative agent of pyogenic meningitis may die at cold temperature).

3. Direct Microscopy and Antigen Detection

Gram staining of smear reveals a large number of polymorphs and typical organism. In case of meningitis,

presumptive diagnosis may be made by finding Gram positive diplococci which may be intracellular as well as extracellular in CSF smear. Capsule swelling may be observed under microscope, when pneumococci are mixed with type specific antisera.

Capsular polysaccharide antigen can be demonstrated by counterimmunoelectrophoresis. This has been employed in blood, urine and cerebrospinal fluid. Antigen may also be detected by immunochromatographic assay, latex agglutination or coagglutination.

4. Culture

Specimen is inoculated on blood agar and incubated at 37°C for 24 hours in the presence of 5–10% CO_2. Typical colonies develop with α–haemolysis. Organisms from the isolated colony are identified by Gram staining and biochemical reactions.

5. Colony Morphology and Staining

Colonies are usually small (0.5–1 mm), with alpha haemolysis around them. On prolonged incubation, colonies have draughtsman appearance.

On Gram staining pneumococci are Gram positive, small (1 μm diameter), diplococci, They are flame shaped or lanceolate in appearance. The capsule may be demonstrated as a clear halo in India ink preparation (Fig. 25.2).

6. Biochemical Reactions

Important biochemical tests are inulin fermentation and bile solubility tests. Another test which has a great value is optochin sensitivity test. Details of these tests have already been described. *Str. pneumoniae* can be differentiated from *Str. viridans* by various characteristics (Table 25.1).

7. Animal Pathogenicity Test

From specimens where organisms are expected to be scanty, intraperitoneal inoculation in mice may be used. Inoculated mice die in 24–48 hours. Heart blood and peritoneal exudate of the animal shows pneumococci. The test may be negative with occasional strains which are avirulent for mice (type 14 strains).

8. Antibiotic Sensitivity Test

It is especially useful in strains which are resistant.

K. Treatment

The antibiotic of choice is parenteral penicillin. Cephalosporin is indicated in case of penicillin resistant strains.

L. Prophylaxis

Immunity is type specific and is associated with antibody to the capsular polysaccharide. The existence of more than 90 serotypes makes prophylactic immunisation impracticable. But, as a limited number of serotypes cause most of the pneumonia cases, vaccination is possible. Three pneumococcal vaccines are available.

A polysaccharide vaccine containing prevalent serotypes (23 serotypes) is administered by a single dose injection. Such vaccines are used only in those persons who are at enhanced risk of pneumococcal infection. These include persons with absent or dysfunctional spleen, nephrotic syndrome, sickle cell anaemia, multiple myeloma, hepatic cirrhosis, diabetes mellitus and immunodeficiencies including HIV infection. Vaccination is contraindicated in lymphomas.

The heptavalent pneumococcal conjugate vaccine (7 serotypes) is composed of purified polysaccharides of seven serotypes conjugated to a diphtheria protein is now available for use in children from two months to two years. More recently, a new 13-valent vaccine that contain six additional serotypes was approved for young children. However, protection would depend on the principle that serotypes prevalent in the community and serotypes used in the vaccine are the same.

Table 25.1 Differential Characteristics of Str. pneumoniae and Str. viridans

Characteristic	Str. pneumoniae	Str. viridans
Morphology	Capsulated, lanceolate diplococci (in pairs)	Non-capsulated, oval or round cocci in chains
Colonies on blood agar	Initially dome shaped with α–haemolysis, later 'draughtsman' colonies	Dome shaped with α–haemolysis
Colonies in liquid medium	Uniform turbidity	Granular turbidity, powdery deposit
Bile solubility	Positive	Negative
Inulin fermentation	Positive	Negative
Optochin sensitivity	Positive	Negative
Animal pathogenicity (Intraperitoneal inoculation in mice)	Fatal infection	Non-pathogenic

KEY POINTS

1. Pneumococci are *Gram positive*, small, slightly elongated cocci arranged in pairs (*diplococci*) with broad ends in apposition. Each coccus has one end broad or rounded and other pointed (*flame shaped* or *lanceolate appearance*). They are *capsulated.*
2. They have complex nutritional requirement and grow only in *enriched media* especially supplemented with blood. Their growth is improved by *5-10 per cent* CO_2.
3. On blood agar, the colonies are usually small, dome shaped with an area of greenish discolouration (*alpha haemolysis*) around them. On prolonged incubation, the colonies become flat, with raised edges and central umbonation which creates a *draughtsman appearance* (concentric rings are seen when viewed from above).
4. Pneumococci differ from streptococci in their *morphology* (*diplococci*), *bile solubility* and *optochin sensitivity*.
5. They may cause *pneumonia*, *meningitis*, *empyema*, *otitis media* etc.

YOU MUST KNOW

1. Morphology and culture characteristics of Pneumococcus.
2. Quellung reaction.
3. Laboratory diagnosis of pneumococcal infections.
4. Differences between *Str. pneumoniae* and *Str. viridans*.

STUDY QUESTIONS

1. Describe the laboratory diagnosis of pneumococcal infections.
2. Differentiate between *Str. pneumoniae* and *Str. viridans* in a tabulated form.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Draughtsman colony is a characteristic feature of:
   (a) *Streptococcus pyogenes* (b) *Strep. pneumoniae*
   (c) *Enterococcus facecalis* (d) Viridans streptococci
2. Capsule of *Streptococcus pneumoniae* can be demonstrated by:
   (a) India ink staining (b) Quellung reaction (c) Both of the above (d) None of the above
3. *Streptococcus pneumoniae* may cause:
   (a) Lobar pneumonia (b) Bronchopneumonia (c) Meningitis (d) All of the above
4. Pneumococcal antigen in CSF can be demonstrated by:
   (a) Counterimmunoelectrophoresis (b) Latex agglutination
   (c) Coagglutination (d) All of the above
5. Which of the following tests can be useful to identify *Streptococcus pneumoniae*?
   (a) Bile solubility test (b) Inulin fermentation
   (c) Optochin sensitivity test (d) All of the above
6. Which of the following bacteria produce alpha haemolysis on blood agar?
   (a) *Staphylococcus aureus* (b) *Streptococcus pyogenes*
   (c) *Streptococcus pneumoniae* (d) All of the above

Answers (MCQs):

1. (a) 2. (c) 3. (d) 4. (d) 5. (d) 6. (c)

Chapter 26

NEISSERIA AND MORAXELLA

The genus *Neisseria* consists of Gram negative, aerobic, oxidase positive, non-motile diplococci (arranged in pairs). They may be classified into pathogenic and non-pathogenic (commensals). The two pathogenic species are *N. meningitidis* (causes pyogenic meningitis) and *N. gonorrhoeae* (causes gonorrhoea). The non-pathogenic species include *N. flavescens*, *N. sicca*, *N. subflava* and other species. *N. meningitidis* and *N. gonorrhoeae* are two important species causing human infections.

I. NEISSERIA MENINGITIDIS (MENINGOCOCCUS)

Meningococcus was first isolated by *Weichselbaum* in 1887, from the spinal fluid of a patient.

A. Morphology

They are Gram negative, spherical or oval cocci, 0.6–0.8 μm in size, arranged in pairs with the adjacent sides flattened (Fig. 26.1). They are non-motile. The cocci are generally intracellular when isolated from lesions.

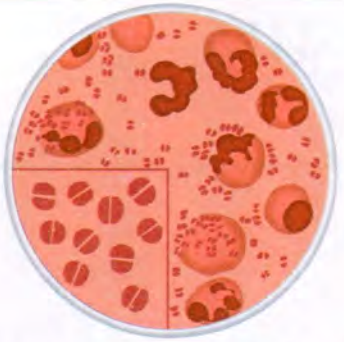


Fig. 26.1 *N. meningitidis* in cerebrospinal fluid. Inset—enlarged view showing flat adjacent sides of cocci.

B. Culture

They do not grow on ordinary media but have exacting growth requirements. Growth occurs on media enriched with blood or serum. These substances promote growth by neutralising certain inhibitory substances found in culture media rather than by providing additional nutrients. Blood agar, chocolate agar and Mueller-Hinton agar are commonly used media. Thayer-Martin (TM) medium with antibiotics (vancomycin, colistin, and nystatin), modified Thayer-Martin (MTM) medium with antibiotics (vancomycin, colistin, nystatin and trimethoprim), Martin-Lewis (ML) medium and New-York City (NYC) medium are selective media used for its isolation. Thayer-Martin medium and Martin-Lewis (ML) medium are chocolate agar based media, while New-York City medium is a clear peptone-cornstarch agar based medium. All the three media contain antibiotics to inhibit bacteria, yeasts and moulds. These media can also be used to grow *N. gonorrhoeae*.

They are strict aerobes and cannot grow anaerobically. The optimum temperature and pH for growth are 35–36°C and 7.4–7.6 respectively. A moist environment with 5–10% CO_2 is must for growth to occur.

On solid media, the colonies are small (1 mm in diameter), round, convex, grey, translucent and with entire edges. The colonies are butyrous in consistency and easily emulsifiable. In liquid media, it produces a granular turbidity.

C. Biochemical Reactions

N. meningitidis is catalase and oxidase positive. The prompt oxidase reaction helps to identify neisseria (both meningococci and gonococci) in mixed cultures. Glucose and maltose are fermented with acid production but no gas (gonococci ferment glucose but not maltose). They do not ferment lactose or sucrose. Indole and H_2S are not produced and nitrates are not reduced.

Oxidase Test

When freshly prepared oxidase reagent (1% tetramethyl paraphenylene diamine hydrochloride) is poured on the culture plate, neisseria colonies become deep purple. These colonies can be picked up for subcultures immediately, as the organism dies on prolonged exposure to the reagent. The test may also be performed by rubbing bacterial growth with a loop on a filter paper strip moistened with the oxidase reagent (*Kovac's method*). A deep purple colour is a positive reaction.

D. Antigenic Structure

N. meningitidis has been divided into 12 serogroups on the basis of immunological specificity of the capsular polysaccharide. These serogroups are A, B, C, X, Y, Z, W-135, 29E, H, I, K and L. Groups A, B and C are the most important. Groups 29E, W-135 and Y also frequently cause meningitis. These six groups (A, B, C, 29E, W-135 and Y) are responsible for the large majority of meningitis.

E. Resistance

These are very delicate organisms, being highly susceptible to heat, desiccation and to disinfectants. They are susceptible to penicillin, ampicillin, chloramphenicol, macrolides and ciprofloxacin. Strains resistant to penicillin have now been reported from several countries.

F. Pathogenesis

N. meningitidis causes pyogenic meningitis in all ages, but is most common in children and young adults. The other common bacterial agents responsible for pyogenic meningitis include *H. influenzae* and *Str. pneumoniae*.

Meningococci are strict human pathogens. The infection is acquired by droplet spread via the carriers and the cases. Human nasopharynx is the reservoir of *N. meningitidis*. The incubation period of the disease is about 3 days. The spread of cocci from the nasopharynx to the meninges is controversial. The organism may spread along the perineural sheath of the olfactory nerve, through the cribriform plate to the subarachnoid space or through the blood stream.

Meningococcaemia presents as acute fever with petechial rash. A few develop fulminant meningococcaemia (*Waterhouse–Friderichsen syndrome*) characterised by shock, disseminated intravascular coagulation and multisystem failure. It is usually a fatal condition.

Deficiency of complement components (C5–C9) favours the meningococcal infections.

Bacterial endotoxin appears to play the pathogenic role. The vascular endothelium is sensitive to the endotoxin. All major inflammatory cascade systems as well as cytokines are triggered.

G. Epidemiology

The humans are the only reservoir of the meningococcus. Asymptomatic nasopharyngeal carriers serve to infect their contacts. Transmission is by airborne droplets, or less often by fomites. The carrier rate is about 5–10 per cent which rises during epidemics. Meningitis is more common in children below the age of five years and in males. Epidemic usually occurs in overcrowded area. Serogroup A is usually associated with epidemics. Serogroup B causes both epidemics and outbreaks, while serogroup C is associated mostly with localised outbreaks. Group A meningococcus affected Delhi during epidemic in early 1985.

H. Laboratory Diagnosis

1. Specimens

- CSF
- Blood
- Petechial lesions specimen
- Nasopharyngeal swab—especially to detect carriers.

2. Collection and Transport

Collection of specimen is done under sterile conditions by lumbar puncture for CSF and by venepuncture for blood. Blood is injected into blood culture bottles (glucose broth and sodium taurocholate broth) through the hole in the bottle cap. Nasopharyngeal specimen is collected by using a sterile swab. All the specimens must be transported immediately. CSF should never be refrigerated as *H. influenzae* (another common causative agent of meningitis) may die at the cold temperature. The nasopharyngeal swab should be held in a transport medium (e.g. Stuart's) until it is inoculated on a culture medium.

3. Direct Microscopy and Antigen Detection

CSF is divided into three portions. One portion is centrifuged and smear is prepared from the deposit for Gram staining. Meningococci are seen as Gram negative diplococci present mainly inside polymorphs (intracellular), but may also be present extracellularly. A large number of pus cells are also seen. The supernatant fluid may be used for detection of meningococcal antigen (capsular polysaccharide) by rapid tests, such as countercurrent immunoelectrophoresis (CIEP), coagglutination or latex agglutination test. Antigen detection is useful when organisms are scanty.

The second portion of the CSF is used for direct culture while the third portion of the CSF is incubated overnight after adding an equal volume of glucose broth and then subcultured on blood agar or chocolate agar.

4. Culture

(i) CSF

Centrifuged deposit is inoculated on blood agar or chocolate agar. The plate is incubated at 35–36°C under 5–10% CO_2. Colonies appear after 18–24 hours. These are identified by colony morphology, Gram staining and biochemical reactions.

(ii) Blood

Blood culture is often positive in meningococcaemia and in early cases of meningitis. Blood culture bottles (glucose broth and sodium taurocholate broth) are incubated at 35–36°C for 24 hours. Subcultures are made from these broths on to blood agar and chocolate agar. These plates are again incubated at 35–36°C under 5–10% CO_2 for 18–24 hours. Identification of organism is done by colony morphology, Gram staining and biochemical reactions. Blood culture should be incubated for 4–7 days, with daily subcultures.

(iii) Other specimens

Other specimens (petechial lesions, nasopharyngeal swab, autopsy specimens) are inoculated on blood agar and chocolate agar and are processed in similar way as described for CSF. Specimens may be collected from the meninges, lateral ventricles or brain and spinal cord in case of autopsy. Meningococci may die if specimens are not collected within 12 hours of the death of the patient.

5. Colony Morphology and Gram Staining

On solid media, colonies are small (1 mm in diameter), round, convex, grey, translucent and with entire edges. Smear is prepared from the suspected colony and Gram staining is done. On Gram staining, they are Gram negative, spherical or oval cocci, 0.6–0.8 μm in size, arranged in pairs (diplococci) with the adjacent sides flattened (Fig. 26.1).

6. Biochemical Reactions

N. meningitidis is catalase and oxidase positive. Glucose and maltose are catabolised with acid production but no gas. This breakdown of glucose and maltose occurs by oxidation and not by fermentation.

7. Slide Agglutination

Direct slide agglutination of the organism may be done with specific antisera.

8. Serological Diagnosis

This may be attempted in chronic meningococcal septicaemia where no organism has been isolated. Specific antibodies to capsular polysaccharide may be demonstrated by haemagglutination test.

9. Polymerase Chain Reaction

Meningococcal DNA in CSF or blood can be amplified and then detected. It is a rapid method.

I. Treatment

Meningococci are uniformly sensitive to penicillin. Chloramphenicol is used in persons allergic to penicillin. Penicillin G in high doses is given intravenously or intrathecally if necessary. Cefotaxime or ceftriaxone are as effective as chloramphenicol and the possibility of blood dyscrasia may be avoided.

J. Prophylaxis

1. Chemoprophylaxis

It is indicated for close contacts of patients for eliminating the bacteria from nasopharynx. Rifampicin or ciprofloxacin are recommended.

2. Immunoprophylaxis

Meningococcal vaccines prepared from polysaccharides of serogroups A, C, W-135 and Y are available. Single dose is given intramuscularly. The protection is group specific and lasts for at least three years but does not prevent meningococcal carriage. No vaccine is available against group B meningococci because capsular polysaccharide of this group is a poor immunogen.

II. NEISSERIA GONORRHOEAE (GONOCOCCUS)

N. gonorrhoeae causes the sexually transmitted disease, gonorrhoea. The organism was first described by *Neisser* in 1879 in gonorrheal pus. *N. gonorrhoeae* and *N. meningitidis* are closely related and have 70% DNA homology.

A. Morphology

They are Gram negative oval cocci arranged in pairs (diplococci) with adjacent sides concave (pear or bean shaped). In smear from purulent material, they are intracellular within polymorphs, some cells containing as many as a hundred cocci (Figs. 26.2 and 26.3).

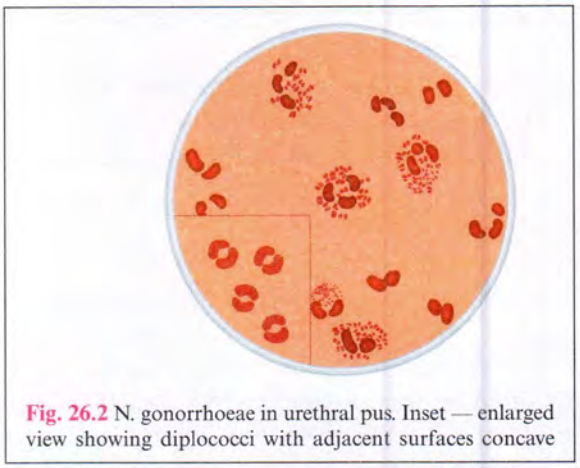


Fig. 26.2 N. gonorrhoeae in urethral pus. Inset — enlarged view showing diplococci with adjacent surfaces concave

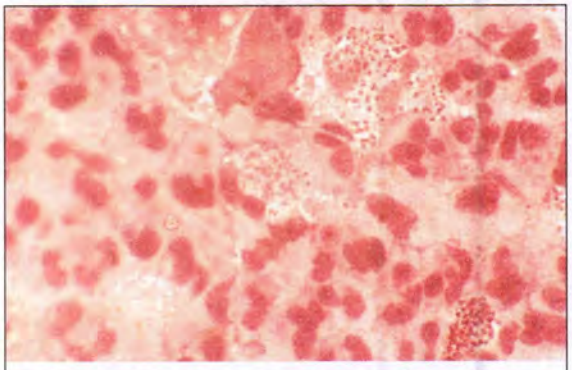


Fig. 26.3 Gram-negative intracellular diplococci (*N. gonorrhoeae*)

Gonococci possess pili on their surface. These pili facilitate adhesion of cocci to host cells and promote virulence by resisting phagocytosis. Pili of different strains are antigenically different. Piliated gonococci agglutinate human erythrocytes and the haemagglutination is not inhibited by mannose.

B. Culture

This organism is more difficult to grow than *N. meningitidis*. They are aerobic, but may grow anaerobically as well. They grow best at a temperature of 35–36°C in presence of 5–10% CO_2 and at pH 7.2–7.6. They require an enriched medium like chocolate agar for their growth. A popular selective medium is the *Thayer-Martin medium* which contains chocolate agar with antibiotics (vancomycin, colistin and nystatin). It inhibits most contaminants including commensal neisseria. Trimethoprim may be added to Thayer-Martin medium to inhibit swarming of Proteus sp. This medium is known as *modified Thayer-Martin medium*.

Colony Morphology

Colonies are small, round, grey, translucent, convex with finely granular surface. They are easily emulsifiable. *N. gonorrhoeae* produces five types of colonies, T1, T2,

T3, T4 and T5. Type 1 and 2 produce small brown colonies and possess pili. These are autoagglutinable and virulent strains. Type 3, 4 and 5 form larger, granular, non-pigmented colonies and are non-piliated. They form smooth suspensions and are avirulent. T1 and T2 are also known as P^{+} and P^{++} respectively, while T3, T4 and T5 are named as P^{-}.

C. Biochemical Reactions

N. gonorrhoeae is oxidase positive. Gonococci ferment glucose (with acid production only) and not maltose.

Rapid carbohydrate utilisation test (RCUT): These rapid tests also detect acid production from various carbohydrates. These are based on the presence of preformed enzymes for carbohydrate utilisation rather than on bacterial growth. They take 2-4 hours rather than 24 to 72 hours required for the conventional carbohydrate tests.

D. Antigenic Structure

The antigenic structure is complex. The surface structures of *N. gonorrhoeae* include the following :

1. Capsule

It is polyphosphate and not polysaccharide. Capsule is most evident in freshly isolated gonococci. It is loosely associated with cell surface. The capsule inhibits phagocytosis. Presence of capsule and its role requires further elucidation.

2. Pili

These are hair like structures extending from the surface. The piliated organisms produce altered appearance of colonies on culture. Pili enhance attachment of the organism to host cells and resist phagocytosis. They act as virulence factor. They are made up of pilin proteins. These pilin proteins are antigenically different in almost all strains and a single strain can produce several antigenically distinct pilins.

3. Lipooligosaccharide (LOS)

There is absence of long O-antigenic side chains in the chemical structure of lipooligosaccharide (LOS). This property differentiates it from lipopolysaccharide (LPS) of Gram negative bacilli. Toxicity in gonorrhoea is largely due to the endotoxic effects of lipooligosaccharide (LOS).

4. Proteins

The outer membrane antigens (proteins) are the porins.

(i) Protein I (por)

It forms pores on surface. Each strain of gonococcus expresses one type of protein I. It helps in serotyping of gonococci. There are two variants of protein I, named IA and IB. Any one strain carries either IA or IB but not both. 24 serovars of type IA and 32 serovars of type IB have been demonstrated.

(ii) Protein II (opa)

One part of protein II is in outer membrane and the rest is exposed on the surface of bacteria. This protein takes part in adhesion of bacterium and its attachment to host cell. Type II protein is present in those strains which form opaque colonies, therefore, it is also called the *opacity associated* (OPA) protein.

(iii) Protein III

It is associated with protein I in the formation of pores on the cell surface and hence plays a role in the exchange of molecules across the outer membrane.

5. Other Proteins

IgA1 protease produced by gonococci as well as meningococci splits and inactivates IgA which plays a major role in mucosal defence. Two other proteins H8 and iron binding protein are described but their role in pathogenicity is not known.

E. Resistance

The gonococcus is a very delicate organism. It is strict parasite and dies rapidly outside the human host, in 1 to 2 hours in exudate and in 3 to 4 days in culture. It survives at 35°C if kept in slant cultures under sterile paraffin oil. Cultures may also be stored for years if frozen quickly and kept at –70°C.

Gonococci are readily killed by heat, drying and antiseptics. Formerly, they were highly susceptible to sulphonamides and penicillin but have steadily developed resistance to many antibiotics. PPNG (penicillinase producing *Neisseria gonorrhoeae*) strains are resistant to penicillin due to production of β-lactamase (penicillinase) enzyme by these strains.

F. Pathogenesis

1. Gonorrhoea

It is a sexually transmitted disease involving urethra in both sexes but in females, the endocervix is the primary site of infection. The incubation period is 2–8 days. The disease is an acute urethritis characterised by purulent urethral discharge. Asymptomatic infection is common in women. In males, the acute urethritis may extend to the prostate, testes, seminal vesicles and epididymis. If untreated, it may lead to fibrosis and stricture. The infection may spread to the periurethral tissues, causing abscesses and multiple discharging sinuses ('**watercan**

perineum') In females, the primary infection may spread from urethra and cervix to Bartholin's glands, uterus, fallopian tubes, ovaries and may cause pelvic inflammatory disease resulting in sterility. In some patients, who develop pelvic inflammatory disease, there is inflammation of liver capsule (Fitz-Hugh-Curtis syndrome). Occasionally hematogenous spread of bacteria may lead to septicaemia, endocarditis or acute purulent arthritis.

2. Ophthalmia Neonatorum

It is a nonvenereal gonococcal conjunctivitis in the newborn through infected birth canal.

3. Gonococcal Vulvovaginitis

In adult women the vagina usually is resistant to gonococcal infection because of the acidic pH of the vaginal secretions, but vulvovaginitis can occur in prepubertal girls.

4. Other Infections

Sometimes the disease may involve rectum or oropharynx following rectal intercourse or by orogenital contact respectively. Involvement of oropharynx may lead to gonococcal pharyngitis.

G. Epidemiology

Gonorrhoea is an exclusively human disease. The only source of infection is a human carrier or less often a patient. Asymptomatic infection in woman makes them a reservoir to spread infection among their male contacts. The mode of infection is almost exclusively venereal. Ophthalmia neonatorum is a non-venereal gonococcal infection.

H. Laboratory Diagnosis

The diagnosis is readily made in acute stage, as urethral discharge contains large number of gonococci. It is difficult to detect gonococci from chronic cases or from patients with metastatic lesions such as arthritis.

1. Specimens

Urethral discharge and cervical discharge (in females) are collected in acute urethritis. The meatus is first cleaned with a gauze soaked in saline. A sample of the urethral discharge is then collected with a platinum loop or by a sterile swab. Calcium alginate and some cotton swabs have been shown to be inhibitory to gonococci, so Dacron or Rayon swabs are preferred. In women, cervical swab is collected in addition to urethral discharge. High vaginal swabs are not satisfactory.

In chronic urethritis, urethral discharge is observed only in few cases. In these cases, some exudate obtained after prostatic massage or morning drop of secretion may be examined. Centrifuged deposits of urine is examined in cases where no urethral discharge is available.

2. Transport

All the specimens should be transported and processed immediately. If this is not possible, specimens should be collected with charcoal coated swabs and transported to the laboratory in Stuart's transport medium. Another transport medium is Amies medium with charcoal.

Several commercial transport systems such as JAMBEC plates (James E. Martin Biological Environmental Chamber), Bio-Bag, Gono-Pak, and Transgrow, contain selective media and a carbon dioxide atmosphere to provide optimal conditions for the specimens during transport.

3. Direct Microscopy

Gram staining of smear provides a presumptive evidence of gonorrhoea in men. Gram negative intracellular diplococci are found in smear of at least 95% cases of acute gonococcal urethritis in males. In females, diagnosis of gonorrhoea by smear examination is unreliable as some of the normal genital flora have similar morphology. Fluorescent antibody tests are more sensitive and specific methods for diagnosis by microscopy especially in females.

4. Detection of Antigen or Nucleic acid

The gonococcal antigens can be detected by ELISA in clinical specimens. Nucleic acid can be directly detected in urethral discharge using DNA probe.

5. Culture

The specimens should be inoculated directly on preheated plates immediately on collection. Chocolate agar is used for culture of the specimens and incubated at 35°C to 36°C under 5–10% CO_2 for 48 hours. In chronic cases, where mixed infection is usual and in lesions like proctitis, selective medium such as Thayer-Martin medium or modified Thayer-Martin medium is used. Typical translucent colonies appear on culture medium.

6. Colony Morphology and Gram Staining

On solid medium, colonies are small, round, grey, translucent, convex with finely granular surface. They are easily emulsifiable. Smear is made from the colony and Gram staining is done. Gonococci are Gram negative cocci arranged in pairs (diplococci) with adjacent sides concave (pear or bean shaped).

7. Superoxol Test

Superoxol test is helpful in rapid presumptive identification of *N. gonorrhoeae*. Superoxol is 30% hydrogen peroxide (not the 3% solution used routinely for catalase test).

N. gonorrhoeae produce immediate brisk bubbling when some colonies of this bacteria are emulsified with 30% hydrogen peroxide reagent on a glass slide. In contrast, both *N. meningitidis* and *N. lactamica*, produce weak, delayed bubbling.

8. Biochemical Reactions

They are oxidase positive. They breakdown glucose with acid only by oxidation. They do not catabolise maltose unlike meningococci.

9. Serology

It may not be possible to isolate gonococci in culture from some chronic cases or from some patients with metastatic lesions such as arthritis. Serological tests may be of value in such cases. Complement fixation test, immunofluorescence and ELISA tests have been used to detect antibodies. However, no serological test has been found useful for routine diagnostic purposes.

I. Treatment

The organism is sensitive to large doses of penicillin (intramuscular) or doxycycline.

Two types of penicillin resistance have been observed:

1. Low Level Resistance

The bacterial strains are sensitive to higher concentrations of penicillin and are not related to beta-lactamase production. The resistance is chromosomally determined. These resistant gonococci are designated as 'chromosomally-mediated resistant *Neisseria gonorrhoeae*' (CMRNG).

2. High Level Resistance

It is due to production of enzyme beta-lactamase by the bacteria and is plasmid coded. In such cases, the strains are totally resistant to penicillin. It was first recognised in 1976. Since then these penicillinase producing *Neisseria genorrhoeae* (PPNG) strains have spread widely.

In penicillin-resistant gonorrhoea, cefotaxime, ceftriaxone, ciprofloxacin, tetracycline or spectinomycin are used.

In 1985, plasmid mediated high level tetracycline resistance was recognised. These gonococci are named as 'tetracycline resistant *Neisseria gonorrhoeae*' (TRNG). Chromosome mediated spectinomycin resistance has also been known in gonococci. This is a high level resistance due to mutation. In 1991 'quinolone-resistant *N. gonorrhoeae*' (QRNG) was identified. Mechanism of resistance is mutation.

Currently a single dose of ceftriaxone intramuscular or cefixime orally is recommended for treatment of uncomplicated gonococcal infections. Coinfection with *C. trachomatis* is common in patients with gonorrhoea therefore dual therapy is frequently recommended. Dual therapy includes ceftriaxone/cefixime (for gonorrhoea) and azithromycin/doxycycline (for *C. trachomatis*).

J. Prophylaxis

Control of gonorrhoea consists of early detection of cases, tracing of contacts, health education and other general measures. Vaccination has no role in prophylaxis.

III. NON-GONOCOCCAL (NON-SPECIFIC) URETHRITIS

It is a condition of chronic urethritis where gonococci cannot be demonstrated. It is known as non-gonococcal urethritis (NGU).

A. Causative agents

1. Bacterial

- *Chlamydia trachomatis* is the most common.
- *Ureaplasma urealyticum*
- *Mycoplasma hominis*
- *Gardnerella vaginalis*
- *Acinetobacter lwoffi*

2. Viral

Herpes virus
Cytomegalovirus

3. Fungal

Candida albicans

4. Protozoal

Trichomonas vaginalis

B. Treatment

Tetracycline is effective for both *C. trachomatis* and *Ureaplasma urealyticum* infections.

IV. COMMENSAL NEISSERIAE

This group of bacteria inhabits the normal respiratory tract. These commensals (*N. flavescens*, *N. sicca* and *N. subflava*) are differentiated from the pathogenic *Neisseriae* by following methods.

1. Commensals can grow on ordinary agar not enriched with blood or serum at 22°C.
2. Their primary isolation does not require presence of CO_2.

3. These bacteria produce pigmented (yellow to greenish yellow) colonies and ferment a number of carbohydrates.

V. MORAXELLA (BRANHAMELLA) CATARRHALIS

Neisseria catarrhalis was initially classified as *Branhamella catarrhalis* but now reclassified as *Moraxella catarrhalis.* It is part of the normal flora of the upper respiratory tract and genital tract.

A. Morphology

Gram negative diplococci, 0.6–1μm oval with adjacent sides flattened.

B. Culture

It grows on ordinary medium like nutrient agar.

C. Biochemical Reactions

It does not ferment any carbohydrate but hydrolyses tributyrin—a test for its identification. It is catalase and oxidase positive.

D. Pathogenesis

It causes lower respiratory tract infections, especially in adults with chronic obstructive airway disease. It has also been isolated in cases of otitis media, less commonly in meningitis, endocarditis and sinusitis. Some strains of *B. catarrhalis* produce beta-lactamase and are resistant to penicillin.

VI. MORAXELLA LACUNATA (MORAX-AXENFELD BACILLUS)

This was formerly included in the genus *Haemophilus* but as it does not require X or V factor, it has been separated into the genus *Moraxella*. It was described by *Morax* (1896) and *Axenfeld* independently from cases of subacute conjunctivitis. The most important species is *M. lacunata.*

A. Morphology

It is a Gram negative, short (2–3 μm) ovoid, non-motile bacillus arranged in pairs.

B. Culture

They require blood or serum for growth and are strict aerobes. On loeffler's serum slope, the colonies form pit or lacunae (hence the name lacunata).

C. Biochemical Reactions

It does not ferment sugars and is oxidase and catalase positive.

D. Pathogenesis

Moraxella are parasitic and their habitat includes mucosa of upper respiratory tract, genital tract and conjunctiva. *M. lacunata* causes catarrhal conjunctivitis (angular conjunctivitis). A few species of *Moraxella* occasionally give rise to septic arthritis, meningitis and endocarditis.

E. Treatment

M. lacunata is very sensitive to zinc salts. They are sensitive to penicillin and most other antibiotics.

KEY POINTS

1. The genus *Neisseria* consists of *Gram negative, aerobic, oxidase positive,* non-motile *diplococci.*
2. The two pathogenic species are *N. meningitidis* (causing *pyogenic meningitis*) and *N. gonorrhoeae* (causing *gonorrhoea*).
3. They grow on media enriched with blood or serum. *Chocolate agar* is the commonly used medium. *Modified Thayer-Martin medium* and New-York City medium are selective media used for their isolation.
4. *N. meningitidis* is *strict aerobe* and cannot grow anaerobically. A moist environment with *5-10%* CO_2 is must for growth to occur.
5. Both *N. meningitidis* and *N. gonorrhoeae* are *catalase* and *oxidase positive*. *N. meningitidis* ferments both *glucose* and *maltose* but *N. gonorrhoeae* only ferments *glucose*.
6. *N. meningitidis* has been divided in 13 serogroups (*A, B, C, D, X, Y, Z, W-135, 29E, H, I, K and L*) on the basis of immunological specificity of the capsular polysaccharide. Serogroups *A, B, C, 29E, W-135* and *Y* are responsible for the large majority of meningitis.
7. *Meningococcal vaccines* prepared from polysaccharides of serogroups A, C, W-135 and Y are available. *Single dose* is given *intramuscularly*.
8. *N. gonorrhoeae* is *pear or bean* shaped. It is more difficult to grow than *N. meningitidis*. It is aerobic but may grow anaerobically as well. It can grow on all those culture media used for growth of *N. meningitidis*.

9. Penicillin resistance in *N.gonorrhoeae* may be due to production of enzyme *beta-lactamase* (*penicillinase*). It is *plasmid* coded. These strains are named penicillinase producing *Neisseria gonorrhoeae* (*PPNG*).
10. *Non-gonococcal urethritis* (*NGU*) is a condition of *chronic urethritis* where gonococci cannot be demonstrated.
11. Non-pathogenic species of Neisseria include *N. flavescens*, *N. sicca* and *N. subflava*. They are commensals and inhabit the normal respiratory tract.

YOU MUST KNOW

1. Morphology, culture characteristics, biochemical reactions and antigenic structure of *Neisseria meningitidis*.
2. Laboratory diagnosis of meningococcal meningitis.
3. Meningococcal vaccines.
4. Morphology, culture characteristics, biochemical reactions of *Neisseria gonorrhoeae*.
5. Laboratory diagnosis of gonorrhoea.
6. Penicillinase producing *N. gonorrhoeae* strains (PPNG).
7. Non-gonococcal urethritis (NGU).
8. *Moraxella catarrhalis*.

STUDY QUESTIONS

1. Describe the laboratory diagnosis of meningococcal meningitis,
2. Discuss the laboratory diagnosis of gonorrhoea.
3. Write short notes on:
   (a) Non-gonococcal urethritis (NGU) (b) Antigenic structure of N.gonorrhoeae
   (c) Mo*raxella catarrhalis.*

MULTIPLE CHOICE QUESTIONS (MCQs)

1. All of the following bacteria are non-pathogenic except:
   (a) *Neisseria flavescens* (b) *N. sicca* (c) *N. subflava* (d) *Moraxella catarrhalis*
2. Which of the following is a selective medium for isolation of *Neisseria gonorrhoeae*?
   (a) Modified Thayer-Martin medium (b) Blood agar
   (c) MacConkey's agar (c) Potassium tellurite blood agar
3. All of the following bacteria are oxidase positive except:
   (a) *Neisseria gonorrhoeae* (b) *Neisseria meningitidis* (c) *Vibrio cholerae* (d) *Enterobacter*
4. How many serogroups of *Neisseria meningitidis* are known?
   (a) Nine (b) Twelve (c) Thirteen (d) Fifteen
5. Which of the following rapid tests may be used to detect meningococcal antigen?
   (a) Countercurrent immunoelectropheresis (b) Coagglutination test
   (c) Latex agglutination test (d) All of the above
6. Waterhouse-Friderichsen syndrome is caused by:
   (a) *Neisseria meningitidis* (b) Leptospira (c) *Streptococcus pyogenes* (d) Borrelia
7. The causative agent/s of non-gonococcal urethritis is/are:
   (a) *Chlamydia trachomatis* (b) *Ureaplasma urealytium*
   (c) *Mycoplasma hominis* (d) All of the above

Answers (MCQs):

1. (d) 2. (a) 3. (d) 4. (c) 5. (d)
6. (a) 7. (d)

Chapter 27

CORYNEBACTERIUM

Corynebacteria are Gram positive, non-acid-fast, non-sporing, non-motile bacilli with average size 3 μm × 0.3 μm. They frequently show club-shaped swellings (*coryne* means club shaped). The most important member of the genus is *C. diphtheriae* which causes diphtheria in humans but other species are increasingly assuming a pathogenic role particularly in immunocompromised individuals. *Bretonneau* (1826) called the disease 'diphtherite' because of leathery pseudo-membrane produced in the disease (*diphtheros* meaning leather). The diphtheria bacillus was first described by *Klebs* (1883), but was first cultivated by *Loeffler* (1884). It is also known as Klebs-Loeffler bacillus. *Roux and Yersin* (1888) discovered diphtheria exotoxin. The antitoxin was discovered by *von-Behring* (1890) who was awarded the Nobel Prize for this work.

I. CORYNEBACTERIUM DIPHTHERIAE

A. Morphology

These are thin, slender, Gram positive bacilli (but tend to be decolourised easily) and measure approximately 3–6 μm × 0.6–0.8 μm. They are pleomorphic. They are club-shaped due to the presence of metachromatic granules at one or both ends. With Loeffler's methylene blue stain, granules take up a bluish purple colour and hence they are named *metachromatic granules*. These granules are also called *volutin or Babes-Ernst granules*. These are composed of polymetaphosphates and represent energy storage depots. The bacilli are usually seen in angular fashion resembling the letters V or L. This has been called *Chinese letter* or *cuneiform arrangement*. This typical arrangement is due to incomplete separation of the daughter cells after binary fission (Fig. 27.1).

Special stains like Albert (malachite green and toludine blue), Neisser or polychrome methylene blue are used for staining the bacilli. The bacilli look green and metachromatic granules appear bluish black when Albert stain is used. They are non-capsulated, non-acid-fast and non-motile.

B. Cultural Characteristics

C. diphtheriae are grown best on media enriched with blood, serum or egg. Growth is scanty on ordinary

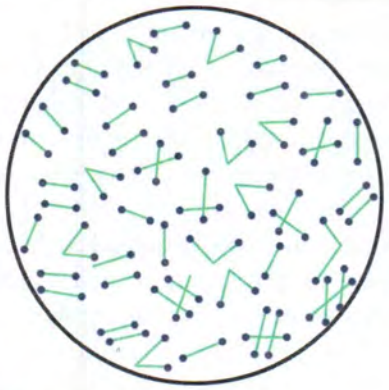


Fig. 27.1 Morphology of *C. diphtheriae*

media. They are aerobic and facultatively anaerobic. The optimum temperature for growth is 37°C (range 15–40°C) and optimum pH 7.2. The following are the usual media employed for cultivation of diphtheria bacillus.

1. Hiss's Serum Water

This is a liquid medium containing serum. Growth is seen as a turbidity and pellicle formation.

2. Loeffler's Serum Slope

Diphtheria bacilli grow on this medium very rapidly. Colonies appear after 6 to 8 hours of incubation, long before other bacteria grow. The colonies are small, circular, white or creamy and glistening.

3. Tellurite Blood Agar Medium

It contains potassium tellurite (0.04%) which inhibits most other bacteria and thus acting as a selective agent. The organisms grow slowly on this medium and form grey or black colonies due to reduction of potassium tellurite to tellurium (Fig. 27.2). The colonies may take two days to appear on this medium. Based on colony morphology on tellurite medium and other properties, three main biotypes of *C. diphtheriae—gravis, intermedius* and *mitis* are distinguished (Table 27.1).

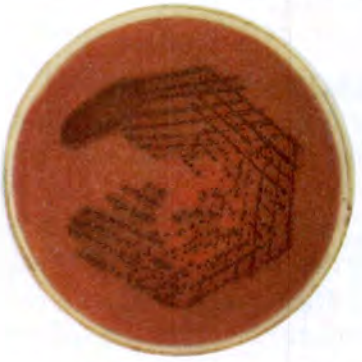


Fig. 27.2 *C.diphtheriae* on potassium tellurite blood agar

Tinsdale agar, which contains sheep's blood, bovine serum, cystine and potassium tellurite, is one of the selective media used for growing *C. diphtheriae*. Black or brown colonies are formed on this medium. A brown halo surrounding the colony is a useful differentiating feature, because only *C. diphtheriae*, *C. ulcerans* and *C. pseudotuberculosis* produce a brown halo. On this medium staphylococci can also grow as black colonies but without haloes around the colonies.

Table 27.1 Type Differentiation of C. diphtheriae Strains

	Character	Gravis	Intermedius	Mitis
1.	Morphology	Short rods, few metachromatic granules, uniform staining	Long, poor granulation, barred forms with clubbed ends, pleomorphic	Long rods, curved, prominent granules, pleomorphic
2.	Colony on tellurite blood agar	In 18 hours, 1–2 mm, grey, raised centre. In 48–72 hours, flat colony with raised centre and crenated edge with radial striation—*daisy head* colony	In 18 hours, 1 mm in size. In 48 hours, no change in size, dull granular centre, more glistening periphery and a lighter ring near the edge—*frog's egg* colony	Size variable, shiny black. In 48–72 hours, flat colony with central elevation—*poached egg* colony
3.	Consistency of colonies	Brittle, not easily picked out or emulsifiable, like *cold margarine*	Intermediate between gravis and mitis	Soft, buttery, easily emulsifiable
4.	Haemolysis	Variable	Non-haemolytic	Usually haemolytic
5.	Growth in broth	Surface pellicle	Turbidity in 24 hours, clearing in 48 hours, with fine granular sediment	Diffuse turbidity
6.	Biochemical tests			
	Glucose	Acid without gas	Acid without gas	Acid without gas
	Glycogen	Acid without gas	Negative	Negative
	Starch	Acid without gas	Negative	Negative
7.	Virulence	Severe	Moderate	Mild

C. Biochemical Reactions

They ferment glucose and maltose with the production of acid but without gas. They do not ferment lactose, mannitol or sucrose. The gravis biotype ferments starch and glycogen while intermedius and mitis have no such action (Table 27.1). It is necessary to employ Hiss's serum water for testing fermentation of sugars. Proteolytic activity is absent. *C. diphtheriae* reduces nitrates to nitrites. They do not hydrolyse urea or form phosphatase. Gelatin is not liquefied.

PYRAZINAMIDASE (PYZ) TEST

Pyrazinamide is converted into pyrazinoic acid by the organisms which produce pyrazinamidase (PYZ). This test is helpful to distinguish '*C. diphtheriae*' (PYZ-negative) from other corynebacterium species (mostly PYZ-positive). However, *C. ulcerans* and *C. pseudotuberculosis* are also PYZ-negative but they are urease test positive which differentiate them from *C. diphtheriae* (urease negative).

D. Toxin

The pathogenicity is due to production of a very powerful exotoxin by virulent strains of diphtheria bacilli. About 90–95% gravis and intermedius strains are toxigenic, while only 80–85% of mitis strains are so. Avirulent strains are frequent among convalescents, carriers and contacts. The strain most widely used for toxin production is *Park–Williams 8* strain which has been described as a mitis strain (Topley and Wilson) and as an intermedius (Cruickshank) strain.

The toxigenicity of the diphtheria bacillus depends on the presence of a *tox* gene which can be transferred from one bacterium to another by lysogenic bacteriophages, of which *beta phage* is the most important. Non-toxigenic strains may be rendered toxigenic by infecting them with *beta* phage. This process is known as *lysogenic or phage conversion*. The toxigenicity remains as long as the bacteria is lysogenic. When the bacteria is freed of its phage, as by growing it in the presence of antiphage serum, it loses the toxigenicity and becomes non-toxigenic strain. In addition to beta phage, other phages (α, P, L, γ) are also known to carry tox genes.

1. Properties

The diphtheria toxin is a protein and has a molecular weight of about 62,000. It consists of two fragments A and B, of molecular weights 24,000 and 38,000 respectively. Both fragments are required for toxicity. Fragment A has all the enzymatic activity whereas fragment B is responsible for binding the toxin to the cells. The antibody to fragment B prevents the binding of toxin to cells and is thus protective. The toxin is heat labile. It is extremely potent (0.0001 mg kills a guinea pig of 250 gm weight). It is converted into toxoid by heat (at 37°C for 4 to 6 weeks), treatment with 0.2 to 0.4% formalin or by acidic pH. The toxoid is a toxin that has lost its toxicity but has retained the antigenicity. It is capable of producing antitoxin.

Toxin production is also influenced by the concentration of iron. 0.1 mg of iron per litre of culture medium is optimum level for toxin production, while a concentration of 0.5 mg or more per litre inhibits the toxin production. Reason for this is not known. The repressor of the tox gene appears to be an iron containing protein. When level of iron is more, suppressor is formed which inhibits toxin production.

2. Mode of Action

It acts by inhibiting protein synthesis. It inhibits polypeptide chain elongation in the presence of nicotinamide adenosine dinucleotide (NAD) by inactivating the elongation factor, EF-2. Fragment A splits NAD to form nicotinamide and adenosine diphosphoribose (ADPR). ADPR binds to and inactivates EF-2 which is an enzyme required for elongation of polypeptide chain.

$$NAD^+ + \underset{\text{Active}}{\text{EF-2}} \rightleftharpoons \underset{\text{Inactive}}{\text{ADPR-EF2}} + \text{Nicotinamide} + H^+$$

E. Resistance

It readily dies when exposed to a temperature of 58°C for 10 minutes or 100°C for one minute. It is resistant to drying. The organism remains virulent in blankets and floor dust for five weeks. It is quite susceptible to penicillin, erythromycin and broad spectrum antibiotics.

F. Antigenic Structure

C. diphtheriae possesses heat labile type-specific protein antigens (K antigen) and heat stable polysaccharide 'O' antigen. By agglutination, gravis strain has 13, intermedius 4 and mitis 40 types. A group specific heat stable polysaccharide O antigen is shared by all the strains.

G. Bacteriophage Typing

About 15 bacteriophage types have been described, type I to III strains are mitis, IV to VI intermedius, VII avirulent gravis and the remainder virulent gravis strains. The only other corynebacteria which are found susceptible to the *C. diphtheriae* typing phages are *C. ulcerans* and *C. pseudotuberculosis*. Bacteriocin (diphthericin) typing has also been described. Other typing methods include DNA restriction patterns, bacterial polypeptide analysis and hybridisation techniques.

H. Pathogenesis

Diphtheria is most commonly seen in children of 2 to 10 years. Infection is confined to humans only. The incubation period is 3 to 4 days, but may on occasion be as short as one day. Infection occurs by way of droplet spread. Diphtheria may be of the following clinical types depending upon the site of infection.

1. Faucial
2. Laryngeal
3. Nasal
4. Conjunctival
5. Otitic
6. Vulvovaginal
7. Cutaneous mainly around mouth and nose.

Faucial diphtheria is the commonest type. The toxin has both *local* as well as *systemic* effects.

1. Local effects

The bacilli remain confined to the site of entry (usually upper respiratory tract), where they multiply and start producing toxin. The toxin causes local necrotic changes along with superficial inflammatory reaction. The necrosed epithelium together with fibrinous exudate, leucocytes, erythrocytes and bacteria, constitute the *pseudomembrane*, which is a characteristic feature of diptheritic infection. The mechanical complications of diphtheria are due to the pseudomembrane, whereas the systemic effects are due to toxin.

2. Systemic effects

The diphtheria toxin diffuses into the blood stream and causes toxaemia. The toxin has got affinity for cardiac muscle, adrenals and nerve endings. It acts systemically on the cells of these tissues. The bacilli themselves do not play any part in systemic effects because they neither penetrate into the tissues nor pass into blood stream producing bacteraemia.

Complications

1. Local

The pseudomembrane may extend to the larynx which may lead to laryngeal obstruction, asphyxia and death.

2. Systemic

(a) Diphtheritic myocarditis which may terminate in heart failure and death.
(b) Polyneuropathy and post-diphtheritic paralysis of palatine and ciliary muscles.
(c) Degenerative changes in adrenals, kidney and liver may occur.

I. Laboratory Diagnosis

Laboratory confirmation of diphtheria is necessary for control measures and epidemiological studies, but not for the treatment of cases. Specific treatment should be started immediately after clinical diagnosis without waiting for laboratory reports. Any delay may be fatal. Laboratory diagnosis consists of *isolation of organism* and demonstration of its toxicity by *virulence tests*.

1. Isolation of Organism

(i) Collection of specimen

Two swabs from the lesions (throat, nose, larynx, ear, conjunctiva, vagina, or skin) are collected. One swab is used for smear examination and other for culture.

Local lesion is usually in the throat. Swabs are collected prior to start of antibiotics and application of antiseptics in form of gargles. The swabs are rubbed over the affected area and pseudomembrane, if formed, should be scraped with swab. If there is no definite localised lesion, the swabs should be rubbed over tonsils and the posterior pharyngeal wall without touching the mouth parts.

(ii) Direct microscopy

Smears are stained with both Gram and Albert stain. Diphtheria bacilli show beaded slender green rods in typical Chinese letter pattern on Albert's staining. However, they cannot be confidently differentiated from some commensal corynebacteria normally present in the throat. Hence it is reported as "the organisms resembling *C. diphtheriae*" seen in direct smear examination. Gram staining is done to identify Vincent's spirochaetes and fusiform bacilli (other causes of sore throat).

(iii) Culture

The swabs are inoculated on the following culture media:

(a) Loeffler's serum slope

Growth appears within 6–8 hours on this medium. Subculture from Loeffler's serum slope is made on tellurite blood agar and plate is incubated at 37°C for 48 hours.

(b) Tellurite blood agar

These plates have to be incubated at 37°C for at least 48 hours before declaring these as negative, as growth may sometimes be delayed.

(c) Blood agar

It is useful for differentiating streptococcal or staphylococcal pharyngitis, which may simulate diphtheria.

It may also help to differentiate mitis biotype which shows haemolysis.

(iv) Colony morphology and staining

On Loeffler's serum slope, the colonies are small, circular, white or creamy. Diphtheria bacilli grow as black or grey coloured colonies on tellurite blood agar.

Smears are prepared from suspected growth from various media. These smears are stained with Albert and Gram stain to confirm the morphology of *C. diphtheriae*. Albert staining shows green bacilli with bluish black metachromatic granules. Gram staining reveals Gram positive bacilli that tend to be decolourised easily. Diphtheroids cannot be decolourised as easily as *C. diphtheriae*.

(v) Biochemical reactions

Hiss's serum water is used for testing fermentation of carbohydrates. Biochemical reactions of *C. diphtheriae* are as follows:

Glucose	Lactose	Mannitol	Sucrose
A	–	–	–
Maltose	NO_3 reduction	Indole	Urease
+	+	–	–
phosphatase	Catalase	Oxidase	Glycogen*
–	+	–	+
Starch*			
+			

*Only Gravis biotype is positive

2. Virulence Tests

These tests demonstrate the production of exotoxin by bacteria isolated on culture. Virulence testing may be done by *in vivo* or *in vitro* methods.

IN VIVO TESTS

Guinea pigs and rabbits are susceptible to toxin produced by *C. diphtheriae*. Two types of test are used viz. subcutaneous and intracutaneous.

(a) Subcutaneous test

The growth from an overnight culture on Loeffler's serum slope is emulsified in 2–5 ml broth and 0.8 ml of this emulsion is injected subcutaneously into two guinea pigs, one of which has received an intramuscular injection of 500 units of diphtheria antitoxin 18–24 hours previously (this protected animal acts as a control). If the strain is virulent, the unprotected animal will die within 2 to 3 days with evidence of haemorrhage in the adrenal glands which is the pathognomonic feature. Other features which can be observed at autopsy are as follows:

- haemorrhagic oedema and, often, necrosis at the site of inoculation,
- swollen and congested lymph nodes,
- congested abdominal viscera,
- peritoneal and pleural exudate.

(b) Intracutaneous test

Two guinea pigs (or rabbits) are injected intracutaneously with 0.1 ml emulsion from growth on Loeffler's serum slope, one of these animals is protected with 500 units antitoxin the previous day (control) and the other is given 50 units of antitoxin intraperitoneally four hours after the skin test, in order to prevent death. If the strain is toxigenic (virulent), the inflammatory reaction at the site of injection, progresses to necrosis in 48 to 72 hours in the test animal but there is no change in the control animal. An advantage in the intracutaneous test is that 8 to 10 strains can be tested at a time on a pair of animals and the animals do not die.

IN VITRO TESTS

(a) Elek's gel precipitation test

This is an immunodiffusion test described by *Elek* (1949). A rectangular strip of filter paper soaked in diphtheria antitoxin (1000 units per ml) is placed on the surface of a 20% horse serum agar plate while the medium is still fluid. When the agar solidifies, the test strain is streaked at right angle to the filter paper strip. The positive and negative controls are also put up. The plate is incubated at 37°C for 24 to 48 hours. The toxin produced by the bacterial growth diffuses in the agar and produces a line of precipitation where it meets the antitoxin at optimum concentration (Fig. 27.3). Non-toxigenic strains will not produce any precipitation line.

(b) Tissue culture test

This is done by incorporating the test strains into the agar overlay of cell culture monolayers. The toxin produced diffuses into the underlying cells and kills them.

(c) Other tests

Enzyme linked immunosorbent assays (ELISA) and immunochromatographic strip assays are also available for the detection of toxin. These are rapid tests.

(d) Polymerase chain reaction (PCR)

C. diphtheriae tox gene can be detected by PCR. This test can also be applied directly to clinical specimen.

J. Prophylaxis

1. Active Immunisation

One attack of diphtheria provides life long immunity.

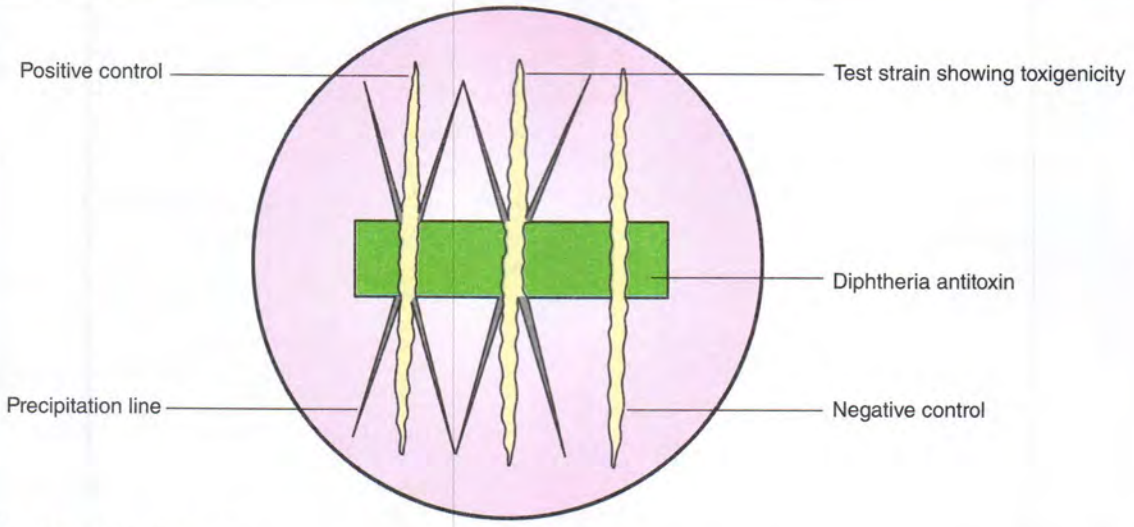


Fig. 27.3 Elek's gel precipitation test

For active immunisation, alum precipitated toxoid (APT), purified alum precipitated toxoid (PAPT) and toxoid antitoxin floccules (TAF) are used. Children are generally immunised with formol toxoid.

Active immunisation is started at 6 weeks of age by toxoid in combination with tetanus toxoid and pertussis vaccine (DPT, Triple vaccine). Three doses of 0.5 ml each are given by intramuscular route at an interval of 4–6 weeks. Booster doses of DPT of are given at 18 months and at 5 years.

2. Passive Immunisation

This is an emergency measure when susceptible persons are exposed to infection, as when a case of diptheria is admitted to paediatric wards. In such cases 500–1000 units of antitoxin (antidiphtheric serum, ADS) is administered subcutaneously. As this is a horse serum, precautions should be observed against hypersensitivity reactions.

3. Combined Immunisation

All persons receiving ADS as prophylactic measure should receive combined immunisation. An alum containing preparation like APT or PAPT should be preferred for combined immunisation as the plain formol toxoid (FT) response is unsatisfactory when given with antitoxin (ADS).

K. Schick Test

This is an intradermal test to demonstrate circulating diphtheria antitoxin. This antitoxin may be present either due to previous infection (clinical or subclinical) or immunisation. This test demonstrates immunity or susceptibility of a person against diphtheria and is one example of neutralisation test (toxin-antitoxin).

1. Method

A test dose of 0.2 ml diphtheria toxin containing 1/50 minimum lethal dose (MLD) is injected intradermally on one forearm and a similar amount of inactivated toxin (inactivated at 70°C for 30 minutes) is injected on another forearm. MLD is defined as the least amount of toxin required to kill a guinea pig weighing 250 gm within 96 hours by subcutaneous inoculation.

2. Results

Results are read after 1, 4 and 7 days. There may be four types of reaction.

(i) Positive reaction

An area of swelling and erythema appears at the site of injection of toxin after 24–48 hours, reaching maximum in 4–7 days, when it measures 1–5 cm and then fading. The control forearm injected with heated toxin will show no reaction. A positive test signifies that the person is susceptible to diphtheria due to either absence or lack of adequate amount of circulating antitoxin.

(ii) Negative reaction

There is absence of any reaction in either forearm (control and test). It indicates that the toxin has been neutralised by sufficient amount of antitoxin present in the blood and that the person is immune to diphtheria.

(iii) Pseudoreaction

There is erythema occurring in both forearms within 6–24 hours and disappearing completely from both forearms within four days. This indicates that the individual is immune to diphtheria and he is also hypersensitive to the components of diphtheria bacilli.

(iv) Combined reaction

The initial reaction is similar to that of pseudoreaction. Erythema disappears in the control forearm within four days but it progresses in the test forearm to a typical positive reaction. It indicates that the individual is susceptible to diphtheria and hypersensitive to bacillus. It is necessary to immunise such persons but the vaccine may likely induce reaction.

L. Treatment

C. diphtheriae is sensitive to penicillin, erythromycin and other antibiotics. Diphtheria patients are given a course of penicillin, to stop cases from becoming carriers. Erythromycin is more active in the treatment of carriers.

The antibiotics are of little value as these cannot inactivate the toxin already present in patient's body. When a case is suspected as diphtheria, antidiphtheric serum should be given immediately as the mortality rate increases with delay in starting antitoxin treatment. In moderate cases, the dosage recommended is 20,000 units intramuscularly and in severely ill cases 50,000 to 100,000 units are injected, half the dose being given intravenously.

II. OTHER PATHOGENIC CORYNEBACTERIA

C. ulcerans is a bacillus related to *C. diphtheriae*, which can cause localised ulcerations in throat, clinically resembling diphtheria. It resembles the gravis type but it liquefies gelatin, ferments trehalose slowly and does not reduce nitrate. It is PYZ-negative and urease positive. It produces two types of toxins, one is identical with diphtheria toxin and the other resembling the toxin of *C. pseudotuberculosis*. It is pathogenic to guinea pigs, the lesions produced are similar to those produced by *C. diphtheriae*. It causes infections in cows and human infections may occur through cow's milk. Some consider *C. ulcerans* as a variant of diphtheria bacillus. Diphtheria antitoxin is protective.

C. pseudotuberculosis (Preisz-Nocard bacillus) also referred to as *C. ovis* causes pseudotuberculosis in sheep and suppurative lymphadenitis in horses. It is PYZ-negative and urease positive. It produces a toxin which differs from that of *C. diphtheriae*. The toxin kills guinea pig in 24 hours. *C. bovis*, commensal of cow's uddar, is a doubtful agent for mastitis. Many of them cause infections in immunocompromised patients (Table 27.2). *C. jeikeium* is an important pathogen in neutropenic patients and those receiving antibiotics. It is usually multiresistant and responds only to vancomycin.

Table 27.2 Medically Important Non–Diphtheria Corynebacteria and Diseases Caused by these Organisms

	Disease	Organisms
1.	Acute pharyngitis	*C. ulcerans*
2.	Cutaneous infections	*C. jeikeium*
3.	Lymphadenitis	*C. pseudotuberculosis*
4.	Infective endocarditis	*C. jeikeium* *C. xerosis* *C. bovis*
5.	Shunt or cannula infections	*C. jeikeium* *C. xerosis* *C. bovis*

C. minutissimum and *C. tenuis* are associated with superficial skin infection.

Some bacteria which were initially in genus Corynebacteria include *Arachnobacterium haemolyticum* (formerly *C. haemolyticum*) and *Rhodococcus equi* (formerly *C. equi*). *A. haemolyticum* may cause pharyngitis, peritonsillar abscess, cervical lymphadenitis, osteomyelitis and brain abscess. *R. equi* is found in soil. Human infection is rare,although an increased incidence in immunocompromised patients, especially AIDS patients, has been reported.

III. DIPHTHEROIDS

Commensal corynebacteria are normally present in the throat, skin, conjunctiva and other areas. These may sometimes be mistaken for *C. diphtheriae* and are called diphtheroids (Table 27.3). The common diptheroids include *C. xerosis* (found in the conjunctival sac) and *C. pseudodiphtheriticum* (*C. hofmannii*) which is found in the throat. In general, diphtheroids possess few or no metachromatic granules and are arranged in palisades (parallel rows) rather than in Chinese letter pattern. Some diphtheroids are indistinguishable from diphtheria bacilli microscopically and require to be differentiated by biochemical tests and more reliably by virulence tests.

Table 27.3 Distinguishing Features of ***C. diphtheriae*** and Diphtheroids

Feature	C. diphtheriae	Diphtheroids
1. Morphology	(i) Weakly Gram positive and thin bacilli	Strongly Gram positive, short and thick bacilli
	(ii) Metachromatic granules present	Few or absent
	(iii) Chinese letter pattern	Pallisade arrangement
	(iv) Pleomorphism present	Very little pleomorphism present
2. Culture	Grow on special enriched media	Can grow on ordinary media
3. Biochemical tests	Ferments glucose only and does not ferment sucrose	Ferments both glucose and sucrose
4. Toxin production	Toxic	Non-toxic

KEY POINTS

1. *Corynebacterium diphtheriae* are bacilli usually seen in angular fashion resembling the letters V or L. This has been called *Chinese letter*. These bacilli contain *metachromatic granules*.
2. Special stains like *Albert*, *Neisser* or *polychrome methylene blue* are used for staining the bacilli. The bacilli look *green* and metachromatic granules appear *bluish black* when Albert stain is used.
3. *C. diphtheriae* are grown best on media enriched with blood, serum or egg. *Loeffler's serum slope* (*LSS*) and *potassium tellurite blood agar medium* are employed for cultivation of this bacillus.
4. Potassium tellurite blood agar is a *selective medium*. The organisms grow slowly on this medium and form grey or black colonies due to reduction of *tellurite to tellurium*.
5. *C. diphtheriae* causes *diphtheria*. The pathogenicity is due to production of a very powerful exotoxin. The toxigenicity of the diphtheria bacillus depends on the presence of a *beta phage* which can be transferred from one bacterium to another by *lysogenic conversion*. Diphtheria toxin acts by *inhibiting protein synthesis*. It inhibits polypeptide chain elongation by inactivating the elongation factor, EF-2.
6. *Virulence tests* demonstrate the production of exotoxin by bacteria isolated on culture. These may be done by *in vivo* or *in vitro* methods. *Guinea pigs* and *rabbits* are susceptible to toxin produced by *C. diphtheriae*. Two types of tests are used i.e. *subcutaneous* and *intracutaneous*. In vitro methods include *Elek's gel precipitation test* and *tissue culture* test.
7. *Triple vaccine* (*DPT*) is used for active immunisation. This vaccine contains diphtheria toxoid, tetanus toxoid and pertussis vaccine.
8. Commensal corynebacteria are normally present in the throat, skin, conjunctiva and other areas. These may sometimes be mistaken for *C. diphtheriae* and are called *diphtheroids*. In general, diphtheroids possess few or *no metachromatic granules* and are arranged in *pallisades* (parallel rows) rather than in Chinese letter pattern. *C. xerosis* and *C. pseudodiphtheriticum* are two *examples* of diphtheroids.

YOU MUST KNOW

1. Morphology, culture characteristics and biochemical reactions of *Corynebacterium diphtheriae*.
2. Differences between three biotypes gravis, intermedius and mitis of *C. diphtheriae*.
3. Laboratory diagnosis of diphtheria.
4. Virulence tests for demonstration of diphtheria toxin.
5. DPT and antidiphtheric serum (ADS).
6. Schick test.
7. Differences between *C. diphtheriae* and diphtheroids.

STUDY QUESTIONS

1. Name the different species of genus Corynebacterium. Discuss in detail the laboratory diagnosis of diphtheria.
2. Write short notes on:
   (a) Diphtheria toxin
   (b) Schick test
   (c) Corynebacteria other than *C.diphtheriae*
   (d) Diphtheroids.
   (e) Toxigenicity tests of *C.diphtheriae.*

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Chinese letter arrangement of bacilli is a characteristic feature of:
   (a) *Mycobacterium tuberculosis* (b) *M. leprae*
   (c) *Corynebacterium diphtheriae* (d) *Bacillus cereus*
2. Babes-Ernst granules are present in:
   (a) *Mycobacterium tuberculosis* (b) *M. laprae*
   (c) *Corynebacterium diphtheriae* (d) *Bacillus cereus*
3. What is the colour of colonies of *C. diphtheriae* on tellurite blood agar medium?
   (a) White (b) Golden yellow
   (c) Grey to black (d) Cream
4. Which is the selective medium used for isolation of *C. diphtheriae*?
   (a) Tellurite blood agar (b) Loeffler's serum slope
   (c) Lowenstein-Jensen medium (d) Chocolate agar
5. Which biotype of *Corynebacterium diphtheriae* ferments starch and glycogen?
   (a) Gravis (b) Intermedius
   (c) Mitis (d) None of the above
6. Diphtheria toxin has affinity for the following tissues:
   (a) Cardiac muscle (b) Adrenals
   (c) Nerve endings (d) All of the above
7. Production of diptheria toxin by bacteria can be demonstrated by:
   (a) Subcutaneous test (b) Intracutaneous test
   (c) Elek's gel precipitation test (d) All of the above
8. The most common site affected by *Corynebacterium diphtheriae* is:
   (a) Upper respiratory tract (b) Conjunctiva
   (c) Skin (d) Vulvovaginal
9. Schick test is an example of:
   (a) Precipitation (b) Agglutination
   (c) Neutralisation (d) None of the above

Answers (MCQs):

1. (c)	2. (c)	3. (c)	4. (a)	5. (a)
6. (d)	7. (d)	8. (a)	9. (c)	

Chapter 28

BACILLUS

Sporogenous, rod shaped Gram positive bacteria are divided into two groups, the aerobic *Bacillus* and the anaerobic *Clostridium*. The spores of *Bacillus* (unlike the genus *Clostridium*) are of the same width as that of bacteria and do not produce a bulge in the bacterial cell. Members of the genus *Bacillus* are ubiquitous, present in soil, dust, air and water and are frequently isolated as contaminants in bacteriological culture media. *B. anthracis*, the causative agent of anthrax, is the most important pathogen of the group. *B. cereus* can cause food poisoning. They are generally motile with peritrichous flagella except the anthrax bacillus which is non-motile.

I. BACILLUS ANTHRACIS

It is the causative agent of anthrax, a disease primarily of animals, and man gets infected secondarily. Considerable historical interest is attached to the anthrax bacillus due to the following reasons:

1. It was the first pathogenic bacterium to be seen under the microscope (*Pollender*, 1849).
2. The first communicable disease to be transmitted by inoculation of infected blood was anthrax (*Davaine*, 1850).
3. It was the first bacterium to be isolated in pure culture and shown to possess spores (*Robert Koch*, 1876).
4. *B. anthracis* was the first bacterium used for the preparation of attenuated vaccine (*Louis Pasteur*, 1881).

A. Morphology

Bacillus anthracis is a Gram positive, non-acid-fast, non-motile, large (3–10 μm × 1–1.6 μm), rectangular, spore forming bacillus. The spores are refractile, oval and central in position and are of the same width as the bacillary body so that they do not cause bulging of vegetative cell (Fig. 28.1). In infected tissues, the bacilli are found singly, in pairs or in short chains, the entire chain

being surrounded by capsule. The capsule is polypeptide (polymer of d-glutamic acid) in nature.

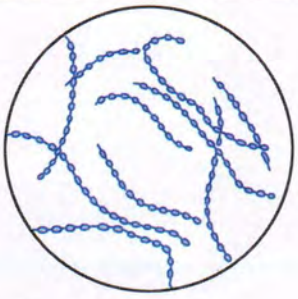


Fig. 28.1 Anthrax bacilli

Spores are never found in the animal body during life but are formed in culture or in the soil. Sporulation occurs under unfavourable conditions for growth and is encouraged by distilled water and 2% NaCl. It is inhibited by calcium chloride. In contrast, the capsules are formed in the tissues but are usually lacking in ordinary conditions of culture. Capsules are formed only if the media contain added bicarbonate or are incubated under 10–25 per cent CO_2. Capsule formation may occur in the absence of CO_2, when this bacterium is grown in media containing serum, albumin, charcoal or starch.

When blood films containing anthrax bacilli are stained with polychrome methylene blue for 10–12 seconds and examined under the microscope, an amorphous purple material is noticed around the bacilli. This represents the disintegrated capsular material and is characteristic of the anthrax bacillus. This is known as **McFadyean's reaction** and is employed for presumptive diagnosis of anthrax. When stained with Giemsa stain, the bacillus stains purple and capsule red.

In cultures, the bacilli are arranged end-to-end in long chains. The ends of the bacilli are truncated or often concave and somewhat swollen so that a chain of bacilli presents a **bamboo-stick** appearance.

B. Culture

B. anthracis is an aerobe and facultative anaerobe, with a temperature range for growth being 12–45°C (optimum 35–37°C).

1. Nutrient Agar Media

Colonies are round, 2–3 mm in diameter, raised, opaque and greyish white. Under low power of the microscope, the edge of the colony is found to be composed of long, interlacing chains of bacilli, resembling locks of matted hair, the so called the **Medusa head appearance** (Fig. 28.2).

Fig. 28.2 Medusa head appearance of colony on nutrient agar

2. Blood Agar Media

The colonies are non-haemolytic, though occasional strains produce a narrow zone of haemolysis.

3. Gelatin Stab Culture

Gelatin is liquefied mostly at the top due to aerobic environment and gives a characteristic *inverted fir tree* appearance (Fig. 28.3).

Fig. 28.3 Inverted fir tree appearance in gelatin stab culture

4. Selective Medium

A selective medium (PLET medium) consisting of heart infusion agar with polymyxin, lysozyme, ethylene diamine tetracetic acid (EDTA) and thallous acetate has been devised for isolation of *B.anthracis* from mixtures containing other spore-bearing bacilli.

5. Solid Medium Containing Penicillin

When *B. anthracis* is grown on a solid medium containing 0.05–0.5 units penicillin per ml, in 3–6 hours the cells become large, spherical, and occurs in chains on the surface of the agar, resembling a string of pearls. This *string of pearls reaction* is useful in differentiation of *B. anthracis* from *B. cereus* and other aerobic spore bearers. *B. anthracis* is susceptible to *gamma phage*. This is another characteristic which differentiates it from *B. cereus*.

C. Biochemical Reactions

Glucose, maltose and sucrose are fermented with production of acid only. Catalase is formed and nitrates are reduced to nitrites.

D. Resistance

1. *Vegetative form:* It gets killed at 60°C in 30 minutes.
2. *Spore form:* Spores of many strains resist dry heat at 140°C for 1–3 hours and boiling for 10 minutes. They remain viable for years in environment such as in soil. Spores are destroyed by:
   (*i*) Four per cent potassium permanganate solution in 15 minutes.
   (*ii*) Autoclaving (at 121°C, 15 lbs/in^2 for 15 minutes)

They survive in 5% phenol for weeks. Destruction of the spores in animal product is achieved by *duckering* in which 2% formaldehyde is used at 30–40°C for 20 minutes for disinfection of wool while 0.25% solution is used at 60°C for six hours for animal hair and bristles.

The anthrax bacillus is susceptible to penicillin, streptomycin, erythromycin, tetracycline and chloramphenicol.

E. Antigens

These are of three types:

1. *Capsular antigen*: It is present in virulent strain and consists of a polypeptide and acts as hapten.
2. *Cell wall antigen*: polysaccharide
3. *Somatic antigen*: It is heat labile protein present in bacterial body. It stimulates immune system to produce the antibody which is protective in nature.

F. Toxin

The virulence of anthrax bacilli depends on two factors—an extracellular toxin and a capsular polypeptide. The toxin consists of three components namely the oedema factor (OF or Factor-I), the protective antigen (PA or Factor-II) and the lethal factor (LF or Factor-III). These are not toxic individually, but a mixture of all three produces the *anthrax toxic complex*. The production of toxin is controlled by a plasmid (p × 01). These factors have been characterised and cloned. PA binds to the receptor on the target cell surface, and provides attachment sites for OF or LF. OF is an adenyl cyclase which is activated only inside the target cells and lead to intracellular accumulation of cyclic AMP. This is thought to be responsible for the oedema and other effects of the toxin. LF causes cell death but the mechanism of action is not known.

The capsular polypeptide is controlled by a plasmid (p × 02) and aids virulence by inhibiting phagocytosis.

G. Pathogenesis

Anthrax is primarily a disease of animals like cattle and sheep, and less often of horses and swine. Infection occurs in susceptible animals by ingestion of the spores present in the soil. Direct spread of disease from animal to animal is rare. Infected animals discharge large number of bacilli from the mouth, nose and rectum. These bacilli sporulate in soil and remains as the source of infection. The disease is usually septicaemic but may sometimes be localised.

Human Anthrax

Humans are occasionally secondarily infected from diseased animals. There are three clinical type of disease based on route of infection—cutaneous, pulmonary and intestinal anthrax. All types lead to septicaemia and meningitis.

Cutaneous anthrax follows entry of the spores through the abraded skin. The face, neck, hands and back are commonly affected sites. This is commonly found in farmers and persons handling carcasses. The lesion starts as a papule which becomes vesicle containing fluid (pustule). The acute inflammatory reaction leads to congestion and oedema of the area with a central necrotic lesion, which is covered by a **black eschar**. The name anthrax, meaning coal, comes from the eschar which is black coloured. The lesion is called 'malignant pustule'. Cutaneous anthrax may resolve spontaneously, but may sometimes lead to fatal septicaemia and meningitis.

Pulmonary anthrax occurs due to inhalation of the dust or filaments of wool from infected animals, particularly in wool factories. This is also called **wool sorter's disease**. The infection is characterised by haemorrhagic bronchopneumonia often followed by septicaemia. It has a high fatality rate.

Intestinal anthrax is rare in man and is found in those who consume improperly cooked infected meat. Ingestion of spores causes violent enteritis with bloody diarrhoea with high case fatality.

Cutaneous, intestinal or pulmonary anthrax, if not treated in time, lead to septicaemic anthrax and death occurs.

H. Laboratory Diagnosis

1. Specimens

Swabs, fluid or pus from pustules; sputum and blood from pulmonary and septicaemic anthrax are generally collected. Faecal specimen or peritoneal fluid can be

collected from intestinal anthrax. Laboratory personnel should take additional protective precautions against infection while handling the material.

2. Microscopy

Gram stained smear from the specimen shows often chain of large Gram positive bacilli. Capsule appears as a clear halo around the bacterium by India-ink staining.

Direct fluorescent antibody test (DFA) for capsule specific staining and for polysaccharide (cell wall) antigen confirms the identification.

McFadyean's Reaction

When blood films are stained with the polychrome methylene blue for 10–12 seconds and examined under the microscope, an amorphous purple material is noticed around the bacilli. This represents the disintegrated capsular material and is a characteristic of *B. anthracis*. This reaction is employed for the presumptive diagnosis in animals.

3. Culture

Specimen is inoculated on nutrient agar medium and incubated at 37°C for overnight. Medusa head colonies appear on the medium. Smears made from these colonies show typical Gram positive spore bearing bacilli.

Gelatin stab culture shows inverted fir tree appearance.

4. Animal Inoculation

White mouse or guinea pigs are injected with exudate or culture. Animal dies in 36–48 hours. Smears made from heart blood and sputum show bacilli.

5. Serology (Ascoli's Thermoprecipitin Test)

The tissues are ground up in saline and boiled for 5 minutes and filtered. When this extract is layered over the anti-anthrax serum in a narrow tube, a ring of precipitate appears at the junction of two liquids within five minutes in a positive case. It is mainly used for rapid diagnosis when the sample received is putrid and viable bacilli are unlikely to be found.

6. Polymerase Chain Reaction

It is used for confirmation of anthrax bacillus.

In the wake of bioterrorism experience in the USA in 2001, the Centers for Disease Control (CDC) have prepared guidelines for identification of *B. anthracis* as mentioned below.

1. For presumptive identification of anthrax

(i) Large gram positive bacillus with the general morphology of anthrax bacillus and non-motile.
(ii) Non-haemolytic on blood agar and cultural features of anthrax bacilus.
(iii) Catalase positive.

2. For initial confirmation

(i) Lysis by gamma phage.
(ii) Direct fluorescent antibody test (DFA).

3. For further confirmation

(i) Polymerase chain reaction.

I. Treatment

Doxycycline and ciprofloxacin are used for treatment. Antibiotics are ineffective once toxin is formed, although it may reduce the case fatality rate.

J. Prophylaxis

Prevention is mainly by general methods such as improvement of factory hygiene and proper sterilization of animal products like wool. Carcasses of animals suspected to have anthrax are buried deep in lime.

Animals are to be protected by active immunisation. As spore is the common infective form in nature, *Sterne vaccine* contains spores of a non-capsulated avirulent mutant strain. This avirulent mutant strain is obtained by loss of two plasmids (p × 01 and p.×.02) which encode the anthrax toxin (p.×.01) and capsular polypeptide (p.×.02) production. The animal is protected for a year with a single injection of spore vaccine. It is extensively used in animals, however, it is not safe for human use.

Anthrax vaccines have been used in persons occupationally exposed to anthrax infection. There are two types of vaccines used in humans, namely cell free protective antigen (PA) vaccines and live attenuated vaccines. In PA vaccines, protective antigen (PA) component of the anthrax toxic complex is either adsorbed (AVA) on to aluminium hydroxide or precipitated (AVP) on to aluminium potassium phosphate. Both AVA and AVP vaccines are given by intramuscular route. Five doses (0 and 4 weeks and at 6, 12, and 18 months) are administered in case of AVA vaccine while four doses are given over a period of 8 months for AVP vaccine. Annual booster is required in both the vaccines.

Live attenuated vaccines contain spores from attenuated strains of *B.anthracis*. Two doses are administered subcutaneously (Table 28.1).

Table 28.1 Anthrax vaccines for humans

Types	Vaccines	Doses
Cell free PA vaccines	• Anthrax vaccine adsorbed (AVA) • Anthrax vaccine precipitated (AVP)	Five doses, one booster. Four doses, one booster
Live vaccines	• Russian vaccine • PR China vaccine	Two doses

II. ANTHRACOID BACILLI

Aerobic spore bearing bacilli resembling *B. anthracis* are called anthracoid or pseudoanthrax bacilli. Some of them are frequent laboratory contaminants and have to be differentiated from *B. anthracis*. *B. subtilis* may act as opportunistic pathogen, causing eye infections and septicaemia. *B. licheniformis* has also been incriminated in patients of food poisoning. Spores of *Bacillus stearothermophilus* are used to test the efficiency of sterilisation by autoclaves. Some species of bacillus are important as producers of antibiotics such as bacitracin, tyrothricin and polymyxin. Most of anthracoid bacilli can be differentiated easily from the anthrax bacillus, but some, like non-motile strains of *B. cereus* may resemble *B. anthracis*. The main differentiating features between anthracoid bacilli and *B. anthracis* are shown in Table 28.2.

III. BACILLUS CEREUS

B. cereus has assumed importance as a cause of food poisoning. It is widely distributed in nature such as soil, vegetables, milk, cereals, spices, meat and poultry. Some spores survive cooking and germinate into vegetative bacilli which produce *enterotoxin* that cause food poisoning.

A. Types of Food Poisoning

1. Short Incubation Period Type (1–5 Hours)

It is characterised by acute nausea and vomiting, 1–5 hours after the meal. Diarrhoea is not common. It is usually associated with consumption of cooked rice, usually fried rice from Chinese restaurants. *B. cereus* is present in large numbers in the cooked rice as well as in faecal samples of these patients.

2. Long Incubation Period Type (8–16 Hours)

It is characterised by acute abdominal pain and diarrhoea, 8–16 hours after ingestion of contaminated food. Vomiting is rare. The faecal samples contain small number of *B. cereus*.

B. Pathogenesis

The disease is due to *enterotoxin* production. The long incubation type (diarrhoeal disease) is caused by serotypes 2, 6, 8, 9, 10 or 12 while the short incubation type (emetic type) is caused by serotypes 1, 3 or 5 of *B. cereus* strains. Isolates from the diarrhoeal type of disease produce an enterotoxin which resembles heat labile enterotoxin of *Esch.coli*. Strains that cause the emetic type of disease produce a toxin which resembles staphylococcal enterotoxin. Two mechanisms of action, one involving stimulation of CAMP system and the other

Table 28.2 Differentiating Features of ***B. anthracis*** and Anthracoid Bacilli

Feature	B. anthracis	Anthracoid Bacilli
1. Motility	Non-motile	Generally motile
2. Capsule	Capsulated	Non-capsulated
3. Chain formation	Long chains	Short chains
4. Colony on nutrient agar	Medusa head colony	Not present
5. Growth in broth	No turbidity	Uniform turbidity
6. Gelatin stab culture	Inverted fir tree appearance and slow gelatin liquefaction	Rapid gelatin liquefaction
7. Haemolysis on blood agar	Absent or weak	Usually well marked
8. Growth in Penicillin agar (10 units/ml)	No growth	Grow usually
9. Growth at 45°C	No growth	Grow usually
10. Fermentation of salicin	Negative	Usually positive
11. Susceptibility to gamma phage	Susceptible	Not susceptible
12. Pathogenicity test in animals	Pathogenic	Not pathogenic
13. McFadyean's reaction	Positive	Negative
14. Ascoli's precipitin test	Positive	Negative
15. Fluorescent antibody test with anthrax antiserum	Positive	Negative

independent of it, have been described for the enterotoxin of *B. cereus*.

C. Diagnosis

Suspected food, faeces and vomitus are cultured on ordinary media or a special mannitol-eggyolk-phenol red-polymyxin agar (MYPA) medium. Spore bearing Gram positive bacilli may be seen on smear from colonies. *B. cereus* is a motile bacillus, non-capsulated, not susceptible to gamma phage and does not react with fluorescent antibody conjugate. It produces lecithinase and ferment glucose but not mannitol.

D. Treatment

Disease is mild and self limiting, requiring no specific treatment.

E. Control

Adequate food hygiene is to be maintained while storing cooked food especially rice and reheating should be rapid.

KEY POINTS

1. Members of the genus *Bacillus* are ubiquitous, present in soil, dust, air and water and are frequently isolated as contaminants in bacteriological culture media. They are *sporogenous*, rod shaped, *aerobic*, Gram positive bacteria.
2. *B. anthracis*, the causative agent of anthrax, is the most important pathogen of the bacillus group. *B. cereus* can cause food poisoning.
3. *B. anthracis* is a *capsulated* organism and *McFadyean's reaction* is positive. It grows as '*Medusa head*' appearance on nutrient agar medium.
4. *Ascoli's thermoprecipitin test* has been used for rapid diagnosis of anthrax.
5. *Alum precipitated toxoid* has been used in persons occupationally exposed to anthrax infection. It is given in three doses intramuscularly at intervals of 6 weeks and 6 months. A booster dose may be given after one year.
6. Aerobic spore bearing bacilli resembling *B. anthracis* are called *anthracoid bacilli*.
7. *B. cereus* causes food poisoning usually associated with *fried rice from Chinese restaurants*. It produces enterotoxin. The diseases is mild and self limiting, requiring no specific treatment.

YOU MUST KNOW

1. Morphology and culture characteristics of *Bacillus anthracis*.
2. Laboratory diagnosis of anthrax.
3. *Bacillus cereus*.
4. Anthracoid bacilli.

STUDY QUESTIONS

1. Discuss the laboratory diagnosis of anthrax.
2. Write short notes on:
   (a) Anthracoid bacilli. (b) *Bacillus cereus* food poisoning

MULTIPLE CHOICE QUESTIONS (MCQs)

1. What is the chemical nature of capsule of *B. anthracis*?
   (a) Polysaccharide (b) Polypeptide (c) Fatty acid (d) None of the above
2. McFadyean's reaction is used for presumptive diagnosis of:
   (a) Tetanus (b) Anthrax (c) Cholera (d) Gas gangrene

3. 'Medusa head' appearance of colonies is a characteristic feature of:
   (a) *Clostridium perfringens* (b) *Bacillus anthracis*
   (c) *Mycoplasma hominis* (d) *Ureaplasma urealyticum*
4. 'Malignant pustule' lesion is found in:
   (a) Cutaneous anthrax (b) Pulmonary anthrax
   (c) Intestinal anthrax (d) None of the above
5. Ascoli's thermoprecipitin test is helpful in the laboratory diagnosis of:
   (a) Tetanus (b) Anthrax
   (c) Cholera (d) Gas gangrene
6. Which of the following characteristics can differentiate *Bacillus cereus* from *Bacillus anthracis*?
   (a) Non-capsulated (b) Motile
   (c) Not susceptible to gamma phage (d) All of the above

Answers (MCQs):

1. (b) 2. (b) 3. (b) 4. (a) 5. (b) 6. (d)

Chapter 29

CLOSTRIDIUM

The genus *Clostridium* consists of anaerobic, spore forming, Gram positive bacilli. The spores are wider than the bacterial bodies, giving the bacillus a swollen appearance resembling a spindle. The name Clostridium is derived from the word 'Kloster' (meaning a spindle).

Most species are saprophytes found in soil, water and decomposing plant and animal matter. Some of the pathogens (e.g. *Cl. tetani* and *Cl. perfringens*) are

commensals of the intestinal tract of human and animals. The genus contains bacteria that cause three major diseases of man—*tetanus*, *gas gangrene* and *food poisoning*. Clostridial pathogenicity is mainly due to production of a powerful exotoxin. *Cl. botulinum* is non-invasive, *Cl. tetani* is slightly invasive and clostridia causing gas gangrene are not only toxigenic but are also invasive.

Most species of the genus are motile with peritrichous flagella except *Cl. perfringens* and *Cl. tetani* type VI which are non-motile. All clostridia are non-capsulated with the exception of *Cl. perfringens* and *Cl. butyricum*.

I. CLASSIFICATION

Clostridia of medical importance may be classified on the basis of diseases they produce:

A. Tetanus	*Cl. tetani*
B. Gas gangrene	
1. Established pathogens	*Cl. perfringens* (*Cl. welchii*)
	Cl. novyi (*Cl. oedematiens*)
	Cl. septicum
2. Less pathogenic	*Cl. histolyticum*
	Cl. fallax
3. Doubtful pathogens	*Cl. bifermentans*
	Cl. sporogenes
C. Food poisoning	
1. Gastroenteritis	*Cl. perfringens* (Type A)
2. Necrotising enteritis	*Cl. perfringens* (Type C)
3. Botulism	*Cl. botulinum*
D. Acute colitis	*Cl. difficile*

II. GENERAL FEATURES

A. Morphology

Clostridia are Gram positive, spore forming rods, usually 3–8 μm × 0.4–1.2 μm in size and are highly pleomorphic. The shape and position of spores varies in different species and is useful in identification of clostridia. Spores may be (1) central or equatorial in *Cl. bifermentans* (2) sub-terminal in *Cl. perfringens* (3) Oval and terminal in *Cl. tertium* (4) spherical and terminal, giving a drumstick appearance, in *Cl. tetani*.

B. Culture

Clostridia grow well on ordinary media under anaerobic conditions. Inoculated culture (e.g. blood agar) is placed in anaerobic jar (McIntosh Filde's jar) from which air is removed and replaced with a mixture of nitrogen and carbon dioxide. The anaerobic jar containing culture media is incubated at 37°C for 2–3 days. Most clostridia produce colonies surrounded by haemolysis on blood agar.

Liquid media like cooked meat broth (CMB) or thioglycollate media (containing reducing agent thioglycollate and 0.1% agar) are very useful for growing clostridia. CMB contains unsaturated fatty acids which take up oxygen, the reaction is catalysed by haematin in the meat and also sulphydryl compounds which lower down the redox potential. Growth appears as turbidity in the medium. Proteolytic clostridia turn the meat black and produce foul odour whereas saccharolytic species turn the meat pink.

C. Biochemical Reactions

Most clostridia ferment a variety of sugars and hydrolyse protein.

D. Resistance

The vegetative cells of clostridia do not differ from other non-sporing bacilli in their resistance to various physical and chemical agents. The spores exhibit a variable resistance. Spores of *Cl. tetani* persist for years in dried earth. *Cl. perfringens* spores are destroyed by boiling for less than five minutes, but spores of some strains (type A) that cause food poisoning survive for several hours. Spores of *Cl. botulinum* are not killed completely even at 105°C for less than 100 minutes. However all spores are killed at 121°C within 20 minutes (autoclaving). Halogens and glutaraldehyde (2%) are very effective in killing spores. Aqueous iodine solution (1%) kills spores within three hours. Spores are resistant to phenolic disinfectants. Formaldehyde is not very active.

Clostridia are susceptible to metronidazole, penicillin, chloramphenicol and erythromycin but are resistant to aminoglycosides and quinolones.

E. Toxin

Most clostridia produce one or more protein toxins which are responsible for pathogenic effects. Toxins of *Cl. tetani* and *Cl. botulinum* attack nervous system and are called *neurotoxin*. The gas gangrene clostridia toxin attacks soft tissues and is known as *histotoxic*.

III. CLOSTRIDIUM PERFRINGENS (CLOSTRIDIUM WELCHII)

The organism was originally cultivated by *Achalme* (1891) and was first described in detail by *Welch and Nuttal* (1892). *Cl. perfringens* is the most important and common aetiological agent of gas gangrene (60%), followed by *Cl. novyi* (30–40%) and *Cl. septicum* (10–20%). *Cl. perfringens*

also produces food poisoning and necrotising enteritis in man.

Cl. perfringens is a commensal in the large intestines of man and animals. The spores are commonly found in soil and dust.

A. Morphology

It is a large, stout, Gram positive bacillus measuring 4–6 μm × 1μm with subterminal spore (Fig. 29.1). It usually occurs singly or in chains and is pleomorphic. Involution forms are common. It is capsulated and non-motile. The spores are rarely seen in artificial culture or in tissue and their absence is one of the characteristic features of *Cl. perfringens*. Spore formation can be induced only on special media.

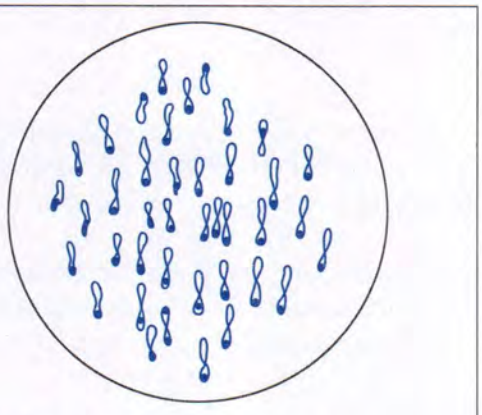


Fig. 29.1 *Clostridium perfringens*

B. Culture

It grows on blood agar, cooked meat broth (CMB) and thioglycollate broth within 24–48 hours. It grows best in carbohydrate containing media like glucose blood agar. It is anaerobic and grows over a pH range of 5.5–8.0 and wide temperature range of 20°C–50°C. The optimum temperature for growth is 37°C.

On blood agar, colonies of most strains show a **target haemolysis**, resulting from a narrow zone of complete haemolysis caused by theta-toxin and a much wider zone of incomplete haemolysis due to alpha toxin. In cooked meat broth, the meat pieces turn pink but are not digested.

C. Biochemical Reactions

Cl. perfringens is predominantly saccharolytic but also have mild proteolytic action (gelatin liquefaction). It ferments glucose, lactose, sucrose and maltose with the production of acid and gas. It is indole negative, H_2S is formed abundantly. MR is positive and VP negative. Most strains can reduce nitrates.

In litmus milk, lactose fermentation leads to formation of acid, which changes the colour of litmus from blue to red. The acid coagulates the casein (acid clot) and the clotted milk is disrupted due to vigorous gas production and this is known as **stormy fermentation**.

D. Resistance

Spores are usually destroyed within five minutes by boiling but those of the 'food poisoning' strains (type A and certain type C strains) resist boiling for 1–3 hours. Autoclaving at 121°C for 15 minutes is lethal. Spores are resistant to commonly used antiseptics and disinfectants.

E. Classification

Cl. perfringens produce at least 12 distinct toxins but on the basis of production of four major toxins (*alpha*, *beta*, *epsilon* and *iota*), these are classified into five types A to E.

1. Type A strains produce *alpha* toxin.
2. Type B strains produce *alpha*, *beta* and *epsilon* toxins.
3. Type C strains produce *alpha* and *beta* toxins.
4. Type D strains produce *alpha* and *epsilon* toxins.
5. Type E strains produce *alpha* and *iota* toxins

Type A causes gas gangrene in man and some strains of type A produce enterotoxin which causes food poisoning.

Alpha (α) Toxin

It is produced by all types of *Cl. perfringens* but most abundantly by type A strains. Chemically it is a phospholipidase (lecithinase.C) and is responsible for profound toxaemia in gas gangrene. It is relatively heat stable, lethal, dermonecrotic and haemolytic. The toxin is haemolytic for red blood cells of most animal species, except those of horse and goat. The haemolysis is of the hot-cold variety i.e. best seen after incubation at 37°C followed by chilling at 4°C. The toxin splits lecithin, an important constituent of mammalian cell membrane. This specific effect of toxin is utilised for rapid detection of *Cl. perfringens* in clinical specimens (Nagler reaction).

Nagler reaction

Cl. perfringens is grown on a medium containing 6% agar, 5% Fildes' peptic digest of sheep blood and 20% human serum or 5% egg yolk in a plate. Neomycin sulphate may be added to the medium to make it more selective by inhibiting aerobic spore bearers and coliforms. To one half of the plate, antitoxin is spread on the surface. The inoculated culture plate is incubated at 37°C for 24 hours. Colonies on the half plate without the antitoxin

will be surrounded by opacity while colonies on the other half with antitoxin shows no opacity, due to specific neutralisation of the alpha toxin (Fig. 29.2). Alpha toxin (lecithinase C) splits lecithin into phosphoryl choline and a diglyceride (lipid). The lipid deposits around the colonies resulting in opacity.

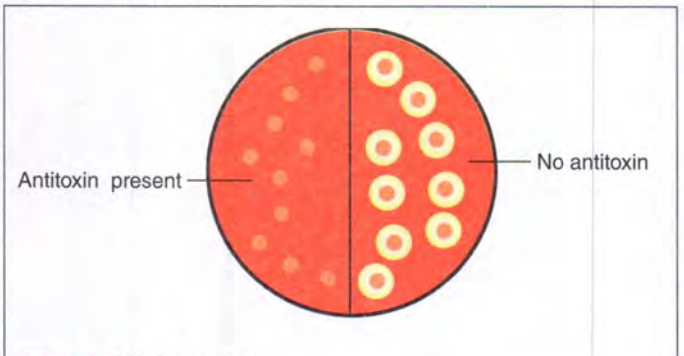


Fig. 29.2 Nagler reaction

Certain lecithinase forming bacteria (*Cl. novyi*, some vibrios, some aerobic spore bearers) may produce opacity in egg yolk media but is not neutralised by *Cl. perfringens* antitoxin, except with *Cl. bifermentans* which produces a serologically related lecithinase.

Reverse CAMP test

This test is similar to the CAMP test for identifying group B streptococci (Refer Chapter 24) except that *Clostridium sp.* is inoculated in place of *Staph. aureus* and a known group B streptococcus is used. Although group B streptococci may show some enhanced haemolysis with other clostridia but only *Cl. perfringens* exhibits accentuated zone of haemolysis as butterfly appearance (Fig. 29.3).

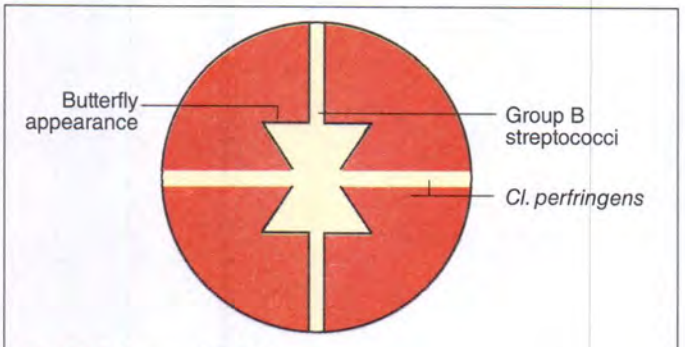


Fig. 29.3 Reverse CAMP test

Other Major Toxins

Beta (β), *epsilon* (ε) and *iota* (ι) toxins have lethal and necrotising properties.

Minor Toxins

Gamma (γ) and eta (η) toxins have only minor lethal actions. The delta (δ) toxin is lethal and haemolytic. The theta (θ) toxin is oxygen labile and is antigenically related to streptolysin O. The kappa (κ) toxin is a collagenase. The lambda (λ) toxin is a proteinase and gelatinase. The mu (μ) toxin is a hyaluronidase. The nu (ν) toxin is a deoxyribonuclease.

Enterotoxin

Some strains of type A produce enterotoxin which causes diarrhoea and other symptoms of food poisoning.

Other Soluble Substances

Haemagglutinins, neuraminidase, fibrinolysin, haemolysin and histamine are produced.

F. Pathogenesis

Cl. perfringens produces the following human infections:

1. Gas Gangrene

Cl. perfringens type A is the predominant bacterial agent causing gas gangrene. When a wound gets contaminated by faecal matter or soil, it may lead to 'simple wound contamination', anaerobic cellulitis or myonecrosis (gas gangrene proper). It is only when muscle tissues are invaded that gas gangrene results. The incubation period varies from six hours to six weeks.

2. Food Poisoning

Some strains of type A produce food poisoning. Incubation period varies from 8 to 12 hours. A heat labile enterotoxin is liberated in the small intestine after ingestion of a contaminated food, usually cooked meat and poultry. The toxin acts like enterotoxin of *V. cholerae* and leads to fluid accumulation in the rabbit ileal loop.

3. Necrotising Enteritis

A severe and fatal necrotising jejunitis (enteritis necroticans) is caused by type C strains of *Cl. perfringens*. Active immunistation with *Cl. perfringens* type C toxoid offers protection against this condition.

4. Other Diseases

These include gangrenous appendicitis, urogenital infections, brain abscess, meningitis, panophthalmitis and puerperal infection.

G. Laboratory Diagnosis

The diagnosis of gas gangrene must be made primarily on clinical grounds and the laboratory only confirms the clinical diagnosis. The specimens to be collected are exudates from wound, necrotic tissue and muscle fragments.

1. Direct Microscopy

Gram stained smears give presumptive diagnosis. Large number of Gram positive bacilli without spores is strongly suggestive of *Cl. perfringens*. 'Citron bodies' and boat or leaf shaped pleomorphic bacilli with irregular staining suggest *Cl. septicum* and large bacilli with oval, subterminal spores indicate *Cl. novyi*. In case of anaerobic streptococcal myositis, which may be indistinguishable from gas gangrene clinically in the early stages, Gram stained smears shows large number of streptococci and pus cells, but no bacilli.

2. Culture

The specimens are inoculated on fresh and heated blood agar and cooked meat broth (CMB). Growth in CMB is subcultured on blood agar plates after 24–48 hours. The blood agar is incubated anaerobically for 48–72 hours. Most strains produce beta haemolysis on blood agar and few are non-haemolytic. A plate of serum or egg yolk agar is used for Nagler reaction. The bacterial isolates are identified by morphology, cultural characteristics, biochemical reactions and reverse CAMP test. Toxigenicity of the strain can be done by animal pathogenicity.

3. Animal Pathogenicity

0.1 ml of 24 hours growth in cooked meat broth is injected into a healthy guinea pig by intramuscular route. The animal dies within 24 hours. A control animal protected with antiserum prior to test is also included. On autopsy, bacteria can be recovered from heart and spleen of the test animal.

Laboratory diagnosis of food poisoning is made by isolating heat resistant *Cl. perfringens* Type A from the faeces and food. Laboratory methods are same but selective media are used for direct plating. CMB broth is inoculated and heated at 100°C for 30 minutes. After cooling, it is incubated at 37°C for 18 hours and subcultured on selective medium which is then incubated anaerobically at 37°C for 18 hours. The bacterial isolates are identified by morphology, cultural characteristics, biochemical reactions and Nagler reaction. As *Cl. perfringens* may be present in normal intestines, isolation from faeces, except in large numbers is not meaningful.

H. Prophylaxis

1. *Surgery*: As a prophylatic measure, all damaged tissue should be removed promptly and the wound is irrigated with antiseptic solution to remove blood clots, necrotic tissue and foreign materials. In established gas gangrene, uncompromising excision of all affected tissue may save life.
2. *Antibiotics*: Gas gangrene organisms are susceptible to metronidazole, penicillin, sulphonamide, tetracycline and amoxycillin.
3. *Antitoxin*: Passive immunisation with *anti-gas gangrene serum* is used prophylactically in cases with extensively soiled wounds. Precautions must be taken to guard against hypersensitivity to horse serum.
4. *Hyperbaric oxygen*: Hyperbaric oxygen is introduced in the depth of wound to reduce anaerobiosis.
5. *Active immunisation*: Toxoids have been found, experimentally, to induce antitoxic response, but it has not come into practical use.

IV. CLOSTRIDIUM TETANI

Cl. tetani is the causative agent of tetanus, a disease, which has been known since very early times. *Cl. tetani* is widely distributed in soil and intestine of man and animals. It is ubiquitous.

A. Morphology

It is a Gram positive, slender bacillus (measuring 4–8 μm × 0.5 μm) with spherical, terminal spores giving the bacillus the characteristic *drumstick* appearance (Fig. 29.4). It is non-capsulated and motile (except *Cl. tetani* type VI) with peritrichate flagella. The young cultures are stongly Gram positive but older cultures may show variable staining and may even be Gram negative.

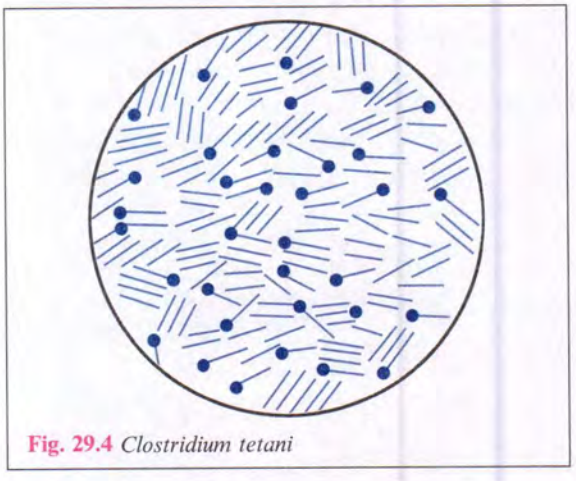


Fig. 29.4 *Clostridium tetani*

B. Culture

It is an obligate anaerobe which grows on ordinary media. Growth is improved by the addition of blood or serum. The optimum temperature for growth is 37°C and pH 7.4. It can grow well in cooked meat broth (CMB), thioglycollate broth, nutrient agar and blood agar. In CMB, growth occurs as turbidity and there is also some gas formation. The meat is not digested but becomes black on prolonged incubation. The bacilli

produce a swarming (thin spreading film) growth on blood agar. However, on horse blood agar, they produce α-haemolytic colonies which subsequently develop into β-haemolytic, due to the production of a haemolysin (tetanolysin).

C. Biochemical Reactions

Cl. tetani has slight proteolytic, but no saccharolytic property. Gelatin liquefaction occurs very slowly. It does not ferment any sugar. It forms indole but is MR and VP negative. H_2S is not formed. Nitrates are not reduced. A greenish fluorescence is produced on media containing neutral red (e.g. MacConkey's medium).

D. Resistance

The resistance of *Cl. tetani* spores to heat vary in different strains. Most of the strains are killed by boiling for 10–15 minutes but some resist boiling for three hours. However, autoclaving (at 121°C for 20 minutes) kills the spores of most strains. Spores are also killed with Iodine (1% aqueous solution), hydrogen peroxide (10 volumes) and glutaraldehyde (2%) within a few hours. The spores may survive in soil for years.

E. Classification

Ten serological types (types I to X) of *Cl. tetani* have been recognised based on type specific flagellar (H) antigens by agglutination test. Type VI strains are non-flagellated. All the types produce the same toxin, which can be neutralised by antitoxin produced against any one type.

F. Toxins

Cl. tetani produces two distinct toxins—*tetanolysin* (haemolysin) and *tetanospasmin* (neurotoxin).

1. Tetanolysin

Tetanolysin is a heat labile, oxygen labile toxin which causes lysis of erythrocytes of several animal species, especially the rabbit and the horse. It may act as a leucotoxin but its pathogenic role is not clear.

2. Tetanospasmin

Tetanospasmin is a heat labile, oxygen stable, powerful neurotoxin and rapidly gets destroyed by proteolytic enzymes. It is protein in nature. It is plasmid coded. This is responsible for clinical manifestations of tetanus. On its release from the bacillus, it is autolysed to form a heterodimer consisting of a large polypeptide chain (93,000 MW) and a smaller polypeptide chain (52,000 MW) joined by a disulphide bond. It is lethal to mice and guinea pigs in minute doses (0.0000001 mg for mouse). Tetanospasmin is a good antigen and is specifically neutralised by the antitoxin.

G. Pathogenesis

Cl. tetani has little invasive power. Tetanus develops following the contamination of wound with *Cl. tetani* spores. The source of infection may be soil, dust, faeces etc. Infection strictly remains localized in the wound. Germination of spores and toxin production occur only if favourable conditions exist, such as reduced O-R potential, devatilised tissues and foreign bodies. Pathogenic effects are mainly due to tetanospasmin (neurotoxin) of *Cl. tetani*. The tetanus toxin specifically blocks synaptic inhibition in the spinal cord. The toxin acts presynaptically. The abolition of spinal inhibition leads to uncontrolled spread of impulses initiated anywhere in the central nervous system. This results in muscle rigidity and spasms due to the simultaneous contraction of agonists and antagonists.

H. Laboratory Diagnosis

The diagnosis of tetanus should always be made clinically and laboratory tests are done only to confirm it. Laboratory diagnosis may be made by demonstration of bacilli by microscopy, culture or by animal inoculation. Specimens generally collected are wound swab, exudate or tissue from the wound.

1. Microscopy

Gram staining may show Gram positive bacilli with drumstick appearance but these are indistinguishable from morphologically similar non-pathogenic bacilli like *Cl. tetanomorphum* and *Cl. sphenoides*. Hence microscopy alone is unreliable but diagnosis by culture is more dependable.

2. Culture

Specimen is inoculated on freshly prepared blood agar and incubated at 37°C for 24–48 hours under anaerobic conditions. The incorporation of polymyxin B in culture medium makes it more selective as clostridia are resistant to this antibiotic. *Cl. tetani* produces a swarming growth. The specimen is also inoculated in three tubes of cooked meat broth. One of these tubes is heated at 80°C for 15 minutes, the second tube for five minutes and the third left unheated. Heating for different periods is to kill vegetative bacteria, while leaving tetanus spores undamaged. These cooked meat broths are incubated at 37°C and subcultured on blood agar plates daily for upto four days.

Gram stained smear from culture shows typical Gram positive bacilli with drumstick appearance.

3. Toxigenicity Test

Pathogenicity of the isolated organism is established with demonstration of toxin production. It is best tested in animals. 0.2 ml of 2 to 4 days old cooked meat culture is injected into the root of the tail of one mouse (test animal), same amount is injected into another animal (control) that has received tetanus antitoxin (1000 units) an hour earlier.

In positive case, the test animal develops symptoms within 12–24 hours, beginning with stiffness of the tail. Rigidity proceeds to the leg on the inoculated side, the another leg, trunk and forelimbs, in that particular order. Death occurs within two days, but may be killed earlier as the ascending tetanus is very much diagnostic. The control animal does not show any change due to neutralisation of toxin by antitoxin.

I. Prophylaxis

The available methods are:

1. Surgical
2. Antibiotics
3. Immunisation

(1) Surgical

It aims at removal of foreign body, blood clots etc., in order to prevent anaerobic conditions favourable for the bacillus. Depending on the type of wound, surgical prophylaxis may vary from simple cleansing to radical excision.

(2) Antibiotics

Antibiotics destroy or inhibit tetanus bacilli and other pyogenic bacteria in wounds and thus the production of toxin is prevented. Long acting penicillin injection or erythromycin may be given. Antibiotic prophylaxis does not replace immunisation but is a useful adjunct.

(3) Immunisation

Tetanus is a preventable disease.

(a) Active immunisation

It is the most effective method of prophylaxis. *Tetanus toxoid* (formol toxoid), which is available either as 'plain toxoid', or adsorbed on aluminium hydroxide or phosphate (APT), is commonly used for active immunisation. Three doses of 0.5 ml tetanus toxoid (APT) each are given intramuscularly, with an interval of 4 to 6 weeks between first two doses and 6–12 months between the second and third dose. A full course of three doses confers immunity for a period of at least 10 years. A 'booster dose' of toxoid is recommended after 10 years.

Tetanus toxoid is given along with diphtheria toxoid and pertussis vaccine (DPT) in children. Pertussis vaccine acts as adjuvant. Three doses are given intramuscularly at interval of 4–6 weeks, starting at age as early as 6 weeks. Booster doses are given at age of 18 months and then at five years.

(b) Passive immunisation

Antitetanus serum (ATS), prepared by immunising horses with toxoid, has been used for preventing tetanus. The dose is 1500 IU by intramuscular route immediately after the person is wounded.The dose is same for adults and children. Being a horse serum, it carries the risk of hypersensitivity reaction, therefore, a skin test (subcutaneous injection of 0.5 ml ATS) is recommended before administersing ATS.

Homologous serum prepared from humans, human antitetanus immunoglobulins (HTIG), is now being used without the risk of hypersensitivity. Dose of 250 units is used in prophylaxis.

(c) Combined prophylaxis

In non-immune person, it is ideal to immunise with first dose of tetanus toxoid in one site along with administration of ATS or HTIG in another arm, followed by the second and third doses of tetanus toxoid at monthly interval. In combined prophylaxis, adsorbed toxoid should be used as the immune response to plain toxoid may be interfered with antitetanus serum.

J. Treatment

Tetanus patients are treated in special isolated units. The reason for isolation is to protect them from noise and light which may provoke convulsions. These patients are not infectious and person to person transmission does not occur at all.

Treatment consists of controlling spasms, maintaining airway by tracheostomy and attention to feeding. The antitoxin may be used to neutralise unbound toxin. Antibiotic therapy with penicillin or metronidazole should be started and continued for a week or more. Patients recovering from tetanus should be immunised with full course of toxoid, as an attack of the disease does not confer immunity.

V. CLOSTRIDIUM BOTULINUM

Clostridium botulinum causes a severe form of food poisoning named botulism. The term botulism means sausage (*botulus*, Latin for sausage) as poorly cooked sausages were formerly associated with this type of food

poisoning. It is a widely distributed saprophyte and is found in soil, animal manure, vegetables and sea mud.

A. Morphology

It is a Gram positive, non-capsulated bacillus about 5 μm × 1 μm, motile by peritrichate flagella and produces subterminal, oval, bulging spores (Fig. 29.5).

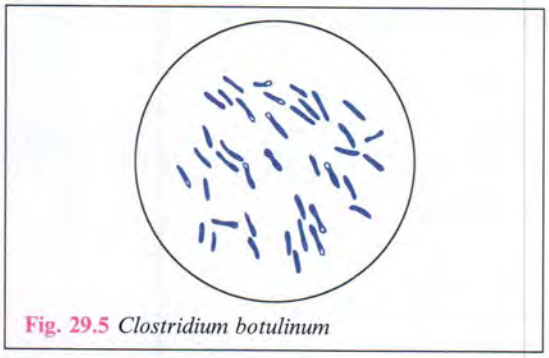


Fig. 29.5 *Clostridium botulinum*

B. Culture

It is a strict anaerobe and can grow on ordinary media. Optimum temperature is 35°C but some strains may grow at 1-5°C. Commonly used media are blood agar and cooked meat broth. Colonies are large, irregular, semitransparent, with fimbriate border. On blood agar, haemolysis around the colonies is observed.

C. Resistance

The spores are highly resistant, and can withstand heat for several hours at 100°C and for upto 10 minutes at 120°C, whereas spores of types B, E and F are less resistant to heat. They are also resistant to radiation.

D. Classification

Eight types—A, B, C1, C2, D, E, F and G have been identified on the basis of their antigenically distinct toxins. The toxins of different types are identical in their pharmacological action, but are neutralised only by the homologous antiserum.

E. Toxin

Cl. botulinum forms a powerful exotoxin. The toxin differs from other exotoxins in that it is not released during the life of the bacterium. It is produced intracellularly and appears in the medium on autolysis of the cell. It is believed to be synthesised as a non-toxic protoxin or progenitor toxin. Progenitor toxin is converted to active toxin by trypsin and other proteolytic enzymes. The toxin has been isolated as a pure crystalline protein with a M.W. 70,000. It has a lethal dose for mice 0.000,000,033 mg and a lethal dose for humans is probably one microgram. It is a neurotoxin and acts slowly, therefore, takes several hours to kill. Type A toxin is the most potent. Botulinum toxin is the most toxic substance known so far.

The toxin is relatively stable, being inactivated only after 30–40 minutes at 80°C and 10 minutes at 100°C. Preformed toxin in food is destroyed by boiling for 20 minutes. It is a good antigen and can be neutralised specifically by its antitoxin.

The toxin acts by blocking the production or release of acetylcholine at synapses and neuromuscular junctions. Death occurs due to respiratory failure.

The toxin production is probably determined by the presence of bacteriophage.

F. Pathogenesis

Cl. botulinum is non-invasive and its pathogenicity is due to the action of preformed toxin, the manifestations of which are collectively called botulism. Botulism is of three types—foodborne, infant and wound botulism.

1. Foodborne Botulism

It is due to preformed toxin in food contaminated with *Cl. botulinum*. Human disease is usually caused by types A, B, E and very rarely by types C, F and G. The source of botulism is usually various preserved foods—meat, fish, vegetables etc. The contaminated food usually exhibits signs of spoilage, and cans may be inflated and show bubbles on opening. Often the food may look normal and there is no alteration in taste.

The symptoms appear 12–36 hours after ingestion of contaminated food. Vomiting, thirst, constipation, ocular paresis, difficulty in swallowing, speaking and breathing are the common symptoms. Diarrhoea is not a symptom. Fatality rate is 25–70% and death results from respiratory failure.

2. Infant Botulism

It affects infants, usually below 6 months. It is a disease due to ingestion of food contaminated by spores of *Cl. botulinum*. Honey has been incriminated as a source of infection. It is a toxico-infection. The disease is characterised by constipation, weakness, lethargy and cranial palsies. Infants exhibit flaccid paralysis, usually with a weak sucking response, generalised loss of tone ("the floppy baby syndrome") and respiratory complications. Spores and toxin are excreted in the faeces of the patient. Management is by supportive care and assisted feeding. Severity of illness varies from mild illness to fatal disease.

3. Wound Botulism

It is a very rare condition which results from wound infection with *Cl. botulinum*. Toxin produced is absorbed

into the blood and the symptoms similar to foodborne botulism occur except for the gastrointestinal components which are absent. Type A has been recovered from most of the cases studied.

G. Laboratory Diagnosis

Diagnosis may be confirmed by demonstration of the bacillus or the toxin in suspected residual food or in faeces.

1. Demonstration of the Organism

Smears made from suspected food or faeces are examined by Gram's staining which may show Gram positive sporing bacilli. Culture is done on blood agar or cooked meat broth. Growth on culture media may be confirmed by Gram's staining. Presence of bacilli in food or faeces in absence of toxin is of no significance. Hence, toxin in culture fluid must be demonstrated by toxigenicity test in mice.

2. Demonstration of Toxin

Specimens (stool, food and vomitus) are macerated in sterile saline and the filtered extract is divided into three parts. One portion of extract is heated at 100°C for 10 minutes and other two kept unheated. Two mice or guinea pigs are injected with unheated filtrate; one of them (control) is protected with polyvalent botulinum antitoxin prior to injection. The third animal is injected with heated filtrate. The test animal (unprotected) develops dyspnoea, flaccid paralysis and dies within 24 hours whereas control animal (protected) remains healthy. The animal injected with heated filtrate also remains free of any toxic symptoms.

Typing of *Cl. botulinum* is done by using a series of animals protected with type specific antitoxins instead of polyvalent antitoxin.

H. Prophylaxis and Treatment

As botulism follows consumption of canned or preserved food, control may be achieved by proper canning and preservation. By the time symptoms appear, the toxin is fixed to nervous tissue and it can no longer be inactivated by antitoxin. Intensive supportive therapy may bring down the mortality rate. However, if outbreak occurs, prophylactic dose of antitoxin should be given intramuscularly to all who have consumed the suspected food. Antitoxin may be tried for treatment. Polyvalent antiserum to types A, B and E may be given. Supportive therapy with maintenance of respiration is also very important.

If immunisation is needed, as in laboratory workers exposed to the risk, two injections of toxoid may be administered at an interval of ten weeks, followed by a booster dose a year later.

VI. CLOSTRIDIUM DIFFICILE

Cl. difficile was first isolated from the faeces of newborn infants. It was so named due to unusual difficulties involved in its isolation.

A. Morphology

It is a long, slender, Gram positive bacillus containing oval and terminal spores. There is a tendency to lose its Gram reaction.

B. Toxins

It produces two toxins, an *enterotoxin* (toxin A) and a *cytotoxin* (toxin B). The enterotoxin is primarily responsible for diarrhoea whereas cytotoxin is capable of producing cytopathogenic effects in several tissue culture cell lines.

C. Pathogenesis

The pathogenic role of *Cl. difficile* was established in 1977, when it was found to be responsible for antibiotic associated colitis. Acute colitis with or without membrane formation is an important complication in patients on oral antibiotic therapy. Many antibiotics have been incriminated but lincomycin and clindamycin are particularly prone to cause pseudomembranous colitis. Active multiplication of *Cl. difficile* and the production by it of an enterotoxin as well as a cytotoxin is responsible for antibiotic associated colitis. If the condition is not recognised early and properly treated, it terminates fatally.

D. Laboratory Diagnosis

1. Demonstration of Toxin

Toxin can be demonstrated in the faeces by its characteristic effect on human diploid cells and HEp–2. ELISA can also be used for the demonstration of toxin. The toxin is specifically neutralised by the *Cl. sordelli* antitoxin.

2. Nucleic Acid Amplification Tests

Nucleic acid amplification tests (NAATs) which can determine the presence of toxins A and B genes in faeces are becoming the new gold standard for the detection of toxin producing *C. difficile*.

3. Isolation of Bacilli

Cl. difficile can be grown from the faeces of patients with subsequent test for toxigenicity. Cycloserine-

cefoxitin-fructose agar (CCFA) is a selective medium to primarily isolate *Clostridium difficile* from faeces of patients suspected of antibiotic associated colitis or pseudomembranous colitis. *C. difficile* colonies appear yellow due to fructose fermentation.

E. Treatment

Cl. difficile are generally resistant to most antibiotics, but these are susceptible to vancomycin. Clindamycin and lincomycin should be avoided. Metronidazole is the drug of choice.

KEY POINTS

1. The genus *Clostridium* consists of *anaerobic, spore forming, Gram positive bacilli.*
2. This genus contains bacteria that cause three major diseases of man—*tetanus, gas gangrene* and *food poisoning.*
3. Clostridial pathogenicity is mainly due to production of a powerful exotoxin.
4. The shape and position of spores varies in different species and is useful in identification of clostridia, spores may be *subterminal* in *Cl. perfringens* and *drumstick* appearance in *Cl. tetani.*
5. Clostridia grow well on blood agar medium under *anaerobic* conditions. Liquid media like *cooked meat broth* (*CMB*) is very useful for growing clostridia.
6. *Nagler reaction* and *reverse CAMP test* are useful in identification *Cl. perfringens*, a causative agent of gas gangrene and food poisoning.
7. *Cl. tetani* produces two distinct toxins—*tetanolysin* (haemolysin) and *tetanospasmin* (neurotoxin). It is the causative agent of *tetanus.*
8. *Tetanus toxoid* is used for prophylaxis of tetanus.
9. *Cl. botulinum* forms a powerful exotoxin which is responsible for the disease *botulism.*
10. *Cl. difficile* has been found to be responsible for antibiotic associated colitis.
11. As clostridia are toxin producing bacteria, antitoxins are used for passive immunisation in the diseases associated with them. *Anti-gas gangrene* and *human antitetanus immunoglobulins* (*HTIG*) are two examples of antitoxins used in gas gangrene and tetanus respectively.

YOU MUST KNOW

1. Diseases produced by different Clostridia species.
2. Morphology, culture and biochemical reactions of *Clostridium perfringens.*
3. Nagler reaction.
4. Laboratory diagnosis of gas gangrene.
5. Morphology, culture characteristics and biochemical reactions of *Cl. tetani.*
6. Toxins produced by *Cl. tetani.*
7. Laboratory diagnosis of tetanus.
8. Immunisation against tetanus.
9. Morphology and culture characteristics of *Cl. botulinum.*
10. *Cl. botulinum* toxin.
11. Laboratory diagnosis of botulism.
12. *Clostridium difficile.*

STUDY QUESTIONS

1. Classify clostridia. Discuss the laboratory diagnosis of gas gangrene.
2. Write short notes on:
   (a) Nagler reaction
   (b) Laboratory diagnosis of tetanus
   (c) Tetanospasmin
   (d) Prophylaxis against tetanus

(e) Stormy clot reaction
(f) Alpha toxin
(g) *Clostridium botulinum* exotoxins
(h) Laboratory diagnosis of botulism
(i) *Clostridium difficile.*

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Which of the following species of *Clostridium* is non-motile?
   (a) *Clostridium perfringens* (b) *Clostridium botulinum* (c) *Clostridium novyi* (d) None of the above
2. Which of the following species of *Clostridium* is capsulated?
   (a) *Clostridium perfringens* (b) *Clostridium botulinum* (c) *Clostridium novyi* (d) None of the above
3. All of the following species of *Clostridium* may cause gas gangrene except:
   (a) *Clostridium perfringens* (b) *Cl. novyi* (c) *Cl. septicum* (d) *Cl. difficile*
4. Following tests may be helpful in identification of *Clostridium perfringens*?
   (a) Nagler reaction (b) Reverse CAMP test (c) Both of the above (d) None of the above
5. *Clostridium perfringens* may produce the following infections:
   (a) Gas gangrene (b) Food poisoning (c) Necrotising enteritis (d) All of the above
6. Drumstick appearance of spores is a characteristic feature of:
   (a) *Clostrdium perfringens* (b) *Cl. tetani* (c) *Cl. septicum* (d) *Cl. novyi*
7. Which type of *Clostridium perfringens* strains is responsible for food poisoning?
   (a) Type A (b) Type B (c) Type C (d) Type E
8. Stormy fermentation is useful in identification of:
   (a) *Clostridium perfringens* (b) *Cl. tetani* (c) *Cl. botulinum* (d) *Cl. difficile*
9. Which of the following species of Clostridium shows swarming on blood agar?
   (a) *Clostridium perfringens* (b) *Cl. novyi* (c) *Cl. tetani* (d) *Cl. difficile*
10. Which of the following toxins is the most toxic?
    (a) Botulinum toxin (b) Tetanus toxin (c) Diphtheria toxin (d) Cholera toxin
11. Floppy baby syndrome is associated with:
    (a) *Clostridium botulinum* infection (b) *Cl. perfringens* infection
    (c) *Cl. difficile* infection (d) *Cl. novyi* infection
12. Which of the following bacteria is responsible for pseudomembranous enterocolitis?
    (a) *Clostridium perfringens* (b) *Cl. tetani* (c) *Cl. botulinum* (d) *Cl. difficile*
13. Which of the following types of toxins is/are most commonly associated with foodborne botulism in man?
    (a) Type A (b) Type B (c) Type E (d) All of the above
14. Botulism is due to
    (a) Preformed toxin of *Clostridium botulinum* (b) Invasion of *Cl. botulinum* in the intestine
    (c) Both of the above (d) None of the above
15. Selective medium used for isolation of *Clostridium difficile* from faeces is:
    (a) Cycloserine-cefoxitin-fructose agar (b) Monsur's gelatin taurocholate trypticase tellurite agar
    (c) Cefsulodin-irgasan-novobiocin agar (d) All of the above

Answers (MCQs):

1. (a)	2. (a)	3. (d)	4. (c)	5. (d)
6. (b)	7. (a)	8. (a)	9. (c)	10. (a)
11. (a)	12. (d)	13. (d)	14. (a)	15. (a)

Chapter 30

NONSPORING ANAEROBES

I. INTRODUCTION

Non-sporing anaerobes have been recognised as an important cause of human and animal infections. Many of these bacteria form commensal flora of man and animals in mouth, oropharynx, gastro-intestinal tract and genital tracts. In the gut the anaerobic bacteria outnumber the aerobes (*Escherichia coli*) by a ratio of 1000:1. Even in such seemingly aerobic situations as the mouth and the skin, anaerobic bacteria are more frequent than aerobes (ratio may be 10:1 to 30:1). The number of anaerobes have been estimated to be 10^4 to 10^5 per ml in the small intestine, 10^8 per ml in saliva and 10^{11} per gm in the colon. Some of these anaerobic bacteria act as opportunistic pathogens when body resistance is lowered. These non-sporing anaerobes are particularly likely to set up infections where there is damaged and necrotic tissue. Anaerobic infections of the head, neck and respiratory tract are often associated with commensals in the mouth, while infection in the abdominal and pelvic regions are more commonly associated with those of gut.

On the basis of morphology and staining characters, they have been classified as given in Table 30.1.

Spirochaetes are described separately in Chapter 45.

II. ANAEROBIC COCCI

The anaerobic cocci occur as normal flora of skin, mouth, intestine and vagina. Recently, changes have been made in classifying anaerobic cocci but there is no change in

Table 30.1 Classification of Nonsporing Anaerobes

A. Cocci	
1. Gram positive cocci	(*i*) *Peptococcus* (*ii*) *Peptostreptococcus* (*iii*) *Parvimonas* (*iv*) *Peptoniphilus* (*v*) *Anaerococcus* (*vi*) *Finegoldia* (*vii*) *Gallicola* (*viii*) *Atopobium* (*ix*) *Blantia* (*x*) *Ruminococcus*
2. Gram negative cocci	(*i*) *Veillonella* (*ii*) *Acidaminococcus* (*iii*) *Megasphaera* (*iv*) *Negativococcus* (*v*) *Anaeroglobus*
B. Bacilli	
1. Gram positive bacilli	(*i*) *Eubacterium* (*ii*) *Lactobacillus* (*iii*) *Bifidobacterium* (*iv*) *Propionibacterium* (*v*) *Actinomyces* (*vi*) *Mobiluncus*
2. Gram negative bacilli	(*i*) *Bacteroides* (*ii*) *Prevotella* (*iii*) *Porphyromonas* (*iv*) *Fusobacterium* (*v*) *Leptotrichia*
C. Spirochaetes	
	(*i*) *Treponema* (*ii*) *Borrelia*

genus *Peptococcus*. Genus *Peptostreptococcus* contains only two species *Pst. anaerobius* and *Pst. stomatis*. Other species of *Peptostreptococcus* are reclassified in different genera such as *Peptoniphilus*, *Anaerococcus*, *Parvimonas*, *Finegoldia*, *Gallicola* and *Slackia*.

A. Peptococcus

Peptococci are Gram positive, non-sporing, anaerobic cocci that do not form chains. They are small spherical measuring 0.5–1 μm in size, arranged singly or in pairs or in clumps. They are coagulase negative. They may cause pyogenic infections of wounds, puerperal sepsis and urinary tract infection. This genus contains only one species i.e., *P. niger*.

B. Peptostreptococcus and Others

Peptostreptococci are small Gram positive cocci, 0.3 to 1 μm in size, arranged in chains. Carbohydrates are fermented with the production of acid, gas or both. They are the commonest anaerobic cocci isolated from human infections such as pleuropulmonary disease, brain abscess and puerperal infections. *Peptostreptococcus anaerobius* is most often responsible for puerperal sepsis. *Finegoldia magna* (formerly *Pst. magnus*) is commonly isolated anerobic cocci from wound infections. *Peptoniphilus asaccharolyticus* (formerly *Pst. asaccharolyticus*), *Anaerococcus prevoti* (formerly *Pst. prevoti*) and *Anaerococcus tetradius* (formerly *Pst. tetradius*) are some other anaerobic cocci commonly found in clinical specimens.

Anaerobic cocci are present in large number in pus from suppurative lesions, so a Gram stained smear may be helpful in diagnosis. Infections are usually mixed, the anerobic cocci being present along with clostridia or other anaerobic Gram negative bacilli.

C. Veillonella and Others

Veillonellae are small (0.3–2.5 μm), Gram negative cocci, occurring in short chains, pairs or clumps. They are usually non-pathogenic but occasionally invade blood stream. Other important genera of anaerobic Gram negative cocci include *Acidaminococcus* and *Megasphaera*.

III. ANAEROBIC GRAM POSITIVE BACILLI

A. Eubacterium

Eubacterium species are strictly anaerobic and grow very slowly. They are members of the normal mouth and intestinal flora. Some species (*E..brachy*, *E..nodatum*) are commonly seen in periodontitis.

B. Lactobacillus

Lactobacilli are Gram positive bacilli that frequently show bipolar and barred staining. Most strains are non-motile. They form considerable amount of lactic acid from carbohydrates and grow best at pH of 5 or less.

Lactobacilli are normally present in the mouth, gut and vagina. They are widely distributed as saprophytes and ferment material such as milk and cheese. Several species of lactobacilli are present in the intestine, the commonest being *L. acidophilus*, they synthesise vitamins such as biotin, vitamin B_{12} and vitamin K, which may be absorbed by the host. In the mouth lactobacilli have been incriminated in the pathogenesis of dental caries. It is believed that the mineral components of enamel and dentine are dissolved by acid formed by the fermentation of sucrose and other dietary carbohydrates by lactobacilli.

Lactobacilli of several species occur in the vagina and these are collectively known as Doderlein's bacilli. They ferment the glycogen deposited in the vaginal epithelial cell and form lactic acid, which accounts for the highly

acidic pH of the vagina. They protect adult vagina from infections. In prepubertal and postmenopausal vagina, lactobacilli are scanty.

They grow best in media enriched with glucose or blood in presence of 5% CO_2 and at pH 6. Lactobacilli are generally non-pathogenic, however, they can be involved in serious infections, especially in immunocompromised individuals. Lactobacilli are particularly associated with advanced dental caries.

C. Bifidobacterium

Bifidobacteria are Gram positive, non-sporing, pleomorphic, non-motile bacilli showing true and false branching. The name is derived from frequent bifid Y-shaped cells. They occur as normal flora in the mouth and gastrointestinal tract and are occasionally pathogenic. Dental caries is the most common clinical entity in which *B. dentium* may have a pathogenic role.

D. Propionibacterium

Propionibacteria are Gram positive, pleomorphic, non-motile bacilli. They are related to corynebacteria and are usually labelled as *anaerobic diphtheroids*. The commonest species isolated in the clinical laboratory is *P. acnes* (*Corynebacterium acnes*). This is commensal found abundantly on the normal skin. It is regularly found in acne and was thought to be its causative agent. It has also been isolated from abscesses, in some cases of infective endocarditis and in infections associated with implanted prosthesis. Pathogenic role of *Propionibacteria* is not definite.

E. Actinomyces

Genus *Actinomyces* is described in Chapter 47.

F. Mobiluncus

Mobiluncus species are motile, curved, anaerobic bacilli. They may appear as Gram variable. They have been isolated from the vagina in bacterial vaginosis, along with *Gardnerella vaginalis*. Mobiluncus is described in Chapter 35.

IV. ANAEROBIC GRAM NEGATIVE BACILLI

Anaerobic Gram-negative, non-spore forming bacilli of clinical relevance belong to families *Bacteroidaceae*, *Porphyromonadaceae*, *Prevotellaceae*, *Rikenellaceae* and *Fusobacteriaceae*. Important genera include *Bacteroides*, *Porphyromonas*, *Prevotella*, *Fusobacterium* and *Leptotrichia*. Besides these genera many new genera have also been included in these families.

They were grouped previously in the Family *Bacteroidaceae* and classified into the following genera :

A. Bacteroides (bacilli with rounded ends)

1. Fragilis group
   B. fragilis, *B. vulgatus*, *B. distasonis*, *B. ovatus*, *B. thetaiotaomicron* (*Sacchrolytic*)
2. Melaninogenicus group
   B. melaninogenicus, B. oralis (*Moderately sacchrolytic*)
3. Asacchrolytic group
   B. asacchrolyticus, B. gingivalis

The new genus names for melaninogenicus group is *Prevotella* and for asacchrolytic group is *Porphyromonas.*

B. Fusobacterium (bacilli with pointed ends)

Species are *F. necrophorum*, *F. nucleatum*, *F. necrogenes*

C. Leptotrichia (large bacilli)

L. buccalis is the only species.

A. Bacteroides

Bacteroides occur as commensals in the mouth, gastrointestinal and female genital tracts. Normal stools contain 10^{11} organisms per gram. The most common are *B. fragilis* group isolated from the large intestine. *P. melaninogenica* is isolated particularly from the oropharynx, gut and vagina. These are Gram negative, strict anaerobes, non-sporing, non-motile bacilli and are very pleomorphic. They require enriched media containing blood or haemin for growth. They grow readily in media such as brain-heart infusion agar in atmosphere containing 10% carbon dioxide.

They cause peritonitis following bowel injury and pelvic inflammatory disease (PID). They are also found in abdominal and brain abscesses and in empyema. Pus is often foul smelling. *B. fragilis* is probably the most frequent among non-sporing anaerobes isolated from clinical specimens. *P. melaninogenica* is recognised easily because of black or brown coloured colonies. The colour is due to a haemin derivative and not by a melanin pigment, as was once thought. Cultures of *P. melaninogenica* give a characteristic red fluorescence when exposed to ultraviolet light. It has been isolated from lung or liver abscess, mastoiditis, lesions of intestine, mouth and gums. Anaerobic infections are generally mixed infections containing other organisms that are often part of the normal flora.

Porphyromonas gingivalis is responsible for periodontal disease while *P. endodontalis* causes dental root canal infections.

B. Fusobacterium

Fusobacteria are Gram negative bacilli which are strict anaerobes. Some species of *Fusobacterium* produce long slender rods that are wide at the centre and taper towards the ends (fusiform). They are usually non-motile. They can be grown on blood agar containing neomycin and vancomycin.

Fusobacterium species are commensals in the mouth and also cause infections of mouth and related sites. *F. nucleatum* is the most common species. It may cause infections of head and neck, dental and periodontal infections and cerebral abscess. Some of *Fusobacterium* species are commensals in the gastrointestinal and genitourinary tracts. These species along with *F. nucleatum* may cause intraabdominal infections, osteomyelitis and various soft tissue infections.

C. Leptotrichia

Leptotrichia is a long, straight or slightly curved Gram negative, anaerobic, non-sporing, non-motile bacillus. It measures 5–15 μm × 1–1.5 μm in size. The genus has only one species *Leptotrichia buccalis*. It is normally present in mouth and gastrointestinal tract. In association with *Borrelia vincentii* it is believed to cause acute ulcerative gingivitis or Vincent's angina. Diagnosis can be made by direct microscopy. They can also be grown on blood agar at 37°C in presence of 5–10% CO_2 under anaerobic conditions. *Leptotrichia buccalis* was previously named as *Fusobacterium fusiforme*.

V. ANAEROBIC INFECTIONS

Anaerobic infections are usually endogenous and are caused by normal flora of body surfaces. Anaerobic bacteria are normally present on the skin, mouth, nasopharynx, intestines and vagina (Table 30.2). They produce disease when the host's resistance is lowered. Trauma, tissue necrosis, diabetes, malnutrition, malignancy or prolonged treatment with antibiotics may act as predisposing factors. Anaerobic infections are usually polymicrobial, more than one anaerobe is involved along with aerobic organisms. Infections are usually localised but general dissemination may occur by bacteraemia. Pus produced by anaerobes is usually putrid with a nauseating odour. Cellulitis is a common feature of anaerobic wound infections. Toxaemia and fever are not marked. Bacteria responsible for common anaerobic infections are listed in Table 30.3.

Table 30.2 Normal Anaerobic Flora

Bacteria	Skin	Mouth and nasopharynx	Intestine	Vagina
Clostridium			++	
Gram positive cocci		++	++	++
Gram negative cocci		++	+	++
Bifidobacterium		+	++	+
Propionibacterium	++			
Actinomyces		+		
Bacteroides fragilis			++	
P. melaninogenica		++	+	++
Fusobacterium		++	+	
Spirochaetes		+		

VI. LABORATORY DIAGNOSIS

As anaerobic bacteria form part of the normal flora of the skin and mucous surfaces, their isolation from clinical specimens has to be interpreted cautiously. The mere presence of an anaerobe does not prove it as a causative agent.

A. Specimens

Specimens are to be collected in such a manner to avoid normal resident flora. For example, material collected by aspiration in case of lung abscess is acceptable but not the sputum. As these anaerobic bacteria die on exposure to oxygen, care has to be taken to minimise contact of specimen with air during collection, transport and handling of specimens. A satisfactory method of collection is to aspirate the specimen into an airtight syringe. After collection, the needle is plunged into a sterile rubber cork and is sent immediately to the laboratory. Pus and other fluids may be collected in small bottles with air-tight caps and transported immediately. Specimens should fill the bottles completely without leaving any airspace. Swabs are unsatisfactory specimens, but where they are to be used, they should be sent in Stuart's transport medium. Other clinical samples unsuitable for anaerobic culture include sputum, urine, faeces and secretions obtained by nasotracheal suction. Specimens should be inspected for characteristics that strongly indicate the presence of anaerobes, such as foul odour; sulphur granules associated with *Actinomyces* sp., *Propionibacterium* sp. or *Eubacterium*. Other clues suggestive of anaerobic infection include:

1. Failure to isolate organisms from pus (sterile pus)
2. Deep abscesses.
3. Pus which shows red fluorescence under UV light.
4. Failure to respond to conventional antimicrobial treatment.

B. Direct Microscopy

Examination of a Gram stained smear is very useful. Pus in anaerobic infection shows a variety of organisms

Table 30.3 Bacteria Responsible for Common Anaerobic Infections

Site	Type of infection	Bacteria responsible
Central nervous system (C.N.S.)	Brain abscess	*B. fragilis, Peptostreptococcus*
Ear, Nose, Throat (E.N.T.)	Chronic otitis media, mastoiditis, orbital cellulitis	Fusobacteria
Mouth and Jaw	Vincent's angina, dental abscess, cellulitis, abscess and sinus of jaw	Fusobacteria, spirochaetes, Mouth anaerobes, Actinomyces
Respiratory tract	Lung abscess, empyema, bronchiectasis, aspiration pneumonia	Fusobacteria, *P. melaninogenica, Peptostreptococcus, Peptococcus*
Abdominal	Subphrenic abscess, hepatic abscess, appendicitis, peritonitis, ischiorectal abscess, wound infection after colo-rectal surgery.	*B. fragilis*
Female genital tract	Puerperal sepsis, tubo-ovarian abscess, Bartholin's abscess, septic abortion, wound infection following genital surgery	*P. melaninogenica, B. fragilis, Peptostreptococcus, Peptococcus*
Skin and tissue	Infected sebaceous cyst, axillary abscess, breast abscess, cellulitis, diabetic ulcer, gangrene	*Peptococcus, Peptostreptococcus, P. melaninogenica, B. fragilis*

and numerous pus cells. Examination of specimen under ultraviolet light may show the bright red fluorescence in cases of *P. melaninogenica*.

C. Culture

Freshly prepared blood agar with neomycin, yeast extract, haemin and vitamin K is a suitable culture medium. Culture plates are incubated at 37°C in anaerobic jar with 10 per cent CO_2. Anaerobiosis can also be maintained by Gas-Pak system. Plates are examined after 24–48 hours. Parallel aerobic cultures (such as *Pseudomonas aeruginosa*) should always be set up to ensure the proper anaerobiosis inside the anaerobic jar. To verify the anaerobic conditions in the jar, reduced methylene blue indicator is generally used for this purpose. This indicator remains colourless anaerobically but turns blue on exposure to oxygen.

Other anaerobic media, such as cooked meat broth (CMB) and thioglycollate broth, may also be used for incoculating the specimens. For more details about the anaerobic jar, and the CMB, refer to Chapter 6. Specimens should also be put up for growth of aerobic bacteria as in most anaerobic infections aerobic bacteria are also involved.

Definitive identification of the anaerobes depends on colony morphology and various biochemical tests. It takes time and is difficult, but it is possible to report on the following (1) whether the infection is solely aerobic, anaerobic or mixed (2) the identification of the commoner anaerobes, particularly of *B. fragilis* (3) an indication of antimicrobial agents likely to be used. Antibiotic sensitivity tests can be done by disc or dilution methods.

D. Other Anaerobic Techniques

Gloved anaerobic chambers (refer Chapter 6) with continuous gas flow may be used for culture of specimens. Pre-reduced anaerobically sterilised media (PRAS) can also be employed but are not essential for rountine diagnostic procedures. Gas liquid chromatography (GLC) is also useful to identify anaerobes in specimens.

VII. TREATMENT

Besides antibiotic therapy, most anaerobic infections also require surgical intervention. Penicillin is effective against most anaerobes except *B. fragilis*. Tetracycline and chloramphenicol are also used. Clindamycin is effective, but is not commonly used because it is prone to cause pseudomembranous colitis. Metronidazole is the drug of choice. It is also useful as a prophylactic measure in large bowel surgery.

KEY POINTS

1. Non-sporing anaerobes form *commensal flora* of man and animals in mouth, oropharynx, gastrointestinal tract and genital tracts. Some of these anaerobic bacteria act as *opportunistic pathogens* when body resistance is lowered. These are particularly likely to set up infections where there is damaged and necrotic tissue.
2. *Peptococcus*, *Peptostreptococcus* and *Veillonella* are some examples of nonsporing anaerobic *cocci*.

3. *Bacteroides*, *Prevotella*, *Porphyromonas* and *Fusobacterium* are some examples of nonsporing anaerobic *bacilli*.
4. Anaerobic bacteria can be grown on blood agar under anaerobic conditions. *Anaerobic jar* is generally used for producing anaerobiosis. *Cooked meat broth* (CMB) may also be used to grow these bacteria.

YOU MUST KNOW

1. Classification of nonsporing anaerobes.
2. Bacteria responsible for anaerobic infections.
3. Laboratory diagnosis of anaerobic infections.

STUDY QUESTIONS

1. Classify non-sporing anaerobes. Discuss the laboratory diagnosis of infections caused by non-sporing anaerobes.
2. Write short notes on:
   (a) Anaerobic cocci (b) Bacteroides (c) Propionibacterium.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Which of the following bacteria are Gram negative cocci?
   (a) *Peptococcus* (b) *Peptostreptococcus*
   (c) *Veillonella* (d) All of the above
2. Which of the following bacteria protect adult vagina from infections?
   (a) Lactobacilli (b) *Peptococcus*
   (c) *Veillonella* (d) *Bacteroides*
3. Which is the commonest species of *Bacterioides* in fragilis group responsible for human infections?
   (a) *Bacterioides fragilis* (b) *B. distasonis*
   (c) *B. thetaiotamicron* (d) *B. vulgatus*
4. Which of the following bacterial cultures show red fluorescence when exposed to UV light?
   (a) *Bacteriodes fragilis* (b) *Prevotella melaninogenica*
   (c) *Prophyromonas gingivalis* (d) *Fusobacterium nucleatum*

Answers (MCQs):

1. (c) 2. (a) 3. (a) 4. (b)

Chapter 31

ENTEROBACTERIACEAE

Members of the family *Enterobacteriaceae* are aerobic and facultative anaerobic Gram negative enteric bacilli. They are motile by peritrichous flagella or are non-motile. They grow readily on ordinary media, ferment glucose with production of acid or acid and gas, reduce nitrates to nitrites, oxidase negative except. *Pleiomonas sp.* and catalase positive except *Sh. dyseneriae* type 1 which is catalase negative. They are non-capsulated and non-sporing. Their natural habitat is usually the intestinal tract of man and animals. Some species are commensals and some are pathogenic for humans.

I. CLASSIFICATION

The classification of *Enterobacteriaceae* has been controversial. There has been successive changes in the nomenclature. The oldest method was to classify these bacteria on the basis of their action on lactose i.e. lactose fermenters, late lactose fermenters and non-lactose fermenters. The lactose fermenting property is observed on a medium (MacConkey's agar) containing lactose and neutral red. The organisms fermenting lactose form acid and in acidic pH, neutral red (indicator) becomes red in colour, therefore, the bacterial colonies are red or pink and those of non-lactose-fermenting bacteria are pale. The major intestinal pathogens (*Salmonella, Shigella*) are non-lactose fermenters. The majority of the commensal intestinal bacilli are lactose fermenting and the most common member is the 'colon bacillus' or *Escherichia coli*. All lactose fermenting enteric bacilli (*Escherichia, Klebsiella, Enterobacter* and *Citrobacter*) were called *coliform bacilli*. The heterogenous group of late lactose fermenters was called *paracolon bacilli*. Although classification based on lactose fermentation is not acceptable yet this had a practical value in diagnostic bacteriology to differentiate members of enterobacteriaceae.

Three widely used classification of *Enterobacteriaceae* (Bergey's manual; Kauffmann; Edwards-Ewing) have certain differences but the basic approach is the same. The family is first classified into group or tribe. Each tribe consists of one or more genera and each genus has one or more subgenera and species.

Tribe	***Genus***
Escherichiae	*Escherichia, Shigella*
Edwardsielleae	*Edwardsiella*
Salmonelleae	*Salmonella*
Citrobactereae	*Citrobacter*
Klebsielleae	*Klebsiella, Entrobacter, Hafnia, Pantoea, Serratia*
Proteeae	*Proteus, Morganella, Providencia*
Yersinieae	*Yersnia*

The species are further classified into biotypes, serotypes, bacteriophage types and colicin types. The differentiating features of various genera of enterobactriaceae are shown in Table 31.1.

II. ESCHERICHIA COLI

The genus is named after *Escherich* who first isolated the bacillus under the name *Bacterium coli commune* (1885). *Esch. coli* is the type species of the genus *Escherichia*. Unlike other coliforms, *Esch. coli* is a parasite inhabiting only in the human or animal intestine.

A. Morphology

Esch. coli is a Gram negative bacillus measuring 1–3 μm × 0.4–0.7 μm. Most strains are motile by peritrichate flagella. It is non-sporing and non-capsulated.

Table 31.1 Differentiating Features of Important Genera of Enterobacteriaceae

Properties	Escherichia	Edwardsiella	Citrobacter	Salmonella	Shigella	Klebsiella	Enterobacter	Serratia	Proteus	Morganella	Providencia
Gas in glucose	+	+	+	+	–	+	+	V	+	+	V
Acid from lactose	+	–	+	–	–	+	+	–	–	–	–
Motility	+	+	+	+	–	–	+	+	+	+	+
Indole	+	+	V	–	V	–	–	–	V	+	+
Urease	–	–	–	–	–	+	V	–	+	+	V
PPA test	–	–	–	–	–	–	–	–	+	+	+
Citrate	–	–	+	+	–	+	+	+	V	V	V
H_2S	–	+	+	+	–	–	–	–	+	–	–
Lysine decarboxylase	+	+	–	+	–	V	V	+	–	–	–

V, Variable
Important exceptions:
1. *S. typhi* does not produce gas from sugars.
2. *Sh. sonnei* is late lactose fermenter.
3. *S. typhi, S. paratyphi A* and some other species of salmonella are citrate negative.

B. Culture

It is an aerobe and facultative anaerobe and grows on ordinary culture medium at optimum temperature of 37°C (temperature range 10–40°C) in 18–24 hours. Colonies of some strains show beta haemolysis on blood agar. On MacConkey's medium, colonies are pink due to lactose fermentation (LF or lactose fermenter colonies). In general, colonies are circular, moist, smooth with entire margin and non-mucoid unlike colonies of Klebsiella which are mucoid. In liquid medium, growth occurs as uniform turbidity.

C. Biochemical Reactions

They ferment most of the sugars (glucose, lactose, mannitol, maltose) with production of acid and gas. Typical strains do not ferment sucrose. Indole and methyl red (MR) reaction are positive but Voges–Proskauer (VP) and citrate utilisation tests are negative (IMViC ++ – –). Urea is not split, gelatin is not liquified, H_2S is not formed and growth does not occur in KCN medium. The important biochemical reactions are summarised as follows:

Glucose	Lactose	Mannitol	Sucrose	
AG	+	+	–	
Indole	Urease	Citrate	MR	VP
+	–	–	+	–

D. Antigenic Structure

Serotyping of *Esch. coli* is based on the presence of O (somatic antigen), K (capsular antigen) and H (flagellar antigen) antigens detected by agglutination reactions. Another antigen present is F (fimbrial) antigen.

1. Somatic Antigen (O Antigen)

These are heat stable, lipopolysaccharide antigens of cell wall and 173 O antigens have been described which are designated as 1, 2, 3, 4 and so on. For O agglutination, the cultures should be boiled to overcome inagglutinability caused by K antigens. Numerous cross-reactions occur between individual *Esch. coli* O antigens, and between these and O antigens of other genera of family *Enterobacteriaceae* (*Citrobacter*, *Salmonella*, *Shigella* and *Yersinia*). The normal colon strains of *Esch. coli* belong to 'early' O groups (1, 2, 3 etc.) and the enteropathogenic strains belong to the 'latter' group (26, 55, 86, 111 etc.).

2. Capsular Antigen (K Antigen)

This term was used collectively for the surface or capsular antigens that cause O inagglutinability. These 'capsular' structures are not demonstrable by light microscopy. Thus K antigens were previously divided into three classes as L, A and B on the basis of (i) effect of heat on agglutinability (ii) antigenicity (iii) antibody binding power of bacterial strains carrying them. Only one K antigen, L, A or B is present in any one strain.

(*i*) L antigen: It is thermolabile and its capacity to combine with specific antibody is lost.

(*ii*) A antigen: It is thermostable and is usually associated with well marked capsule.

(*iii*) B antigen: It is heat labile but antibody binding power remains unaffected.

In the modern usage the term 'K' antigen refers to the acidic polysaccharide capsular (surface) antigens. 103 K antigens of *Esch. coli* are described. 'K' antigen is now divided into two groups—I and II (Table 31.2). Group I and Group II antigens correspond to the old A and L antigens.

Table 31.2 Differentiating Properties of Group I and II antigens of ***Esch. coli***

Properties	Group I	Group II
Molecular weight	More than 100,000	Less than 50,000
Heat stability (100°C)	Stable	Labile
O groups	O8, O9	Many
Electrophoretic mobility	Low	High
Acidic content	Hexuronic acid, pyruvate	Glucuronic acid, phosphate, KDO NeuNac
KDO	–	Ketodeoxyoctonate
NeuNac	–	N-acetylneuraminic acid

Role of K antigens

(*i*) They cause 'O' inagglutinability by homologous antisera.

(*ii*) K antigens act as virulence factor by protecting bacilli from the killing action of antibody and complement and also by impending phagocytosis.

Most strains of intestinal *Esch. coli* do not possess K antigens. Of the strains possessing these antigens, most are of the L type.

3. Flagellar Antigen (H Antigen)

These are thermolabile and 75 H antigens have been described. All of them are monophasic. For H antigen determination, the organisms have to be grown in semisolid agar. There are very few cross-reactions between H antigens of *Esch. coli* and those of other members belonging to the family *Enterobacteriacae*.

4. Fimbrial Antigen (F Antigen)

These are thermolabile proteins and heating the organisms at 100°C leads to detachment of fimbriae. The F antigen has no role in antigenic classification of *Esch. coli*.

Esch. coli possesses common fimbriae (pili) which are chromosomally determined and sex pili, which are determined by conjugative plasmids and appear to be organ of conjugation. Type I fimbriae mediate adhesion of bacterium to cells that contain mannose residue. Such adhesion enhances bacterial pathogenicity e.g. type I fimbriae and its possible role in urinary tract infection.

Several fibrin structures resembling fimbriae are described in *Esch. coli*. These cause a mannose-resistant haemagglutination. These structures probably play a significant role in the pathogenesis of diarrhoeal disease and in urinary tract infection. These include K88, K99 and colonisation factor antigens (CFAs). They are plasmid determined. K88 causes enteritis of piglets and K99 produces diarrhoea in calves and lambs. CFAs are detected in some enterotoxigenic strains of *Esch. coli* that cause human diarrhoea. Till now, four CFAs designated as CFA I, CFA II, CFA III and CFA IV have been described. They can be detected by serological tests (agglutination and immunodiffusion) with specific antisera.

E. Antigenic Typing

On the basis of O antigen, *Esch. coli* has been subdivided into a number of O groups. Each O group is then divided into subgroups on the basis of K antigens. Each of these subgroups includes strains with different H antigens. Thus, the antigenic pattern of a strain is recorded as the number of the particular antigen it carries, as for example O111 : K58 : H12.

F. Resistance

Esch. coli is excreted in faeces of humans and animals and contaminate soil and water. It is killed by moist heat at 60°C usually within 30 minutes. It can be killed by 0.5–1 part per million (ppm) chlorine in water. It can survive for several days in soil, water, dust and air.

G. Toxins

Some strains of *Esch. coli* produce *enterotoxins*, *haemolysin* and *Verocytotoxin*.

1. Enterotoxins

Enterotoxigenic strains of *Esch. coli* (ETEC) produce one or both of two enterotoxins, a heat-labile toxin (LT) and a heat-stable toxin (ST). Production of both LT and ST is plasmid (*ent* plasmid) mediated.

(i) Heat labile toxin (LT)

It is a heat labile protein and closely resembles enterotoxin produced by *V. cholerae*. LT is composed of one enzymatically active polypeptide A (A for active) subunit and five identical B (B for binding) subunits. The B subunits bind to the Gm_1 ganglioside receptor at the brush border of epithelial cells of small intestine and facilitates the entry of subunit A. The subunit A is activated to yield two fragment—A_1 and A_2. The A_1 fragment activates adenyl cyclase in the enterocyte. The activation of adenyl cyclase converts adenosine triphosphate (ATP) to cyclic adenosine 5'–monophosphate (cAMP). The marked increase of cAMP results in intense and prolonged hypersecretion of water and chlorides and inhibits the reabsorption of sodium. The intestinal lumen is distended with fluid and hypermotility leads to profuse watery diarrhoea.

Occasional strains of *Esch. coli* produce two types of LT i.e. LT–I and LT–II. LT–II has similar biological action as LT–I but does not react with LT–I antiserum.

(ii) Heat-stable toxin (ST)

In contrast to LT, ST is a low molecular weight polypeptide and poorly immunogenic. Two types of ST are known, STI (or ST_A) and STII (or ST_B). STI stimulates fluid secretion in the gut through the mediation of cyclic guanosine monophosphate (cGMP). The mode of action of STII is not known. ST acts more rapidly than LT. STII cannot be detected by ligated rabbit ileal loop test or infant mouse intragastric test, however these tests are useful in detection of STI.

Poorly immunogenic nature of ST initially prevented the development of immunological assays. This problem was overcome by preparing antiserum from toxin coupled to a hapten. Subsequently ELISA with ST monoclonal antibodies specific for ST have become available.

2. Haemolysin

Some strains of *Esch. coli* produce a haemolysin which can lyse erythrocytes of some species. A larger proportion of *Esch. coli* recovered from extra-intestinal lesions of man are haemolytic than those isolated from faeces.

3. Verocytotoxin (VT)

It is also called Shiga like toxin (SLT). Biological, physical and antigenic properties of VT are similar to Shiga toxin produced by *Sh. dysentriae* type 1. It is of two types, VT1 which is neutralised by antiserum to Shiga toxin and VT2 that resists neutralisation by this antiserum. They are cytotoxic to *Vero* and *Hela* cells, enterotoxic in rabbit ileal loop and show paralytic-lethality in mouse. VT is

also composed of A and B subunits. VT production is found to be phage encoded in several *Esch. coli* strains.

Besides VT1 and VT2, there is another toxin named VT2v which is human and porcine variants. VT2v toxin differs from VT1 and VT2 in that it is not cytotoxic to Hela cells and is not phage encoded (Table 31.3).

Table 31.3 Differentiating features of verocytotoxins

	VT1	VT2	VT2v*
Synonym	SLT1	SLT2	SLT2v
Cytotoxicity			
Vero cells	+	+	+
Hela cells	+	+	–
Phage encoded	+	+	–

*Human and porcine variants.

H. Pathogenesis

Esch. coli forms a part of normal intestinal flora of man and animal. There are four major types of clinical syndromes which are caused by *Esch. coli:* (1) urinary tract infection (2) diarrhoea (3) pyogenic infections, and (4) septicaemia.

Pathogenic *Esch. coli* strains are grouped into two categories, namely extraintestinal pathogenic *Esch. coli* (ExPEC) and intestinal or diarrhoeagenic *Esch. coli*. ExPEC contains two groups 'urinary pathogenic *Esch. coli*' (UPEC) and meningitis/sepsis associated *Esch. coli* (MNEC). UPEC strains are a major cause of community acquired urinary tract infections. MNEC strains cause neonatal meningitis. There are five groups under diarrhoeagenic *Esch. coli*, namely EPEC, ETEC, EIEC, EHEC and EAEC. These have been described under heading 'Diarrhoea'.

1. Urinary Tract Infection

Esch. coli is the commonest organism responsible for urinary tract infection (UTI). Most frequent O serotypes of *Esch. coli* causing UTI include 01, 02, 04, 06, 07, 018 and 075. These are also named as nephritogenic strains. Special nephropathogenic potential of these strains appears to be due to following factors :

(*i*) Polysaccharides of O and K-antigens protect the organism from the bactericidal effects of complement and phagocytes. Strains possessing K1 or K5 antigen appear to be more virulent.

(*ii*) Fimbriae mediate the adherence of the organism to uro-epithelial cells. The receptor to which it attaches, is believed to be a part of the P blood group antigen and therefore it is termed as *P fimbriae*.

Esch. coli that causes UTI often originates in the intestine of the patient. Route of infection to reach urinary tract is either the ascending route or the haematogenous route. The ascending route is through faecal flora spreading to the perineum and from there they ascend into the bladder.

The other commonly encountered bacteria in UTI are *Klebsiella*, *Proteus*, *Citrobacter* and those which rarely produce UTI are salmonellae, edwardsiellae and *Enterobacter*. The Gram positive organisms that can cause UTI include *Staph. aureus*, coagulase negative staphylococci, *Str. faecalis, Str. pyogenes, Str. milleri, Str. agalactiae*, other streptococci and anaerobic streptococci. Rarely *Gardnerella vaginalis* may cause UTI. Among fungal causes, *Candida albicans* may cause UTI in immunocompromised patients. The hospital-associated infection following instrumentation, catheterization and other procedures, is mostly caused by *Pseudomonas* and *Proteus*.

2. Diarrhoea

Esch. coli causing diarrhoeal diseases are of five groups. They produce diarrhoea with different pathogenic mechanisms.

(i) Enteropathogenic Esch. coli (EPEC)

They cause enteritis in infants, especially in tropical countries. In past, several serious institutional outbreaks of diarrhoea had occurred in babies less than 18 months old. EPEC adhere tightly to enterocytes, leading to inflammatory reactions and epithelial degenerative changes. Common serogroups of EPEC are, 026, 055, 086, 0111, 0114, 0119, 0125, 0126, 0127, 0128 and 0142.

(ii) Enterotoxigenic Esch. coli (ETEC)

These are the strains that form a heat- labile enterotoxin (LT) or a heat-stable enterotoxin (ST) or both. They are now known to be a major cause of diarrhoea in children in developing countries and are the most important cause of *travellers diarrhoea* or *turista*. The name 'travellers' diarrhoea refers to diarrhoea in persons from the developed countries within a few days of their visit to one of the developing countries. ETEC sometimes causes a diarrhoea similar to that produced by *Vibrio cholerae*.

ETEC also possess *colonisation factors* which promote their virulence by adhering the organisms to the epithelium of the small intestine. These colonisation factors may be pili or special type of protein K antigens. Most strains of ETEC belong to the serogroups : 06, 08, 015, 025, 027, 063, 078, 0115, 0148, 0153, 0159 and 0167.

The identification of ETEC strains only depends on the demonstration of toxins. The methods available for the detection of the toxins are listed in Table 31.4. There is no biochemical markers available to differentiate ETEC strains from other *Esch. coli*. In ELISA test, plates are coated with ganglioside GM_1 which is used to capture

Table 31.4 Methods for Detection of Enterotoxins of *Esch. coli*

Test	Heat-labile toxin (LT)	Heat-stable toxin (ST)
In vivo tests		
Ligated rabbit ileal loop		
Reading at 6 hours	±	+
Reading at 18 hours	+	–
Infant rabbit bowel	+	+
Infact mouse intragastric test (fluid accumulation)	–	+
Adult rabbit skin for vascular permeability factor	+	–
In vitro tests		
Steroid production in Y1 mouse adrenal cell culture	+	–
Changes in Chinese hamster ovary (CHO) cells	+	–
Enzyme linked immunosorbent assay (ELISA)	+	+•
Solid phase radioimmunoassay (RIA)	+	–
DNA probes	+	+
Biken test	+	–

• ST ELISA with monoclonal antibodies

LT if present in the specimen. This bound LT is detected by adding toxin (LT) specific rabbit antibodies.

Biken test

It is a precipitin test to detect LT directly on bacterial colonies. Bacteria are grown on a special agar medium containing rabbit antibodies specific for LT. As bacteria grow and secrete LT, this toxin binds to the anti-LT antibodies to form a precipitin line.

(iii) Enteroinvasive Esch. coli (EIEC)

Some strains of *Esch. coli* invade the intestinal epithelial cells as do dysentery bacilli and produce disease identical to shigella dysentery. These have been named enteroinvasive *Esch. coli* (EIEC). They belong to serogroups : 028, 0112, 0124, 0136, 0143, 0144, 0152 and 0164. On instillation into the eyes of guinea pigs, EIEC cause keratoconjunctivitis, this diagnostic test for EIEC is called *Sereny test*. Another diagnostic method is the invasion of HeLa or HEp-2 cells in tissue culture. This ability to penetrate cells is determined by a large plasmid. The plasmid codes for outer membrane antigens called the *virulence marker antigens* (VMA) which can be detected by ELISA test. Recently DNA probes have become available to screen faeces specimens for EIEC.

EIEC strains are often atypical in biochemical reactions such as these may be late lactose fermenter or non-lactose fermenter and anaerogenic. These also resemble antigenically to shigellae. Because of their biochemical and antigenic similarity to shigellae, many EIEC outbreaks have been mistaken for shigellosis.

(iv) Enterohaemorrhagic Esch. coli (EHEC) or Verocytotoxin producing Esch. coli (VTEC)

These strains cause haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS). It is most common in infants and young children but can occur in all ages. It is characterised by marked haemorrhage but fever is not always present. Toxin responsible is called 'Verotoxin' because of its effect on vero cells in culture. These strains belong to serogroup O157.

(v) Enteroaggregative Esch. coli (EAEC)

These strains are so named because they appear aggregated in a 'stacked brick' formation on HEP–2 cells or glass. Most of the strains are O-untypable, but many are H-typable. They form a heat stable enterotoxin called *enteroaggregative heat stable enterotoxin–1 (EAST 1)*.

3. Pyogenic Infections

Esch. coli may cause wound infection, peritonitis, cholecystitis and neonatal meningitis. It is an important cause of neonatal meningitis.

4. Septicaemia

Esch. coli is a very common cause of septicaemia in many hospitals and leads to fever, hypotension and disseminated intravascular coagulation (endotoxic shock). This condition usually occurs in debilitated patients and mortality is very high.

I. Laboratory Diagnosis

URINARY TRACT INFECTION

Normal urine is sterile, but during voiding may become contaminated with commensals of genital tract.

1. Specimen Collection

(i) Midstream urine specimen (MSU)

It is collected preferably prior to administration of antibiotics. Specimen is collected in a sterile container. Before collecting a sample, genitalia should be cleaned with soap and water and men are instructed to retract the foreskin of glans penis whereas women should keep the labia apart. The first portion of urine is allowed to pass, then without interrupting the urine flow, mid-portion of the stream is collected. The first portion of urine adequately flushes out the normal urethral flora.

(ii) Catheter specimen

Urine should be collected directly from the catheter and not from the collection bag. The catheter should not touch the container. Although a catheter specimen yields excellent results but catheterisation to obtain urine is not justified because of risk of introducing infection.

(iii) Urine specimens from infants

A clean catch specimen after cleansing of genitalia is preferred. Another procedure of collecting specimen in infants is by supra-pubic aspiration.

2. Transport

As urine is a good culture medium, specimens after collection should reach the laboratory with minimum delay, if it is not possible, the specimen is to be refrigerated at 4°C.

3. Laboratory Methods

Part of the specimen is used for bacteriological culture and the rest is examined immediately under the microscope.

(i) Microscopy

Urine is centrifuged and deposit is examined under the microscope for detecting pus cells, erythrocytes, epithelial cells and bacteria.

(ii) Culture

Most laboratories use a semiquantitative method (standard loop technique) for culture of urine specimens.

Standard loop technique

A standard calibrated loop is used to culture a fixed volume of uncentrifuged urine. Blood agar and MacConkey's agar are used and incubated at 37°C for 24 hours. Next day, the number of colonies grown is counted and total count per ml is calculated. The blood agar gives a quantitative measurement of bacteriuria, while MacConkey's medium enables the presumptive diagnosis of the bacterium.

Interpretation of results

Kass (1956) gave a criterion for active bacterial infection of urinary tract as follows:

Significant bacteriuria — When bacterial count is more than 10^5/ml of a single species.

Doubtful significance — between 10^4 to 10^5 bacteria per ml. Specimen should be repeated for culture.

No significant growth — < 10^3 bacteria per ml and are regarded as contaminant.

(iii) Identification

The organisms are identified by colony characters, Gram's staining, motility, biochemical reactions and slide agglutination test.

(iv) Antibiotic sensitivity test

Esch. coli and other common urinary pathogens develop multiple drug resistance and of the transferable variety. Antibiotic sensitivity is necessary to administer proper antibiotics.

(v) Other methods of diagnosing urinary tract infection

Several screening methods have been introduced and these are described in Chapter 69.

DIARRHOEA

Laboratory diagnosis of *Esch. coli* diarrhoea has been complex as there is no single test which can identify all strains of *Esch. coli* responsible for diarrhoea. Faeces or rectal swab is plated directly on blood agar and MacConkey's medium. After overnight incubation, growth on culture media is identified by colony morphology, Gram staining, motility and biochemical reactions. *Esch. coli* colonies are emulsified in saline on a slide and tested by agglutination with polyvalent and monovalent OB antisera against entero-pathogenic (EPEC) serotypes. It is essential to test at least ten isolated colonies by agglutination as more than one serogroups may be present in faecal plates.

Special indicator media may be employed for specific serotypes with distinctive properties. A modified MacConkey's medium in which sorbitol is incorporated instead of lactose, has been employed for the detection of serotypes O157:H7, 055:B5 and 0111:B4 as these do not ferment sorbitol unlike most other *Esch. coli* strains.

The identification of ETEC depends on demonstration of LT and ST. The tests for detection of toxins are shown in Table 31.4.

VTEC can be detected by its cytotoxic effect on *Vero* and *HeLa* cells, enterotoxin effect in rabbit ileal loop and its lethality in mouse. It can also be detected by DNA probes for the genes encoding VT1 and VT2. PCR with VT specific primers has also been used to detect VTEC.

The demonstration of EIEC is more difficult. It can be detected by sereny test. Simpler tests using *HeLa* cells or *HEp2* cells have now been standardised. Monolayers of these cells are exposed to suspension of organisms. After an appropriate infection period, cells are examined microscopically for the presence of intracellular organisms.

EAEC can be detected on HEp-2 cells which are exposed to *Esch. coli* strains and 'allowed' to adhere to these cell monolayers in-vitro. The pattern of adhesion is observed by microscopy of these exposed cells.

An aggregative adhesion gene probe has also been used as a rapid means of screening these strains.

For more details on diarrhoeal diseases, refer to Chapter 70.

PYOGENIC INFECTIONS

The specimens are usually pus and wound swab. Cultures are made on MacConkey's agar and the isolate is identified by colony morphology, staining, motility and biochemical reactions.

SEPTICAEMIA

Diagnosis depends on the isolation of the organism by blood culture and its identification by colony morphology, staining, motility and biochemical reactions.

Alkalescens–Dispar Group

A group of non-motile bacilli associated with dysentery were called the Alkalescens–Dispar (AD) group. They were believed to be related to *Shigella*. These are lactose fermenting (similar to *Sh. sonnei*) but are indole positive. Antigens of AD group appear to be identical with those of *Esch. coli*. The AD group is now considered as non-motile anaerogenic *Esch. coli* EIEC. It is now named as **Esch. coli inactive**.

Escherichia albertii

It is a new species and has been associated with diarrhoeal diseases in Bangladeshi children. It ferments mannitol but is indole negative. It was previously classified as *Shigella boydii* serotype 13.

III. EDWARDSIELLA

Genus *Edwardsiella* is separated from *Escherichia* by its ability to produce hydrogen sulphide. *E. tarda* is the only species of the genus and the term tarda refers to slow or weak fermentation of sugars by this bacteria. It is a motile, Gram negative bacillus, non-capsulated and ferments only glucose and maltose. It forms indole and H_2S, utilises citrate and decarboxylates lysine and ornithine.

E. tarda is normal intestinal inhabitant of cold blooded animals. It has been recovered occasionally from the faeces of healthy persons. It is an occasional cause of wound infection in man. However, its role in causation of diarrhoea, meningitis has yet to be established.

IV. CITROBACTER

These motile, Gram negative, citrate positive bacilli are normal inhabitants of intestine. They grow well on ordinary media producing smooth convex colonies, 2–4 mm in diameter. On MacConkey agar, they form pale colonies. They ferment lactose late or not at all. They are indole positive or negative, MR positive, VP negative, urease weak positive and do not decarboxylate lysine but most strains decarboxylate ornithine.

Citrobacter has three species. *C. freundii*, *C. koseri* (*C. diversus*) and *C. amalonaticus*. *C. freundii* gives typical reaction with production of H_2S whereas *C. amalonaticus* does not form H_2S.

Some strains (Ballerup-Bethesda group) exhibits extensive antigenic sharing with salmonellae and may lead to confusion in diagnostic laboratory. Vi antigen of Bhatnagar strain of citrobacter is serologically identical with the Vi antigen of *S. typhi*, and *S. paratyphi C*. However, these citrobacter strains can be distinguished from salmonellae by their negative lysine decarboxylase and positive KCN reactions.

Citrobacter may cause urinary tract, gallbladder and middle ear infections. *C. koseri* may occasionally cause neonatal meningitis.

V. KLEBSIELLA

The genus *Klebsiella* consists of Gram negative, capsulated, non-sporing, non-motile bacilli that grow well on ordinary media, produce pink mucoid colonies on MacConkey's agar. They are widely distributed in nature, occurring as commensals in human and animal intestines and also as saprophytes in soil.

A. Classification

The classification of Klebsiella has undergone various modifications. They have been classified into *K. pneumoniae*, and *K. oxytoca* (Table 31.5). *K. pneumoniae* is further subdivided into three subspecies, namely, pneumoniae, ozaenae and rhinoscleromatis. Unlike other subspecies, *K. oxytoca* is indole positive. A third species *Klebsiella*

Table 31.5 Biochemical Reactions of Klebsiella Species

Property	K. pneumoniae subspecies			K. oxytoca
	pneumoniae	ozaenae	rhinoscleromatis	
Gas from glucose	+	V	–	+
Acid from lactose	+	V	–	+
Urease	+	–	–	+
Indole	–	–	–	+
Citrate	+	V	–	+
Malonate	+	–	+	+
MR	–	+	+	V
VP	+	–	–	+
Lysine decarboxylase	+	V	–	+

V, variable

granulomatis has been included. This was previously known as *Calymmatobacterium granulomatis*. It causes donovanosis and has been described in Chapter 48.

B. Morphology

These are short, plump, Gram negative, capsulated, non-motile bacilli. They are about 1–2 μm × 0.5–0.8 μm in size.

C. Culture

Klebsiellae grow well on ordinary media at optimum temperature of 37°C in 18–24 hours. On MacConkey's agar, the colonies appear large, mucoid and pink to red in colour. Mucoid nature of colonies is due to capsular material produced by the organism.

D. Biochemical Reactions

They ferment sugars (glucose, lactose, sucrose, mannitol) with production of acid and gas. They are urease positive, indole negative, MR negative, VP positive and citrate positive (IMViC – – ++). These reactions are typical of *K. pneumoniae subsp. pneumoniae.*

Glucose	Lactose	Mannitol	Sucrose	
AG	+	+	+	
Indole	Urease	Citrate	MR	VP
–	+	+	–	+

E. Antigenic Structure

1. Capsular (K) Antigen

On the basis of capsular (K) antigens, the klebsiellae have been classified into 80 (1–80) serotypes. Identification of these capsular antigens is usually done by capsular swelling reaction with capsular antiserum. Serotypes 1–6 are found most frequently in human respiratory tract infection.

2. Somatic (O) Antigen

Klebsiellae contain five (01–05) different somatic or O antigens in various combinations. Four of these (01, 03, 04 and 05) are identical or closely related to O antigens of *Esch. coli.*

F. Methods of Typing

Phage typing, biotyping, bacteriocin (klebocin or pneumocin) typing and resistotyping have been tried. Many *Klebsiella* strains produce bacteriocins known as klebocins or pneumocins which show a narrow range of activity on other *Klebsiella* strains. Klebocin typing can be done by the help of liquid preparations of bacteriocins. Klebocin typing and capsular serotyping together may be very useful for epidemiological studies.

G. Pathogenesis

K. pneumoniae subsp. *pneumoniae* is the second most populous member next to *Esch. coli* of aerobic bacterial flora of intestine of humans. It is responsible for severe bronchopneumonia, urinary tract infections, nosocomial infections, wound infections, septicaemia, meningitis and rarely diarrhoea. *Klebsiella* pneumonia is a serious disease with high case fatality. Positive blood cultures can be obtained in about 25 per cent cases of pneumonia. It is very important pathogen causing nosocomial infections of the lower respiratory tract. Some strains of *K. pneumoniae* isolated from cases of diarrhoea produce an enterotoxin similar to the heat-stable toxin of *Esch. coli.*

K. pneumoniae subsp. ozaenae has been associated with atrophic rhinitis, a condition known as *ozaena*. *K. pneumoniae subsp. rhinoscleromatis* causes a granulomatous disease of the nose and pharynx called *rhinoscleroma*.

H. Laboratory Diagnosis

Laboratory diagnosis is done by culturing appropriate specimens on blood agar and MacConkey agar. The isolate is identified by colony morphology, Gram staining, test for motility and biochemical reactions.

I. Treatment

Clinical isolates of *Klebsiella* are resistant to a wide range of antibiotics like ampicillin, amoxycillin and carbenicillin. This resistance is due to R plasmid. Klebsiellae are usually sensitive to cephalosporins, trimethoprim, nitrofurantoin, co-amoxiclav and gentamicin. However, plasmid mediated resistance to gentamicin and various cephalosporins have been reported in hospital strains.

VI. ENTEROBACTER

Enterobacter is a motile, capsulated, lactose fermenting bacillus which is indole and MR negative and VP and citrate positive. These characteristics are similar to those of klebsiella species except motility. Two species are most commonly encountered in clinical specimens, *E. cloacae* and *E. aerogenes*.

They are widely distributed in water, sewage, soil and on vegetables. They are also found in human and animal faeces. They may cause urinary tract infections and hospital infections. They are occasionally associated with meningitis and septicaemia. Aminoglycosides are often effective in the treatment of *Enterobacter* infections.

VII. HAFNIA

Hafnia is a motile, non-lactose fermenting bacillus which is indole and MR negative and VP and citrate positive. Only one species of this genus is recognised, *H. alvei*. Biochemical reactions are evident best at 22°C instead of 37°C. It is found in human and animal faeces, sewage, soil and water. It is believed to be an opportunistic pathogen and has been recovered from wounds, sputum, abscesses, urine and blood.

VIII. SERRATIA

Serratia is characterised by forming a pink, red or magenta, non-diffusible pigment called prodigiosin. Pigment is formed optimally at room temperature. It is small motile, Gram negative bacillus and ferments lactose very slowly. It is a saprophytic, found in water, soil and food. Only one species is of medical importance—*S. marcescens*. Infections due to this species are on increase in recent years particularly among hospital patients. It can lead to serious nosocomial infections particularly in the newborn and the debilitated persons. These include infections of urinary tract, respiratory tract, wound and meninges. Septicaemia, endotoxic shock and endocarditis have also been reported. In hospital strains, multiple drug resistance is common.

IX. PROTEUS, MORGANELLA, PROVIDENCIA

The tribe *Proteeae*, in the family *Enterobacteriaceae*, contains three genera: *Proteus, Morganella, Providencia*. The single biochemical character, phenyl pyruvic acid test (PPA test), distinguishes this tribe from all other members of *Enterobacteriaceae*. These bacteria are PPA test positive i.e. they contain an enzyme phenyl alanine deaminase which converts phenyl alanine to phenyl pyruvic acid (PPA). Most strains of *Proteus*, *Morganella* and *Providencia* are widely distributed in nature such as sewage, soil, decomposing organic matter, faeces of humans and animals.

A. Classification

The genus *Proteus* was previously classified into five biotypes namely *Pr. vulgaris, Pr. mirabilis, Pr. morganii, Pr. rettgeri* and *Pr. inconstans*. Because of high G+C content of DNA and low level of genetic homology, *Pr. morganii, Pr. rettgeri* and *Pr. inconstans* are placed under new genera *Morganella* and *Providencia*. *Pr. vulgaris* and *Pr. mirabilis* which are very similar in their properties are included in the genus *Proteus*. Another clinically relevant species is *Proteus penneri*.

B. Proteus

These are motile, Gram negative bacilli, characterised by *swarming* growth on agar. They were named *Proteus* because of their pleomorphic character, after the Greek God Proteus who could assume any shape.

1. Morphology

These are Gram negative bacilli measuring 1–3 μm × 0.5 μm in size. They are non-capsulated, non-sporing and actively motile. They possess peritrichate flagella. They may present as short coccobacillary forms, long and filamentous type especially in young cultures. Many strains possess fimbriae.

2. Culture

They are aerobic and facultatively anaerobic. They grow on ordinary media and culture emits a characteristic putrefactive (fishy or seminal) odour. When grown on nutrient agar or blood agar, *Pr. vulgaris* and *Pr. mirabilis* exhibit 'swarming', but it does not occur with *Morganella* and *Providencia* species. Swarming of *Proteus* appears to be due to vigorous motility of the bacteria although the exact cause is yet not established.

Swarming growth presents problems in the diagnostic laboratory when mixed growth is obtained in which *Proteus* is also present. Several methods have been used to inhibit swarming. These are (i) increasing the concentration of agar in the medium, raising it to 6% instead of 1 to 2% (ii) by incorporation of chloral hydrate (1:500), sodium azide (1:500), boric acid (1:1000) in the medium.

They form smooth, pale or colourless (NLF) colonies on MacConkey's agar and do not swarm on this medium. In liquid medium (peptone water), *Proteus* produces uniform turbidity with a slight powdery deposit and an ammonical odour.

3. Dienes Phenomenon

When two different strains of *Proteus* species are inoculated at different places of the same culture plate, swarming of the two strains remain separated by a narrow, visible furrow. However, in case of two identical strains of *Proteus*, swarming of two coalesce without signs of demarcation. It is known as *Dienes phenomenon*. It has been used to determine the identity or non-identity of different strains of *Proteus*.

4. Biochemical Reactions

The distinctive characters of this genus are:

(*i*) Deamination of phenyl alanine to phenyl pyruvic acid (PPA test)—It is always positive.

(*ii*) Hydrolysis of urea by enzyme urease—It is another characteristic of *Proteus*, but is negative in some Providencia strains.

They ferment glucose by producing acid and gas. Lactose is not fermented. Indole is formed by *Pr. vulgaris* but is negative in *Pr. mirabilis*. H_2S is produced by *Pr. vulgaris* and *Pr. mirabilis*. They are MR positive and VP negative.

Species of *Morganella* and *Providencia* can be differentiated from *Proteus* by different biochemical reactions (Table 31.6).

5. Antigenic Structure

The bacilli possess thermostable, 'O' (somatic) and thermolabile 'H' (flagellar) antigens, based upon which, several serotypes have been recognised. *Weil and Felix* (1916) observed that flagellated strains of *Proteus* bacilli grew on agar as a thin surface film resembling the mist produced by breathing on glass and named this variety as 'Hauch' form (from Hauch, meaning 'film of breath'). Non-flagellated variant strains grew as isolated colonies without the surface film and were named 'Ohne Hauch' form (meaning 'without film of breath'). These names are abbreviated as the 'H' and 'O' forms. Subsequently, the terms H and O were used to refer the flagellar and somatic antigens of other bacilli as well. On the basis of O and H antigens, several serotypes have been recognised within the different species.

Weil and Felix also noted that certain non-motile strains of *Pr. vulgaris*, called the 'X' strains were agglutinated by sera of typhus patients. The sharing of polysaccharide antigens of *Proteus* with some rickettsiae forms the basis of the Weil and Felix reaction for the diagnosis of some rickettsial infections. Three non-motile strains of *Proteus* are employed as the antigens for this test—*Pr. vulgaris* strains OX 2, OX 19 and *Pr. mirabilis* OX K.

6. Pathogenesis

Proteus species are saprophytic and widely distributed in nature. They also occur as commensals in the intestine. They are opportunistic pathogens and may cause many types of infections such as urinary tract infection (UTI), pyogenic lesions, infection of ear, respiratory tract infection and nosocomial infections. Pyelonephritis due to *Proteus* is particularly toxic as the ammonia produced by the organism interferes with complement and other defence mechanisms. *Pr. mirabilis* is the predominant and accounts for the most infections in man.

Multiple drug resistant strains carrying R plasmids have become very important in nosocomial infections.

Table 31.6 Biochemical Reactions of Tribe Proteeae

Property	Pr. vulgaris	Pr. mirabilis	Morg. morganii	Prov. alcalifaciens	Prov. stuartii	Prov. rettgeri
Gas from Glucose	+	+	+	+	–	–
Indole	+	–	+	+	+	+
Phenyl pyruvic acid (PPA) test	+	+	+	+	+	+
Urease	+	+	+	–	±	+
H_2S production	+	+	–	–	–	–
Ornithine decarboxylase	–	+	+	–	–	–
Fermentation of adonitol	–	–	–	+	±	±
Fermentation of trehalose	±	+	±	–	+	–

7. Laboratory Diagnosis

(*i*) *Specimens*:
  (*a*) Mid stream urine sample in UTI;
  (*b*) Pus in pyogenic lesions

(*ii*) *Collection*: Specimen should be collected in sterile container under all aseptic conditions and transported immediately.

(*iii*) *Culture*: It is cultured on MacConkey agar or blood agar with 6% agar to inhibit swarming. Culture media are incubated at 37°C for 18–24 hours. NLF colony are seen on MacConkey agar. Peptone water is also inoculated.

(*iv*) *Gram's staining*: Gram negative bacilli which are non-capsulated and non-sporing.

(*v*) *Hanging drop preparation*: Actively motile bacilli are observed.

(*vi*) *Biochemical reactions*: Most important are PPA and urease tests. PPA test is positive in all *Proteus* species. Urease test is also positive in all *Proteus* species with exception of *Providencia* which is negative. Other biochemical reactions can be used to differentiate various species (Table 31.6).

(*vii*) *Agglutination test*: Strain may be agglutinated with polyvalent group specific sera to confirm it.

(*viii*) *Antibiotic susceptibility test*: It is important as proteus bacilli are resistant to many of the common antibiotics.

C. Morganella

The genus *Morganella* has one species, *M. morganii*. It does not swarm. It causes urinary tract infection and nosocomial wound infections.

D. Providencia

This genus contains three species seen in clinical infections. *Prov. alcalifaciens* (formerly *Pr. inconstans*) is sometimes found in diarrhoeal stools though its aetiological role is uncertain. *Prov. stuartii* is a common cause of urinary tract infection and of infection in burns. *Prov. rettgeri* (formerly *Pr. rettgeri*) sometimes causes nosocomial infection of the urinary tract, wounds and burns, Providencia are very resistant, particularly *Prov. stuartii* which is also resistant to disinfectants, making it a major pathogen in burn units.

E. Laboratory Diagnosis of Morganella and Providencia

Laboratory diagnosis of species of these two genera is similar to that of *Proteus* species. However, these can be differentiated on the basis of different biochemical reactions (Table 31.6).

KEY POINTS

1. Members of family *Enterobacteriaceae* are aerobic and facultative anaerobic *Gram negative* enteric bacilli. They are motile by *peritrichous flagella* or are non-motile. They grow readily on ordinary media, ferment glucose with production of acid or acid and gas, reduce nitrates to nitrites, *oxidase negative* and catalase positive except *Sh. dysenteriae type I* which is catalase negative.
2. Some important genera of family *Enterobacteriaceae* include *Escherichia, Salmonella, Shigella, Klebsiella, Proteus, Morganella* and *Providencia*.
3. *Escherichia coli* forms pink colonies (*lactose fermenter colonies*) on MacConkey's medium. It ferments most of the sugars (glucose, lactose, mannitol, maltose) with production of acid and gas. Typical strains do not ferment sucrose. Indole and methyl red (MR) reactions are positive but Voges-Proskauer (VP) and citrate utilisation tests are negative (*IMVIC* ++—).
4. Toxins produced by *Esch. coli* include *enterotoxins*, *haemolysin* and *verocytotoxin*.
5. Enterotoxigenic strains of *Esch. coli* (*ETEC*) produce one or both of two enterotoxins, a *heat-labile toxin* (LT) and *a heat-stable toxin* (ST).
6. *Urinary tract infection*, *diarrhoea*, *pyogenic infections* and *septicaemia* are the diseases caused by *Esch. coli*.
7. *Midstream urine specimen* (*MSU*) is collected for diagnosis of urinary tract infection.
8. *Esch. coli* causing diarrhoeal diseases are of five types namely *Enteropathogenic Esch. coli* (*EPEC*), *Enterotoxigenic Esch coli* (*ETEC*), *Enteroinvasive Esch. coli* (*EIEC*), *Enterohaemorrhagic Esch. coli* (*EHEC*) or *Verocytotoxin producing Esch. coli* (*VTEC*) and *Enteroaggregative Esch. coli* (*EAEC*).
9. *Klebsiella* is a short, *plump*, *Gram negative*, *capsulated*, *non-motile bacillus*. It forms large, *mucoid*, *pink to red colonies* (*lactose fermenter* colonies) on MacConkey's agar. It ferments sugars (glucose, lactose, sucrose, mannitol) with production of acid and gas. It is *urease positive*, *indole negative*, *MR negative*, *VP positive* and *citrate positive* (*IMVIC* – – + +).

10. *K. pneumoniae* and *K. oxytoca* are two species of genus *Klebsiella*.
11. The tribe *Proteeae* in the family *Enterobacteriaceae* contains three genera : *Proteus*, *Morganella*, and *Providencia*. *Phenyl pyruvic acid test* (*PPA test*) distinguishes this tribe from all other members of *Enterobacteriaceae*. Bacteria of tribe Proteeae are *positive* for *PPA test*.
12. There are two species of proteus i.e. *Pr. vulgaris* and *Pr. mirabilis*. These species exhibit '*swarming*' when grown on blood agar. They form smooth, *pale or colourless colonies* (*non-lactose fermenter colonies*) on MacConkey's agar. They are *Gram negative*, *actively motile bacilli*.
13. *Hydrolysis of urea* by enzyme urease is another characteristic of *Proteus*, but is negative in some providencia strains.
14. Proteus may cause *urinary tract infections* (*UTI*), *pyogenic lesions*, *infection of ear*, *respiratory tract infection* and *nosocomial infections*.

YOU MUST KNOW

1. Classification of Enterobacteriaceae.
2. Morphology, culture characteristics and biochemical reactions of *Escherichia coli*.
3. Enterotoxins and verocytotoxin of *Escherichia coli*.
4. Diseases caused by *Esch. coli*.
5. Different groups of *Esch. coli* causing diarrhoeal diseases.
6. Differences between heat-labile toxin (LT) and heat stable toxin (ST) of *Esch. coli*.
7. Laboratory diagnosis of urinary tract infection and diarrhoea caused by *Esch. coli*.
8. Morphology, culture characteristics and biochemical reactions of *Klebsiella sp.*
9. Morphology, culture characteristics and biochemical reactions of *Proteus sp.*
10. Differences between *Proteus sp.*, *Morganella sp.* and *Providencia sp.*

STUDY QUESTIONS

1. Discuss the laboratory diagnosis of urinary tract infections caused by *Esch. coli*.
2. Describe in detail the laboratory diagnosis of diarrhoea caused by *Esch. coli*.
3. Write short notes on:
   (a) Antigenic structure of *Esch. coli*
   (b) Enterotoxins of *Esch. coli*
   (c) Verocytotoxin of *Esch. coli*.
4. Describe the various mechanisms by which *Esch. coli* produces diarrhoea.
5. Write briefly about:
   (a) *Citrobacter*
   (b) *Klebsiella pneumoniae*
   (c) Enterobacter
   (d) Serratia.
6. Write short notes on:
   (a) Classification of tribe *Proteeae*
   (b) Dienes phenomenon
   (c) Genus *Morganella*
   (d) Genus *Providencia*.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Which of the following bacteria is/are member/s of the family *Enterobacteriaceae*?
   (a) *Salmonella typhi* (b) *Citrobacter freundii* (c) *Yersinia enterocolitica* (d) All of the above
2. Which of the following bacteria is/are called coliform bacilli?
   (a) *Escherichia* (b) *Enterobacter* (c) *Citrobacter* (d) All of the above

3. Heat labile toxin (LT) of *Escherichia coli* activates:
   (a) Adenyl cyclase (b) Guanyl cyclase (c) Both of the above (d) None of the above
4. Verocytotoxin 1 (VT1) of *Escherichia coli* is similar to:
   (a) Shiga toxin (b) Cholera toxin
   (c) Enterotoxin of *Staphylococcus aureus* (d) Toxin of *Bacillus cereus*
5. Travellers diarrhoea is caused by:
   (a) Enteropathogenic *Escherichia coli* (EPEC) (b) Enterotoxigenic *Esch. coli* (ETEC)
   (c) Enteroinvasive *Esch. coli* (EIEC) (d) Enterohaemorrhagic *Esch. coli* (EHEC)
6. Biken test is used for the detection of:
   (a) Heat-labile toxin (LT) of *Escherichia coli* (b) Heat-stable toxin (ST) of *Esch. coli*
   (c) Both of the above (d) None of the above
7. Sereny test is used for the identification of:
   (a) Enteropathogenic *Escherichia coli* (EPEC) (b) Enterotoxigenic *Esch. coli* (ETEC)
   (c) Enteroinvasive *Esch. coli* (EIEC) (d) Enterohaemorrhagic *Esch. coli* (EHEC)
8. The term 'significant bacteriuria' is used in urinary tract infection when bacterial count is:
   (a) more than 10^5 bacteria per ml (b) between 10^4 to 10^5 bacteria per ml
   (c) less than 10^3 bacteria per ml (d) None of the above
9. MacConkey's medium with sorbitol instead of lactose is used for the detection of *Escherichia coli* strains:
   (a) 055 : B5 (b) 015 : B11 (c) 08 : B70 (d) None of the above
10. Klebsiella may cause:
    (a) Pneumonia (b) Ozaena (c) Rhinoscleroma (d) All of the above
11. Which of the following bacteria produces indole?
    (a) *Klebsiella pneumoniae* subspecies *pneumoniae* (b) *K. pneumoniae* subspecies *ozaenae*
    (c) *K. pneumoniae* subspecies *rhinoscleromatis* (d) *K. oxytoca*
12. Which of the following bacteria produces red pigment (progidiosin)?
    (a) *Enterobacter cloacae* (b) *Hafnia alvei* (c) *Serratia marcescens* (d) *Klebsiella pneumoniae*
13. Which of the following genera are included in the tribe *Proteeae* of family *Enterobacteriaceae*?
    (a) *Proteus* (b) *Morganella* (c) *Providencia* (d) All of the above
14. Which of the following has/have positive phenyl pyruvic acid test (PPA test)?
    (a) *Proteus* (b) *Morganella* (c) *Providencia* (d) All of the above
15. Which of the following methods can be used to inhibit swarming of *Proteus strains* on solid culture media?
    (a) Increasing the concentration of agar in the medium
    (b) Incorporation of chloral hydrate in the medium
    (c) Incorporation of boric acid in the medium
    (d) All of the above
16. Dienes phenomenon is useful to find out the identity or non-identity of two strains of:
    (a) *Proteus* (b) *Escherichia coli* (c) *Citrobacter* (d) *Shigella*
17. Which of the following *Proteus* strains is/are used in Weil-Felix reaction?
    (a) *Proteus vulgaris* OX2 (b) *Proteus vulgaris* OX19
    (c) *Proteus mirabilis* OXK (d) All of the above
18. All of the following are urease test positive except:
    (a) *Proteus vulgaris* (b) *Proteus mirabilis* (c) *Helicobacter pylori* (d) *Escherichia coli*

Answers (MCQs):

1. (d)	2. (d)	3. (a)	4. (a)	5. (b)	6. (a)	7. (c)	8. (a)	9. (a)	10. (d)
11. (d)	12. (c)	13. (d)	14. (d)	15. (d)	16. (a)	17. (d)	18. (d)		

Chapter 32

SHIGELLA

Shigella

A. Morphology
B. Culture
C. Resistance
D. Antigenic Structure
E. Classification
F. Toxins
G. Bacteriophage and Colicin Typing
H. Pathogenesis
I. Laboratory Diagnosis
J. Treatment
K. Prevention and Control

SHIGELLA

The organisms of genus *Shigella* are exclusively parasites of human intestine and other primates and cause bacillary dysentery in man. The name *Shigella* is named after *Shiga* who isolated the first member of this genus in 1896 from epidemic dysentery in Japan.

A. Morphology

Shigellae are short, Gram negative bacilli measuring about 1–3 μm × 0.5 μm. They are non-motile, non-capsulated and non-sporing.

B. Culture

They are aerobes and facultative anaerobes and can grow on ordinary media. Optimum temperature for growth is 37°C and pH 7.4. They can grow at temperature range of 10–40°C. After overnight incubation, colonies are about 2 mm in diameter, circular, convex, smooth and translucent. Colonies on MacConkey's agar and deoxycholate citrate agar (DCA) are colourless (non-lactose fermenting–NLF) except in case of *Sh. sonnei* which forms pink colonies due to late lactose fermentation. DCA is a useful selective medium to isolate these organisms from faeces. However, xylose lysine deoxycholate (XLD) agar is a better selective medium than DCA. It is less inhibitory to *Sh. dysenteriae* and *Sh. flexneri*. On this medium colonies of shigella appear red without black centres. The organisms which decarboxylate lysine, forming alkaline amines produce red colonies. The bacteria which also produce H_2S have black centres in the colonies.

Salmonella-Shigella (SS) agar is a highly selective medium for the isolation of *Salmonella* and *Shigella*. Colonies of Shigella on this medium are colourless (due to non-lactose fermentation) with no blackening, while those of *Salmonella* are colourless with black centres. This medium contains bile salts, sodium citrate, sodium thiosulphate, ferric citrate, lactose and neutral red (indicator). The high bile salt concentration and sodium citrate, in this medium, inhibit all Gram positive bacteria and coliforms. Sodium thiosulphate is a source of sulphur. Bacteria that produce H_2S are detected by the black precipitate formed with ferric citrate. Lactose fermentation can be detected by the presence of neutral red. Lactose fermenting colonies appear red.

Hektoen-enteric (HE) agar is a direct plating medium for the isolation of *Salmonella* and *Shigella* from the

Table 32.1 Differentiating features of Shigella species

Subgroup	Fermentation of				Indole	Lysine decar-boxylase	Ornithine decar-boxylase	Serotypes
	Lactose	Mannitol	Sucrose	Dulcitol				
Sh. dysenteriae	–	–	–	–	d	–	–	15
Sh. flexneri	–	A	–	–	d	–	–	6+2 variants
Sh. boydii	–	A	–	d	d	–	–	19
Sh. sonnei	A*	A	A*	–	–	–	+	One

A–Acid; d–Variable; *–Late fermenter

faeces. Colonies of *Shigella* appear green with colour fading to the periphery. *Salmonella* colonies are blue-green typically with black centres. This medium contains bile salts, lactose, sucrose, sodium thiosulphate, ferric ammonium citrate, acid fuchsin and thymol blue. The high bile salt concentration inhibits Gram-positive bacteria and many coliforms. Sodium thiosulphate is a sulphur source and H_2S gas is detected by ferric ammonium citrate.

Enrichment medium

Selenite F broth: Sodium selenite in this medium inhibits coliform bacilli while permitting salmonellae and shigellae to grow. It is recommended for the isolation of these organisms from faeces.

Gram-Negative (GN) broth: Most strains of *Shigella* and *Salmonella* species grow in this medium. It is very useful enrichment medium for the isolation of these organisms from faeces.

C. Resistance

Shigellae are killed at 56°C in one hour and by 1% phenol in 30 minutes. They remain viable in water and ice for 1 to 6 months. Boiling or chlorination of water and pasteurisation of milk are effective and destroy the bacilli.

D. Antigenic Structure

Shigellae possess a large number of somatic 'O' antigens and some strains produce K antigens which cover the O-antigen. K antigens are not relevant in typing but may interfere with agglutination by O antisera. Fimbrial antigens are also present. There is some antigenic sharing within some members of the genus as well as between shigellae and *Esch. coli*. It is, therefore, important to identify shigellae on the basis of antigenic and biochemical properties and not by slide agglutination alone.

E. Classification

Shigellae are divided into four subgroups (A, B, C & D) based on a combination of biochemical and serological specificity. Mannitol fermentation reaction distinguishes subgroup A (mannitol negative) from subgroups B, C, and D (all of them are mannitol positive) (Table 32.1).

1. Subgroup A (Sh. dysenteriae)

It is divided into 15 serotypes. Serotype 1 is the bacillus originally described by Shiga (*Sh. shigae*). It is the only member of the family that is always catalase negative. Serotype 2 is also known as *Sh. schmitzi*. It forms indole in contrast to type 1 which is indole negative. Serotype 3–7 used to be known as *Large–Sachs group* after the name of Large and Sachs who described these serotypes in India.

2. Subgroup B (Sh. flexneri)

This subgroup is named after *Flexner*, who first isolated *Sh. flexneri* in Philippines (1900). It is antigenically the most complex among shigellae. They have been classified into six serotypes (1–6) based on specific antigens. Each of these is further divided into subtypes (e.g. 1a and 1b). In addition to six serotypes, two antigenic 'variants' (X and Y) are recognised, which do not possess type specific antigens.

Serotype 6 occurs in three biotypes, some of which form gas from sugars (Table 32.2). Gas formation is an important characteristic as all other Shigellae are anaerogenic.

Table 32.2 Biotypes of ***Sh. flexneri*** type 6

Biotype	Indole	Fermentation of	
		Glucose	Mannitol
Boyd 88	–	A	A
Manchester	–	AG	AG
Newcastle	–	A or AG	–

A–Acid; AG–Acid and Gas

3. Subgroup C (Sh. boydii)

It is named after *Boyd*, who first described these strains from India (1931). It resembles *Sh. flexneri* biochemically but not antigenically. Nineteen serotypes (numbered 1 to 20 except 13) of *Sh. boydii* have been identified. *Sh. boydii* serotype 13 has been reclassified as *Esch. albertii*.

4. Subgroup D (Sh. sonnei)

Sonne described *Sh. sonnei* in Denmark (1915). It is indole negative but ferments lactose and sucrose late. It is antigenically homogeneous but may occur in two forms—Phase I and Phase II. *Sh. sonnei* has been classified into many colicin types. It causes the mildest form of bacillary dysentery. In many cases, the disease may occur as a mild diarrhoea. *Sh. sonnei* infection is the most common shigellosis in advanced countries.

F. Toxins

1. Endotoxin

All shigellae release endotoxin after autolysis. Endotoxin has irritating effect on intestinal wall which causes diarrhoea and subsequently intestinal ulcers.

2. Exotoxin

Sh. dysenteriae type 1 produces a powerful exotoxin. It acts as enterotoxin and as well as neurotoxin. As enterotoxin, it induces fluid accumulation, and as neurotoxin it damages the endothelial cells of small blood vessels of the central nervous system which results in polyneuritis, coma and meningism. The exotoxin is not primarily a neurotoxin.

3. Verocytotoxin (VT)

Sh. dysenteriae type 1 also produces a cytotoxin which acts on Vero cells and is named *verocytotoxin* or VT. Two verocytotoxins, VT1 and VT2 are recognised. VT1 is identical to exotoxin produced by *Sh. dysenteriae* type 1. VT1 comprises two subunits—A and B. Subunit B binds the cytotoxin to cells while subunit A inhibits protein synthesis. Genes coding for VT production are located in the chromosome. VT1, VT2 or both are also produced by certain strains of enterohaemorrhagic *Esch. coli* causing haemorrhagic colitis.

G. Bacteriophage and Colicin Typing

The determination of serotype within subgroup A, B and C is sufficient for epidemiological study and further subdivision is not necessary. However, each serotype of *Sh. flexneri* is further subdivided into 123 phage types by bacteriophage typing.

Sh. sonnei consists of a single serotype which can be further subtyped either by phage typing or by colicin typing. Phage typing being cumbersome, therefore, colicin typing has been adopted by most centres. *Sh. sonnei* strains are classified into 26 colicin types and each type is characterised by the production of a specific colicin.

H. Pathogenesis

Shigellae cause bacillary dysentery. Man is the only natural host of shigellae. The minimum infective dose is as few as 10–100 bacilli. *Sh. dysenteriae* type 1 causes most severe form of dysentery usually associated with toxaemia. The infection due to *Sh. flexneri* and *Sh. boydii* are less severe and prevalent in tropical countries including India. *Sh. sonnei* causes the mildest form of the disease and is common in Western countries like Britain. It is more frequently seen in children.

The incubation period of the disease is usually less than 48 hours but varies between 1–7 days. Mode of infection is by ingestion of contaminated food.

The food or drink is contaminated by faeces of cases or of carriers. The organisms infect the epithelial cells of the terminal ileum and colon and multiply inside them. The distal parts of the colon are severely affected. Then they spread laterally into adjacent cells as well as to lamina properia. Inflammatory reaction develops and there is necrosis of surface epithelial cells. The necrotic epithelia become soft and friable and are sloughed out leaving behind transverse superficial ulcers. The organisms tend to remain localised in the gut wall and usually do not lead to bacteraemia. Invasive property of *Shigella* is related to the presence in the bacillus of large plasmids coding for outer membrane proteins which are responsible for cell penetration. These proteins are named *virulence markers antigens* (VMA). VMA can be detected by ELISA. The invasive property can be demonstrated by its ability to penetrate HeLa or Hep-2 cells.

Sh. dysenteriae type 1 causes toxaemia due to production of exotoxin. The toxin causes accumulation of fluid leading to severe diarrhoea (enterotoxic action) and may cause complications like polyneuritis, arthritis, conjunctivitis and parotitis. Haemolytic uraemic syndrome may occur as a rare complication in severe cases. The severity of the disease varies from acute fulminating dysentery to mild diarrhoea. The term *shigellosis* has been employed to include the whole spectrum of the disease caused by shigellae. In India, *Sh. flexneri* (50–85%) is the most common isolate followed by *Sh. dysenteriae* (8–25%), *Sh. sonnei* (2–24%) and *Sh. boydii* (0–8%). Several localised outbreaks of *Sh. dysenteriae* type 1 were observed in India from 1974, followed by extensive epidemics in various states. These epidemic strains showed multiple drug resistance.

I. Laboratory Diagnosis

Diagnosis depends upon isolating the bacillus from faeces.

1. Specimens

Fresh stool is collected. Rectal swabs are not satisfactory. The ideal specimen is a direct swab of an ulcer taken under sigmoidoscopic examination.

2. Transport
Specimens should be transported immediately and inoculated without delay. If delay is inevitable, specimens should be transported in a suitable medium such as Sach's buffered glycerol saline, pH 7.0–7.4. Alkaline transport media used for vibrios are inhibitory for shigellae.

3. Direct Microscopy
Saline and iodine preparation of faeces show large number of pus cells, erythrocytes and macrophages. Parasitic causes of dysentery may also be excluded by this examination.

4. Culture
Specimen is inoculated on selective media like MacConkey's agar, DCA or XLD agar. Selenite F broth (0.4%) is used as enrichment medium which permits rapid growth of enteric pathogens while inhibiting the growth of normal flora like *Esch. coli* for 6–8 hours. Subculture is done on solid media from selenite F broth. All the solid media are incubated at 37°C for 24 hours.

5. Colony Morphology and Staining
Colourless (NLF) colonies appear on MacConkey's agar which are further confirmed by Gram staining, hanging drop preparation and biochemical reactions. Shigellae are Gram negative bacilli and are non-motile.

6. Biochemical Reactions
Any non-motile bacillus that is urease, citrate, H_2S and KCN negative should be further confirmed by various biochemical tests (Table 32.1).

7. Slide Agglutination Test
Identification of shigella is confirmed by slide agglutination with polyvalent antisera and monovalent sera. Then type specific antisera belonging to subgroups A, B or C is used for agglutination test.

8. Colicin Typing
It is done for subgroup D (*Sh. sonnei*) strains.

J. Treatment
Uncomplicated shigellosis is a self limiting condition that usually recovers spontaneously. Dehydration has to be corrected promptly particularly in infants and young children.

Routine antibacterial treatment is not indicated but antibiotics should be reserved for the severe toxic cases. In very serious infections, nalidixic acid has been life saving. The wide prevalence in shigellae of R factors conferring resistance to multiple antibiotics makes antibiotic therapy futile. Many strains are still sensitive to nalidixic acid or norfloxacin. There is no convincing evidence that antibacterial therapy either hasten recovery or prevent the carrier state.

K. Prevention and Control
As man is a major source of infection, control consists essentially in improvement of environmental sanitation.

The role of vaccination in the prevention of dysentery is still uncertain. Killed vaccines proved to be toxic and ineffective. Many live oral vaccines have been tested including avirulent mutants, streptomycin dependent strain and *Shigella-Esch. coli* hybrids. The presence of several sero-groups and types would appear to preclude effective immunisation.

KEY POINTS
1. The organisms of genus *Shigella* cause *bacillary dysentery* in man. They are short, *Gram negative*, *non-motile* bacilli.
2. Shigella has four species i.e. *Sh. dysenteriae*, *Sh. flexneri*, *Sh. boydii* and *Sh. sonnei*.
3. They form colourless colonies (*Non-lactose fermenting—NLF colonies*) on MacConkey's agar. Exception is *Shigella sonnei* which forms pink colonies due to late lactose fermentation.
4. *Sh. dysenteriae* type 1 produces a powerful *exotoxin* which acts as *enterotoxin* and as well as *neurotoxin*. It also produces a cytotoxin which acts on Vero cells and is named *Verocytotoxin* or VT.
5. The term *shigellosis* has been employed to include the whole spectrum of the disease caused by shigellae.
6. Uncomplicated shigellosis is a *self limiting* condition that usually recovers spontaneously.

YOU MUST KNOW

1. Morphology and culture characteristics of *Shigella sp.*
2. Subgroups of *shigellae.*
3. Laboratory diagnosis of dysentery caused by shigella.

STUDY QUESTIONS

1. Discuss the laboratory diagnosis of dysentery caused by shigella.
2. Write short notes on:
   (a) Classification of shigella
   (b) Colicin typing.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Which of the following is used as an enrichment medium for the isolation of *Shigella* from the faeces?
   (a) Selenite F broth
   (b) Alkaline peptone water
   (c) Taurocholate tellurite peptone water
   (d) None of the above
2. Which of the following *Shigella* species does not ferment mannitol?
   (a) *Shigella dysenteriae*
   (b) *S. boydii*
   (c) *S. sonnei*
   (d) None of the above
3. Which of the following biotypes of *Shigella flexneri* type 6 ferment mannitol with acid and gas?
   (a) Boyd 88
   (b) Manchester
   (c) Newcastle
   (d) None of the above
4. Which of the following *Shigella* is also known as *Shigella shigae*?
   (a) *Shigella dysenteriae* serotype 1
   (b) *Shigella dysenteriae* serotype 2
   (c) *Shigella dysenteriae* serotype 3
   (d) *Shigella dysenteriae* serotype 4
5. Which of the following *Shigella* is also known as *Shigella schmitzi*?
   (a) *Shigella dysenteriae* serotype 1
   (b) *Shigella dysenteriae* serotype 2
   (c) *Shigella dysenteriae* serotype 3
   (d) *Shigella dysenteriae* serotype 4
6. Large-Sachs group of Shigella contains:
   (a) *Shigella dysenteriae* serotype 1 and 2
   (b) *Shigella dysenteriae* serotype 3 to 7
   (c) *Shigella flexneri*
   (d) None of the above
7. Which of the following serotype of *Shigella dysentriae* produce verocytotoxin?
   (a) Serotype 1
   (b) Serotype 2
   (c) Serotype 3
   (d) Serotype 7
8. Which *Shigella* species is late fermenter of lactose and surcose?
   (a) *Shigella dysenteriae*
   (b) *Sh. flexneri*
   (c) *Sh. boydii*
   (d) *Sh. sonnei*

Answers (MCQs):

1. (a) 2. (a) 3. (b) 4. (a) 5. (b)
6. (b) 7. (a) 8. (d)

Chapter 33

SALMONELLA

The salmonellae are primarily intestinal parasites of vertebrates and which infect man, leading to *enteric fever, gastroenteritis* and *septicaemia*. The most important member is *Salmonella typhi*, the causative agent of typhoid fever. Man is the only natural host for *S. typhi* and *S. paratyphi A* whereas most of the other salmonellae are chiefly pathogenic in animals like poultry, pigs, cattle etc.

Salmon and Smith (1885) isolated the hog cholera bacillus (*S. cholerae-suis*) and the term *Salmonella* was coined from the name of Salmon. Interestingly, hog-cholera disease was subsequently proved to be a viral infection in which *S. cholerae-suis* was a common secondary invader.

I. SALMONELLA

Salmonellae produce three main types of diseases in man:

1. Enteric Fever

The causative agents of enteric fever are *Salmonella typhi* (causing typhoid fever) or *S. paratyphi A, B, and C* (causing paratyphoid fever). The term enteric fever includes both typhoid and paratyphoid fever.

2. Gastroenteritis

Salmonellae under this group are essentially animal parasites but can also infect man. These include *S. typhimurium, S. enteritidis, S. newport, S. dublin and S. thompson.*

3. Septicaemia

The commonly associated Salmonella is *S. choleraesuis* but other species may also cause septicaemia.

A. Morphology

Salmonellae are Gram negative bacilli measuring 1–3 μm × 0.5 μm. They are motile, non-sporing and non-capsulated. Motility is due to the presence of peritrichous flagella except *S. gallinarum* and *S. pullorum* which are non-motile.

B. Culture

Salmonellae grow on ordinary culture media at optimum temperature of 37°C (range 15–41°C), pH 6–8 and are aerobic and facultatively anaerobic. They produce colonies of 2–3 mm in diameter, circular, translucent, low convex and smooth. On MacConkey's agar and deoxycholate citrate agar (DCA), colonies are colourless due to non-lactose fermentation (NLF). On Wilson and Blair bismuth sulphite medium (selective medium for salmonellae), jet black colonies with metallic sheen are formed due to formation of hydrogen sulphide. *S. paratyphi A* and other species which do not form H_2S produce green colonies. Xylose lysine deoxycholate (XLD) agar is another medium used for isolation of this organism. Most strains of salmonellae produce red colonies with black centres, when grown on this medium. H_2S negative serotypes of Salmonella produce red colonies without black centres.

Selenite F broth and tetrathionate broth (TTB) are commonly used enrichment media for inoculation of specimens especially faeces.

C. Biochemical Reactions

Salmonellae ferment glucose, mannitol and maltose forming acid and gas except *S. typhi* which produces only acid and no gas. They do not ferment lactose or sucrose. Indole is not produced. Most salmonellae produce H_2S in triple sugar iron (TSI) agar except *S. paratyphi A* and *S. cholerae-suis*. They utilise citrate (except *S. typhi* and *S. paratyphi A*) and are MR positive and VP negative. Urea is not hydrolysed. Common biochemical reactions of salmonellae are shown in Table 33.1.

D. Resistance

The salmonellae are killed at 60°C in 15 minutes. Boiling, chlorination of water and pasteurisation of milk destroy the bacilli. They survive in water, ice and snow for weeks and months. They are killed within five minutes by mercuric chloride (0.2%) or phenol (5%).

E. Antigenic Structure

Salmonellae possess three types of antigens based on which they are classified. These are (1) flagellar antigen 'H', (2) somatic antigen 'O', and (3) a surface antigen 'Vi', found in some species (Fig. 33.1). Several strains possess fimbriae. Fimbrial antigens are not important in identification, but confusion may be created due to their non-specific nature and widespread sharing among enteric bacteria.

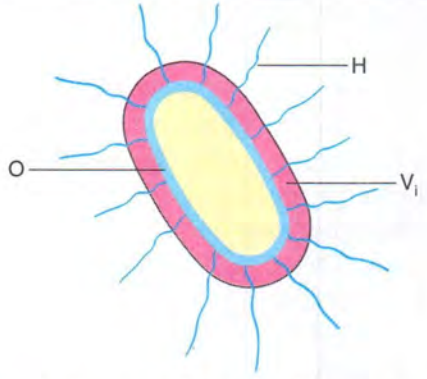


Fig. 33.1 Cellular locations of H, O and Vi antigens

1. H-Antigen

This antigen is present on the flagella and is a heat labile protein. The H antigens of *Salmonella* are not shared by other enterobacteria. It is destroyed by boiling or by treatment with alcohol and acids, but not by formalin. It is strongly immunogenic. When mixed with antisera, H agglutination occurs rapidly, producing large, loose, fluffy clumps. Flagellar antigens are present in two forms called *phase* 1 and *phase* 2.

2. O-antigen

The somatic O antigen is lipopolysaccharide complex

Table 33.1 Biochemical Reactions of ***S. typhi*** and ***S. paratyphi***

	Glucose	Mannitol	Lactose	Sucrose	Indole	Citrate	MR	VP	H_2S	Xylose	d–tartrate	Mucate
S. typhi	A	A	–	–	–	–	+	–	+	d	A	d
S. paratyphi A	AG	AG	–	–	–	–	+	–	–	–	–	–
S. paratyphi B	AG	AG	–	–	–	+	+	–	+	AG	–	AG
S. paratyphi C	AG	AG	–	–	–	+	+	–	+	AG	AG	–

A–Acid, AG–Acid & gas, d–delayed

which forms an integral part of the cell wall. It is identical with endotoxin. It is heat stable and is present both in motile and non-motile bacilli. It is resistant to boiling, alcohol and weak acids. When mixed with antisera, O *antigen* suspensions produce compact, chalky, granular clumps. O antigen is less immunogenic than *H antigen*. The titre of O antibody in serum after infection or immunisation is generally less than that of H antibody. Anti O antibody is pre-dominantly IgM. O agglutination occurs more slowly and at a higher optimum temperature (50–55°C) than H agglutination (37°C). Sixty seven different O antigens have been recognised and they are designated as 1, 2, 3 etc.

3. Vi-antigen

Many strains of *S. typhi* possess surface antigen enveloping the O antigen, referred to as Vi antigen. Hence, this antigen interferes with agglutination of freshly isolated strains with O antiserum. *Felix* and *Pitt* who first described the antigen believed that it was related to virulence (Vi for virulence). It is analogous to the K antigens of coliform bacteria. Vi antigen is heat labile and is destroyed by boiling or heating at 60°C for one hour. It is also destroyed by N HCl, 0.5N NaOH and phenol. It is unaffected by 0.2% formol or alcohol.

Besides *S. typhi*, the Vi antigen with similar specificity is also present in *S. paratyphi C*, *S. dublin* and certain strains of *Citrobacter* (the Ballerup–Bethesda group). The Vi antigen is lost on serial subculture. The Vi antigen acts as a virulence factor by inhibiting phagocytosis, resisting complement activation and lysis of bacteria by the alternative pathway. It has been demonstrated that strains with Vi antigen produce clinical disease more consistently than those without Vi antigen.

The Vi antigen is poorly immunogenic and induces production of low titres of antibody following infection. Detection of Vi antibody is not helpful for diagnosis of enteric fever and hence is not employed in the Widal test. The total absence of Vi antibody in a proven case of typhoid fever indicates poor prognosis. The Vi antibody disappears in early phase of convalescence. Persistence of this antibody indicates the development of the carrier state. Specific Vi bacteriophages are used for epidemiological typing of *S. typhi*.

In prophylactic vaccination with TABC, no Vi antibody is induced by the phenolised vaccine, though low titres of the antibody are produced by the alcoholised vaccine.

F. Antigenic Variation

The antigens of salmonellae undergo several types of phenotypic and genotypic variations.

1. H ⟶ O Variation

Motile strains lose their flagella and become non-motile. When salmonellae are grown on nutrient agar media containing phenol (1:800), flagella are inhibited. This change is phenotypic and temporary since flagella reappear when the strain is subcultured on normal media without phenol. Salmonellae may rarely lose flagella by mutation. A stable non-motile mutant is 901–O strain of *S. typhi* which is widely used for the preparation of O-agglutinable bacterial suspensions.

Generally, loss of flagella is not total but only a diminution in the number of flagella occurs. Such bacterial cultures contain small number of flagellated cells. To obtain motile bacteria, rich in H antigen from such cultures, selection may be carried out by use of Craigie's tube. This consists of a wide tube containing 0.2% nutrient agar (soft agar) in which a short narrow tube open at both ends is kept embedded in such a way that one end of it projects above the agar. The strain is inoculated into the inner tube and after incubation, subcultures are taken from the top of agar outside the central tube. This yields motile bacteria with rich H antigen (Fig. 33.2).

Instead of the Craige's tube, a U-tube of soft agar may also be used. Inoculation of strain is made into one limb of U-tube and subculture is taken from the other limb.

2. Phase Variation

The flagellar antigens of most salmonellae occur in one of two phases, phase 1 or phase 2. Phase 1 antigens are either specific or shared by a few species only and hence phase 1 is called *specific* phase. Phase 2 antigens are widely shared by several unrelated species of salmonellae.

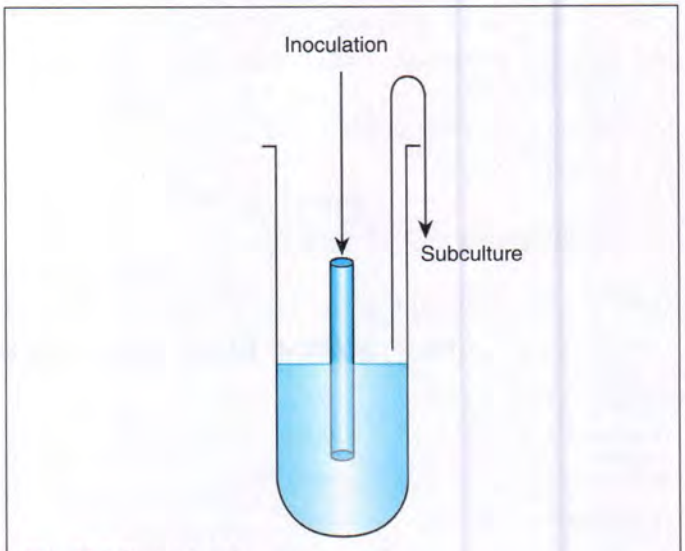


Fig. 33.2 Craigie's tube

Hence phase 2 is called the *non-specific* or *group* phase. Phase 1 antigens are designated as a, b, c, d etc. up to z and after z, as z1, z2, z3, up to z14. Phase 2 antigens are fewer and termed as 1, 2, 3, etc. In some species, antigens of phase 1 may occur as phase 2 antigens (e.g., e, n, x and z15). Strains that possess both phases are termed *diphasic* while some strains (*S. typhi, S. enteritidis, S. dublin* and *S. paratyphi A*) occur in phase 1 only and are called *monophasic*.

A culture will contain flagellar antigens of both phases, but generally one of the two phases predominates so that the culture is agglutinated only by one of the phase antisera. For identification of a serotype, it is necessary to identify the flagellar antigens of both phases. A culture in one phase can be converted to the other phase by passing the bacteria through a Craigie's tube containing the homologous phase antiserum incorporated in the agar (Fig. 33.2).

3. V ⟶ W Variation

Fresh isolates of *S. typhi* carry Vi antigen that completely masks the O antigen. These isolates are agglutinable with Vi antiserum but not with the O antiserum. These are called the *V forms*. Organisms lost their Vi antigen either partially or completely after a number of subcultures. When there is a complete loss of Vi antigen, such cultures are agglutinable by O-antiserum but not with Vi antiserum. These are called *W forms*. With partial loss of Vi antigen, the bacilli are agglutinable with both Vi and O antisera and are called *VW forms*.

Other Vi containing bacilli (*S. paratyphi C and S. dublin*) seldom have the O antigen completely masked by Vi antigen.

4. S ⟶ R Variation

The smooth-to-rough variations occur due to mutation and are associated with the change in the colony morphology from smooth to rough, loss of the O antigen and of virulence of the strain. The rough (R) variants have defective capacity to synthesise somatic (O) antigen and loss of O antigen may be partial or total. R forms may be common in laboratory strains maintained by serial subcultures. S ⟶ R variation may be prevented to some extent by maintaining laboratory cultures on Dorset's egg media in cold or by lyophilisation.

5. Variations in O Antigen

Changes in the structural formulae of O antigen may be induced by lysogenisation with some bacteriophages, resulting in the alteration of the bacterial serotypes. Thus, *S. anatum* (serotype, 3,10: e, h : 1,6) is converted into S. *newington* (serotype, 3,15: e, h : 1,6) by phage 15. *S. newington* is further converted into *S. minneapolis* (serotype, 3, 15, 34 : e, h : 1,6) by phage 34. Such changes are taking place in nature which might be responsible for so many serotypes of salmonellae.

G. Classification

The genus Salmonella is classified broadly into four subgenera on the basis of biochemical reactions (Table 33.2).

Table 33.2 Classification of Salmonellae on the Basis of Biochemical Properties

Property	Subgenera of Salmonella			
	I	II	III	IV
Lactose	–	–	+	–
Dulcitol	+	+	–	–
Malonate	–	+	+	–
d–Tartrate	+	–	–	–
Salicin	–	–	–	+
KCN	–	–	–	+

Subgenus I is the largest and the most important group containing all the species that are commonly responsible for human and animal infections. Other subgenera have little importance in human disease. Subgenera II is common in reptiles and subgenera III in lizards. Subgenera IV strains are rarely encountered and are considered as atypical members of subgenera II. The bacilli of subgenera III, formerly designated as Arizona and later included in the genus salmonella because of their antigenic similarity. About 60 per cent of them ferment lactose promptly.

Modern taxonomical techniques have shown that all the members of the genus *Salmonellae* belong to two species, *S.enterica* and *S. bongori. S. enterica* is classified into six subspecies namely *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae* and *indica*. Subspecies *enterica* corresponds to former subgenus I. *S. enterica* subsp. *enterica* includes the typhoid, parathypoid bacilli and most other serotypes responsible for human diseases. According to this, the taxonomically correct name for the typhoid bacillus would be '*Salmonella enterica*, subspecies enterica, serotype 'Typhi.' Serotype name should be given in Roman and not in italics. This classification and nomenclature is taxonomically correct but would be too complicated for use in clinical bacteriology. Therefore, the old species names are still being used in clinical bacteriology.

Salmonellae are classified serologically by the Kauffmann-White scheme.

H. Kauffmann-White Scheme

It forms the basis of serotyping of salmonellae based on identification of O and H antigens by agglutination. Salmonellae are classified into groups based on distinctive O antigen factors. The distinctive factor for group A is 2, for group B, 4 and for group D, 9. Any strain possessing factor 2 will be classified as group A and any strain possessing factor 9 as group D. Salmonellae are divided into 67 O-groups, each is designated by letters A to Z and those discovered later by the numbers. Within each group, further identification and differentiation is by phase 1 and phase 2 flagellar (H) antigens (Table 33.3).

In Kauffmann-White scheme each serotype is given a species status. There are about 2300 serotypes of salmonellae. Most serotypes of medical importance belong to groups A to E.

The Salmonella species were originally named after the place of isolation (*S. newport, S. panama, S. poona*), after the disease caused by them (*S. schottmulleri*) and after the name of the person from whom the first strain was isolated (*S. thompson*).

Sometimes, more than one species (*S. gallinarum* and *S. pullorum*) may have the same antigenic formulae and cannot be distinguished serologically. Differentiation can be done by biochemical reactions (*S. gallinarum* is anaerogenic and ferments dulcitol unlike *S. pullorum*). Important pathogens can be further typed for epidemiological purposes by phage susceptibility, biochemical reactions, bacteriocin production and antibiogram.

I. Toxin

Like all other Gram negative bacilli, the cell walls of salmonellae contain lipopolysaccharide which acts as endotoxin.

J. Pathogenesis

S. typhi, *S. paratyphi A* and usually *S. paratyphi B* are confined to human beings. The majority of other salmonellae are primarily infective for animals and human beings are secondarily infected.

Salmonellae cause three types of clinical syndrome in human beings, enteric fever, septicaemia and gastroenteritis.

1. Enteric Fever

The term enteric fever includes typhoid fever (*S. typhi*) and paratyphoid fever (*S. paraptyphi A, B, C*). Infections due to *S. typhi* and *S. paratyphi A* are prevalent in India.

(i) Typhoid fever

The infection is acquired by ingestion through contaminated food and water. The incubation period is usually 7–14 days but appears to be related to the dose of infection. On reaching the small intestine the bacilli attach to the epithelial cells of the intestinal villi and penetrate to the lamina propria and submucosa. They are phagocytosed by neutrophils and macrophages. These bacteria resist intracellular killing and multiply within these cells. They enter the mesenteric lymph nodes, multiply there and, via the thoracic duct, enter the blood stream. A transient bacteraemia follows and internal organs like liver, gall bladder, spleen, bone marrow, lungs, lymph nodes and kidneys are infected. Towards the end of the incubation period, a massive bacteraemia occurs from these sites of multiplication and clinical disease sets in.

Table 33.3 Illustration of the Kauffmann-White Scheme.

O group		Serotype	O antigens*	H antigens	
New	Old			Phase 1	Phase 2
2	A	*S. paratyphi A*	1, **2**, 12	a	–
4	B	*S. paratyphi B*	1, **4**, 5, 12	b	1, 2
		S. typhimurium	1, **4**, 5, 12	i	1, 2
7	C1	*S. paratyphi C*	**6**, **7**, (Vi)	c	1, 5
		S. cholerae–suis	**6**, **7**	c	1, 5
8	C2	*S. muenchen*	**6**, **8**	d	1, 2
		S. newport	**6**, **8**, 20	e, h	1, 2
9	D	*S. typhi*	**9**, 12, (Vi)	d	–
		S. enteritidis	1, **9**, 12	g, m	–
		S. gallinarum	1, **9**, 12	–	–
		S. pullorum	1, **9**, 12	–	–
3, 10	E1	*S. anatum*	**3**, **10**	e, h	1, 6

Vi ⟶ V antigen *Number in bold type are the antigens characterising the O group

Salmonellae multiply abundantly in the gall bladder as bile is a good culture medium for the bacillus. These bacteria are discharged continuously into the intestine involving the Peyer's patches and lymphoid follicles of the ileum. These become inflamed, necrosed and slough off, leaving behind the typhoid ulcers. These ulcers may lead to two major complications—intestinal perforation and haemorrhage. During the course of the disease (3–4 weeks), the intestinal lesions undergo healing.

The clinical course may vary from a mild pyrexia to a fatal fulminating disease. The illness is usually gradual, with headache, anorexia and congestion of mucous membranes. The characteristic features are hepatosplenomegaly, step-ladder pyrexia with relative bradycardia and leucopaenia. Skin rashes known as rose-spots may appear during the second or third week. The infecting organisms appear in stool during second to third week and in urine during third to fourth week. 'Rose spots' appear on the skin during the second or third week.

(ii) Paratyphoid fever

Paratyphoid fever resembles typhoid fever but is milder. *S. paratyphi A, B and C* cause paratyphoid fever. *S. paratyphi C* more often leads to a frank septicaemia with suppurative complications.

Some other salmonellae have also been reported to cause enteric fever occasionally. These include *S. dublin, S. barielly, S. sendai, S. enteritidis, S. typhimurium, S. eastbourne, S. saintpaul, S. oranienburg* and *S. panama.*

2. Septicaemia

Salmonella septicaemia is commonly caused by *S. cholerae-suis* or *S. paratyphi C* and occasionally by other salmonellae. Infection occurs through oral route. There is early invasion of blood stream and it produces local suppuration in different organs. It may cause osteomyelitis, pneumonia, pulmonary abscess, meningitis or endocarditis.

3. Gastroenteritis

Salmonella gastroenteritis or food poisoning is caused by ingestion of food like meat, milk, egg contaminated by certain salmonellae which are primarily animal pathogens. Eggs and egg products are of great concern. Salmonellae can enter through the shell if eggs are left on faeces or contaminated feed of chicken. *S. typhimurium* is the most frequently isolated in food poisoning. The other common species responsible are *S. enteritidis, S. newport, S. senftenberg, S. dublin, S. heidelberg* and *S. indiana.*

The incubation period is 12 to 24 hours. The illness is characterised by fever, vomiting, abdominal pain and diarrhoea. Salmonella food poisoning is of infective type in which the organisms not only grow in the food before ingestion but also in the intestine. Generally, there is no bacteraemia.

K. Epidemiology

Enteric fever is endemic in all parts of India. Typhoid fever is commoner than paratyphoid fever. *S. paratyphi.A* is prevalent in India. *S. paratyphi B* is rare and C very rare. Enteric fever occurs at all ages but is probably most common in the 5–20 years age group.

The faeces of carriers are important source of contamination of food and drink rather than the frank clinical cases. Sewage contaminated by a carrier is responsible for polluting drinking water. Food material gets contaminated via polluted water or by the hands of carriers. Mary Mallon ('Typhoid Mary'), a cook in USA, was a famous carrier who caused several outbreaks over a period of 15 years.

L. Laboratory Diagnosis

Bacteriological diagnosis of enteric fever consists of

1. Isolation of bacilli
2. Demonstration of antibodies
3. Demonstration of circulating antigen
4. Other laboratory tests

1. Isolation of Bacilli

This may be done by culture of specimens like blood, faeces, urine, aspirated duodenal fluid etc. Selection of relevant specimen depends upon duration of illness which is very important for the laboratory diagnosis of enteric fever (Table 33.4, Fig. 33.3).

Table 33.4 Relevance of Examination of Different Specimens at Different Phases of Enteric Fever

Duration of disease	Specimen examination	% Positivity
1st Week	Blood culture	90
2nd Week	Blood culture	75
	Faeces culture	50
	Widal test	low titre
3rd Week	Widal test	80–100
	Blood culture	60
	Faeces culture	80

BLOOD CULTURE

Blood cultures are positive in approximately 90 per cent of cases in first week of fever, 75 per cent in the second week and 60 per cent in the third week. Positivity rate declines thereafter and blood cultures remain positive in 25 per cent of cases till the subsidence of pyrexia.

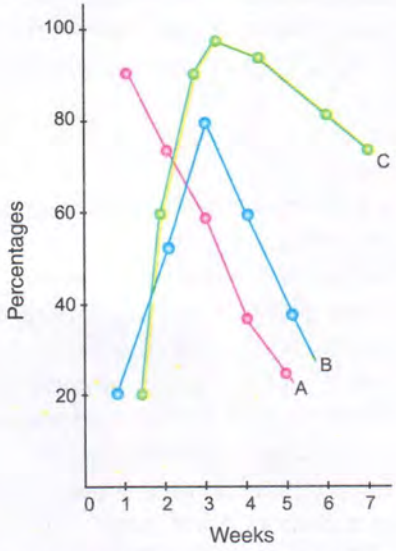


Fig. 33.3 Bacteriological diagnosis in typhoid fever. The approximate percentage of positivity during different weeks of disease A—Blood culture, B—Faeces culture C—Widal test.

10 ml of blood is collected by venepuncture under aseptic conditions and transferred into blood culture bottles (glucose broth and taurocholate broth). Before transferring blood into blood culture bottles, caps of these bottles should be thoroughly cleaned with spirit or alcohol. Blood should be transferred through a hole in a cap by inserting the needle of the syringe rather than opening the bottle, thus it avoids contamination from the external environment.

Blood contains substances that inhibit the growth of the bacilli and hence it is essential to dilute out these substances (5 ml blood into the 50 ml culture media, 1:10 dilution). Alternative is addition of liquoid (sodium polyanethol sulphonate) which counteracts the bactericidal action of these substances.

Both blood culture bottles are incubated at 37°C for overnight. The glucose broth is subcultured on blood agar and the taurocholate (bile) broth on MacConkey's agar. Taurocholate broth is inhibitory to Gram positive bacteria, therefore, Gram negative bacilli are selected out. Pale non-lactose fermenting (NLF) colonies appear on MacConkey's agar and are picked out for biochemical reactions and motility. Salmonellae will be Gram negative, motile bacilli and fermenting glucose, mannitol but not lactose or sucrose. *S. typhi* will be anaerogenic but paratyphoid bacilli will form acid and gas from carbohydrates (sugars). Final identification of the isolate is by slide agglutination with O and H antisera.

If salmonellae are not obtained from the first subculture from taurocholate broth, subcultures should be done every other day up to ten days before declaring the culture as negative. Sometimes, Castaneda's method of culture is practised instead of routine blood culture.

CLOT CULTURE

It is an alternative to blood culture. 5 ml of blood is withdrawn aseptically into a sterile container and allowed to clot. The serum is separated and used for the Widal test. The clot is broken up with a sterile glass rod and added to bile broth containing streptokinase (100 units/ml) which digests the clot causing its lysis and thereby the bacteria are released from the clot. Serum used for Widal test may be negative due to the early stage of the disease but it may be useful as baseline titre against which the results of tests performed later may be evaluated.

FAECES CULTURE

Salmonellae are shed in the faeces throughout the disease and even in convalescence, due to lesions present in the intestine. Hence, faecal cultures may be helpful in patients as well as for the detection of carriers. These are particularly valuable during antibiotic therapy when blood culture shows no growth of organisms. The antibiotic does not eliminate the bacilli from the gut as rapidly as it does from the blood. Faeces cultures are generally positive after the second week of illness.

As normal flora is present in faeces, successful culture will depend on use of enrichment and selective media. Faecal samples are inoculated into one tube each of selenite and tetrathionate broth (both enrichment media) and are also plated directly on MacConkey's agar, DCA, XLD and Wilson-Blair media. Salmonellae appear as pale yellow (NLF) colonies on MacConkey's agar and DCA media. On Wilson-Blair medium, *S. typhi* forms large black colonies with a metallic sheen whereas *S. paratyphi A* produces green colonies due to the absence of H_2S production.

Enrichment media (selenite F and tetrathionate broths) are incubated for 6–8 hours before subculture on to selective media such as MacConkey's agar and DCA. These selective media are then incubated at 37°C for overnight.

URINE CULTURE

Urine culture is less frequently positive than the culture of blood or faeces. Cultures are generally positive only in the second and third weeks. After third week, only about 25 per cent cases may be positive. Repeated cultures improve the chances of isolation.

Urine samples are centrifuged and the deposit is inoculated into enrichment and selective media.

OTHER SPECIMENS FOR CULTURE

Bone marrow culture is valuable as it is positive even when blood cultures are negative. Culture of bile is usually positive and may be useful in detection of chronic carriers. Other materials which may be used for culture are rose spot's discharge, pus from suppurative lesions, CSF and sputum. At autopsy, cultures may be done from the gall bladder, liver, spleen and mesenteric lymph nodes.

Colony Morphology and Staining

On MacConkey's agar or DCA, salmonellae grow as pale yellow, non-lactose fermenting (NLF) colonies. Gram staining from these colonies show Gram negative bacilli and on hanging drop preparation, these are motile bacilli.

Biochemical Reactions

Salmonellae are catalase positive, oxidase negative, nitrate reduction positive and ferment glucose, mannitol but not lactose or sucrose. *S. typhi* ferments glucose and mannitol with production of acid only but paratyphoid bacilli (*S. paratyphi A, B* and *C*) form acid and gas.

Slide Agglutination Test

A loopful of the growth from a nutrient agar slope is emulsified in two drops of saline on a microscopic slide. One emulsion acts as a control and other as a test. Control is to show that the strain is not autoagglutinable. Agglutination is first carried out with the polyvalent O and the polyvalent H antisera. Positive agglutination indicates that the isolate belongs to genus *Salmonella*. Further agglutination tests are done with single factor sera for determining the O and H antigens. If *S. typhi* is suspected (i.e. when no gas is formed from glucose and mannitol), agglutination with O antiserum (factor 9) is done. Prompt agglutination indicates that the isolate belongs to group-D salmonella. Its identity as *S. typhi* is established by agglutination with H antiserum (anti–d serum). Sometimes fresh isolates of *S. typhi* are in V form and do not agglutinate with O antiserum. Such strains should be agglutinated with anti–Vi serum. Alternatively, the bacterial suspension in saline is boiled for 20 minutes which removes the Vi antigens from the organism. The boiled bacterial suspension is then tested with poly O–antiserum.

When the isolate is a non-typhoid salmonella producing gas from carbohydrates, it is tested for agglutination with O and H antisera for groups A, B and C. For identification of unusual serotypes, the help of the National Salmonella Reference Centre at the Central Research Institute (CRI) Kasauli, may be sought. The reference centre for salmonellae of animal origin is located at the Indian Veterinary Research Institute, Izatnagar.

2. Demonstration of Antibodies

WIDAL TEST

It is an agglutination test for detection of agglutinins (H and O) in patients with enteric fever. Salmonella antibodies start appearing in the serum at the end of first week (Fig. 33.3) and rise sharply during the third week of enteric fever. Two specimens of sera at an interval of 7 to 10 days are preferred to demonstrate a rising antibody titre.

Procedure

Two types of tubes were originally used for the test—Dreyer's tube (narrow tube with a conical bottom) for the H agglutination and Felix tube (short, round bottomed tube), for the O agglutination. Equal volumes (0.4 ml) of serial dilutions of the serum (1:10 to 1:640) and the H and O antigens are mixed and incubated in a water bath at 37°C for 4 hours and read after overnight refrigeration at 4°C. Control tubes containing the antigen and normal saline are included to check for autoagglutination. H agglutination leads to the formation of loose, cottonwool clumps, while O agglutination appears as a granular deposit at the bottom of the tube. The highest dilution of the serum showing agglutination (carpet formation) indicates the antibody titre against that particular antigen (Fig. 33.4). Control tubes show a compact deposit (button formation).

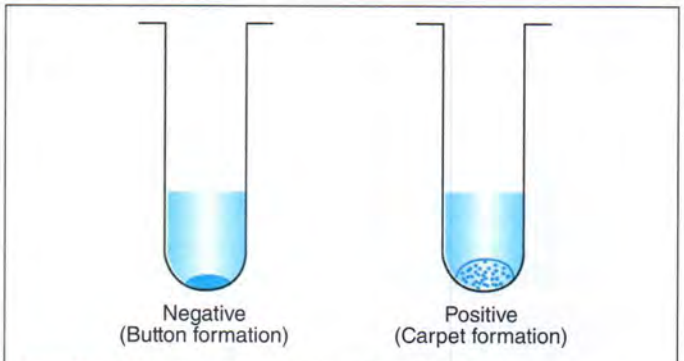


Fig. 33.4 Widal test (Negative and Positive tubes)

The antigens used in the test are the H and O antigens of *S. typhi* (TH and TO antigens) and H antigens of *S. paratyphi A* (AH antigen) and *S. paratyphi B* (BH antigen). The paratyphoid O antigens are not employed as they cross react with *S. typhi* O antigen (TO) due to their sharing of factor 12.

Preparation of widal antigen

The H agglutinable suspension of bacteria is prepared by adding 0.1 per cent formalin to a 24 hour broth culture or saline suspension of an agar culture. For preparation of O suspension of bacteria, the bacillus is cultured on phenol agar (1:800). The growth is scraped off in a small volume

of saline and mixed with 20 times its volume of absolute alcohol. It is then heated in a water bath at 40°C–50°C for 30 minutes, centrifuged and the deposit resuspended in saline to the appropriate density. Chloroform is then added as a preservative.

S. typhi 901, O and H strains, are used for preparation of antigens. Each batch of prepared antigen should be compared with a standard. Widal kits of stained antigens are available commercially.

Result

The highest dilution (titre) of patient's serum in which agglutination occurs is noted i.e. if the dilution is 1 in 160, the titre is 160.

Interpretation of widal test

(a) The agglutinin (antibody) titre depends on the stage of the disease. Agglutinins usually start appearing in the serum by the end of the first week, so that blood specimen taken earlier than first week may give a negative result. The titre rises steadily till the third or fourth week after which it declines.

(b) Rising titre: Demonstration of a four-fold or greater rise in titre of both H and O antibodies at an interval of 7–10 days is more meaningful and diagnostic than a single test.

(c) Single test: O titre of 1:100 or more and H titre of 1:200 or more signifies presence of active infection, but that has to be interpreted with caution considering the following factors:

- Local titre: In endemic area, low titre of agglutinins is present in the serum of normal persons. This normal titre differs from place to place. Local titre of that place should be known before interpreting the results of a single test.
- Immunisation: In immunisation with TAB vaccine, individuals may show high titres of antibody to *S. typhi, S. paratyphi A* and *B*, while in case of infection, antibodies will be seen only against the infecting organism.
- H–agglutination: In non-immunised individuals, presence of H agglutinin in serum indicates enteric fever or a latent infection. H agglutinin is more reliable than O agglutinin as O antigen is common in all the salmonellae.
- Anamnestic reaction: Persons who have had past enteric infection or immunisation may develop anamnestic reaction during unrelated fever like malaria etc. In such cases, a transient rise in H antibody occurs, whereas the rise is sustained in enteric fever. This may be differentiated by repeating the widal test after a week.
- Fimbrial antigens: Bacterial suspensions used as antigens may contain fimbrial antigen which may produce false positive results.
- Effect of treatment: Cases treated early with chloramphenicol may show a poor antibody response; if the antibody is already present, no further rise in titre is expected.
- Carriers: Test may be positive in many healthy carriers.

OTHER SEROLOGICAL TESTS

ELISA is a sensitive method of measuring antibody against the lipopolysaccharide of salmonellae. Indirect haemagglutination test and CIEP are other serological methods of diagnosis.

Detection of porins, the outer membrane proteins of *S. typhi*, by ELISA method is useful for early serodiagnosis of typhoid fever.

3. Demonstration of Circulating Antigen

Typhoid bacilli antigens are present in the blood in the early phase of the disease, and also in the urine of patients. The antigen can be detected by coagglutination test. *Staph. aureus* (Cowan I strain) containing protein A, is first stabilised with formaldehyde and then coated with *S. typhi* antibody. These sensitised staphylococcal cells (1% suspension) are mixed on slide with patient serum. The typhoid antigen present in the serum combines with the antibody coated on staphylococci producing visible agglutination within two minutes. The test is rapid, sensitive and specific, but is positive only during the first week of the disease.

Counterimmunoelectrophoresis (CIEP) and ELISA have also been employed to detect typhoid antigens in blood and urine.

4. Other Laboratory Tests

(i) Total leucocyte count (TLC)

Leucopaenia with a relative lymphocytosis is found.

(ii) Diazo test in urine

This test becomes positive generally between 5th and 14th day of fever and remains positive till the fever subsides.

Procedure

Equal volumes of patient's urine and the diazo reagent are mixed and a few drops of 30% ammonium hydroxide are added. On shaking the mixture, a red or pink froth develops, if the test is positive.

Diazo reagent

Solution A — Sulphanilic acid
Conc. H_2SO_4
Distilled water

Solution B — Sodium nitrite
Distilled Water

For use, 40 parts of solution A are added to one part of solution B.

DIAGNOSIS OF CARRIERS

Carriers are important sources of infection and their detection is necessary for epidemiological and public health purposes. Laboratory methods are also very useful in screening food handlers for detecting carrier state.

Even after clinical recovery, about 2 to 3% patients in convalescence phase (convalescent carriers) and some persons with subclinical infection (healthy carriers) continue to excrete the bacilli in faeces for about two months to a year. Chronic carriers harbour the bacilli in gall bladder or rarely in intestine or urinary tract. Most of these carriers continue excreting organisms after one year of convalescence and may continue to excrete the bacilli for several years or throughout life. In most of these cases, there is intermittent shedding of bacilli in stool, therefore, repeated stool cultures may be helpful to detect the bacilli. Cholagogue purgatives increase the chance of isolation from stool specimens. Other useful specimens for culture are bile and duodenal drainage. For the detection of urinary carriers, repeated urine cultures should be done.

Widal test is of no value in detection of carriers in endemic countries like India. Antibody to Vi antigen in serum is claimed to indicate the carrier state. This may be used for mass screening, however, confirmation should always be made by culture.

'Sewer-swab' technique is used for the tracing of carriers for epidemiological purposes. Gauze pads left in sewers and drains are cultured and a positive swab may lead to a house harbouring a carrier. Organism isolated from carrier is to be phage typed to establish the carrier as a cause of an epidemic.

Another method of isolation is filtration of sewage through millipore membranes and culturing the membranes on highly selective medium e.g. Wilson and Blair medium.

M. Bacteriophage Typing

For epidemiological purposes, strains may be differentiated into a number of phage types. *Craigie and Yen* (1937) originally observed that a bacteriophage (Vi phage II) was acting on Vi antigen of *S. typhi*. The parent phage is known as phage A. It could be made specific for a particular strain of *S. typhi* by serial propagation in that particular strain. As phage typing of *S. typhi* depends on the presence of Vi antigens, some Vi negative strains will be untypable. A phage type is stable. At present, 97 Vi II phage types of *S. typhi* are known, which are designated by letter or number. Phage types A and E1 are the most common in India. However, prevalence in different regions is subject to change from time to time. Phage typing is carried out at National Phage Typing Centres. The National Salmonella Phage Typing Centre for India is situated at the Lady Hardinge Medical College, New Delhi.

Bacteriophage typing schemes for *S. paratyphi A, S.paratyphi.B, S. typhimurium, S. enteritidis, S. dublin* have also been developed. The predominant phage types of *S. paratyphi A* isolated from India belong to types 1 and 2.

N. Biotyping

The serotypes can be subdivided into biotypes based on fermentation of xylose and arabinose.

O. Plasmid Typing

Plasmids present in salmonellae are separated electrophoretically and analysed.

P. Treatment

Chloramphenicol has been the antibiotic of choice for enteric fever. Amoxycillin, ampicillin, furazolidone and cotrimoxazole are also effective. Resistance to chloramphenicol and other antibiotics has been reported. Fluoroquinolones (ciprofloxacin, pefloxacin, ofloxacin) and the third generation cephalosporins (ceftazidime, cefotaxime, ceftriaxone cefoperazone) are useful in such multiresistant cases.

Antibacterial therapy has been very effective in the treatment of patients but it has been disappointing in the treatment of carriers. A prolonged course of antibacterial agents along with the vaccine has been tried in the eradication of carrier state. Cholecystectomy is indicated for chronic carriers who do not show any response to antibacterials.

Q. Drug Resistance

Resistance to chloramphenicol became a problem in 1972, when resistant strains emerged in Mexico and in Kerala (India). In Mexico, the resistant strain caused an explosive epidemic.

In India, chloramphenicol resistant typhoid fever appeared as epidemic in Calicut (Kerala) in 1972. It became endemic and remain confined to Kerala till 1978. Subsequently, resistant *S. typhi* strains were reported in

many other parts of India. Resistance was originally confined to phage type D1-N, but later to types C5, A and O. Resistance is due to a transmissible plasmid carrying r determinants to chloramphenicol, streptomycin, sulphonamide and tetracycline.

Multiple drug resistant *S. typhi* is increasingly being recognised in many countries and has become a problem in India, too. R factors carrying multiple drug resistance have become widely disseminated among salmonellae. These may cause nosocomial salmonellosis particularly in neonates as septicaemia, meningitis and suppurative lesions. In India, hospital outbreaks of neonatal septicaemia caused by multiple drug resistant salmonellae have occurred in recent years. Mortality in neonates is very high unless early treatment is started with antibiotics.

R. Prophylaxis

1. General Measures

Typhoid fever can be effectively controlled by sanitary measures for disposal of sewage, clean water supply and supervision of food processing and handling. Infected meats and eggs should be thoroughly cooked.

2. Carriers

Carriers should not be engaged in food preparation and should observe strict personal hygiene.

3. Vaccination

Vaccine is indicated for travellers or who live in endemic areas.

(i) TAB vaccine

It is heat-killed whole cell vaccine which contains *S. typhi.* 1,000 millions, *S. paratyphi A* and *B*, 750 millions each per ml and preserved in 0.5 per cent phenol.

Dose schedule: The vaccine is given subcutaneously in two doses of 0.5 ml at an interval of 4–6 weeks followed by booster every three years.

Protection: It varies from 3–7 years with an efficacy of 60–80%.

Side Effects: Local discomfort and fever may occur for one or two days.

In India, a divalent vaccine containing *S. typhi* and *S. paratyphi A* are now in use. In Europe and USA, monovalent vaccine containing *S. typhi* is employed as paratyphoid A and B infections are rare in these countries.

(ii) Live oral (Ty 21a) typhoid vaccine

Avirulent mutant strain of *S. typhi* (Ty 21a) lacking the enzyme UDP–galactose–4–epimerase (Gal E mutant) has been used as a live oral vaccine. These mutants initiate infection in the intestine but 'self- destructs' after four to five cell divisions and therefore cannot induce any illness.

Dose schedule: Three doses are given on alternate day. The oral vaccine (*typhoral*) is available in capsule form containing 10^9 viable lyophilised mutant bacilli.

Protection: It gives 65–96% protection for 3–5 years and is safe.

(iii) Purified Vi polysaccharide vaccine (typhim–Vi)

It contains purified Vi antigen.

Dose schedule: It is injected intramuscularly in a single dose of 25 μg. The efficacy is about 75%.

II. SALMONELLA GASTROENTERITIS

Salmonella gastroenteritis or food poisoning is generally a zoonotic disease, therefore the source of infection is animal product. All salmonellae can cause the disease except *S. typhi.* A large number of salmonellae have been identified from cases of gastroenteritis and food poisoning. *S. typhimurium* is the commonest species. Other common species include *S. enteritidis*, *S. heidelberg*, *S. indiana*, *S. newport*, *S. haldar*, *S. agona*, *S. senftenberg*, *S. virchow* and *S. anatum*.

A. Source of Infection

The most important and frequent sources are poultry, meat, milk, cream and eggs. It is due to ingestion of contaminated food. Food contamination may result from droppings of small animals like rats or lizards. Salmonellae can enter through the shell of egg and grow inside. It happens when eggs are left on contaminated chicken feed or faeces. Human carriers do occur but their role is minimal. Cross infections in hospitals may lead to gastroenteritis without food poisoning.

B. Clinical Features

It presents with diarrhoea, vomiting, abdominal pain and fever. It has a short incubation period of 24 hours or less. Disease usually subsides in 2–4 days, but in some, a prolonged enteritis develops.

C. Laboratory Diagnosis

It depends on isolation of salmonellae from faeces and from food.

D. Control

It requires the prevention of food contamination. It may be achieved by proper cooking of food and control of natural infection in animals.

E. Treatment

Symptomatic treatment should be given. Antibiotics should not be used in uncomplicated, non-invasive salmonellosis. They may not hasten recovery but may actually increase the period of faecal shedding of the bacilli. But for serious invasive cases, antibiotics are needed.

III. SALMONELLA SEPTICAEMIA

Certain salmonellae may cause septicaemia with focal suppurative lesions, such as deep abscesses, osteomyelitis, pneumonia, endocarditis and meningitis. Among all salmonellae, *S. cholerae-suis* is particularly very important causative agent of salmonella septicaemia. It is a fulminating blood infection. Infection occurs by oral route.

Salmonellae may be isolated from the blood or from the pus from the suppurative lesion. Rarely, faeces culture may be positive for salmonellae. These septicaemic cases should be treated with chloramphenicol or appropriate antibiotics as determined by susceptibility tests.

KEY POINTS

1. Salmonellae are Gram negative, motile bacilli. They grow on ordinary culture media. On MacConkey's agar and deoxycholate citrate agar (DCA), colonies are colourless due to non-lactose fermentation (NLF).
2. On Wilson and Blair bismuth sulphide medium (*selective medium for salmonellae*), *jet black colonies with metallic sheen* are formed due to formation of hydrogen sulphide. *S. paratyphi A* and other species which do not form H_2S produce green colonies.
3. Xylose lysine deoxycholate (XLD) agar is another medium for isolation of this organism. Most strains of salmonellae produce red colonies with black centres, when grown on this medium. H_2S negative serotypes of Salmonella produce red colonies without black centres.
4. Selenite F broth and tetrathionate broth (TTB) are commonly used enrichment media for inoculation of specimens especially faeces.
5. Salmonellae ferment *glucose* and *mannitol* forming acid and gas except *S. typhi* which produces *only acid* and *no gas*.
6. Most salmonellae produce H_2S in triple sugar iron (TSI) agar except *S. paratyphi A* and *S. cholerae-suis*. They utilise citrate except *S. typhi* and *S. paratyphi A*.
7. Salmonellae possess three types of antigens namely flagellar antigen 'H', somatic antigen 'O', and surface antigen 'Vi' (found in some species).
8. The antigens of salmonellae undergo several types of phenotypic and genotypic variations.
9. Modern taxonomical techniques have shown that all the members of the genus *Salmonellae* belong to two species *S. enterica* and *S. bongori*. *S. enterica* is classified into six subspecies namely *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae* and *indica*.
10. *Kauffmann-white scheme* forms the basis of serotyping of salmonellae based on identification of O and H antigens by agglutination.
11. Salmonellae cause three types of clinical syndrome in human beings, *enteric fever*, *septicaemia* and *gastroenteritis*.
12. The term enteric fever includes typhoid fever (*S. typhi*) and paratyphoid fever (*S. paratyphi A, B, C*). Infections due to *S. typhi* and *S. paratypi A* are prevalent in India.
13. Salmonella septicaemia is commonly caused by *S. cholerae-suis* or *S. paratyphi C* and occasionally by other salmonellae.
14. *S. typhimurium* is the most frequently isolated in salmonella food poisoning. The other common species responsible are *S. enteritidis*, *S. newport*, *S. senftenberg*, *S. dublin*, *S. heidelberg* and *S. indiana*.
15. The faeces of carriers are important source of contamination of food and drink. Mary Mallon ('*Typhoid Mary*'), a cook in USA, was a famous carrier who caused several outbreaks over a period of 15 years.
16. Laboratory diagnosis of enteric fever consists of *isolation of bacilli*, *demonstration of antibodies*, and *demonstration of circulating antigen*.

17. Isolation of bacilli may be done by culture of specimens like *blood, faeces, urine, aspirated duodenal fluid* etc. Selection of relevant specimen depends upon duration of illness which is very important for laboratory diagnosis of enteric fever.
18. Blood culture is very important specimen in first week of enteric fever.
19. Faeces culture may be helpful in diagnosis of enteric fever as well as for the detection of carriers.
20. Widal test is an *agglutination test* for detection of *antibodies* in patients with enteric fever. Salmonella antibodies start appearing in the serum at the *end of first week*.
21. Multiple drug resistant *S. typhi* is increasingly being recognised in many countries and has become a problem in India too.
22. TAB vaccine is available for prophylaxis. It is *heat killed whole cell vaccine* which contains *S. typhi* 1,000 millions, *S. paratyphi A and B*, 750 millions each per ml.
23. Live oral (Ty 21a) typhoid vaccine is another vaccine available.

YOU MUST KNOW

1. Morphology, culture characteristics and biochemical reactions of *Salmonella sp.*
2. Antigenic structure and antigenic variations of Salmonella.
3. Kauffmann–White scheme.
4. Diseases caused by *Salmonella sp.*
5. Laboratory diagnosis of enteric fever.
6. Vaccination for prevention of enteric fever.

STUDY QUESTIONS

1. Describe in detail the laboratory diagnosis of enteric fever.
2. Write briefly about:
   (a) Vi-antigen
   (b) Clot culture
   (c) Kauffmann-White scheme
   (d) Antigenic variations in Salmonella
   (e) Widal test
   (f) Salmonella septicaemia
   (g) Salmonella gastroenteritis
   (h) Vaccination against enteric fever

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Which of the following disease/s is/are produced by *Salmonella*?
   (a) Enteric fever (b) Gastroenteritis (c) Septicaemia (d) All of the above
2. Which of the following culture media is/are used as selective media for *Salmonella*?
   (a) Wilson and Blair bismuth sulphite medium (b) Bile salt agar
   (c) Potassium tellurite blood agar (d) None of the above
3. Which of the following *Salmonella* ferments glucose with acid but no gas?
   (a) *S. typhi* (b) *S. paratyphi A* (c) *S. paratyphi B* (d) *S. cholerae-suis*
4. Which of the following *Salmonella* does/do not produce H_2S in triple sugar iron (TSI) agar?
   (a) *Salmonella paratyphi A* (b) *S. cholera-suis*
   (c) Both of the above (d) None of the above
5. Which of the following *Salmonella* does/do not utilise citrate?
   (a) *S. typhi* (b) *S. paratyphi A* (c) Both of the above (d) None of the above
6. Which of the following *Salmonella* possess flagellar antigen in one phase (monophasic)?
   (a) *S. typhi* (b) *S. paratyphi A* (c) *S. enteritidis* (d) All of the above
7. Vi antigen is present in the following bacteria:
   (a) *S. typhi* (b) *S. paratyphi C* (c) *S. dublin* (d) All of the above

8. Which of the following *Salmonella* is/are non-motile?
   (a) *Salmonella pullorum* (b) *S. paratyphi B* (c) *S. typhimurium* (d) All of the above
9. Which of the following *Salmonella* is/are primarily infective to human beings?
   (a) *S. typhi* (b) *S. paratyphi A* (c) *S. paratyphi B* (d) All of the above
10. Bacteriological diagnosis of enteric fever consists of:
   (a) Isolation of bacilli (b) Demonstration of antibodies
   (c) Demonstration of antigen (d) All of the above
11. Which of the following *Salmonella* is/are responsible for food poisoning?
   (a) *Salmonella typhimurium* (b) *S. enteritidis*
   (c) *S. senftenberg* (d) All of the above
12. Enteric fever can be diagnosed by isolation of *Salmonella typhi* from:
   (a) Blood (b) Faeces (c) Urine (d) All of the above
13. The most important specimen for isolation of *Salmonella typhi* in first week of enteric fever is:
   (a) Blood (b) Faeces (c) Urine (d) Pus
14. What type of test is Widal test?
   (a) Slide agglutination test (b) Tube agglutination test
   (c) Co-agglutination test (d) Latex agglutination test
15. Which of the following specimens is the most important for diagnosis of carriers in enteric fever?
   (a) Blood (b) Faeces (c) Urine (d) Pus
16. Which of the following vaccines can be used for prevention of enteric fever?
   (a) TAB vaccine (b) Live oral vaccine
   (c) Purified Vi polysaccharide vaccine (d) All of the above

Answers (MCQs):

1. (d) 2. (a) 3. (a) 4. (c) 5. (c) 6. (d) 7. (d) 8. (a) 9. (d) 10. (d)
11. (d) 12. (d) 13. (a) 14. (b) 15. (b) 16. (d)

Chapter 34

VIBRIO

Vibrios are Gram negative, oxidase positive, short, rigid, curved rods that are actively motile by a polar flagellum. The name 'vibrio' is derived from the characteristic vibratory motility of the bacilli (*Vibrare* meaning to vibrate). The most important member of the genus is *Vibrio cholerae*. It is the causative agent of cholera. It was first observed by *Pacini* (1854) and later on *Koch* (1883) isolated the bacillus from cholera patients.

I. CLASSIFICATION

Vibrios are grouped under family *Vibrionaceae* which initially contained three genera, *Vibrio, Aeromonas* and *Plesiomonas;* now a days family *Vibrionaceae* contains single genus *Vibrio*. *Aeromonas* and *Plesiomonas* belong to separate families, *Aeromonadaceae* and *Enterobacteriaceae* respectively. Vibrio species are susceptible to the compound O/129 (2, 4,-diamino-6, 7 diisopropylpteridine phosphate) at strength of 150 µg, which differentiates them from aeromonas species which is resistant.

Genus *Vibrio* has about 33 species and the important pathogens of man include:

1. Non-Halophilic Vibrios

These vibrios may grow in media without salt (NaCl).

(*i*) *V. cholerae*: O1 classical and El Tor biotype

(*ii*) *Non O1 V. cholerae*: Non-cholera vibrio (NCV) or non-agglutinating vibrio (NAG) (For details refer page 280)

V. mimicus is a non-halophilic vibrio which is similar to *V. cholerae* except that it is sucrose negative.

2. Halophilic Vibrios

They cannot grow in media without addition of salt (NaCl).

(*i*) *V. parahaemolyticus*
(*ii*) *V. alginolyticus*
(*iii*) *V. vulnificus*

Heiberg (1934) classified vibrios on the basis of fermentation of mannose, sucrose and arabinose. There are eight groups (I to VIII). Cholera vibrios belong to Group I, fermenting mannose and sucrose with acid production but not the arabinose.

Gardner and Venkatraman (1935) classified vibrios based on antigenic structure. Details of this classification have been described under heading 'Antigenic structure' of this chapter.

Modern taxonomical criteria, particularly DNA studies, have recognised that all the cholera vibrios that belong to Group A of Gardner and Venkatraman's classification, constitute a single species *Vibrio cholerae*. This can be further classified into serogroups (or serovars), biotypes and serotypes. The present nomenclature of *V. cholerae* strain may be written as *V. cholerae* serovar O1, biotype Eltor, serotype Ogawa.

Phage typing can further classify these strains. Typing of strains can also be done by molecular methods like ribotyping.

II. VIBRIO CHOLERAE

A. Morphology

V. cholerae is a Gram negative, curved or comma-shaped rod, non-sporing, non-capsulated, about 1.5 μm × 0.2–0.4 μm in size (Fig. 34.1).

The organism is very actively motile with a single polar flagellum and movement is named as *darting motility*. Because of its typical *comma shaped* appearance, it is also named *Vibrio comma*. S shaped or spiral forms may be seen due to end to end attachment of two or more cells. In old cultures, they are frequently pleomorphic.

In stained mucous flakes of cholera cases, the vibrios are arranged in parallel rows, described by Koch as the *fish in stream* appearance.

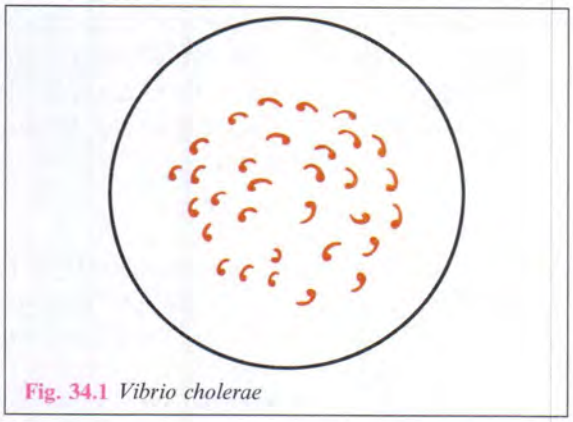


Fig. 34.1 *Vibrio cholerae*

B. Culture

V. cholerae is strongly aerobic, growth being scanty and slow anaerobically. It grows within a temperature range of 16°–40°C but optimum temperature is 37°C. It grows best in alkaline media, the optimum pH being 8.2 (pH range 7.4–9.6). The organism is extremely sensitive to an acidic pH which kills it. *V. cholerae* is a non-halophilic vibrio, therefore, cannot grow in media with a concentration of sodium chloride more than 7%. However it can grow in media without sodium chloride.

1. Ordinary Media

(i) Nutrient agar

After overnight incubation, the colonies are moist, translucent, round disks, 1–2 mm in diameter, with a bluish tinge in transmitted light.

(ii) MacConkey's agar

The colonies are colourless or pale at first, but become reddish or pink on prolonged incubation due to late fermentation of lactose.

(iii) Blood agar

V. cholerae, classical biotype, does not produce hemolysis although some strains produce greenish discolouration around colonies which later becomes clear due to haemodigestion. However, colonies of El Tor biotype produce haemolysis on blood agar.

(iv) Peptone water

It grows as a surface pellicle because of its aerobic nature.

(v) Gelatin stab culture

At first a white line of growth appears along the track of inoculation. Gelatin liquefaction begins at the top which spreads downward in a funnel shaped form (infundibuliform or napiform) in 4 days at 22°C.

2. Special Media

The special media are classified as follows:

(*i*) Transport or holding media
(*ii*) Enrichment media
(*iii*) Plating media

TRANSPORT OR HOLDING MEDIA

Venkatraman-Ramakrishnan (VR) medium

It contains 20 gm common salt and 5 gm peptone in one litre of distilled water and pH is adjusted to 8.6–8.8. It is dispensed in screw-capped bottles in 10–15 ml amount. About 1–3 ml faeces is added to each bottle. Vibrios do not multiply, but remain viable for several weeks.

Cary-Blair medium

This medium is prepared by adding disodium phosphate, sodium thioglycollate, sodium chloride and calcium chloride and pH is adjusted at 8.4. It is a suitable transport medium for *Salmonella, Shigella* and *Vibrio*.

If a transport medium is not available, a strip of thick blotting paper is soaked in the faecal matter, then placed in a sealed plastic bag, and sent to the laboratory.

ENRICHMENT MEDIA

Alkaline paptone water (APW)

It is a peptone water at pH 8.6. Besides enrichment medium, it is also an excellent transport medium.

Monsur's taurocholate tellurite peptone water

It contains peptone, sodium chloride, sodium taurocholate in one litre of distilled water and pH is adjusted at 9.2. To this medium, sterile potassium tellurite solution is added to give a final concentration of 1: 200,000. Like APW, it is not only a good enrichment medium but is transport medium as well.

PLATING MEDIA

Alkaline bile salt agar (BSA); pH 8.2

It is modified nutrient agar medium containing 0.5% sodium taurocholate (bile salt). The colonies are similar to those on nutrient agar.

Monsur's gelatin taurocholate trypticase tellurite agar (GTTA) medium; pH 8.5

The colonies are small, translucent with a greyish black centre and a turbid halo around the colonies due to hydrolysis of gelatin. The colonies become 3–4 mm in size after 48 hour incubation.

Thiosulphate citrate bile sucrose (TCBS) agar; pH 8.6

It is the most widely used selective medium for isolation of vibrios. It contains sodium thiosulphate, sodium citrate, bile salts, sucrose, bromothymol blue (indicator), yeast extract, peptone, sodium chloride, ferric citrate and water. *Vibrio cholerae* forms yellow colonies due to sucrose fermentation, while non-sucrose fermenters such as *V. parahaemolyticus* produces green colonies. The colonies of *V. cholerae* are large, yellow, convex and turn green on continued incubation.

C. Biochemical Reactions

Carbohydrate breakdown is fermentative, producing acid, but no gas. It is catalase and oxidase positive. It ferments glucose, mannitol, sucrose, maltose and mannose, but not lactose, though lactose may be split very slowly. It is indole positive and reduces nitrates to nitrites. These two properties contribute to the *cholera red reaction* which is tested by adding a few drops of concentrated sulphuric acid to peptone water culture. In case of *Vibrio cholerae*, a reddish pink colour is developed due to the formation of nitrosoindole. Other indole producing organisms can also reduce nitrates and thus give this reaction positive e.g. *Esch. coli*. It is methyl red (MR) and urease negative. Gelatin is liquefied. It decarboxylates lysine and ornithine but do not utilise arginine. Voges-Proskauer (VP) reaction and haemolysis of sheep erythrocytes are positive in ELTor biotype and both these tests are negative in classical biotype. Important diagnostic biochemical tests of classical biotype are summarised as follows:

Catalase	Oxidase	Glucose	Lactose	Mannitol
+	+	A	–	+
Sucrose	Maltose	Mannose	Indole	NO_3 reduction
+	+	+	+	+
Urease	Gelatin	MR	VP*	
–	+	–	–	
Lysine	Ornithine	Arginine	Sheep RBCs haemolysis*	
+	+	–	–	

*In case of El Tor biotypes, all biochemical reactions are similar but VP and sheep RBCs haemolysis are positive.

D. Resistance

Vibrio chloerae is susceptible to heat and drying. It is killed at 55°C in 15 minutes. It is sensitive to common disinfectants and a pH less than 5. It survives for months in sea water. In grossly contaminated water, such as the Ganges in India, the vibrios do not survive for any length of time perhaps due to the presence of large number of vibriophages in this water. ELTor vibrios are hardier and survive for longer duration than the classical biotypes. On fruits, they survive for 1–5 days at room temperature and for a week in the refrigerator.

E. Antigenic Structure

V. cholerae contains somatic 'O' and flagellar 'H' antigens. The 'H' antigen is shared by all the strains. *Gardner* and *Venkatraman* (1935) classified vibrios based on antigenic structure (Fig. 34.2).

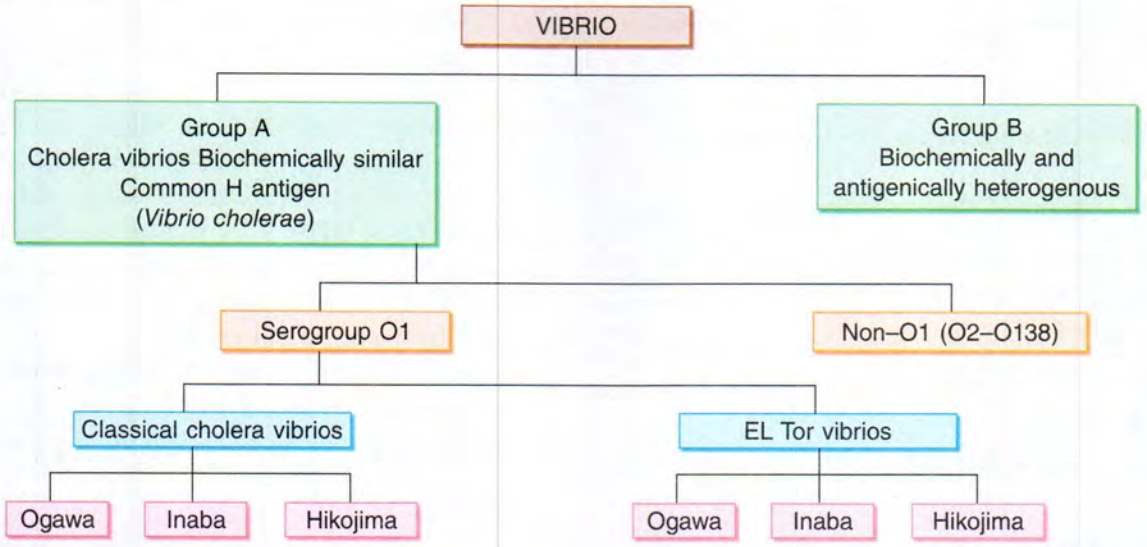


Fig. 34.2 Antigenic classification of vibrios (Gardner and Venkatraman, updated)

Group A vibrios have a common H antigen but distinct 'O' component. On the basis of O antigen, it is divided into subgroups (now named O serogroups or serovars). Both classical and ElTor biotypes belong to serogroups O1 and are antigenically indistinguishable. These are referred as *V. cholerae O1*. On the basis of minor O antigens (A, B, C), *V. cholerae* O1 are subdivided into three serotypes—Ogawa (AB), Inaba (AC) and Hikojima (ABC) (Table 34.1).

Table 34.1 Serotypes of Cholera Vibrios

Serotype	O antigen
Ogawa	AB
Inaba	AC
Hikojima	ABC

At least 139 (O1 to O139) O serogroups are recognised. Serogroups O2 to O138 are called non-O1 *V. cholerae*. Since these organisms were not agglutinated by O-1 antiserum, they were called non-agglutinating (NAG) vibrios. Though NAG vibrios are not agglutinable by O-1 antiserum, they are agglutinated by their specific homologous antisera.

F. Toxins

Besides heat stable *endotoxin, V. cholerae* also produces *exotoxin* (enterotoxin). The enterotoxin is also named as *cholera toxin* (CT), choleragen or cholera enterotoxin. Both *V. cholerae* O1 and O139 produce cholera toxin.

Enterotoxin

It is a heat labile, protein in nature. It has a molecular weight of 90,000. It has two fractions: one A (*active*) subunit and five B (*binding*) subunits. The cholera toxin (CT) resembles heat-labile toxin (LT) of *Esch. coli* chemically, antigenically and in its mode of action. CT is more potent than LT of *Esch. coli*. CT production is determined by phage integrated with bacterial chromosome while LT production is plasmid mediated.

Mechanism of action

The B subunit binds specifically to the Gm_1 ganglioside receptor on the intestinal epithelial cell. The active A subunit then enters the cell and is cleaved to its fragments, A1 and A2. The A2 fragment links the active A1 to the B subunit and thus the A1 fragment stimulates adenyl cyclase activity of the epithelial cell. This in turn, converts ATP into cAMP. The cAMP concentration within the cells causes (i) hypersecretion of water and electrolytes (Na, K, HCO_3^-) within the intestinal lumen and (ii) inhibition of reabsorption of sodium and chloride by cells, which results in purging diarrhoea of *rice water stool*. The fluid secreted is isotonic with plasma but contains more of potassium and bicarbonate.

The enterotoxins of *V. cholerae* and NAG vibrios appear to be identical in antigenic specificity.

ELTOR VIBRIOS

Gotschlich isolated a vibrio from six Haj pilgrims at the Tor quarantine station in Arabia and the organism was called *ElTor Vibrio*. Antigenically it is identical to classical

cholera vibrio and is now proved to be biotype of *V. cholerae–O1*. The following tests are used for identification of El Tor biotype (Table 34.2).

Table 34.2 Differentiating Features of Classical and El Tor Vibrios

Test	Classical	El Tor
Haemolysis of sheep erythrocytes	–	+
Agglutination of chick erythrocytes	–	+
Voges-Proskauer (VP) reaction	–	+
Polymyxin B sensitivity	+	–
Susceptibility to Mukherjee Phage IV	+	–
Modified CAMP test	–	+

1. Chick Red Cell Agglutination Test

A loopful of the organisms from an agar cultures is emulsified in a drop of saline on a slide and a drop of 2.5% chick erythrocyte suspension is added. Clumping of erythrocytes within a minute indicates a positive test. The test is positive with all El Tor strains but classical cholera vibrios are negative.

2. Sensitivity to Polymyxin B

The organism is tested by the disc diffusion method using discs containing 50 units of polymyxin B. All strains of classical cholera vibrio are sensitive and all strains of El Tor vibrio are resistant.

3. Sensitivity to Cholera Phage IV

All strains of classical cholera vibrio are lysed by Mukherjee's group IV phage routine test dilution (RTD), while all El Tor strains are not lysed. This is considered to be the most dependable test for differentiating between El Tor and classical strains.

4. Modified CAMP Test

This test is similar to the CAMP test for identification of group B streptococci. It is performed by inoculating a beta-lysin producing *Staph. aureus* strain onto a blood agar plate by making a single straight line streak and then inoculating the vibrio species to be tested in a line prependicular to it. The test is positive with El Tor strains but classical cholera vibrios are negative. However, O139 strains also demonstrate a strong positive CAMP reaction while non-O1 and non-O139 vibrios give a weak positive reaction.

NON-AGGLUTINATING VIBRIOS (NON-O1 V. CHOLERAE)

The somatic antigen O1 is specific to the *classical* and El Tor biotypes of *V. cholerae*, while vibrios containing antigen other than O1 and O139 are called non-agglutinating (NAG) or non-cholera vibrios (NCV) or Non-O1 *V. cholerae*. There are 139 serogroups containing O antigen, O1 to O139. Serogroup O1 and O139 contain *V. cholerae*, while O2 to O138 are NAG vibrios. It is extremely difficult to distinguish most of the NAG vibrios from *V. cholerae* by means of culture and biochemical reactions. They are not agglutinable by O1 antiserum, therefore, named as non-agglutinable (NAG) vibrios. However, they are readily agglutinable by their homologous antiserum.

V. cholerae O139

V. cholerae serogroup O139 originated in Madras, India, in 1992 and has led to a widespread occurrence of cholera cases throughout India and Bangladesh. Later on *V. cholerae* O139 emerged as aetiological agent in a series of outbreaks of cholera in the entire Indian subcontinent. *V. cholerae* strains of serogroup O1 and serogroup group O139 cause classical cholera. *V. cholerae* O139 is very similar to El Tor biotype of *Vibrio cholerae*. It does not produce the O1 lipopolysaccharide. Like El Tor biotype, it is also positive for modified CAMP test. It makes a polysaccharide capsule like other non-O1 *V. cholerae* strains, while *V. cholerae* O1 does not make a capsule. Like *V. cholerae O1*, it (*V. cholerae O139*) also produces enterotoxin (*cholera toxin*).

G. Phage Typing

The classical strains of cholera vibrios are classified into five types (1 to 5) by using Mukherjee's phages I to IV. Phage IV lyses all classical but not EL Tor strains (Table 34.3). El Tor strains can be divided into six types (1 to 6) on the basis of lysis by five phages (I to V). All El Tor strains are lysed by phage V (Table 34.4). National Institute of Cholera and Enteric diseases (NICED), Kolkata is the International Reference Centre for Vibrio phage typing.

Table 34.3 Phage Types of Classical Biotype of ***V. cholerae***

Phage type	Sensitivity to phage group			
	I	II	III	IV
1	+	+	+	+
2	–	+	+	+
3	+	–	+	+
4	–	–	+	+
5	+	+	–	+

Table 34.4 Phage Types of El Tor Biotype of *V. cholerae*

Phage type	Sensitivity to phage group				
	I	II	III	IV	V
1	+	+	+	+	+
2	+	+	+	–	+
3	+	+	–	+	+
4	+	+	–	–	+
5	+	–	–	–	+
6	–	+	–	–	+

H. Pathogenesis

V. cholerae (both O1 and O139) causes an acute diarrhoeal disease known as cholera and it occurs only in man. The human infection occurs by ingestion of contaminated foods and drink. The ingested organisms pass through the acid barrier of the stomach and multiply in the alkaline medium of the small intestine. The vibrios do not penetrate deep into the gut and there is no bacteraemia. Vibrios become adherent to the epithelium by special fimbria such as the *toxin co-regulated pilus* (TCP). Once epithelial cell attachment occurs, *V. cholerae* produces enterotoxin and the disease. Mechanism of action of enterotoxin has been described earlier. Enterotoxin and TCP are regulated by the Tox R gene product, Tox R protein.

The massive loss of water and electrolytes (sodium and bicarbonates) by action of enterotoxin, leads to:

1. dehydration causing haemoconcentration, anuria and hypovolaemic shock
2. base-deficit acidosis and
3. muscle cramps due to hypokalaemia.

In untreated cases the mortality rate is 60–70% due to renal failure. Both the biotypes of *V. cholerae* O1 produce equally severe disease but in El Tor strains, the incidence of mild and asymptomatic infections is more frequent. The NAG vibrios may produce a clinical disease indistinguishable from cholera.

I. Carrier State

1. Convalescent Carrier

After treatment, few cases may continue to excrete vibrios for 4 to 5 weeks due to survival of the bacilli in gall bladder. These are known as convalescent carriers.

2. Chronic Carrier

It is found in endemic areas and the vibrios are excreted in faeces intermittently from the gall bladder. Chronic carriers have been found in ElTor cholera and serve to perpetuate inter-epidemic infections.

J. Epidemiology

Cholera is an epidemic disease and man is the only natural host of *V. cholerae*. Cholera has been endemic in the Ganges and Brahmaputra deltas in Bengal and neighbouring parts of the Indian subcontinent. Before 1817, cholera was confined to these endemic areas. Between 1817 and 1923 cholera spread to all over the world in six pandemics. All these pandemics were caused by classical biotype of *V. cholerae*. Between 1923 and 1961, the disease remained confined almost to endemic areas except for one isolated epidemic in Egypt in 1947.

The recent pandemic, the seventh, occurred in 1961 and this pandemic has been caused by the EL Tor biotype. The El Tor vibrio is hardier and more capable of surviving in the environment. It leads to mild cases, higher incidence of carriers and greater chances of endemicity as compared to classical vibrio. Whenever the El Tor vibrio has caused infection, it displaces the classical vibrio in all those areas.

Epidemic cholera has a seasonal distribution, the epidemic seasons are different in different areas. In India (Calcutta) the epidemic season is in the hot dry months of March to May and ends with onset of monsoon in June, while in Bangladesh the cholera season (November to February) follows the monsoon rains. Epidemic cholera has been associated with fairs, festivals during which sanitary conditions tend to be unsatisfactory. The only natural reservoir is man in the form of convalescent and chronic carriers. The transmission is maintained by a cycle involving the excretor (carrier) and the environment, particularly a water source. Since vibrios do not survive in water for a long period, so it should be repeatedly contaminated to act as a prolonged source of infection.

In 2012, the World Health Organisation reported an outbreak of cholera in Sierra Leone, causing 280 deaths among 20,700 cases.

K. Laboratory Diagnosis

1. Specimens

(i) Watery stool
(ii) Rectal swab

2. Collection and Transport

Specimens should be collected preferably prior to start of antibiotics. These should not be collected from bedpans due to risk of contamination. Specimens should be immediately transported to the laboratory for processing. In case of delay, stool samples may be preserved in holding media such as VR fluid or Cary-Blair medium for long periods. If the specimen can reach the laboratory in a few hours, enrichment media such as alkaline peptone

water or Monsur's medium may be used as transport media. When transport media are not available, strips of blotting paper may be soaked in watery stool and sent to the laboratory after proper packing in plastic envelopes. If possible, specimens should be plated at bedside and the inoculated plates sent to the laboratory.

3. Direct Microscopy

It is not a reliable method. For rapid diagnosis, the characteristic darting motility of the Vibrio and its inhibition by adding antiserum can be demonstrated under the dark field or phase contrast microscope, using cholera stool.

4. Culture

(i) Stool sample is directly cultured on following media.
   (a) Selective media (BSA, TCBS or Monsur's GTTA) and non-selective media (blood agar and MacConkey's agar) are inoculated. These plates are incubated at 37°C for overnight.
   (b) Enrichment media such as alkaline peptone water or Monsur's liquid media are inoculated. These media are incubated at 37°C for 6–8 hours before subculturing on to selective media.

(ii) When the specimen has been collected in holding medium, it is first inoculated into enrichment medium and incubated at 37°C for 6–8 hours before plating onto a selective medium.

(iii) The specimen collected in enrichment medium should be incubated for 6–8 hours including transit time before subculturing onto a selective medium.

5. Colony Morphology and Staining

After overnight incubation, culture media are examined for typical colonies of *V. cholerae*. On MacConkey's agar, colonies are pale and on Monsur's medium the colony has a black centre with a turbid halo around the colony. TCBS shows yellow colonies and on BSA, translucent colonies are present.

Gram staining from colony shows typical Gram negative comma shaped bacilli. These show darting motility on hanging drop preparation. Further confirmation is done by biochemical reactions and agglutination test.

6. Biochemical Reactions

V. cholerae ferments glucose, mannitol, sucrose maltose, mannose with acid production. Lactose is usually not fermented. Catalase, oxidase and cholera red reactions are positive. The El Tor biotype is usually haemolytic, VP positive, agglutinate chick erythrocytes and is resistant to polymyxin B and group IV cholera phage.

7. Agglutination Test

Colonies are picked up with a straight wire and tested with *V. cholerae* O1 antiserum. If positive, the test is repeated with monospecific *Ogawa* and *Inaba* sera for serotyping. The test should be repeated with at least five colonies as *V. cholerae* O1 and non-O1 *V. cholerae* may co-exist in the same specimen.

The H antigen is shared by both *V. cholerae* O1 and non-O1 *V. cholerae*. Any vibrio which is agglutinated by H antiserum, but not by *V. cholerae* O1 antiserum is considered to be NAG vibrio. These strains should be tested with other O antiserum to establish their identity as they may be belonging to one of O2–O139 serogroups. Specific antiserum against O139 is available.

8. Phage Typing

It is helpful in epidemiological study of cholera. It is of little use in diagnosis.

9. Serological Tests

These are of little use in diagnosis of cholera though it may help in assessing the incidence of cholera in an area. The tests used are agglutination, indirect haemagglutination, vibriocidal tests and antitoxin assay of serum.

DETECTION OF CARRIERS

The procedures are similar to those of an infective case except:

1. repeated stool examination may be needed as the vibrio is excreted intermittently.
2. it gives better result if stool is collected after administration of a purgative.
3. bile may be examined after duodenal intubation.
4. serological tests are useful in detecting chronic carriers.

BACTERIOLOGICAL EXAMINATION OF WATER AND SEWAGE

1. *Water sample*: 900 ml water is added to 100 ml of ten fold concentrated peptone water (pH 9.2) and incubated at 37°C for 6–8 hours. After incubation, it is inoculated on selective media.
2. *Sewage*: It is diluted in saline and filtered through gauze. The filtrate is treated as water.

L. Treatment

1. Oral Rehydration Therapy

The most important is prompt water and electrolyte replacement to correct the severe dehydration and salt depletion. This can be achieved by oral rehydration therapy, either alone or supplemented by intravenous fluids.

2. Antibiotics

Antibiotics are of secondary importance. Tetracycline is useful in reducing the number of stool and it also shortens the period of excretion of vibrios.

M. Prophylaxis

1. General Measures

(*i*) Purification of water supplies.
(*ii*) Better provision for sewage disposal.
(*iii*) Infected patients should be isolated, their excreta disinfected.
(*iv*) Contacts and carriers are followed up.

2. Specific Measures

ACTIVE IMMUNISATION

Killed parenteral vaccine

This vaccine contains 12,000 million *V. cholerae* per ml, composed of equal number of *Inaba* and *Ogawa* strains. It is widely used for active immunisation. Two injections are given intramuscularly at an interval of four weeks. Degree of protection does not exceed 50–60% and the duration of protection is only 3–6 months.

A single dose confers good protection in adults due to its action as a booster on top of prior natural immunisation. In children below five years of age, a single dose is ineffective. There is a good cross protection between classical and El Tor vibros.

The aluminium hydroxide and phosphate adjuvant vaccines have been tried. These vaccines have induced a high degree of immunity, particularly in young children. Cholera toxoid has been tried as vaccine but without any success.

Killed oral vaccine

B subunit whole cell (BS–WC) vaccine has undergone a field trial in Bangladesh. The vaccine contains cholera toxin B subunit, heat killed classical vibrio (both Ogawa and Inaba) and formalin killed ElTor vibrio (both Ogawa and Inaba). Degree of protection was 85% for one year and 60% for two years. This vaccine also conferred significant protection against diarrhoea due to *Esch. coli* (LT–ETEC).

Live oral vaccine

Recombinant DNA vaccine with expression of *V. cholerae* in attenuated strain Ty21a of *Salmonella typhi* has been developed. This vaccine colonises Peyer's patches and induces IgA response by local immunity. Live attenuated vaccine against O139 is also available in some countries.

CHEMOPROPHYLAXIS

In endemic areas, chemoprophylaxis with anti-microbial drugs is useful for attendants, nurses etc.

III. VIBRIO CHOLERAE NON–O1

These strains are identical to *V. cholerae–O1* biochemically and in other characters. Their isolation and identification procedures are same as those of *V. cholerae-O1*. There are 137 (O2–O138) serotypes that occur under this group. Most infections are due to contact with saline environments and consumption of seafood.

Non-O1 V.cholerae are less virulent than *V. cholerae-O1* and cause mild diarrhoea. Occasionally the symptoms may be as severe as cholera. Wound infection with contaminated water, bacteraemia and meningitis are other infections reported by these organisms.

Non-O1 V. cholerae strains may produce entero-toxins, haemolysins and cytotoxins. A few strains produce cholera toxin which can cause cholera like disease.

IV. HALOPHILIC VIBRIOS

Halophilic vibrios cannot grow in media lacking sodium chloride. They grow best in the presence of sodium chloride. Their natural habitat is sea water and marine life. Some halophilic vibrios which cause human diseases are *V. parahaemolyticus, V. alginolyticus* and *V. vulnificus*.

A. Vibrio parahaemolyticus

It was first isolated in Japan in 1951 as causative agent of food poisoning due to consumption of seafood. It was named as *V. parahaemolyticus* by *Sakazaki* (1963) due to haemolysis produced by this organism on blood agar. It has been isolated from shell fish, shrimps, crabs and molluscs in many countries.

Morphologically, it resembles *V. cholerae* and other vibrios. Unlike other vibrios, it produces peritrichous flagella when grown on solid media but polar flagella are formed in liquid media. It grows only in media containing sodium chloride (optimum concentration 2 to 4 per cent). It can tolerate sodium chloride concentration up to 8 per cent but not ten per cent. They do not ferment sucrose, therefore, produce green colonies on TCBS medium. Biochemical reactions are similar to classical *V. cholerae* except it is sucrose negative.

It is killed by heat at 60°C in 15 minutes. It does not grow at 4°C but can survive freezing. Drying destroys it.

All strains of *V. parahaemolyticus* are not pathogenic for man. Strains isolated from environmental sources are nearly always non-haemolytic when grown on a high salt blood agar (*Wagatsuma agar*), while strains isolated

from human cases are almost always haemolytic. This is called the *Kanagawa phenomenon*. It is due to heat stable haemolysin. Kanagawa positive strains are being considered pathogenic for man and negative strains non-pathogenic.

The organism causes food poisoning associated with marine food. It can also cause acute diarrhoea unassociated with food poisoning. Abdominal pain, diarrhoea, vomiting and moderate degree of dehydration are the dominant features. Recovery occurs within three days.

B. Vibrio alginolyticus

It is frequently found in sea fish and its role in human lesion is uncertain. It resembles *V. parahaemolyticus* in many aspects. It has a higher salt tolerance (up to 10 per cent), is VP positive and, ferments sucrose. These features differentiate it from *V. parahaemolyticus*. (Table 34.5).

It has a property to swarm on the surface of non-selective media. It has been incriminated in marine wound infection.

Table 34.5 Differentiating Features of ***V. parahaemolyticus*** and ***V. alginolyticus***

Feature	V. para-haemolyticus	V. algino-lyticus
V.P. reaction	–	+
Sucrose fermentation	–	+
Swarming	–	+
Growth in 0% NaCl	–	–
7% NaCl	+	+
10% NaCl	–	+

C. Vibrio vulnificus

It was previously designated as L+ vibrio for its ability to ferment lactose. It resembles *V. parahaemolyticus* but ferments lactose and has a salt tolerance of less than eight per cent. It may cause wound infection and cellulitis following exposure of wounds to seawater. Following ingestion of under-cooked or raw seafood, it penetrates the gut mucosa without causing gastrointestinal manifestations and enters the blood leading to septicaemia with high mortality.

V. AEROMONAS AND PLESIOMONAS

These are Gram negative, motile bacilli which are oxidase positive. They are aerobes and facultative anaerobes. The medically important species include *Aeromonas hydrophila* and *Plesiomonas shigelloides*.

A. Aeromonas

A. hydrophila is found in water. It is Gram negative bacilli or coccobacilli. It is motile by single polar flagellum. It is catalase and oxidase positive. It can reduce nitrate to nitrite. It grows well on nutrient agar, blood agar, MacConkey agar, DCA and TCBS. It ferments glucose, sucrose, maltose and starch with the production of acid and gas. It produces yellow colonies on TCBS agar. Aeromonas is resistant to the compound O/129 at strength of 150 μg.

It may cause diarrhoea, wound infections, pyogenic infections and septicaemia in humans. It produces an *enterotoxin* which resembles cholera toxin and is neutralised by *V. cholerae* antitoxin.

B. Plesiomonas

The genus *Plesiomonas* has now been classified in family *Enterobacteriaceae*. It has only one species *P. shigelloides*. The strains of *P. shigelloides* share antigens with *Sh. sonnei* and some are agglutinated by *Shigella sonnei*. antiserum. It has derived its name 'shigelloides' from this property. They are Gram negative bacilli. They are motile by polar flagella usually lophotrichous. They are oxidase positive. They grow well on blood agar, MacConkey agar but do not grow on TCBS medium. Most strains ferment inositol with the production of acid only. They do not ferment sucrose.

P. shigelloides is commonly found in surface waters and in soil. It has also been isolated from a variety of mammals. It is rarely recovered from human faeces. Man is infected by ingesting contaminated water or food. It causes gastroenteritis but in immunosuppressed individuals a cholera-like illness may be seen. It may also cause septicaemia, neonatal meningitis and cellulitis.

Aeromonas and plesiomonas are differentiated from *Vibrio cholerae* by biochemical reactions such as utilisation of aminoacids, lysine, arginine and ornithine (Table 34.6). *Vibrio cholerae* is susceptible to the compound O/129, while aeromonas is resistant.

Table 34.6 Utilisation of Aminoacids in Vibrio, Aeromonas and Plesiomonas

Organism	Lysine	Arginine	Ornithine
V. cholerae	+	–	+
Aeromonas	–	+	–
Plesiomonas	+	+	+

KEY POINTS

1. Vibrios are Gram negative, *oxidase positive*, short, rigid, *curved or comma-shaped rods* that are actively motile by a *polar flagellum*.
2. *Vibrio cholerae* is the causative agent of *cholera*. Two serogroups O1 and O139 are responsible for the disease. Both serogroups produce cholera toxin (CT).
3. Two biotypes of *Vibrio cholerae* include *classical* and *El Tor*. Three serotypes are Ogawa (AB), Inaba (AC) and Hikojima (ABC) on the basis of minor 'O' antigens.
4. Special media with *alkaline pH* are required for growth of vibrios.
5. *Venkataraman-Ramakrishnan (VR) medium* and *Cary-Blair medium* are transport media for *V. cholerae* while *alkaline peptone water (APW)* is an enrichment medium.
6. *Alkaline bile salt agar (BSA)* and *Thiosulphate citrate bile sucrose (TCBS)* are two important solid media for growing *V. cholerae*.
7. Classical and El Tor vibrios can be differentiated by haemolysis, agglutination of chick erythrocytes, VP reaction, polymyxin B sensitivity, susceptibility to Mukherjee phase IV and modified CAMP test.
8. Killed parenteral vaccine is widely used for protection against cholera.
9. Non-O1 *V. cholerae* are also named as non-agglutinating vibrios (NAG).
10. *Halophilic Vibrios* cannot grow in media lacking sodium chloride. *V. parahaemolyticus*, *V. alginolyticus* and *V. vulnificus* are some examples of halophilic vibrios. *V. cholerae* is a non-halophilic vibrio.

YOU MUST KNOW

1. Morphology, culture characteristics and biochemical reactions of *Vibrio cholerae*.
2. Serotypes of *V. cholerae*.
3. Mechanism of action of cholera toxin (CT).
4. Differences between classical and El Tor vibrios.
5. Laboratory diagnosis of cholera.
6. Cholera vaccines.
7. Non-agglutinating vibrios.
8. Halophilic vibrios.

STUDY QUESTIONS

1. Discuss the laboratory diagnosis of cholera.
2. Write short notes on:
   (a) El Tor vibrios
   (b) Non-agglutinating vibrios
   (c) Prophylaxis against cholera
   (d) Halophilic vibrios
   (e) Aeromonas
   (f) Plesiomonas.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Which of the following bacteria has darting motility?
   (a) *Escherichia coli*
   (b) *Vibrio cholerae*
   (c) *Salmonella typhi*
   (d) *Pseudomonas aeruginosa*
2. Which of the following media can be used as transport medium for *Vibrio cholerae*?
   (a) Nutrient broth
   (b) Venkatraman-Ramakrishnan medium
   (c) Selenite F broth
   (d) None of the above

3. Which of the following bacteria produce yellow colonies on thiosulphate citrate bile sucrose agar?
   (a) *Vibrio cholerae* (b) *Vibrio parahaemolyticus*
   (c) *Klebsiella* (d) All of the above
4. Which of the following bacteria produce green colonies on thiosulphate citrate bile sucrose agar?
   (a) *Vibrio cholerae* (b) *Vibrio parahaemolyticus*
   (c) *Klebsiella* (d) All of the above
5. Cholera toxin resembles:
   (a) Heat-labile toxin of *Escherichia coli* (b) Heat stable toxin of *Esch. coli*
   (c) Both of the above (d) None of the above
6. Which of the following tests can be used to differentiate Classical and ElTor vibrios?
   (a) Haemolysis of sheep erythrocytes (b) Vogas-Proskauer reaction
   (c) Modified CAMP test (d) All of the above
7. Stool specimens from cholera cases can be collected and transported to the laboratory using:
   (a) Venkatraman-Ramakrishnan medium (b) Cary-Blair medium
   (c) Strips of thick blotting paper (d) All of the above
8. Which of the following vaccines is/are available for prevention of cholera?
   (a) Killed parenteral vaccine (b) Killed oral vaccine
   (c) Live oral vaccine (d) All of the above
9. All of the following are halophilic vibrios except:
   (a) *Vibrio alginolyticus* (b) *V. parahaemolyticus*
   (c) *V. vulnificus* (d) *V. cholerae*
10. *Vibrio parahaemolyticus* strains isolated from environmental sources can be differentiated from those isolated from humans cases by:
   (a) Kanagawa phenomenon (b) Modified CAMP test
   (c) Reverse CAMP test (d) All of the above
11. Which of the following bacteria is associated with food poisoning due to consumption of sea food?
   (a) *Vibrio parahaemolyticus* (b) *V. cholerae*
   (c) *V. vulnificus* (d) All of the above

Answers (MCQs):

1. (b)	2. (b)	3. (a)	4. (b)	5. (a)
6. (d)	7. (d)	8. (d)	9. (d)	10. (a)
11. (a)				

Chapter 35

CAMPYLOBACTER, HELICOBACTER, MOBILUNCUS

I. CAMPYLOBACTER

Campylobacters are slender, spirally curved, Gram negative bacilli. They resemble vibrios but differ in being microaerophilic, not fermenting sugars and having a lower G + C content of DNA.

There are ten species in Genus *Campylobacter* but *C. jejuni, C. coli, C. concisus, C. fetus, C. hyointestinalis, C. lari* and *C. sputorum* are of medical importance.

A. Morphology

Campylobacters are small, curved, Gram negative bacilli measuring 0.5–5 µm × 0.2–0.5 µm. They are motile by single polar flagellum (monotrichate) or amphitrichate flagella.

B. Culture

Growth of campylobacters occurs under strict microaerophilic conditions. They grow readily on simple media in an atmosphere of a mixture of oxygen (5%), CO_2 (10%) and nitrogen (85%). *C. jejuni, C. coli* and *C. lari* grow optimally at 42°C. However, *C. fetus* and most other campylobacters produce visible growth at 37°C. Campylobacters growing at 42°C are referred to as the thermophilic group.

Colonies are circular and convex but those of thermophilic group, particularly *C. jejuni,* are flat and tend to swarm on moist agar. Well formed colonies are seen only after 48 hours.

C. Biochemical Reactions

All produce oxidase and most species are catalase positive. They are biochemically inactive and do not utilise sugars or produce indole. Most species reduce nitrate to nitrite. Of all the species of campylobacter, only *C. jejuni* can hydrolyse sodium hippurate.

D. Pathogenesis

C. jejuni has emerged as the most important human pathogen in diarrhoeal diseases and accounts for 90%

Chapter 36

PSEUDOMONAS, STENOTROPHOMONAS, BURKHOLDERIA

The genus *Pseudomonas* comprises of aerobic, Gram negative, non-fermentative, non-sporing, oxidase positive bacilli which are motile by polar flagella. Many species produce water soluble pigments which diffuse through the culture medium. Majority of them are saprophytic being found in soil, water, sewage or wherever decomposing organic matter is found.

The genus *Pseudomonas* belongs to the family *Pseudomonadaceae* which contains over 200 species. Human disease has been caused by *Ps. aeruginosa, Ps. maltophilia, Ps. mallei, Ps. pseudomallei, Ps. cepacia, Ps. stutzeri, Ps. fluorescens, Ps. multivorans* and *Ps. putida*.

Table 36.1 Differentiating Features of Commonly Encountered Species

Species	Motility	Oxidase	Pyocyanin	Fluorescin	Growth at 42°C	Oxidation of				Lysine decarboxylase	Arginine dihydrolase
						Glucose	Lactose	Mannitol	Maltose		
Ps. aeruginosa	+	+	+	+	+	+	–	–	–	–	+
Ps. stutzeri	+	+	–	–	+	+	–	–	+	–	–
Ps. putida	+	+	–	+	–	+	–	–	–	–	+
Sten. maltophilia	+	–	–	–	+	+/–	+	–	+	+	–
B. mallei	–	–	–	–	+	+	–	–	–	–	+
B. pseudomallei	+	+	–	–	+	+	+	+	+	–	+
B. cepacia	+	+	–	–	+	+	+	+	+	+	–

The most important among these is *Ps. aeruginosa*. Recently, *Ps. mallei, Ps. pseudomallei* and *Ps. cepacia* have been assigned a new genus *Burkholderia* which also belongs to family *Pseudomonadaceae*. New names for these species are *Burkholderia mallei, B. pseudomallei* and *B. cepacia. B. mallei* is non-motile. *B. cepacia* has been included under *B. cepacia* complex which contains about 16 more species of *Burkholderia*. *Pseudomonas maltophilia* has been named as *Stenotrophomonas maltophilia* Differentiating features of commonly encountered species are shown in Table 36.1.

I. PSEUDOMONAS AERUGINOSA

A. Morphology

It is slender, Gram negative bacillus, 1.5–3 μm × 0.5 μm, non-capsulated, non-sporing and is actively motile by a polar flagellum. Most strains possess pili. It is non-capsulated though mucoid strains may sometimes occur. Occasionally strains have two or three polar flagella.

B. Culture

It is a strict aerobe and grows well on ordinary media like nutrient broth and nutrient agar. The optimum temperature for growth is 37°C, but growth occurs at a wide range of temperature 5°C to 42°C.

1. Nutrient Agar

Colonies are smooth, large, translucent, low convex, 2–4 mm in diameter. The organism produces a sweetish aromatic odour. This is due to the production of 2-aminoacetophenone. There is greenish blue pigment which diffuses into the medium (Fig. 36.1).

2. Blood Agar

Colony characters are similar to those on nutrient agar. Many strains are haemolytic on blood agar.

3. MacConkey Agar

Colonies are pale or colourless (non-lactose fermenters, NLF).

4. Cetrimide Agar

It is a selective medium for *Ps. aeruginosa*.

5. Peptone Water

It forms a turbidity with a surface pellicle. Pseudomonas being a strict aerobe tends to collect at the surface for more oxygen hence forming surface pellicle.

C. Pigment Production

Ps. aeruginosa produces a number of pigments which diffuse into surrounding medium. These pigments are:

1. Pyocyanin

It is a bluish-green phenazine pigment soluble in chloroform and water. It is not produced by other species of the genus, hence, it is diagnostic of *Ps. aeruginosa*.

2. Fluorescin (Pyoverdin)

It is a greenish yellow pigment insoluble in chloroform but soluble in water. It may be produced by many other species also.

Fig. 36.1 *Pseudomonas aeruginosa* on nutrient agar

3. Pyorubin
It is a reddish brown pigment that is insoluble in chloroform but soluble in water.

4. Pyomelanin
It is a brown to black pigment and is chemically unrelated to melanin. Its production is uncommon.

D. Biochemical Reactions
Ps. aeruginosa derives energy from carbohydrates by oxidative breakdown rather than a fermentative metabolism. Special O-F medium of Hugh and Leifson must be used to find out oxidative metabolism. It utilises only glucose oxidatively with acid production. Lactose and maltose are not utilised. All strains of *Ps. aeruginosa* are oxidase positive and utilise citrate as the sole source of carbon. They are catalase positive and indole, MR, VP and H_2S tests negative. They reduce nitrates to nitrites and further to gaseous nitrogen. Arginine dihydrolase test is positive. Important biochemical reactions are summarised below.

O/F test	Catalase	Oxidase	NO_3 reduction	Glucose	Lactose
Oxidative	+	+	+	A	–
Mannitol	**Sucrose**	**Citrate**	**Indole**	**Urease**	**H_2S**
–	–	+	–	–/+	–

E. Resistance
It is killed by heating at 55°C for one hour. It is resistant to the chemical disinfectants and can even grow in certain antiseptics like quaternary ammonium compounds, chloroxylenol and hexachlorophane. Indeed, selective media have been devised for isolation of *Ps. aeruginosa* incorporating dettol or cetrimide. It is sensitive to 2% aqueous alkaline solution of glutaraldehyde and also to silver salts. Due to its sensitivity to silver salts, silver sulphonamide compounds has been applied as topical cream in burns.

It is intrinsically resistant to commonly used antibiotics. Examples of clinically effective antibiotics are polymyxin B, colistin, piperacillin, ticarcillin, cefotaxime, gentamicin, azlocillin, tobramycin and ciprofloxacin.

F. Antigenic Structure
O Antigens
It possesses 19 distinct, group specific O antigens. These antigens are heat stable

H antigens
Two heat-labile H antigens have been recognised in *Ps. aeruginosa*.

G. Toxins and Enzymes
Several toxins and enzymes produced by *Ps. aeruginosa* contribute to enhance its virulence.

1. Extracellular Products
Pyocyanin inhibits mitochondrial enzymes in mammalian tissue and causes disruption and cessation of ciliary beat on ciliated nasal epithelium. Thus, this favours colonisation of these organisms in the nasal mucosa.

2. Extracellular Enzymes and Haemolysins
It produces proteases (general protease, alkaline protease and elastase), haemolysins (phospholipase.C and heat stable rhamnolipid) and lipase. These play a key role in producing local lesions.

3. Exotoxin
It produces two exotoxins, A and S. Exotoxin A is a polypeptide of molecular weight 66,000–72,000 and inhibits protein synthesis. Mechanism of action of exotoxin A is similar to that of diphtheria toxin.

4. Endotoxins
It is a lipopolysaccharide (LPS) exhibiting many biological properties of enterobacterial LPS including pyrogenic action etc.

H. Typing Methods
As *Ps. aeruginosa* is an important cause of hospital-acquired infections, it is essential to type the strain for epidemiological study.

1. Bacteriocin (Pyocin) Typing
Three types of bacteriocins (pyocins) are produced by *Ps. aeruginosa*. These pyocins are known as R, F and S. Pyocin-producing strains are resistant to their own pyocins though they may be sensitive to pyocins produced by other strains. Pyocin produced by the test strain is employed to assess the growth inhibition of 13 (1–8 and A to E) indicator strains of *Ps. aeruginosa*. Depending upon the growth inhibition of these 13 indicator strains, 105 types are recognised. Pyocin typing is the most popular method employed for typing of *Ps. aeruginosa*.

2. Phage Typing
Considerable difficulties have been encountered in bacteriophage typing.

3. Serotyping
Based on O and H antigens, 17 serotypes of *Ps. aeruginosa* are recognised. It is said to be reliable but facilities exist only in reference laboratories.

4. Molecular Method

Restriction endonuclease typing with pulsed-field gel electrophoresis (PAGE) is the most reliable typing method.

I. Pathogenesis

It causes infections more common in patients with neutropenia, cystic fibrosis, burns and those on ventilators. It is the most important agent causing nosocomial infections. It is due to its resistance to common antibiotics and antiseptics that it establishes itself widely in hospitals. Equipments such as respirators and endoscopes, articles such as bed pans, and antiseptic or disinfectant solutions may be frequently contaminated. The other common infections caused by it are:

1. Urinary tract infections following catheterisation.
2. Acute purulent meningitis following lumbar puncture.
3. Post-tracheostomy pulmonary infection.
4. Septicaemia in patients who are debilitated due to malignancy or immunosuppresive therapy.
5. Wound and burn infections.
6. Chronic otitis media and otitis externa.
7. Eye infections.
8. Acute necrotising vasculitis which leads to haemorrhagic infection of skin and internal organs.
9. Infantile diarrhoea.

In some tropical areas, *Ps. aeruginosa* has been found to be responsible for a self-limited febrile illness (*Shanghai fever*) resembling typhoid fever.

In spite of its lack of invasiveness, *Ps. aeruginosa* does cause severe disease. The mechanisms of pathogenesis are not clearly understood but it has been claimed that pathological processes seen in infection are caused by exotoxins, proteases, elastases, haemolysins, lipases and enterotoxins. Exotoxin A is a lethal toxin acting like the diphtheria toxin. Elastases may be responsible for haemorrhagic lesions in skin infections. The enterotoxin causes diarrhoeal disease. The slime layer acts as a capsule and enhances the virulence.

The organisms produce blue pus and the term aeruginosa, meaning verdigris which is bluish green in colour and *pyocyanea*, being a literal translation of 'blue pus'.

J. Laboratory Diagnosis

1. Specimens

Pus, wound swab, urine, sputum, blood or CSF

2. Culture

Specimens may be inoculated on nutrient agar, blood agar or MacConkey's agar and incubated at 37°C for 18–24 hours. On nutrient agar, there is bluish green pigment diffused in the medium. On MacConkey's agar they grow as pale colonies (NLF). In peptone water, surface pellicle and green pigment can be observed. Selective media such as cetrimide agar may be necessary to isolate *Ps. aeruginosa* from faeces or other samples with mixed flora. As *Ps. aeruginosa* is a frequent contaminant, isolation of the bacillus from a specimen should not always be taken as aetiological agent. Repeated isolations would help to confirm the diagnosis.

3. Gram Staining and Motility

They are Gram negative bacilli and are actively motile.

4. Biochemical Reactions

The oxidase test is positive within 30 seconds. They are non-fermenter. They break down glucose oxidatively with acid production only. Other biochemical reactions also help to confirm the isolate (Table 36.1).

5. Typing Method

Pyocin typing is the most commonly used method. It is mainly used for epidemiological studies.

6. Antibiotic Sensitivity Test

It is useful to select out proper antibiotic as multiple resistance to antibiotics is quite common in *Ps. aeruginosa*.

K. Treatment

It is intrinsically resistant to most of the commonly used antimicrobial agents. Ciprofloxacin, piperacillin, ticarcillin, azlocillin, cefotaxime, ceftazidime, gentamicin and tobramycin are used in treatment of *Ps. aeruginosa* infections.

L. Control

Cross infections in hospital are to be prevented by constant vigilance and strict attention to asepsis.

A polyvalent vaccine made from the cell surface of 17 recognised serotypes of *Ps. aeruginosa* has been claimed to stimulate active immunity in man.

M. Epidemiology

1. *Cross Infection*: *Ps. aeruginosa* is a saprophyte and its ability to persist and multiply in moist environment of hospital wards, kitchens, equipments and antiseptics or disinfectant solutions is of particular importance in cross infection.
2. *Epidemics*: Epidemics and outbreaks of *Ps. aeruginosa* infection have occurred amongst newborn nurseries and young infants in paediatric wards.

3. *Risk group*: These include burn patients, patients with immunosuppression and patients who have undergone cardiac and renal surgery.

II. OTHER PSEUDOMONAS

Ps. putida, *Ps. stutzeri* and *Ps. fluorescens* are becoming clinically relevant in increasing percentages.

III. STENOTROPHOMONAS MALTOPHILIA (PSEUDOMONAS MALTOPHILIA)

This is an opportunistic pathogen, causing wound infection, urinary tract infection and septicaemia. It is oxidase negative. It acidifies maltose in addition to glucose.

IV. BURKHOLDERIA MALLEI (PSEUDOMONAS MALLEI)

The bacillus was first isolated by *Loeffler* and *Schutz* (1882) from horse dung of glanders (malleus, in latin). It causes a natural disease called glanders in animals (horses, mules and asses), and man occasionally acquires the infection from animals.

A. Morphology

It is a small 2.5 μm × 0.5 μm, non-motile, Gram negative bacillus often giving a beaded appearance.

B. Culture

It is an aerobe and facultative anaerobe, grows on ordinary culture media. Colonies are small and translucent initially become yellowish and opaque on ageing.

C. Animal Pathogenicity

In susceptible animals (horses, mules and asses), *B. mallei* produces two types of lesions:

1. Glanders

In glanders, the respiratory system is affected and the animal develops profuse catarrhal discharge from the nose. Nodule formation starts in the nasal septum and later the nodules break down to form ulcers.

2. Farcy

Farcy follows infection through the skin with involvement of superficial lymph vessels and lymph nodes. The lymph vessels become thickened and stand out as hard cords under the skin which are called *farcy pipes*.

Intraperitoneal inoculation of bacilli in male guinea pig causes testicular swelling in 2–3 days due to inflammation of tunica vaginalis. This is known as *Straus reaction*. This may be followed by death of the animal in 1–2 weeks. The straus reaction is not diagnostic of glanders because similar reactions in guinea pig may also be observed with *Brucella sp.*, *Priesz-Nocard* bacillus, *Actinobacillus* and *B.pseudomallei*.

D. Human Pathogenicity

Humans may become infected via skin abrasions or wounds through contact with discharge of a sick animal. Human disease may present as acute fulminant febrile illness or a chronic indolent infection producing abscesses in respiratory tract or skin. The fatality rate in glanders is high. The laboratory cultures are highly infectious to work with.

E. Laboratory Diagnosis

1. Specimens

Purulent discharge from the lesion.

2. Direct Microscopy

Gram negative beaded bacilli are found on Gram staining of the specimen.

3. Culture

Culture is done on blood agar.

4. Animal Inoculation Test

It is done in guinea pigs. *Straus reaction* can be demonstrated.

5. Allergy Test

Mallein test

Mallein is a preparation from the organism *B. mallei* and is analogous to tuberculoprotein. Intracutaneous or subcutaneous injection of *mallein* evokes a delayed hypersensitivity reaction in animals suffering from glanders.

V. BURKHOLDERIA PSEUDOMALLEI (PSEUDOMONAS PSEUDOMALLEI)

It was formerly known as *Whitmore's bacillus, Actinobacillus whitmori, Malleomyces pseudomallei, Loefflerella pseudomallei.*

It is the causative agent of melioidosis which is a glanders like disease and epizootic in rodents in South-East Asia. The name melioidosis is derived from *melis*, a disease of horses, and *eidos* meaning resemblance. The organism was first described by *Whitmore* and *Krishnaswami* (1912) from a glanders like disease of man in Rangoon.

A. Morphology
It resembles *B. mallei* but differs in being motile.

B. Culture
It is similar to *B. mallei*.

C. Biochemical Reactions
It resembles *B. mallei* but differs in being liquefying gelatin and forming acid from several sugars.

D. Toxins
Two thermolabile exotoxins—one lethal and other necrotising have been identified in culture filtrates.

E. Pathogenesis
B. pseudomallei is a saprophyte of soil and water with a large animal reservoir. Melioidosis occurs in rats, rabbits and guinea pigs. Human infection may occur by inhalation or through skin abrasions, or by arthropod vector. Disease is epidemic in South-East Asia. Subclinical infections are common in man. There may be an acute septicaemia, a subacute typhoid like disease, or pneumonia and haemoptysis resembling tuberculosis. Acute disease has a high case fatality rate. In chronic form, the organism may localize in any tissue producing caseous necrosis or suppurative lesion.

In India, cases of melioidosis have been reported from Maharashtra, Tamil Nadu, Orissa, Kerala, Tripura and West Bengal.

F. Laboratory Diagnosis
The organisms may be detected as small, typical bipolar *safety pin* appearance with methylene blue stain. Small irregularly stained Gram negative bacilli in exudates may be isolated from sputum, pus, urine or blood. Antibody to *B. pseudomallei* may also be detected in patient's serum by ELISA and indirect haemagglutination assay (IHA).

VI. BURKHOLDERIA CEPACIA (PSEUDOMONAS CEPACIA)
Burkholderia cepacia is one member of *B. cepacia* complex which contains about 16 more species.

A. Morphology
It is a slender, Gram negative bacillus which is motile by multitrichous flagella. The bacillus accumulates *poly–β–hydoxy–butyrate* as granules, hence stains irregularly.

B. Culture
It is aerobic and grows well on ordinary nutrient agar optimally at 25–35°C. Colonies appear in 48 hours. Most strains do not grow on DCA medium. Cultures on blood agar die in 3–4 days. On prolonged incubation, colonies become reddish purple due to formation of a non-diffusible phenazine pigment.

C. Pathogenicity
It is a low grade human pathogen and an important cause of nosocomial infection. It is being recognised as an opportunist environmental pathogen, particularly in those with cystic fibrosis, in whom it causes fatal necrotising pneumonia.It is resistant to a large number of antibiotics and contaminate antiseptic solutions including chlorhexidine and cetrimide. It can also survive in distilled water up to a year.

VII. NON-FERMENTERS OTHER THAN PSEUDOMANAS AND BURKHOLDERIA
Several Gram negative bacilli may be non-fermenters of carbohydrates and may be confused with *Pseudomonas* or *Burkholderia* species. Some of these non-fermenters are *Acinetobacter*, *Alcaligenes*, *Achromobacter*, *Flavobacterium* and *Eikinella* species. Some characteristics of these organisms are shown in Table 36.2.

Table 36.2 Some Characteristics of Non–Fermenters

Organisms	Oxidase test	Habitat	Pathogenicity
Acinetobacter spp.	–	Saprophytes of soil, sewage; commensal of skin of groin and axilla of man (10%)	Pneumonia, Respiratory tract infections
Alcaligenes spp.	+	Human faeces	UTI, wound infection
Achromobacter spp.	+	–	CSOM, Post-operative meningitis
Flavobacterium spp.	+	Saprophytes of soil	Opportunistic nosocomial infections, particularly in infants.
Eikenella spp.	+	Commensal of mucosal surfaces	Infections of wounds and soft tissues, endocarditis, meningitis and pneumonia

KEY POINTS

1. The genus *Pseudomonas* comprises of aerobic, Gram negative, *non-fermentative*, non-sporing, *oxidase positive bacilli* which are motile by *polar* flagella.
2. The most important species is *Pseudomonas aeruginosa*. It is a strict aerobe and grows well on ordinary medium like nutrient agar. It produces greenish blue pigment which diffuses into the medium.
3. Different pigments produced by *Ps. aeruginosa* include are *pyocyanin*, *fluorescin*, *pyorubin* and *pyomelanin*.
4. Cetrimide agar is a selective medium for *Ps. aeruginosa*.
5. *Ps. aeruginosa* is the most important agent causing *nosocomial infections*. It is due to its resistance to *common antibiotics* and *antiseptics*. The other common infections caused by it are urinary tract infection, acute purulent meningitis, septicaemia, wound and burn infections.
6. *Stenotrophomonas maltophila* is *oxidase negative*.
7. *Burkoholderia mallei* is *non-motile*. It is the causative agent of *glanders* and *farcy*.
8. *Burkholderia cepacia* is motile by *multitrichous flagella*.

YOU MUST KNOW

1. Morphology, culture characteristics and biochemical reactions of *Pseudomonas aeruginosa*.
2. Pyocin typing.
3. Infections caused by *Pseudomonas aeruginosa*.
4. Diseases caused by *Burkholderia mallei*.

STUDY QUESTIONS

1. Write short notes on:
   (a) Pathogenesis of *Pseudomonas aeruginosa*
   (b) Pyocin typing
   (c) Pigments produced by *Pseudomonas aeruginosa*.
2. Write briefly about:
   (a) *Stenotrophomonas maltophila*
   (b) *Burkholderia mallei*
   (c) *Burkholderia pseudomallei*.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Which of following bacteria is non-motile?
   (a) *Pseudomonas stutzeri* (b) *Burkholderia mallei*
   (c) *B. pseudomallei* (d) *Stenotrophomonas maltophila*
2. Which of the following pigment is diagnostic of *Pseudomonas aeruginosa*?
   (a) Pyocyanin (b) Fluorescin
   (c) Pyorubin (d) Pyomelanin
3. Which of the following tests is/are characteristic feature/s of *Pseudomonas aeruguiosa*?
   (a) Utilization of glucose oxidatively (b) Oxidase positive
   (c) Pyocyanin production (d) All of the above
4. The most popular method employed for typing of *Pseudomonas aeruginosa* is:
   (a) Pyocin typing (b) Phage typing
   (c) Serotyping (d) Antibiogram

5. Which of the following infections can be caused by *Pseudomonas aeruginosa*?
   (a) Urinary tract infection
   (b) Wound and burn infections
   (c) Pulmonary infection
   (d) All of the above
6. The causative agent of *Shanghai fever* is:
   (a) *Pseudomonas aeruginosa*
   (b) *Pseudomonas putida*
   (c) *Burkholderia mellei*
   (d) *B. pseudomallei*
7. Which of the following bacteria was formerly known as *Whitmore's bacillus*?
   (a) *Burkholderia mallei*
   (b) *B. pseudomallei*
   (c) *Pseudomonas putida*
   (d) *Stenotrophomonas maltophila*

Answers (MCQs):

1. (b) 2. (a) 3. (d) 4. (a) 5. (d)
6. (a) 7. (b)

Chapter 37

YERSINIA, PASTEURELLA, FRANCISELLA

These are Gram negative, short bacilli showing bipolar staining with methylene blue. They are non-motile except *Yersinia pseudotuberculosis* and *Y. enterocolitica*. These bacteria are primary pathogens of rodents and men are accidentally infected. They were previously considered together under the genus *Pasteurella*. Based on their cultural, biochemical and antigenic characters, this group has been divided into three genera—Yersinia, Pasteurella and Francisella (Table 37.1). The genus Yersinia contains three medically important species—*Y. pestis* (the causative agent of plague), *Y. pseudotuberculosis* (a primary pathogen of rodents) and *Y. enterocolitica* (causes human diarrhoeal diseases). The name Yersinia is given after *Alexander Yersin*, who discovered the plague bacillus. The genus *Yersinia* now belongs to the tribe *Yersinieae* and the family *Enterobacteriaceae*. The genus *Pasteurella* is now restricted to several related bacteria causing haemorrhagic septicaemia in different species of animals and occasionally producing human infections, grouped under a common species called *P. multocida*. The genus *Francisella*, consisting of the single species, *F. tularensis* is named after *Francis* for his contribution on tularaemia, caused by this bacterium.

Table 37.1 Diseases Caused by Species of Yersinia, Pasteurella, Francisella

Genera	Species	Diseases
Yersinia	*Y. pestis*	Plague in man and rodents
	Y. pseudotuberculosis	Pseudotuberculosis of animals
	Y. enterocolitica	Enteritis in man and animals
Pasteurella	*P. multocida*	Haemorrhagic septicaemia in animals
Francisella	*F. tularensis*	Tularaemia

I. YERSINIA PESTIS

Yersinia pestis, the causative agent of plague, formerly known as *Pasteurella pestis*, was isolated independently and almost simultaneously by *Yersin* (1894) and *Kitasato* (1894).

A. Morphology

Y. pestis is a short, ovoid, Gram negative bacillus, about 1.5 μm × 0.7 μm in size, with rounded ends and convex sides, occur singly, in short chains or in small groups. When stained with methylene blue, it shows bipolar staining (*safety pin appearance*) with two ends darkly or densely stained and the central area clear (Fig. 37.1).

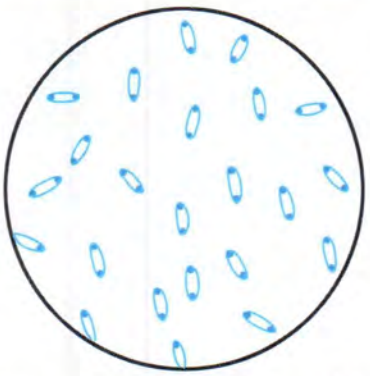


Fig. 37.1. Smear of *Y. pestis* with bipolar staining

Pleomorphism is very common. It is characteristically enhanced in media containing 3% NaCl. In old cultures, involution forms are very common—coccoid, club shaped, filamentous and giant forms.

The bacterial body is surrounded by a slime layer (envelope or capsule). It is non-motile, non-sporing and non-acid-fast.

B. Culture

The organism is aerobic and facultatively anaerobic. The optimum temperature for growth (unlike most pathogens) is 27°C but the envelope develops best at 37°C. It grows on ordinary media.

1. Nutrient Agar

Colonies are small, delicate, transparent after 24–48 hours of incubation.

2. Blood Agar

Colonies are non-haemolytic and dark brown due to the absorption of the haemin pigment. Other characters are same as that of the colonies on nutrient agar.

3. MacConkey Agar

Colourless colonies are formed.

4. Broth

In nutrient broth, a flocculent growth occurs at the bottom and along the sides of the tube, with little or no turbidity. A pellicle may form later.

5. Ghee Broth

When the organisms are grown in a flask with oil or ghee floated on the top (ghee broth), a characteristic growth occurs which hangs down from the under surface of the oil, resembling stalactites (*stalactite growth*).

C. Biochemical Reactions

Y. pestis ferments glucose, mannitol and maltose with the production of acid but no gas. Lactose and sucrose are not fermented. It is catalase positive, indole negative, urease negative, methyl red (MR) positive, Voges Proskauer (VP) and citrate negative. Gelatin is not liquefied.

On the basis of fermentation of glycerol, reduction of nitrate, and breakdown of arabinose; there are four biotypes of *Y.pestis*. This typing is of epidemiological significance because of the different geographical distribution of the types (Table 37.2).

D. Resistance

The plague bacillus is destroyed by heat at 55°C in 15 minutes. It is easily destroyed by sunlight, drying and chemical disinfectants (0.5% phenol in 15 minutes). It can survive for several months in the soil of rodent

Table 37.2 Biotypes of *Y. pestis*

Variety	Glycerol fermentation	Nitrate reduction	Arabinose breakdown	Distribution
Y. pestis var. *orientalis*	–	+	+	Primary foci in India, China and Myanmar. Responsible for wild plague in Western USA, South America, South Africa
Y. pestis var. *antiqua*	+	+	+	Transbaikalia, Manchuria, Mangolia, perhaps responsible for the Justinian plague.
Y. pestis var. *mediaevalis*	+	–	+	South-East Russia
Y. pestis var. *microtus*	+	–	–	

burrows. It remains viable for long periods in cold and moist environments.

E. Antigen Structure

Plague bacilli are antigenically homogenous and serotypes do not exist. The antigenic structure is complex and about 20 different antigens have been identified. These include:

1. A heat-labile protein envelope antigen known as Fraction 1 or F-1. It is best formed in cultures incubated at 37°C. It inhibits phagocytosis. This antigen is generally present only in virulent strains. It has therefore been considered to be a virulence determinant. It has an important role in stimulating protective immunity in mice and man.
2. The V and W proteins have been considered to be virulence factors as they inhibit phagocytosis. These are also formed by virulent strains of *Y. pestis*.
3. Virulent strains produce a bacteriocin (Pesticin I), fibrinolysin and coagulase.
4. Other antigens elaborated by plague bacillus include murine and guinea pig toxins. The endotoxin (lipopolysaccharide) is similar to that of other enteric bacteria. Murine and guinea pig toxins are active in rodents and guinea pigs, their role in natural disease of man is not known. These toxins are termed as *plague toxins*.
5. Virulence has also been associated with the ability to synthesise purines.
6. Production of coloured colonies (on medium containing haemin) also appears to be associated with virulence. It appears that plague bacillus possesses an unidentified surface component which absorbs haemin and basic aromatic dyes in cultured media to form coloured colonies.

F. Pathogenesis

Y. pestis is a natural pathogen of rodents and causes zoonotic disease called *plague*. Infection is transmitted from one animal to another by the bite of flea. Two natural cycles of plague exist, the urban and sylvatic plague. Urban plague involves man and rodents (rats) living with him. In addition cat, goat, sheep, dog and camel are also susceptible. Wild or sylvatic plague occurs in wild rodents most important being mice, squirrels and chipmunk.

Plague is a natural disease of rodents and is transmitted to man via the bite of infected rat flea (*X.cheopis*). The incubation period is 2–6 days. In man, plague occurs in three forms: *bubonic*, *pneumonic* and *septicaemic*.

1. Bubonic Plague

As the plague bacillus usually enters through the bite of infected rat flea on the legs, the inguinal lymph nodes are involved, hence the name bubonic (bubo meaning groin). The inguinal lymph nodes become enlarged and suppurate. Patient develops fever, chills and malaise. Pain may accompany the bubo. The infection may stop up to lymph nodes, but often the bacteria reaches the blood stream and gets widely disseminated.

If the bubonic plague is not treated properly, it may progress to meningitis (meningeal plague) after 7–10 days of the onset of bubonic plague.

2. Pneumonic Plague

It is highly infectious form of plague involving the lungs producing haemorrhagic pneumonia. It can be transmitted from man to man by droplet infection (airborne route) and is virtually always fatal. Primary pneumonic plague is rare but it may sometimes occur secondary to bubonic plague. Pneumonic plague may also occur in epidemic form. Patient develops fever and cough with expectoration.

3. Septicaemic Plague

The presence of bacteria in blood denotes septicaemic plague. This may occur as primary infection but it usually occurs as the terminal event of bubonic and pneumonic plague. Massive involvement of blood vessels results in haemorrhages in the skin and mucosa. Due to this manifestation, the disease is given the name *black death*.

Mortality rate in bubonic plague is 50–75%, in pneumonic plague almost 100% and with treatment it is 5–30%.

Pestis minor or benign plague is also seen in some cases during an epidemic. Only clinical presentation may be slight temperature with one or two swollen glands.

G. Epidemiology

Plague is a *zoonotic disease* and may become epidemic in the rat population. The infection may spread to domestic rats and man by flea bites or contact. The commonest vector is *Xenopsiella cheopis* but other fleas like *X.astia*, *Ceratophylus* may also transmit the infection.

Plague is the greatest killer known to mankind. In 14th century, plague pandemic known as "black death" is believed to have killed about a quarter of all mankind. Several pandemics have occurred between 1500 and 1720. Last pandemic occurred in 1894, causing more than 10 million deaths by 1918. During 1958–77, plague occurred in 29 countries and India remained free of plague from 1967 to 1993.

An outbreak of plague was reported from India in 1994. A total of 876 cases of presumptive plague were diagnosed on the basis of serological tests. These cases were reported from Maharashtra, Gujarat, Delhi, Karnataka, Uttar Pradesh and Madhya Pradesh. Outbreak started in Beed district (Maharashtra) for bubonic plague and Surat (Gujrat) for pneumonic plague. Of the total 54 fatal cases, 52 were in Surat. In 2002, a short outbreak of plague was reported from Shimla, claiming 4 lives.

Plague has several scattered natural foci in many countries. It survives in wild rodents, occasionally causing human infections. At least four such foci of plague are known in India. These include the region near Kolar, Bead-Latur belt in Maharashtra (from where the Surat epidemic started), Rohru in Himachal Pradesh (from where the 2002 Shimla outbreak took place) and a small pocket in Uttaranchal.

H. Laboratory Diagnosis

Laboratory diagnosis of plague includes diagnosis of plague in man as well as rodents. Early detection of infection in animals will help in prevention of plague.

1. Specimens

(*i*) Pus or fluid aspirated from bubo in bubonic plague.
(*ii*) Sputum and blood in pneumonic plague.
(*iii*) Blood in septicaemic plague.
(*iv*) Splenic tissue on postmortem.
(*v*) CSF in meningeal plague.

2. Direct Microscopy

Sputum and aspirate from lymph nodes are stained with Gram's staining and methylene blue. Gram's staining shows characteristic Gram negative coccobacilli. With methylene blue staining, bacilli show typical bipolar staining. Wayson's stain is a methylene blue stain used for direct microscopy.

Microscopic examination of buffy coat smear of blood may show plague bacilli in septicaemic cases.

Other more specific direct staining method include the use of fluorescently labelled antibody to the envelope F-1 antigen.

3. Culture

Culture is made on blood agar medium and incubated at 27°C. Biochemical tests are performed to confirm the diagnosis. On ghee broth, there is characteristic *stalactite* growth.

4. Animal Inoculation

Guinea pigs or white rats are injected subcutaneously with exudates from bubo or with 24 hours broth culture. The animals die within 2–5 days. Postmortem reveals marked local inflammatory reaction with necrosis and oedema. Regional lymph nodes are enlarged and congested. Organisms may be demonstrated in smears from local lesions, lymph nodes, spleen and heart blood, after staining with Gram and methylene blue stains.

5. Antigen Detection

F1 glycoprotein Ag may be detected in aspirated fluid from bubo or sputum by immunofluorescence and ELISA test.

Dipstick test

F1 glycoprotein can be detected by dipstick test using monoclonal antibodies. It is a rapid diagnostic test, which produces reliable results within 15 minutes.

6. Serological Tests

Antibodies to F-1 antigen may be detected by passive haemagglutination or complement fixation tests. Antibodies appear towards the end of first week of the illness. Serological tests are useful for identifying plague foci as the tests remain positive for several years after recovery from plague. It has a limited diagnostic value as the titres reach diagnostic levels (1:16 or four fold rise) only after 2–3 weeks following onset of disease.

7. Polymerase Chain Reaction (PCR)

It is a rapid and sensitive method for diagnosis of plague in clinical specimens and fleas.

I. Diagnosis of Plague in Rats

Before examining rats, immerse them in disinfectant to kill any flea. Necropsy reveals enlargement of lymph nodes with periglandular inflammation and oedema. Generally cervical lymph nodes are involved due to the tendency of the rat flea to bite rat's neck region. Other features include pleural effusion, enlargement of spleen and haemorrhages under the skin and in internal organs.

Specimens (heart blood, lymph nodes and spleen) collected from the animals are utilised for smear examination, culture and animal inoculation test.

J. Treatment

Streptomycin is the drug of choice in both bubonic and pneumonic plague. Early treatment is essential because of high fatality of the disease. Combination of streptomycin and tetracycline is very effective. Chloramphenicol and kanamycin are also effective.

K. Prophylaxis

1. General Measures

General measures such as control of fleas and rodents are of great importance. Spray insecticides (DDT) inside the rodent burrows and houses to kill the fleas. After the fleas have been killed, kill the rats with rodenticides. Attempt should not be made to kill the rats unless spraying has first been done otherwise the fleas will jump from the carcasses of rats to man. Stores especially near railway stations, airports and seaports must be rat-free, otherwise infected rats with rat fleas may travel along the cargo to far off places.

2. Specific Measures

Two types of vaccines can be used—killed and live attenuated.

(i) Killed vaccine

Killed vaccine is widely used for active immunisation. It is a whole bacterial culture antigen of plague bacillus. It is prepared at Haffkine Institute, Mumbai. The plague bacillus (virulent strain) is grown in casein hydrolysate broth for 2–4 weeks at 32°C and killed by 0.05% formaldehyde and preserved with phenyl mercuric nitrate (Sokhey's modification of Haffkine's vaccine). The vaccine contains 2,000 million organisms per ml.

(a) Dose schedule

- 0.5 ml is injected subcutaneously followed by 1 ml after 7–14 days.
- Booster dose is given every 6 months.

(b) Immunity

Immunity appears 5–7 days after vaccination and lasts for 6 months. Vaccination may reduce the morbidity and mortality in bubonic plague but not in pneumonic plague. Vaccine should be given to health workers and to those planning to visit plague infected areas.

(c) Side effects

Fever, headache, lymphadenopathy and erythema at the site of inoculation are some of the side effects. Vaccination is not very effective, therefore, even vaccinated individuals should also be given chemoprophylaxis when exposed to plague.

(ii) Live vaccine

Live vaccines are prepared from two avirulent strains of *Y. pestis*, *Otten's Tjiwidej* strain from Jawa and Girard's EV 76 strain from Malagasey. Since live vaccines are difficult to prepare and carry some risk of unacceptable reactions, killed vaccines are recommended for general use. However, mass vaccination is no more recommended.

3. Chemoprophylaxis

Close contacts of patients with plague should be given a course of tetracycline (500 mg 6 hourly for one week).

II. YERSINIA PSEUDOTUBERCULOSIS AND YERSINIA ENTEROCOLITICA

These two organisms resemble *Y. pestis* in that they are small, Gram negative bacilli with bipolar staining and reservoirs of infection are rodents, wild and domestic animals. They differ from *Y. pestis* by their motility at 22°C (and not at 37°C), non-capsulated, urease positive, oxidase negative and insusceptible to *Y. pestis* bacteriophage (Table 37.3).

A. Yersinia pseudotuberculosis

It ferments rhamnose and melibiose. It is antigenically heterogenous. On the basis of somatic and flagellar antigens, it is divided into six serological groups and nine serotypes. *Y. pseudotuberculosis* exhibits antigenic cross reaction with *Y. pestis* as well as with salmonellae.

Pseudotuberculosis is a zoonosis. The infection occurs in animals by alimentary route resulting in *epizootic* and *enzootic* form of disease. A wide range of animals such as guinea pigs, rabbits, birds etc. are susceptible. The liver, spleen and lungs of infected animals show multiple nodules resembling tuberculosis lesions and hence the name pseudotuberculosis.

Man acquires infection through ingested materials contaminated with animal faeces. Patients develop acute mesenteric lymphadenitis. Occasionally, it may result in a severe generalised disease. The term *yersiniosis* denotes infection with yersiniae other than *Y. pestis*.

Table 37.3 Distinguishing Features of Three Species of Yersinia

Species	Motility at		Fermentation of				Urease	VP test	Ornithine decarboxylase
	22°C	37°C	Sucrose	Rhamnose	Cellobiose	Melibiose			
Y. pestis	–	–	–	–	–	–	–	–	–
Y. pseudotuberculosis	+	–	–	+	–	+	+	–	–
Y. enterocolitica	+	–	+	–	+	–	+	+	+

Laboratory diagnosis includes isolation of the organisms from an excised mesenteric lymph node, or blood and demonstration of antibodies in patient's serum during the acute illness.

B. Yersinia enterocolitica

It is a Gram negative coccobacillus and resembles *Y. pseudotuberculosis* in being motile at 22°C, but differs from it in fermenting sucrose and cellobiose and decarboxylating ornithine. It does not ferment melibiose or rhamnose (Table 37.3).

Many strains are indole and VP positive. It is aerobic and facultative anaerobe. Optimum temperature for growth is 22°C. On blood agar, smooth translucent, non-haemolytic colonies measuring 2–3 mm in diamenter appear after 48 hours incubation at 22°C. On MacConkey agar, it forms pin-point, non-lactose fermenting (NLF) colonies.

Y. enterocolitica has a distinct antigenic structure and so far 60 O-antigenic groups and about 20 heat-labile flagellar (H) antigens have been identified. Most of the human infections are due to serogroups O3, O8 and O9. It can be divided into six biotypes–1A, 1B, 2, 3, 4 and 5 by using biochemical tests.

Y. enterocolitica has been isolated from a wide range of domestic animals. Disease occurs via faeco-oral route by contaminated food and water. In man it may cause gastroenteritis, mesentric lymphadenitis and septicaemia. It affects children and adults of both sexes. The incubation period varies from 5 to 10 days. The early symptoms consist of fever, abdominal pain and diarrhoea. Diarrhoea is probably due to an enterotoxin or invasion of mucosa by the bacteria.

The laboratory diagnosis consists of isolation of the organism and indirectly by demonstration of antibodies in the patient serum. The organisms can be isolated from faeces, blood or from mesenteric lymph nodes. Blood agar and MacConkey agar are used for growing this organism. Cefsulodin-irgasan-novobiocin (CIN) agar is a selective medium for recovery of *Y. enterocolitica* from faeces. Colonies of *Y. enterocolitica* have a bull's eye appearance with a red center on CIN agar.

III. PASTEURELLA MULTOCIDA (P. SEPTICA)

A group of related bacteria isolated from haemorrhagic septicaemia in various animals and birds had, in the past, been named according to their species of origin—*P. boviseptica, P. lepiseptica, P. aviseptica* etc, but now they are regarded as strains of a single species designated *P. multocida*. Pasteur developed first attenuated vaccine from *P. aviseptica*, hence the name *Pasteurella*.

P. multocida is a non-motile, Gram negative rod resembling Yersinia but differs from it in being oxidase positive, producing indole and failing to grow on MacConkey's agar.

The bacillus is usually normal inhabitant of respiratory tract of a variety of animals such as dogs, rats, cats, cattle and sheep. It may sometimes occur as normal commensal in throat in humans. Human infection is rare but it may cause septic wound following animal bites, meningitis following head injury, respiratory tract infections or appendicitis. The bacillus is susceptible to tetracycline, streptomycin and penicillin.

IV. OTHER PASTEURELLA SPECIES

P. canis, P. dagmatis and P. stomatis are other three important species of *Pasteurella*. There species have been isolated from humans.

V. FRANCISELLA TULARENSIS (PASTEURELLA TULARENSIS)

F. tularensis is a causative agent of tularaemia, a major zoonotic disease of rabbits and other rodents. The disease was originally described in Tulare County, California. The disease is transmitted by ticks and several other arthropod vectors among the rodents. Human infection may occur by handling or eating infected meat or drinking contaminated water. It may also occur through tick bites.

It is a capsulated, non-motile, Gram negative bacillus measuring 0.3–0.7 μm × 0.2 μm. It has fastidious growth requirements and special media such as Francis blood dextrose cystine agar is used for its isolation. Minute transparent colonies appear after incubation for 3–5 days.

In humans, tularaemia may present as a local ulceration with lymphadenitis, a typhoid-like fever with glandular enlargement or an influenza like infection. The disease has been reported from North America, Europe and Asia. The organism is highly infectious and laboratory infection has been quite common.

Laboratory diagnosis may be made by smear examination, culture or by inoculation into guinea pigs. Pus from local lesions, blood during bacteraemia and sputum (in pulmonary involvement) are used for diagnosis. Patient's serum may be tested for agglutinating antibodies, a titre of 1:80 or higher is regarded as diagnostic. Since *F. tularensis* shares somatic antigens with both *Br. abortus* and *Br. melitensis*, false positive reactions may occur with brucella agglutinins.

Streptomycin is the drug of choice. An attenuated vaccine is available which can be administered to persons who are subject to high risk of infection.

KEY POINTS

1. Yersiniae are Gram negative, short bacilli showing bipolar staining (*safety pin appearance*) with methylene blue. These are non-motile except *Yersinia pseudotuberculosis* and *Y. enterocolitica*. They are primary pathogens of rodents and men are accidentally infected.
2. The genus yersinia contains three medically important species—*Y. pestis* (the caustive agent of plague), *Y. pseudotuberculosis* (a primary pathogen of rodents) and *Y. entercolitica* (causes human diarrhoeal diseases).
3. *Y. pestis* is Gram negative bacillus, non-motile and capsulated. It grows at 27 °C but envelope develops best at 37 °C. It grows on ordinary media.
4. Envelope antigen known as Fraction 1 or F-1 is an important antigen of *Y. pestis*.
5. In man, plague occurs in three forms: *bubonic*, *pneumonic* and *septicaemic*.
6. Plague is a *zoonotic* disease. It is a natural disease of rodents and is transmitted to man via the bite of infected rat flea (*Xenopsiella cheopis*).
7. Laboratory diagnosis of plague depends upon direct microscopy, culture and antigen detection.
8. *Y. enterocolitica* differs from *Y. pestis* by its motility at 22 °C (and not at 37 °C), *non-capsulated*, *urease positive*, *VP positive*, and *ornithine decarboxylase positive*.
9. In man *Y. enterocolitica* may cause *gastroenteritis*, *mesentric lymphadenitis* and *septicaemia*.
10. Laboratory diagnosis consists of isolation of the organism.

YOU MUST KNOW

1. Morphology, culture characteristics and biochemical reactions of *Yersinia pestis*.
2. Laboratory diagnosis of plague.
3. Vaccines used for prevention of plague.
4. *Yersinia enterocolitica*.

STUDY QUESTIONS

1. Describe the laboratory diagnosis of plague.
2. Write short notes on:
   (a) Prophylaxis against plague
   (b) *Yersinia pseudotuberculosis*
   (c) *Yersinia enterocolitica*
   (d) *Francisella tularensis*.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. 'Safety pin appearance' on methylene blue staining is a characteristic feature of:
   (a) *Yersinia pestis*
   (b) *Pseudomonas aeruginosa*
   (c) *Burkholderia mallei*
   (d) *B. pseudomallei*
2. What is the causative agent of plague?
   (a) *Yersinia pestis*
   (b) *Y. enterocolitica*
   (c) *Y. pseudotuberculosis*
   (d) *Francisella tularensis*
3. Plague is transmitted to man by bite of infected:
   (a) Sandfly
   (b) Rat flea
   (c) Hard tick
   (d) Mite
4. Bubonic plague is transmitted by:
   (a) Infected rat flea
   (b) Infected sandfly
   (c) Inhalation
   (d) Ingestion

5. Pneumonic plague can be transmitted from human to human by:
   (a) Droplet infection
   (b) Ingestion
   (c) Infected sandfly
   (d) All of the above
6. The name '*black death*' has been given to:
   (a) Plague
   (b) Miliary tuberculosis
   (c) Tularensis
   (d) Legionnaire's disease
7. Which vaccine is recommended for prophylaxis against plague?
   (a) Killed vaccine
   (b) Live vaccine
   (c) DNA vaccine
   (d) None of the above
8. *Yersinia enterocolitica* can be differentiated from *Yersinia pestis* by:
   (a) Motility at 22°C
   (b) Non-capsulated
   (c) Decarboxylation of ornithine
   (d) All of the above
9. Which is the selective medium for growing *Yersinia enterocolitica* from faeces?
   (a) Cefsulodin-irgasan novobiocin agar
   (b) Butzler's selective medium
   (c) Tinsdale agar
   (d) All of the above
10. The diseases caused by *Yersinia enterocolitica* in man include:
    (a) Gastroenteritis
    (b) Mesenteric lymphadenitis
    (c) Septicaemia
    (d) All of the above

Answers (MCQs):

1. (a)	2. (a)	3. (b)	4. (a)	5. (a)
6. (a)	7. (a)	8. (d)	9. (a)	10. (d)

Chapter 38

LEGIONELLA

Legionella pneumophila

A. Morphology
B. Culture
C. Biochemical Reactions
D. Susceptibility to Physical and Chemical Agents
E. Serogrouping
F. Pathogenesis
G. Laboratory Diagnosis
H. Treatment
I. Prevention

In 1976, 221 cases of pneumonia occurred in members of the American Legion who attended a convention in Philadelphia. Hence, this form of pneumonia was named as Legionnaire's disease and the causative agent as *Legionella pneumophila*. The aerosols of water from air-conditioning system led to the isolation of the organism. The genus *Legionella* contains 52 species of bacteria. *L.pneumophila* is the most important species. In addition to this, some other species associated with human disease include *L.micdadei, L. longbeachae, L.bozemanii, L.dumoffii, L. gormanii, L.anisa, L. birminghamensis, L. feeleii, L. hackeliae, L. jordanis, L. wadsworthii and L. tucsonensis*.

LEGIONELLA PNEUMOPHILA

A. Morphology

L. pneumophila is a Gram negative bacillus measuring 2–5 μm × 0.3–0.9 μm. In infected tissues, it appears as a short rod or coccobacillus but in culture it becomes longer. It is motile with polar or subpolar flagella. It is non-capsulated, non-sporing and non-acid-fast.

It is Gram negative but stains poorly. It stains better with silver impregnation method but is best visualised by direct fluroescent antibody (DFA) staining with monoclonal or polyclonal sera.

B. Culture

These are fastidious organisms and do not grow on ordinary media but require the presence of cysteine and iron for primary isolation. They are strict aerobe and grow best at pH 6.8 to 7.8 and at 35–37°C temperature. They can be grown on buffered charcoal-yeast extract agar (BCYE) and Mueller-Hinton medium supplemented with ferric salts plus L-cysteine as essential growth factors.

Colonies of *L. pneumophila* usually appear in 3–5 days but some species may require incubation up to 10 days. Colonies are circular, 1–2 mm in diameter, grey or grey blue and low convex with a slightly irregular edge. Colonies have a classical *cut glass* appearance when examined under the plate microscope. With continued incubation, the colonies become larger and more opaque.

C. Biochemical Reactions

L. pneumophila is catalase positive and oxidase variable. It hydrolyses hippurate, starch and gelatin.

D. Susceptibility to Physical and Chemical Agents

L. pneumophila is readily killed by 1% formalin, 2% glutaraldehyde and 70% ethyl alcohol. It can be destroyed by 5 parts per million (ppm) of available chlorine in one minute.

E. Serogrouping

L. pneumophila is divided into more than 14 serogroups. All these serogroups have been associated with human disease but most infections are caused by serogroup 1.

F. Pathogenesis

The natural habitat of *L. pneumophila* is water. The organisms have been isolated from cooling towers, airconditioning systems, humidifiers, whirlpool baths, showers and respiratory ventilators. These sources facilitate transmission of the organism to the human respiratory tract by generating infectious aerosols. Person-to-person transmission has not been demonstrated.

Legionella infection may occur in two main forms which are designated as Legionnaire's disease and Pontaic fever, together known as *legionellosis*.

1. Legionnaire's Disease

It is a pneumonic illness which may also progress to involve virtually every system of the body. The incubation period ranges from 2 to 10 days. Patient develops malaise, fever, headache, myalgia, respiratory distress and non-productive cough. Case fatality may be 15–20 per cent. Smoking, alcohol, diabetes, immunosuppressive therapy and pre-existing lung disease are risk factors for legionnaire's disease.

2. Pontaic Fever

It is a milder, non-fatal 'influenza-like' illness with fever, chills, myalgia and headache.

G. Laboratory Diagnosis

Laboratory diagnosis of *Legionella* infections include:

1. Microscopy

Legionellae can be detected in the clinical specimens (sputum, bronchial aspirate, pleural fluid, lung biopsy or autopsy material) by direct immunofluorescence test using labelled specific monoclonal or polyclonal antisera. Gram-stained smears are of little value as legionellae stain poorly.

2. Culture

Specimen is inoculated on BCYE agar and incubated at 35–37°C up to 10 days. Bacteria from colonies are identified by direct immunofluorescence test with specific antisera as described above.

3. Detection of Legionella Antigen

Legionella antigen in the urine persists for months. This antigen can be detected in the urine by enzyme linked immunosorbent assay (ELISA). This is a rapid and specific test for the identification of *L. pneumophila* in cases of pneumonia.

Immunochromographic assay that detects antigen in urine is also available. It takes only 15 minutes.

4. Serological Test

Antibodies in serum can be detected by ELISA or indirect fluorescent antibody test (IFAT). A four fold or greater rise in antibody titre or a high titre (>256) of antibody may be considered diagnostic of legionellosis.

H. Treatment

Erythromycin is the drug of choice in legionellosis. Rifampicin and ciprofloxacin are also effective.

I. Prevention

Legionellae from water may be eradicated either by heat above 60°C or disinfection with chlorine or other biocides. No vaccine is available against *Legionella* infections.

KEY POINTS

1. *Legionella pneumophila* is the most important species of the genus *Legionella*.
2. It is a Gram negative bacillus, motile with polar or subpolar flagella. It stains better with silver impregnation method but is best visualised by direct fluorescent antibody (DFA) staining with monoclonal or polyclonal sera.
3. The natural habitat of *L. pneumophila* is water. The organisms have been isolated from cooling towers, airconditioning systems, showers and respiratory ventilators. These sources facilitate transmission of the organism to the human respiratory tract by generating infectious aerosols. Legionella infection may occur in two main forms—*Legionnaire's disease* and *Pontaic fever*, together known as *legionellosis*.
4. Laboratory diagnosis depends on microscopy, culture, detection of antigen and by DNA probes.

YOU MUST KNOW

1. Morphology, culture characteristics and biochemical reactions of *Legionella pneumophila*.
2. Diseases caused by *L. pneumophila*.

STUDY QUESTIONS

1. Discuss the laboratory diagnosis of infections with *Legionella pneumophila*.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. The causative agent of Pontaic fever is:
   (a) *Legionella pneumophila* (b) *Yersinia pseudotuberculosis*
   (c) *Pseudomonas putida* (d) *Francisella tularensis*
2. Mode of infection in Legionnaire's disease is/are:
   (a) Infectious aerosols (b) Skin inoculation
   (c) Ingestion (d) All of the above
3. The natural habitat of *Legionella pneumophila* is:
   (a) Water (b) Faeces
   (c) Sputum (d) Urine
4. Which is the antibiotic of choice in *Legionella* infections?
   (a) Erythromycin (b) Cephalosporins
   (c) Penicillins (d) All of the above

Answers (MCQs):

1. (a) 2. (a) 3. (a) 4. (a)

Chapter 39

HAEMOPHILUS AND GARDNERELLA

The genus *Haemophilus* contains non-motile, non-sporing, Gram negative bacilli and require one or both of two accessory growth factors (X and V) present in blood (*Haemophilus* meaning blood loving).

Pfeiffer (1892) isolated the organism from the sputa of patients from the influenza pandemic and proposed this as the causative agent of human influenza. This came to be known as 'influenza bacillus' (Pfeiffer's bacillus). It was renamed as *H. influenzae*. The discovery of influenza virus (causative agent of human influenza) in 1933 established that *H. influenzae* was only a secondary invader.

H. influenzae and *H. ducreyi* are the major pathogens in the genus *Haemophilus*. *H. influenzae* may cause meningitis, pneumonia, epiglottitis, bronchitis, otitis media, septic arthritis and *H. ducreyi* is the causative organism of chancroid.

I. HAEMOPHILUS INFLUENZAE

A. Morphology

It is a small (1.5 μm × 0.3 μm), Gram negative, non-motile bacillus showing considerable pleomorphism. It is non-sporing and non-acid-fast. In young cultures (18–24 hours), the cells are usually coccobacillary, while in older cultures, long filamentous forms may be seen. In sputum, they occur as small clumps of coccobacilli and in CSF from meningitis cases, long and filamentous forms

predominate. Virulent strains possess capsule while the avirulent strains and older cultures are non-capsulated.

B. Culture

H. influenzae has fastidious growth requirements. It grows better in aerobic than in anaerobic conditions. It requires enriched media such as blood agar or chocolate agar because the accessory growth factors known as X ('X' for unknown) and V ('V' for vitamin) present in blood are essential for growth. The optimum temperature for growth is 35–37°C, some strains require 5–10% CO_2. It cannot grow on nutrient agar which lacks the accessory growth factors.

1. X Factor

It is a heat stable protoporphyrin IX, haemin or other iron-containing porphyrin. It is necessary for the synthesis of catalase and other enzymes cytochrome C, cytochrome oxidase, involved in aerobic respiration.

Porphyrin test

The porphyrin test is an alternative method for differentiating the haemin-producing species of *Haemophilus*. This test can be performed in agar, in broth, or on a disc. The principle of the test is based on the ability of the organism to convert the substrate *deltaaminolevulinic* acid (ALA) into porphyrins or porphobilinogen. Porphyrins and porphobilinogens are intermediates in the synthesis of X-factor. Porphobilinogen can be detected by the addition of Kovac's reagent (p-dimethyl aminobenzaldehyde). After adding Kovac's reagent, a red colour develops in the lower acqueous phase if porphobilinogen is present. Porphyrins can be detected using an ultraviolet light (Wood's lamp). Porphyrins fluoresce reddish-orange under ultraviolet light.

Species that are porphyrin negative cannot synthesise haemin and therefore require haemin (X-factor) for their growth.

2. V Factor

It is a heat labile (destroyed at 120°C in 30 minutes) factor, present in red blood cells and in many other animal and plant cells. It is synthesised by some fungi and bacteria (e.g. *Staph. aureus*). It is either nicotinamide adenine dinucleotide (NAD, coenzyme I) or NAD phosphate (NADP, coenzyme II). It appears to act as a hydrogen acceptor in the metabolism of the cell. Ordinary blood agar is not suitable for the growth of *H. influenzae* where growth is scanty, as the V factor is not freely available, being imprisoned inside the red blood cells. V factor is released from erythrocytes in chocolate agar (heated blood agar at 80-90°C). V factor is also synthesised by Staphylococci. Growth on blood agar can be improved by providing a source of V factor (*Staph. aureus*). The differential requirement for X and V factors helps to distinguish various species of *Haemophilus* (Table 39.1). Species with prefix *para-*(*H. parainfluenzae, H. parahaemolyticus* and *H. paraphrohaemolyticus*) and *H. pittmaniae* require V factor only for their growth.

Table 39.1 Requirement of X and V Factor of Haemophilus Species

Species	X factor	V factor	Haemolysis
H. influenzae	+	+	–
H. aegyptius	+	+	–
H. ducreyi	+	–	±
H. parainfluenzae	–	+	–
H. haemolyticus	+	+	+
H. parahaemolyticus	–	+	+
H. paraphrohaemolyticus	–	+	+
H. pittmaniae	–	+	+

Blood agar with *Staph. aureus* streak and chocolate agar are routinely used for the identification of *H. influenzae*. The colonies are small, translucent and non haemolytic on blood agar. Capsulated strains produce distinctive iridescent colonies.

Better growth of *H. influenzae* is also obtained on **Levinthal's medium** (prepared by boiling and filtering a mixture of blood and nutrient broth) or **Fildes agar** (by adding a peptic digest of blood to nutrient agar).

Satellitism

Although blood agar contains X and V factors, colonies of *H. influenzae* are small due to non-availability of V factor. After inoculating suspected *H. influenzae* on a blood agar plate, *Staph. auerus* is streaked across the same blood agar plate and incubated at 37°C for 18–24 hours. The colonies of *H. influenzae* will be large and well developed alongside the streak of staphylococci while those further away from staphylococcal streak are smaller. This phenomenon is called *satellitism* and demonstrates that V factor is available in high concentration near the staphylococcal growth and only in smaller quantities away from it (Fig. 39.1).

C. Biochemical Reactions

H. influenzae is catalase and oxidase positive, ferments glucose and galactose, reduces nitrate to nitrite. It does not ferment sucrose, lactose and mannitol. On the basis of production of indole, urease, ornithine decarboxylase, it is divided into eight (I–VIII) biotypes (Table 39.2). Majority of clinical isolates belong to biotype I to III and

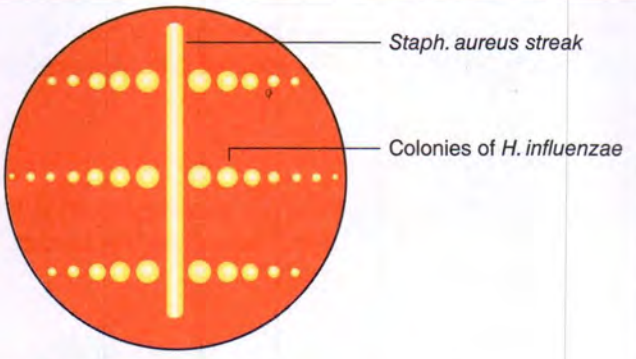


Fig. 39.1 Satellitism

type b organisms belong to biotype I. However, biochemical reactions are not helpful in identification.

Table 39.2 Biotypes of ***H. influenzae***

Biotype	Indole	Urease	Ornithine decarboxylase
I	+	+	+
II	+	–	+
III	–	–	+
IV	–	+	+
V	+	+	–
VI	–	+	–
VII	+	–	–
VIII	–	–	–

D. Antigenic Structure

Isolates occur in capsulated and non-capsulated forms. Three major surface antigens are present—capsular polysaccharide, outer membrane protein (OMP) and lipo-oligosaccharide (LOS).

The capsulated strains produce a capsule which is polysaccharide in nature. On the basis of capsular material, *Pitman* divided *H. influenzae* into 6 serotypes—a, b, c, d, e, and f. Capsular serotype b strains (Hib) are associated with most invasive infections. Serotyping of *H. influenzae* was orginally done by agglutination reaction using type specific antisera. Other methods include, Quellung reaction, coagglutination, CIEP and ELISA. Diagnostic kits for the identification of *H. influenzae* type b (Hib) are available.

The type b capsular polysaccharide has a unique chemical structure containing polyribosyl ribitol phosphate (PRP) which induces IgG, IgM and IgA antibodies. These antibodies are bactericidal, opsonic and protective. Hib PRP is therefore employed for immunisation.

Non-capsulated strains cannot be typed and are called 'non-typable strains'.

OMP antigens of Hib have been classified into 13 subtypes. LOS are antigenically complex. OMP and LOS subtyping are of epidemiological value.

The complete genome of *H. influenzae* has been sequenced.

E. Variation

Colonies of *H. influenzae* show a smooth to rough (S ⟶ R) variation accompanied by loss of capsule and virulence. Genetic transformation has been demonstrated in *H. influenzae* to transfer the characters of capsular antigens and antibiotic resistance. Non-capsulated strains become capsulated by genetic transformation.

F. Sensitivity to Physical and Chemical Agents

It is a delicate organism. It is readily killed by heat (55°C for 30 minutes), refrigeration (at 4°C), disinfectants and drying.Cultures may be preserved on chocolate agar. For long-term preservation, the culture may be lyophilised.

G. Virulence Factors

1. *Capsular polysaccharide*: It resists phagocytosis. Loss of capsule leads to loss of virulence.
2. *Pili (Fimbriae)*: They help in attachment of organisms to epithelial cells.
3. *Outer membrane proteins*: They contribute in adhesion and invasion of host tissues.
4. *IgA1 protease*: *H. influenzae* produces a protease that specifically cleaves the heavy chain of IgA1.

H. Pathogenesis

It is an obligate human parasite. The organism enters by respiratory route. The non-capsulated strains of *H. influenzae* are regular commensal in the nasopharynx or oropharynx. Colonisation by capsulated strains is found in 5–10% cases, of which type b strains are seen in 1–5%. Capsulated strains cause invasive infections, type b accounting for most cases.

The following infections are caused by *H. influenzae*.

1. *Meningitis*

This is the most serious disease occurring in children of 2 months to 2 years of age. The fatality rate is about 90% in untreated patients. The bacilli reach the meninges from the nasopharynx, apparently through the blood stream. Majority of the cases are due to type b strains.

2. *Acute Epiglottitis*

It is the second most common infection caused by *H. influenzae*. This is an acute inflammation of the epiglottis with obstructive laryngeal oedema, seen in children over

two years old. This condition is always associated with bacteraemia and the organisms can be isolated from blood cultures.

3. Pneumonia
It typically occurs in infants and is accompanied by empyema and sometimes meningitis as well. While these primary infections are caused by capsulated strains, bronchopneumonia may occur as secondary infection with non-capsulated strains.

4. Bronchitis
H. influenzae is an important pathogen associated with pneumococci in the acute exacerbations of chronic bronchitis.

5. Suppurative Lesions
Some of the suppurative lesions include septic arthritis, endocarditis, pericarditis and otitis media.

I. Laboratory Diagnosis
1. Specimens
Depending upon the type of lesion, the following specimens may be collected:

(*i*) Cerebrospinal fluid (CSF)
(*ii*) Blood
(*iii*) Throat swab
(*iv*) Sputum
(*v*) Pus
(*vi*) Aspirates from joints, middle ears or sinuses etc.

2. Collection and Transport
Specimens should be collected in sterile containers and under all aseptic conditions. As *H. influenzae* is very sensitive to low temperature, therefore, clinical specimens should never be refrigerated. For optimal yield, specimens should be transported to laboratory without delay and inoculated on culture media immediately.

3. Direct Microscopy
(i) Gram staining
In meningitis, Gram stained smear of CSF shows pleomorphic Gram negative coccobacilli.

(ii) Immunofluorescence and quellung reaction
These can be employed for direct demonstration of *H. influenzae* after mixing with specific type b antiserum.

(iii) Antigen detection
Type b capsular antigen can also be detected in patient's serum, CSF, urine or pus by:

(a) Latex agglutination
Latex particles coated with antibody to type b antigen are mixed with the specimen. In positive test, agglutination occurs.

(b) Coagglutination
Instead of latex particles, *Staph. aureus* is coated with antibody to type b antigen and mixed with specimen. If positive, agglutination occurs.

(c) Counter immunoelectrophoresis (CIE)
Specific antiserum is put in one well of agarose gel and specimen is put in other well. Current is passed. In positive test, precipitation line occurs in between the two wells.

4. Culture
(i) CSF culture
CSF should be plated promptly on blood agar or chocolate agar. A strain of *Staph. aureus* should be streaked across the blood agar plate on which the specimen has already been inoculated. Plates are then incubated at 35–37°C, aerobically with 5–10% CO_2, overnight. The isolate is then identified by its colony morphology, satellitism, Gram staining and serotyping.

(ii) Blood culture
It is usually positive in cases of epiglottitis and pneumonia.

(iii) Sputum culture
Sputum should be homogenised by treatment with pancreatin or by shaking with sterile water and glass beads for 15–30 minutes. Several samples of the sputum are to be cultured to increase the rate of isolation.

5. Colony Morphology and Staining
After overnight incubation, small opaque colonies appear that show satellitism. A smear is made from colony and stained with Gram stain. It shows small Gram negative bacilli or coccobacilli

6. Serotyping
Typing may be done with type specific antisera.

J. Treatment
H. influenzae is susceptible to sulphonamides, chloramphenicol, trimethoprim, ampicillin, tetracycline, ciprofloxacin, cefuroxime, cefotaxime and ceftazidime. Cefotaxime or ceftazidime is the drug of choice for the treatment of *Haemophilus* meningitis.

Beta-lactamase bearing strains have acquired resistance to ampicillin. Amoxycillin-clavulnate or Clarithromycin is more effective.

K. Prophylaxis

1. A purified type b capsular polysaccharide vaccine is used in children of 18–24 months. Vaccine is administered in two doses at an interval of two months.
2. Hib PRP vaccine in which type b capsular polysaccharide is covalently coupled to proteins such as diphtheria toxoid, tetanus toxoid and *N. meningitidis* outer membrane protein. Such Hib PRP are available for use in young children.
3. Rifampicin is given for four days to prevent infection in contacts and also to eradicate carrier state.

II. OTHER HAEMOPHILUS

A. Haemophilus influenzae biotype aegyptius (Koch-Weeks bacillus; H.aegyptius)

It was first observed by *Koch* (1883) in cases of acute conjunctivitis in Egypt and was first cultured by *Weeks* (1887) in New York. It causes a highly contagious form of conjunctivitis (pink eye). It is worldwide in distribution.

The organism, formerly known as *H. aegyptius,* is now thought to be a biotype of *H. influenzae.* It has features similar to that of *H. influenzae* biotype III.

It also causes Brazilian purpuric fever (BPF) which is characterised by conjunctivitis, high fever, vomiting, purpura, petechiae, septicaemia and shock. It responds to combination of ampicillin and chloramphenicol.

B. Haemophilus ducreyi

H. ducreyi was first demonstrated in 1890 by *Ducrey* in chancroid lesions, a venereal disease transmitted by direct contact.

1. Morphology

H. ducreyi is a short, Gram negative coccobacillus, 1–1.5 μm × 0.6 μm in size. They have a tendency to occur in groups or in parallel chains. They frequently take bipolar staining. The microscopic findings has been described as resembling a '*school of fish*' appearance.

2. Culture

It does not grow in ordinary laboratory media but has complex growth requirements. It requires X factor but not V factor for its growth. Primary isolation is difficult. It can be grown on rabbit-blood agar, fresh clotted rabbit blood or chocolate agar enriched with 1% Iso Vitalex, and containing vancomycin as a selective agent. It requires 10% CO_2 and high humidity for primary isolation. Optimum temperature for growth is 35°C for 2–8 days. It may also be grown on chorioallantoic membrane of the chick embryo.

After 24 hours of incubation, the colonies of *H. ducreyi* are small, grey, pin point to 0.5 mm in diameter, translucent, non-mucoid. After 48–72 hours the colonies are 1–2 mm in diameter and become semiopaque.

3. Biochemical Reactions

It is biochemically inert except positive nitrate reduction test.

4. Pathogenesis

H. ducreyi causes chancroid or soft sore, a highly contagious sexually transmitted disease (STD) characterised by tender, non-indurated, irregular ulcers on the genitalia. The infection remains localised, spreading only to the inguinal lymph nodes which are enlarged and painful. Sometimes it leads to inguinal abscess called bubo. There is no immunity following infection but a hypersensitivity develops. Chancroid is also known as *soft chancre.*

5. Laboratory Diagnosis

(i) Specimens

Scrape edge of the ulcer or aspirated material from bubo.

(ii) Direct microscopy

Gram staining of the smear shows typical Gram negative coccobacilli.

(iii) Culture

Specimens are inoculated on chocolate agar with 1% Iso Vitalex. Vancomycin is added to make the medium selective. Incubate the medium at 35°C in a humid atmosphere and 10% CO_2 for 2–8 days and look for characteristic colonies.

(iv) Colony morphology and staining

Colonies are grey, small, translucent and non-mucoid. Gram staining shows Gram negative coccobacilli.

(v) Agglutination

H. ducreyi is antigenically homogeneous and cultures are identified by agglutination with the antiserum.

6. Treatment

Co-trimoxazole or erythromycin is the drug of choice but resistant strains occur. Newer drugs including cefotaxime and ciprofloxacin have been shown to be effective.

C. Haemophilus parainfluenzae

It differs from *H. influenzae* in requiring the V factor only, and not the X factor. It is a commensal in the upper respiratory tract and may occasionally cause acute pharyngitis, subacute bacterial endocarditis and urethritis.

D. Haemophilus haemolyticus

It occurs as a commensal in the upper respiratory tract. It requires both X and V factors. It produces a zone of beta haemolysis around its colonies on blood agar and may be mistaken for haemolytic streptococci. It is non-pathogenic. Similar strains that require only the V factor and not the X factor are termed *H. parahaemolyticus*.

III. HACEK GROUP BACTERIA

HACEK refers to a group of fastidious bacteria, normally resident in the mouth, but can sometimes cause severe infections such as endocarditis. This group contains *Haemophilus sp. (parainfluenzae)*, *Aggregatibacter actinomycetemcomitans, Aggregatibacter aphrophilus* (Previously *Haemophilus aphrophilus* and *paraphrophilus*) *Cardiobacterium hominis, Eikenella corrodens* and *Kingella kingae.* HACEK is an acronym consisting of the first initial of each genus represented in this group. Members of this group are Gram-negative bacilli have in common the need for an increased CO_2 (capnophilic). Blood cultures from patients with these bacteria take 7 to 30 days to become positive. As drug resistance is common, antibiotic sensitivity tests are essential.

IV. GARDNERELLA VAGINALIS (Corynebacterium vaginalis; Haemophilus vaginalis)

As this bacillus does not require X and V factors, it has been placed in the new genus *Gardnerella*. It has been isolated from the normal vagina and male urethra. *G. vaginalis* is primarily known for its association with **bacterial vaginosis** (BV) in humans.

A. Morphology

These are small, 1–2 μm × 0.3–0.6 μm in size, Gram negative, non-sporing, non-capsulated, non-motile, pleomorphic bacilli. They are sometimes Gram variable.

B. Culture

The organisms grow on enriched media such as blood agar or chocolate agar. They are facultative anaerobe and grow well at optimum temperature of 37°C and optimum pH of 6.8. The growth is enhanced by 5% CO_2 and humidity. The inoculated medium is incubated for 48 hours. Small beta- haemolytic colonies develop on human or rabbit (but not horse) blood agar. The medium of choice for *G. vaginalis* is **human blood bilayer Tween (HBT)** agar.

C. Biochemical Reactions

It is catalase, oxidase, indole and urease negative.

D. Pathogenesis

G. vaginalis causes non-specific vaginitis and cervicitis, usually in association with anaerobic bacteria (anaerobic vaginosis). In vaginosis, the vaginal discharge emits a fishy odour. Gram staining shows *clue cells,* which are vaginal epithelial cells covered with many tiny Gram variable rods—the so called *clue cells* of Gardner and Duke.

E. Clinical Features

There is foul smelling vaginal discharge with raised pH (more than 5.0). The odour is due to amines and that gets intensified by mixing with a drop of KOH and is named *amine test*.

F. Diagnosis

Gram staining of vaginal discharge smear shows *clue cells*. *G. vaginalis* can be isolated on blood agar.

G. Treatment

Metronidazole is generally effective.

KEY POINTS

1. The genus *Haemophilus* contains non-motile, non-sporing, Gram negative bacilli and require one or both of two accessory growth factors (*X and V*) present in blood.
2. *H. influenzae* and *H. ducreyi* are the major pathogens in the genus *Haemophilus*.
3. *H. influenzae* may cause *meningitis*, pneumonia, epiglottitis, bronchitis, otitis media, septic arthritis and *H. ducreyi* is the causative organism of *chancroid*.
4. *H. influenzae* requires both X and V factor while *H. ducreyi* requires only X factor.
5. *Satellitism* is an important phenomenon in *H. influenzae*.

6. *Latex agglutination*, *coagglutination* and CIE are *rapid tests* for *H. influenzae* antigen detection in patient's serum, CSF, urine or pus.
7. CSF culture is an important test in laboratory diagnosis of meningitis.
8. *Haemophilus influenzae biotype aegyptius* causes a highly contagious form of conjunctivitis (pink eye).
9. The microscopic findings of *H. ducreyi* has been described as resembling a '*school of fish*' appearance.
10. *H. ducreyi* causes *chancroid* or *soft chancre,* a highly contagious sexually transmitted disease (STD), characterised by *tender*, non-indurated, irregular *ulcers on the genitalia*.
11. *Gardnerella vaginalis* causes *non-specific vaginitis* and *cervicitis*, usually in association with anaerobic bacteria (*anaerobic vaginosis*).
12. In vaginosis, Gram staining of vaginal discharge shows *clue cells*, which are vaginal epithelial cells covered with many tiny Gram variable bacilli.

YOU MUST KNOW

1. Morphology and culture characteristics of *Haemophilus influenzae*.
2. X and V factors.
3. Satellitism.
4. Serotypes of *H. influenzae*.
5. Diseases caused by *H. influenzae*.
6. Laboratory diagnosis of meningitis caused by *H. influenzae*.
7. Koch-Weeks bacillus.
8. Morphology and culture characteristics of *Haemophilus ducreyi*.
9. Disease caused by *H. ducreyi* and its laboratory diagnosis.
10. *Gardnerella vaginalis.*

STUDY QUESTIONS

1. Discuss the laboratory diagnosis of infections caused by *Haemophilus influenzae.*
2. Write short notes on:
   (a) X and V factors
   (b) Satellitism
   (c) *Haemophilus influenzae* biotype *aegyptius*
   (d) *Haemophilus ducreyi.*
   (e) *Gardnerella vaginalis.*

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Accessory growth factor/s required by *Haemophilus influenzae* is/are:
   (a) X factor
   (b) V factor
   (c) Both X and V factors
   (d) Neither X nor V factor
2. Which of the following bacteria shows phenomenon of satellitism?
   (a) *Haemophilus influenzae*
   (b) *Listeria monocytogenes*
   (c) *Streptococcus pyogenes*
   (d) All of the above
3. Which serotype of *Haemophilus influenzae* is associated with most invasive infections?
   (a) Serotype 'a' strains
   (b) Serotype 'b' strains
   (c) Serotype 'c' strains
   (d) Serotype 'e' strains
4. Which of the following infections is/are caused by *Haemophilus influenzae*?
   (a) Meningitis
   (b) Acute epiglottitis
   (c) Both of the above
   (d) None of the above

5. Which of the following rapid methods can be used to diagnose meningitis caused by *Haemophilus influenzae*?
   (a) Latex agglutination (b) Coagglutination
   (c) Counter immunoelectrophoresis (d) All of the above
6. What is the causative agent of Brazilian purpuric fever?
   (a) *Haemophilus influenzae* (b) *H. influenzae* biotype aegyptius
   (c) *H. ducreyi* (d) *H. aphrophilus*
7. *Haemophilus influenzae* biotype aegyptius was formerly known as:
   (a) Koch-Weeks bacillus (b) Klebs-loeffler bacillus
   (c) *Haemophilus vaginalis* (d) *Haemophilus haemolyticus*
8. The causative agent of chancroid is:
   (a) *Haemophilus influenzae* (b) *H. ducreyi*
   (c) *H. haemolyticus* (d) *H. aphrophilus*
9. Which one of the following bacteria is included in HACEK group bacteria?
   (a) *Escherichia coli* (b) *Enterobacter*
   (c) *Eikenella corrodens* (d) *Edwardsiella*
10. '*Clue cells*' in Gram staining can be useful in diagnosis of:
   (a) *Gardnerella vaginalis* (b) *Haemophilus parainfluenzae*
   (c) *Kingella kingae* (d) *Cardiobacterium hominis*

Answers (MCQs):

1. (c)	2. (a)	3. (b)	4. (c)	5. (d)
6. (b)	7. (a)	8. (b)	9. (c)	10. (a)

Chapter 40

BORDETELLA

I. INTRODUCTION

The genus *Bordetella* contains very small, Gram negative coccobacilli which were formerly included in the genus *Haemophilus*. As they do not require X and V factors for growth and are antigenically different from *Haemophilus*, they are separated into another genus *Bordetella*. *Bordet and Gengou* (1906) isolated the organism, *Bordetella pertussis*, from children suffering from whooping cough.

Species

1. *B. pertussis*—causes whooping cough.
2. *B. parapertussis*—responsible for milder form of whooping cough.
3. *B. bronchiseptica*—resposible for about 0.1% cases of whooping cough of milder type.
4. *B. holmesii*—can rarely be isolated from patients with pertussis like symptoms.
5. *B. hinzii*—can rarely be isolated from patients with pertussis like symptoms.
6. *B. avium*—causes respiratory disease in turkeys.
7. *B. trematum*
8. *B. ansorpic*
9. *B. petrii*

II. BORDETELLA PERTUSSIS

A. Morphology

B. pertussis is a small, ovoid, 1–1.5 μm × 0.3 μm, Gram negative coccobacillus. It is non-motile and non-sporing. It is capsulated but tends to lose the capsule on repeated subcultures. Bipolar meta-chromatic granules may be observed on staining with toludine blue.

B. Culture

It is aerobic and cannot grow anaerobically. Optimum temperature for growth is 35–36°C. The organism does not grow on nutrient agar. It requires a complex medium for its primary isolation. Bordet-Gengou (glycerol–potato–blood agar) is a commonly used medium. After incubation for

48–72 hours, colonies on this medium are small, smooth, opaque, greyish white, refractile, resembling *bisected pearls* or *mercury drops*. A hazy zone of haemolysis surrounds the colonies. Blood in the medium is apparently not to provide additional nutritive factors, but neutralises inhibitory substances like toxic fatty acids. Charcoal or starch or both may also be incorporated to neutralise these toxic constituents. Charcoal blood agar medium is an alternative medium used for primary isolation of this organism. Commonly used charcoal blood agar medium is Regan–Lowe (RL) medium. It is available both as semisolid transport medium and as a solid medium for isolation of organism.

C. Biochemical Reactions

B. pertussis is biochemically inactive. It does not ferment sugars, form indole, reduce nitrates, split urea or utilize citrate. It is oxidase positive and usually produces catalase.

D. Resistance

It is killed by heating at 55°C for 30 minutes, drying and disinfectants. It survives outside the body in droplets for few hours. It retains viability at low temperatures (0–4°C).

E. Antigenic Structure

1. Agglutinogens

Freshly isolated strains of *B. pertussis* possess heat labile antigens associated with the capsule (K antigens), designated 1 to 14 factors or agglutinogens. All strains of *B. pertussis* carry factor 1 and one or more of the other factors. Factor 1 to 6 are present only in strains of *B. pertussis* but factor 7 is found in all strains of the three species of *Bordetella*. Factor 12 is specific for *B. bronchiseptica* and factor 14 for *B. parapertussis*. These antigens are useful in serotyping of the strains for epidemiology purposes.

Some of the agglutinogens have been recognised on the fimbriae. *B. pertussis* have eight agglutinogens; 1 to 6, 7 and 13. These type specific antigens are under the control of *vir gene*. Agglutinogens promote virulence by helping organisms to attach to respiratory epithelial cells. As strains causing infection are of types 1, 2 and 3, it is essential that pertussis vaccine strains should have factors 1, 2 and 3.

2. Lipopolysaccharide

It is a heat stable lipopolysaccharide endotoxin of the cell wall. It is not protective. It is common to all smooth strains of all the three species of *Bordetella*.

3. Heat Labile Toxin (HLT)

Heat labile (inactivated at 56°C × 15 minutes) toxin is present in all Bordetellae. Pathogenic role of this toxin is doubtful. It is a cytoplasmic protein.

4. Tracheal Cytotoxin (TCT)

It is derived from the peptidoglycan of cell wall and is present in all Bordetellae. It causes damage to respiratory epithelial cells and therefore makes a person more prone to secondary infection.

5. Pertussis Toxin (PT)

It is a heat labile exotoxin called the pertussis toxin. It is present only in *B. pertussis*. It is a major virulence factor. It is also known as lymphocytosis producing factor, histamine sensitising factor and islet activating protein. It has a wide spectrum of biologic activity. It is apparently responsible for many of the signs and symptoms of pertussis. It also causes profound lymphocytosis in pertussis patients.

PT has a molecular weight of 117,000 and is composed of six polypeptide chains (S1 to S5 with 2 copies of S4). PT has got two units A and B (the A unit being the enzymatically active moiety and B the binding component). The A unit is made up of S1 while B unit consists of the remaining five polypeptide chains. Pertussis toxin can be toxoided. PT toxoid is the major component of acellular pertussis vaccines.

6. Adenylate Cyclase (AC)

Only one of adenylate cyclases has the ability to enter target cells and act as a toxin. This is known as AC toxin (ACT). It acts by catalysing the production of cAMP by various cells. It is produced by all the three mammalian bordetellae.

7. Filamentous Haemagglutinin (FHA)

It is present on the bacillary surface. It mediates the attachment of the bacterium to the ciliated epithelial cells of the respiratory tract. It also adheres to erythrocytes. Antibodies directed against FHA are protective. FHA is used in acellular pertussis vaccines along with PT toxoid.

8. Pertactin

It is an outer membrane protein (OMP) present in virulent strains of *B. pertussis*. It is included in acellular pertussis vaccines. Antibody to pertactin can be detected in blood after infection or immunisation.

F. Variation

Freshly isolated strains are in the smooth form (Phase I). On subculture, they undergo loss of surface antigens and become phase IV, which is the rough avirulent form. They have to pass through phase II and III before becoming phase IV.

A reversible change in the capsular antigen has been described. The organism may occur in one of three 'modes', X, I and C. Each mode has a characteristic surface antigen. These modes refer to the colour of the colonies on the Bordet-Gengou medium—X for *xanthic* (yellow), C for *cyanic* (blue) and I for *intermediate*. On the Bordet-Gengou medium, fresh isolates always occur in X mode.

G. Pathogenesis

B. pertussis infection is predominantly a childhood disease and is known as whooping cough. 95% cases of whooping cough are due to *B pertussis*, 5% by *B. parapertussis* and only in about 0.1% cases, *B. bronchiseptica* is responsible.

In human beings, the source of infection is the patient in the early stage. The incubation period is about one to two weeks. Infection is transmitted by droplets. The disease usually lasts for 6 to 8 weeks. It consists of three stages namely, the catarrhal, paroxysmal and convalescent, each lasting approximately for two weeks. Although cases are maximally infective during the catarrhal stage but clinical diagnosis in this stage is difficult. During the paroxysmal stage, the violent spasms of continuous coughing is followed by a long inrush of air into the almost empty lungs, with a characteristic 'whoop'. During convalescent stage, the frequency and severity of coughing gradually decrease.

Complications

The disease is self-limiting. Complications may be:

(*i*) subconjuctival haemorrhage due to the pressure effects of violent coughing
(*ii*) bronchopneumonia and lung collapse
(*iii*) convulsions and coma.

H. Epidemiology

Whooping cough is predominantly a paediatric disease. The incidence and mortality are highest in the first year of life. The source of infection is the patient, most infective during early stage (catarrhal stage). The mode of infection is via droplets. Whooping cough is one of the most infectious of bacterial diseases. Healthy carriers have not been identified. Although natural infection confers immunity but second attack may occur. Reinfection in adults may be of severe form.

I. Laboratory diagnosis

1. Microscopy

Microscopic diagnosis is made by demonstration of the bacilli in respiratory secretions by the fluorescent antibody technique.

2. Culture

In the early stage (catarrhal), bacilli are most abundant in the upper respiratory tract. They are scanty in the paroxysmal stage and are not demonstrable during convalescence. For culture, following methods have been used for collection of specimens.

(i) The pernasal swab

A swab is passed along the floor of the nasal cavity and material collected from the pharyngeal wall. The swab is inoculated on Bordet-Gengou medium or charcoal blood agar. Nasopharyngeal aspirate collected through a catheter attached to a syringe is a better alternative.

(ii) The cough plate method

A Bordet-Gengou culture plate is held 10–15 cm in front of the patient's mouth during a bout of coughing so that the cough droplets directly inoculate the culture medium.

(iii) The postnasal (peroral) swab

A West's postnasal swab is passed through mouth to collect posterior pharyngeal wall secretions. Precautions should be taken to avoid salivary contamination.

Fatty acids present in cotton may inhibit the growth of the bacilli and so it is better to use dacron or calcium alginate swabs for collection of specimen. The swabs are to be plated without delay. In case of delay, modified Stuart's medium or charcoal agar should be used for transport of the swab.

Medium employed is Bordet and Gengou or charcoal blood agar [Reagan-Lowe (RL) medium]. Incorporation of diamidine, flouride and penicillin (Lacey's DFP medium) into Bordet and Gengou medium makes it more selective. Plates are incubated in high humidity at 35–36°C for 3–5 days. Typical pearl like colonies of *B. pertussis* appear in 48–72 hours. These are confirmed by microscopy and slide agglutination with specific antisera. Immunofluorescence is useful in identifying the bacillus in smears from culture. The differentiating features of different species of bordetella are listed in Table 40.1.

3. Polymerase Chain Reaction (PCR)

PCR is the preferred method for the direct detection of *B. pertussis* and *B. parapertussis* from a nasopharyngeal swab or a nasopharyngeal aspirate.

4. Serology

Rise in titre of antibodies may be demonstrated in paired sera samples by agglutination, complement fixation test, immunofluorescent test and ELISA.

Detection of anti-pertussis toxin (PT) antibody levels can be detected in serum by ELISA. Immunoglobulin G (IgG) anti-PT antibodies levels of >100 to 125 international units/milliliter (IU/mL) can be used as recent contact with PT-producing bacteria. Paired sample serology method can also be used but single-sample serology also provides good sensitivity and specificity.

J. Treatment

The organism is sensitive to various antibiotics such as tetracycline, chloramphenicol, erythromycin and ampicillin. However, erythromycin is the drug of choice.

K. Prophylaxis

Immunisation of infants and children with killed *B. pertussis* vaccine is very effective. Smooth phase I strain of *B. pertussis* is used for vaccine production. Three intramuscular injections at intervals of 4–6 weeks are given before the age of six month, followed by a booster dose at the end of the first year of life. Vaccination is usually started at age of 6 weeks. Pertussis vaccine is generally administered in combination with diphtheria toxoid and tetanus toxoid (DPT). *B. pertussis* acts as an adjuvant for the toxoids, producing better antibody response. Two types of vaccine DwPT (with whole cell pertussis vaccine) and DaPT (with acellular pertussis vaccine) are available.

Rarely neurological complications like encephalopathy and convulsions may occur. Children below four years of age, who are contacts of the case, should receive a booster dose alongwith a chemoprophylaxis of erythromycin for five days.

Acellular pertussis vaccines containing PT, FHA, pertactin, agglutinogens 1, 2, 3 of *B. pertussis* have been developed. They have fewer complications.

Prevention by isolation of cases is seldom practicable, as infectivity is the highest during early stage (catarrhal) of the disease when clinical diagnosis is not easy.

III. BORDETELLA PARAPERTUSSIS

They are responsible for about five per cent of whooping cough cases and generally produce a mild disease. Morphologically they resemble *B. pertussis* but differ by their ability to grow on nutrient agar and pigment production (Table 40.1). They are also antigenically distinct from *B. pertussis*. The pertussis vaccine does not protect against *B. parapertussis* infection.

IV. BORDETELLA BRONCHISEPTICA

It is motile with peritrichate flagella and has been found to cause 0.1% cases of whooping cough in humans. It can grow on nutrient agar and is antigenically related to *B. pertussis* and *Brucella abortus*. Differentiating characters of bordetella species are listed in Table 40.1.

V. BORDETELLA AVIUM

It is the causative agent of coryza in turkeys. It can grow on nutrient agar. It produces HLT and TCT but does not produce ACT and PT.

Table 40.1 Differentiating Characters of Bordetella Species

Character	B. pertussis	B. parapertussis	B. bronchiseptica	B.avium
Motility	–	–	+	+
Growth on nutrient agar	–	+	+	+
Pigment production	–	+	–	–
Oxidase	+	–	+	+
Urease production	–	+	+	–
Citrate utilisation	–	+	+	+
Nitrate reduction	–	–	+	–
Toxins:				
HLT and TCT	+	+	+	+
ACT	+	+	+	–
PT	+	–	–	–

KEY POINTS

1. The genus *Bordetella* contains very small, Gram negative coccobacilli.
2. Three important species of *Bordetella* include *Bordetella pertussis, B. parapertussis* and *B. bronchiseptica.*
3. *B. pertussis* is the main causative agent of whooping cough. It is a small, ovoid, non-motile, Gram negative coccobacillus. It is aerobic and cannot grow anaerobically. *Bordet-Gengou* (glycerol-potato-blood agar) is a commonly used medium. Regan-Lowe (RL) medium is charcoal blood agar used for growth of *B. pertussis*. It is oxidase positive.
4. Pertussis toxin is exotoxin produced by *B. pertussis*.
5. Laboratory diagnosis depends on microscopy and culture.
6. Pertussis vaccine is generally administered in combination with diphtheria toxoid and tetanus toxoid (DPT). *B. pertussis* acts as an adjuvant for the toxoids producing better antibody response. Vaccine is very effective for prophylaxis.
7. Acellular pertussis vaccines containing antigens and toxins are also available.

YOU MUST KNOW

1. Morphology, culture characteristics of *Bordetella pertussis*.
2. Laboratory diagnosis of whooping cough.
3. Pertussis vaccines.

STUDY QUESTIONS

1. Describe the laboratory diagnosis of whooping cough.
2. Write short notes on:
   (a) Antigenic structure of *B. pertussis* (b) Vaccination against pertussis (c) Bordet-Gengou medium.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Which of the following media can be used for isolation of *Bordetella pertussis*?
   (a) Glycerol-potato blood agar (b) Lacey's DFP medium
   (c) Regan-Lowe medium (d) All of the above
2. Which one of the following media is most frequently used for isolation of *Bordetella pertussis*?
   (a) Chocolate agar (b) MacConkey agar (c) Bordel-Gengou medium (d) Tinsdale agar
3. Whooping cough can be caused by:
   (a) *Bordetella pertussis* (b) *B. parapertussis* (c) *B. bronchoseptica* (d) All of the above
4. Which of the following species of Bordetella is motile?
   (a) *Bordetella pertussis* (b) *B. parapertussis* (c) *B. bronchiseptica* (d) None of the above
5. *Bordetella pertussis* infection is transmitted by:
   (a) Droplets (b) Inoculation of skin (c) Contact (d) All of the above
6. Which is the most infective stage in whooping cough?
   (a) Catarrhal stage (b) Paroxymal stage (c) Convalescent stage (d) None of the above
7. Which of the following vaccines is/are available for prophylaxis of whooping cough?
   (a) Whole cell pertussis vaccine (b) Acellular pertussis vaccine
   (c) Both of the above (d) None of the above

Answers (MCQs):

1. (d) 2. (c) 3. (d) 4. (c) 5. (a) 6. (a) 7. (c)

Chapter 41

BRUCELLA

Brucella

A. Morphology
B. Culture
C. Biochemical Reactions
D. Resistance
E. Antigenic Structure
F. Biotypes
G. Phage Typing
H. Classification
I. Pathogenesis
J. Epidemiology
K. Laboratory Diagnosis
L. Treatment
M. Prophylaxis

BRUCELLA

The genus *Brucella* consists of small, non-motile, aerobic, Gram negative coccobacilli which are strict parasite of animals (goats, sheep, cattle, pigs, buffaloes). Man acquires infection by contact with infected animals or through their products. The human disease had been recognised from very early times in the Mediterranean countries and had been known under various names such as Mediterranean fever, Malta fever and undulant fever. *Bruce* (1887) isolated a small microorganism from the spleen of fatal cases in Malta. This has been named *Brucella melitensis* (*Brucella* after *Bruce*; *melitensis* after *Melita*, the Roman name for Malta). *Bang* (1897) described *Br. abortus* in Copenhagen, the cause of contagious abortion in cattle. *Traum* (1914) isolated *Br. suis* from pigs in USA. These three are the major species in the genus. Three additional species *Br. neotomae, Br. ovis* and *Br. canis* have been recognised which are animal pathogens and except for *Br. canis*, they do not infect man.

A. Morphology

Brucellae are Gram negative, non-motile, coccobacilli or short rods measuring 0.5–0.7 μm × 0.6–1.5 μm. They are arranged singly or in short chains. They are non-sporing and non-capsulated.

B. Culture

Brucellae are strict aerobes. *Br. abortus* is capnophilic, requiring 5–10 per cent CO_2 for its growth whereas *Br. suis* growth is unaffected by CO_2. The optimum temperature for growth is 37°C (range 20–40°C) and optimum pH 6.6–7.4.

They may grow on ordinary media, though growth is slow and scanty. Growth is improved by addition of glucose, serum, blood or liver extract. The media employed for culture are serum-dextrose agar, serum-potato-infusion agar, trypticase soy agar or tryptose agar. The addition of bacitracin, cycloheximide, polymyxin B or vancomycin makes these media selective. On solid media, colonies are small, moist and translucent. In liquid media, growth is uniform.

Cultivation in presence of dyes

The three important brucellae (*Br. abortus, Br. melitensis* and *Br. suis*) are differentiated on the basis of their ability to grow in media containing aniline dyes such as basic fuchsin (1: 50,000) and thionin (1: 25,000). *Br. melitensis*

is not inhibited by any of the dyes, while *Br. abortus* is inhibited by thionin and not by basic fuchsin. *Br. suis* is inhibited by basic fuchsin and not by thionin (Table 41.1).

C. Biochemical Reactions

No carbohydrates are ordinarily fermented but they possess oxidative capacity. They are catalase and oxidase positive (except for *Br. neotomae* and *Br. ovis* which are negative). They are urease positive, nitrate reduction positive, citrate negative, indole is not produced and MR and VP tests are negative. Urease test is very rapidly positive particularly in *Br. suis* which is positive within 30 minutes and *Br. abortus* takes 1–2 hour to become urease positive.

D. Resistance

Brucellae are killed by heat at 60°C in 10 minutes, hence in milk they are rapidly destroyed by pasteurisation. They are killed by 1% phenol in 15 minutes. They may survive in soil and manure for several weeks. The organism survives for 10 days in refrigerated milk, for months in butter, one month in ice cream. They die out in butter and cheese undergoing lactic acid fermentation. They are sensitive to direct sunlight and acid. *Br.melitensis* may survive for six weeks in dust and ten weeks in water.

They are resistant to penicillin but are susceptible to streptomycin, tetracycline, chloramphenicol and ampicillin.

E. Antigenic Structure

Two main somatic antigens of brucellae, A and M are present in different amounts in the three major species. Antigen 'A' is dominant (about 20 times as much as 'M' antigen) in *Br. abortus* and antigen 'M' is dominant in *Br. melitensis* (about 20 times of 'A' antigen). *Br. suis* has an intermediate antigenic pattern. Absorption of the minor antigenic components of antiserum will leave most of the major antibody components. Such absorbed A and M monospecific sera are useful for identification by agglutinatation test. The antigenic structure of brucellae is more complex. Some strains behave biochemically as

Table 41.1 Differential Features of Brucella Species and Biotypes

Species	Bio-types	CO_2 requirement	H_2S production	Growth in the presence of		Agglutination by			Lysis by phage		Common host
				Basic fuchsin	Thionin	Monospecific sera		Anti-rough serum	RTD	RTD × 10^4	
						A	M				
Br. melitensis	1	–	–	+	+	–	+	–	–	–	Sheep, goats
	2	–	–	+	+	+	–	–	–	–	
	3	–	–	+	+	+	+	–	–	–	
Br. abortus	1	±	+	+	–	+	–	–	+	+	Cattle
	2	±	+	–	–	+	–	–	+	+	
	3	±	+	+	+	+	–	–	+	+	
	4	±	+	+	–	–	+	–	+	+	
	5	–	–	+	–	–	+	–	+	+	
	6	–	±	+	–	+	–	–	+	+	
	9	±	+	+	–	–	+	–	+	+	
Br. suis	1	–	+	–	+	+	–	–	–	+	Pigs
	2	–	–	±	+	+	–	–	–	±	Pigs, hare
	3	–	–	+	+	+	–	–	–	+	Pigs
	4	–	–	+	+	+	+	–	–	+	Reindeer
	5	–	–	–	+	–	+	–	–	+	Rodents
Br. canis		–	–	–	+	–	–	+	–	–	Dogs
Br. ovis		+	–	+	+	–	–	+	–	–	Sheep
Br. neotomae		–	+	–	–	+	–	–	–	+	Wood rat

Dannish strains of *Br. suis* differ from *American* strains in not producing H_2S.

Br. abortus and serologically as *Br. melitensis* and vice-versa. The battery of other tests in addition to biotyping and serotyping is required for differentiation of various species (Table 41.1).

Antigenic cross reactions exist between brucellae and *Esch. coli* O:116, O:157; *Salmonella* serotype N (O:30 antigen), *V. cholerae*, *Ps. maltophila, F. tularensis* and *Y. enterocolitica*.

F. Biotypes

(*i*) *Br. abortus*–7 biotypes (1 to 9, number 7 and 8 are deleted)
(*ii*) *Br. melitensis*–3 biotypes
(*iii*) *Br. suis*–5 biotypes

Br. suis strains which produce H_2S are called *American strains* and those not producing H_2S are called *Danish strains*.

G. Phage Typing

The Tblisi (Tb) reference phage is used for phage typing. It lyses *Br. abortus* at both RTD (Routine test dilution) and 10,000 RTD but *Br. suis* is lysed at 10,000 RTD only while *Br. melitensis* is not lysed at all.

H. Classification

Brucellae may be classified into six species based on CO_2 requirement, production of H_2S; sensitivity to aniline dyes (basic fuchsin and thionin), agglutination by monospecific sera and phage lysis (Table 41.1).

I. Pathogenesis

All the three major species of brucellae are pathogenic to human beings. *Br. melitensis* is the most pathogenic species followed by *Br. suis* of intermediate pathogenicity and *Br. abortus* is the least pathogenic. Brucellosis is a zoonotic disease.

1. Mode of Infection

Human infection occurs by direct or indirect contact with infected animals. Brucellae are transmitted to humans by:

(*i*) Drinking contaminated (unpasteurised) raw milk or by ingestion of milk products from infected animals.
(*ii*) Direct contact with infected animal tissues: butchers, dairy workers, farm workers and veterinarians are particularly at risk.
(*iii*) Accidental ingestion, inhalation, injection and mucosal or skin contamination may occur in laboratory personnel working with brucellae.

2. Types of Infection

Human infection may be of three types:

(*i*) *Subclinical or latent infection:* There is no clinical evidence of disease but is detectable only by serological tests.
(*ii*) *Acute brucellosis:* It is also known as *undulant fever* or *Malta fever*. It is associated with prolonged bacteraemia.
(*iii*) *Chronic brucellosis:* It is usually non-bacteraemic. The symptoms are generally related to a state of hypersensitivity.

3. Course of Disease

The bacilli after entering the body pass via the lymphatic channels, regional lymph nodes, and thoracic duct to the blood stream causing bacteraemia. Brucellosis is primarily a disease of reticuloendothelial (RE) system, hence, the organisms are localised in the RE system. Brucellae have a special predilection for intracellular growth especially inside phagocytic cells. Cell mediated immunity plays an important role in recovery from brucellosis.

The incubation period varies from 2 to 3 weeks and may extend to 6 months. The onset of symptoms may be acute or insidious. Acute brucellosis is chracterised by fever, chills, shivering, malaise, severe headache, bone and joint pains, mild lymph node enlargement and sometimes hepatosplenomegaly. The name *undulant fever* has been attributed to acute brucellosis because of the periodic nocturnal fever that may occur over weeks, months or years especially in untreated cases. When the disease persists for 6 months or more, it is termed as *chronic brucellosis*.

J. Epidemiology

The disease is worldwide in distribution and endemic in certain areas such as Mediterranean countries (*Br. melitensis*), certain swine rearing areas of USA (*Br. suis*) and Great Britain (*Br. abortus*). In India, *Br. melitensis* is the predominant pathogen of human brucellosis acquired from goats or sheep which is followed by *Br. abortus* of cattle origin.

K. Laboratory Diagnosis

Brucellosis is a febrile disease with varied clinical manifestations and clinical diagnosis is often difficult and laboratory aid is, therefore, essential. Laboratory methods include culture of brucellae, serology and hypersensitivity test.

1. Specimens

Blood culture is the most definitive method for the

diagnosis of acute brucellosis. Brucellae can also be isolated from bone marrow, liver, lymph nodes and occasionally from CSF, urine, sputum, breast milk, vaginal discharge and seminal fluid.

2. Blood Culture

Blood is collected during the pyrexial phase because chances of positive blood culture are more during this period. 5–10 ml of blood is inoculated into 50–100 ml of serum dextrose broth or trypticase soy broth and incubated at 37°C aerobically in presence of 5–10% CO_2. Subcultures are made on solid media every 3–5 days for 8 weeks, and incubated at 37°C aerobically in presence of 5–10% CO_2.

The need for frequent subcultures can be avoided by use of *Castaneda's method* of blood culture. It contains both liquid (trypticase soy broth) and solid (trypicase soy agar) media in the same bottle (Fig. 41.1). The blood is inoculated into the broth (liquid medium) and the bottle incubated in the upright position. For subculture, the bottle is tilted so that the broth flows over the surface of solid agar slant. It is again incubated in the upright position. In case of positive blood culture, colonies appear on the agar slant. This method reduces the chances of contamination and risk of infection to laboratory workers.

Blood cultures are positive in 30–50% cases, even when repeated samples are tested. *Br. melitensis* and *Br. suis* are readily cultured than *Br. abortus*.

BACTEC and BacT Alert are rapid methods for blood culture and majority of samples become positive within 7 days.

3. Identification

It depends on biochemical tests, dye test, agglutination with monospecific sera and lysis by phage.

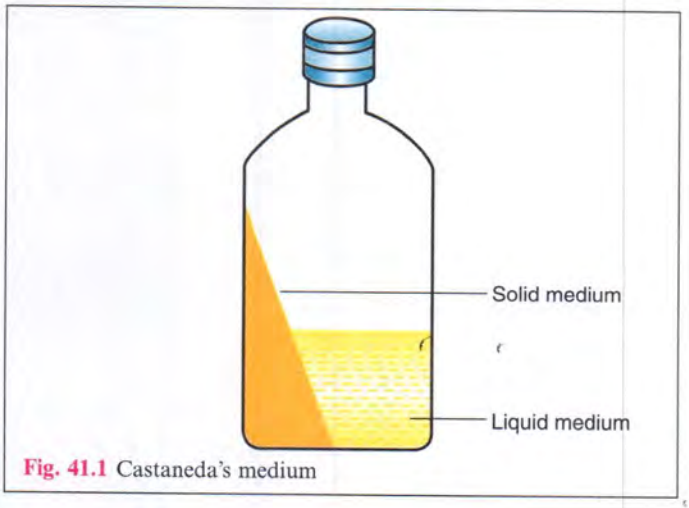


Fig. 41.1 Castaneda's medium

4. Serology

Serological methods are more useful as cultures are negative in high percentage of cases. Antibodies (IgG and IgM) appear in 7–10 days after clinical infection. As the disease progresses, IgM antibodies decline, while the IgG antibodies persist or increase. In chronic brucellosis, only IgG can be demonstrated and IgM may often be absent. The agglutination test identifies mainly the IgM antibody, while both IgM and IgG can fix complement. The IgG and IgA antibodies may act as blocking (incomplete) antibodies and may prevent agglutination. The agglutination test is usually positive in acute infection, but may be negative or weakly positive in chronic cases.

(i) Serum agglutination test (SAT)

Serum agglutination test (SAT) is the most widely used test for diagnosis of brucellosis. This is a tube agglutination test in which equal volumes of serial two fold dilutions (1:20 to 1:640) of patient's serum and the standardised antigen (heat killed *Brucella* suspension) are mixed and incubated at 37°C for 24 hours. A single titre of more than 1:160 is presumptive evidence of *Brucella* infection. The test can also be done in microplate.

Prozone phenomenon is very frequent in brucellosis, therefore it is essential that several serum dilutions should be tested. Another source of error is presence of *blocking* or *non-agglutinating* antibodies in serum. The blocking effect may be removed by heating the serum at 55°C for 30 minutes or by using 4% saline as diluent for the test. The most important method for detecting these incomplete antibodies is the antiglobulin (Coombs) test.

A positive agglutination test may also be produced by cholera, tularaemia or yersinia infection or vaccination. Cholera induced antibodies may be removed by absorption with 2–mercaptoethanol.

Agglutinin titres are expressed in International units. This is done by using a standard reference serum for comparison.

(ii) Castaneda strip test

A loopful of patient's serum is placed on a filter paper strip containing a coloured brucella antigen. Antibody is able to 'fix' the antigen and prevent its movement along the paper. It is simple and suitable test for screening of brucellosis cases.

(iii) Complement fixation test (CFT)

It is more useful in chronic cases as it detects both IgG and IgM.

(iv) ELISA and RIA

These tests are very sensitive and helpful to distinguish acute and chronic brucellosis. ELISA can detect IgM and IgG antibody separately.

(v) Indirect immunofluorescent test

It is specific and sensitive method for detecting antibodies and may be positive even when the agglutination test is negative.

5. Hypersensitivity Test (Brucellin test)

It is delayed (type IV) hypersensitivity to *Brucella* antigen. It is an intradermal test using protein extract of the organisms, called *brucellin*. Positive reaction is indicated by an erythema and induration of 6 mm diameter within 24 hours. This test is not useful for diagnosis of acute infections because test may remain positive for years after acquiring infection. It may be helpful in chronic brucellosis where a positive skin test may sometimes be the only indicator of infection. Persistent negative skin test helps to exclude brucellosis.

6. Detection of Animal Infection

The methods used for the laboratory diagnosis of human infections may also be employed for the diagnosis of animal infections. Culture of milk and urine may give positive results. In addition, rapid methods such as 'rapid plate agglutination test' and the 'Rose Bengal card test' have been employed for the diagnosis of brucellosis in herd of cattle. For detection of infected animals in dairies, besides culture, pooled milk samples may be tested for antibodies by *milk ring test*.

Milk ring test

It detects brucella agglutinins in the milk of infected dairy cattle.

Method

1. A sample of whole milk is mixed well with a drop of the stained brucella antigen (a concentrated suspension of *Br. abortus* or *Br. melitensis* stained with haematoxylin) in a narrow test tube.
2. This mixture of milk and bacterial suspension is incubated in a water bath at 70°C for 40–50 minutes.

Result

In a positive test, the bacilli are agglutinated and rise with the cream to form a blue ring at the top, leaving the milk unstained. When antibodies are absent (negative test), no coloured ring is formed and the milk remains uniformly blue.

Whey agglutination test is another useful test for detecting antibodies in milk.

L. Treatment

Treatment consists of administration of tetracycline along with streptomycin for a period of not less than three weeks. WHO has recommended a regimen of rifampicin (600–900 mg) and doxycycline (200 mg), both taken as a single daily dose for a minimum of 6 weeks.

M. Prophylaxis

1. Persons handling the animals should use protective clothing and gloves.
2. Pasteurisation or boiling of milk should be done.
3. Vaccination: Cattle should be vaccinated with live attenuated *Br. abortus* strain 19 vaccine and goats, sheep with *Br. melitensis* Rev.1 vaccine.
4. Unimmunised infected animals should be slaughtered.
5. *Br. abortus* strain 19–BA, a more attenuated variant of strain 19, has been widely employed for human immunisation in USSR for protection of population exposed to infection. Vaccine is given intradermally.

KEY POINTS

1. The genus *Brucella* consists of small, non-motile, aerobic, Gram negative coccobacilli which are strict parasite of animals (goats, sheep, cattle, pigs, buffaloes).
2. *Brucella melitensis*, *B. abortus* and *B. suis* are the three major species of the genus *Brucella*.
3. Brucellosis is a febrile disease. Man acquires infection by contact with infected animals or through their products. Human infections may be of three types: *subclinical, acute brucellosis, chronic brucellosis*.
4. Brucellae are *strict aerobes*. B. *abortus* is *capnophilic*, requiring 5-10 per cent CO_2 for its growth.
5. The media employed for culture are *serum dextrose agar*, *serum-potato-infusion agar*, *trypticase soy-agar* or *tryptose agar*. The three important species of brucella are differentiated on the basis of their ability to grow in media containing aniline dyes such as *basic fuchsin* and *thionin*.
6. Brucellae are oxidase positive and urease positive. *Urease test* is very *rapidly positive* particularly in *B. suis*.

7. Laboratory diagnosis depends on culture of brucellae and serology. *Castaneda's method* of blood culture is used. *Castaneda's medium* contains both liquid (trypicase soy broth) and solid (trypticase soy agar) media in the same bottle. *Serum agglutination test* is used for detection of antibodies in patient's serum. A single titre of more than 1 : 160 is presumptive evidence of *Brucella* infection.
8. *Brucellin* test may be helpful in chronic brucellosis where a positive skin test may sometimes be the only indicator of infection.

YOU MUST KNOW

1. Morphology, culture characteristics and biochemical reactions of *Brucella sp.*
2. Different species of Brucella.
3. Diseases caused by *Brucella sp.*
4. Laboratory diagnosis of brucellosis.
5. Castaneda's medium.

STUDY QUESTIONS

1. Discuss the laboratory diagnosis of brucellosis.
2. Write short notes on:
   (a) Castaneda's medium
   (b) Serodiagnosis of brucellosis
   (c) Diagnosis of brucellosis in animals.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. The causative agent/s of undulant fever is/are:
   (a) *Brucella abortus* (b) *Br. melitensis* (c) *Br. suis* (d) All of the above
2. Which of the following bacteria is capnophilic?
   (a) *Brucella abortus* (b) *Br. melitensis* (c) *Br. suis* (d) None of the above
3. Which of the following dyes is/are useful for differentiation of various species of Brucella?
   (a) Basic fuchsin (b) Thionin (c) Both of the above (d) None of the above
4. Which of the following media can be employed for isolation of Brucella?
   (a) Serum-dextrose agar (b) Serum-potato-infusion agar
   (c) Trypticase soy agar (d) All of the above
5. Castaneda medium can be used for blood culture in:
   (a) Acute brucellosis (b) Bordetella infections (c) Yersina infections (d) None of the above
6. Brucellae are transmitted to humans by:
   (a) Ingestion of contaminated raw milk
   (b) Direct contact with infected animal tissues
   (c) Accidental mucosal or skin contamination in laboratory personnels
   (d) All of the above
7. Which of the following animals are involved in causing Brucella infection?
   (a) Sheep (b) Goats (c) Cattle (d) All of the above
8. All of the following diseases are zoonotic except:
   (a) Brucellosis (b) Plague (c) Tularensis (d) Floppy baby syndrome
9. 'Rose Bengal Card' test is employed for which of the following infections?
   (a) Brucellosis (b) Salmonellosis (c) Tularensis (d) None of the above
10. Vaccination of cattle against brucellosis is done with:
    (a) *Brucella abortus* vaccine (b) *Br. melitensis* vaccine (c) *Br. suis* vaccine (d) *Br. canis* vaccine

Answers (MCQs):

1. (d) 2. (a) 3. (c) 4. (d) 5. (a) 6. (d) 7. (d) 8. (d) 9. (a) 10. (a)

Chapter 42

MYCOBACTERIUM TUBERCULOSIS

I. INTRODUCTION

Mycobacteria are slender bacilli that sometimes show branching filamentous forms resembling fungal mycelium (*Myces* meaning fungus). They are difficult to stain, but once stained, resist decolourisation with dilute mineral acids and are therefore called *acid-fast bacilli* or *AFB*. These organisms are aerobic, non-motile, non-capsulated and non-sporing. Growth is generally slow. The genus includes obligate parasites, opportunistic pathogens and saprophytic mycobacteria.

Hansen (1868) discovered the first member of the genus i.e. lepra bacillus. *Robert Koch* (1882) isolated the mammalian tubercle bacillus and *Johne* (1895) described *M. paratuberculosis* (Johne's bacillus) causing chronic enteritis in cattle. Subsequently other pathogenic tubercle bacilli such as the murine type (*M. microti*) from voles, the avian type (*M. avium*) from birds and the cold blooded type (*M. piscium, M. marinum*) were isolated.

The saprophytic mycobacteria were isolated from a number of sources e.g. butter (*M. butyricum*), grass (*M. phlei*), dung (*M. stercoris*), smegma (*M. smegmatis*). A group of mycobacteria, distinct from human or bovine tubercle bacilli isolated occasionally from human sources, are grouped together under the term *atypical mycobacteria, anonymous* or *unclassified mycobacteria*. Most of these bacteria occur in soil, water and other sources. They are mostly opportunistic.

Seven species are responsible for mammalian tuberculosis *M. tuberculosis* (human tubercle bacillus), *M. bovis* (bovine tubercle bacillus), *M. microti* (vole tubercle bacillus) *M. africanum* (intermediate form between *M. tuberculosis* and *M. bovis*), *M. caprae*, *M. canettii* and *M. pinnipedii*.

The Mycobacterium Tuberculosis Complex

The M. tuberculosis complex includes *M. tuberculosis*, *M.*

bovis, M. microti, M. africanum M. caprae, M. canettii and *M. pinnipedii*. These species are closely related to each other by antigenic analysis and DNA hybridisation. However, they can be distinguished by certain characters (Table 42.1).

M. tuberculosis and *M. bovis* are typical tubercle bacilli and cause human lesions such as pulmonary tuberculosis.

Table 42.1 Distinguishing Features of M. tuberculosis Complex

	TCH 10 µg/ml	Pyrazinamide 50 µg/ml	Oxygen preference	Cycloserine	Nitrate reduction	Niacin production
M. tuberculosis						
Classical	R	S	Aerobic	S	+	+
South Indian	S	S	Aerobic	S	+	+
M. bovis						
Classical	S	R	Microaerophilic	S	–	–
BCG	S	R	Aerobic	R	–	–
M. africanum						
African I	S	S	Microaerophilic	S	+	+/–
African II	S	S	Microaerophilic	S	+	–

TCH = Thiophen–2–carboxylic acid hydrazide
S = sensitive R = resistant

II. CLASSIFICATION

Mycobacteria causing human diseases may be classified as:

Cultivable

1. Typical tubercle bacilli (mammalian)
   - (*i*) Human type–*M. tuberculosis*
   - (*ii*) Bovine type–*M. bovis*
   - (*iii*) Vole type–*M. microti*
   - (*iv*) African type–*M. africanum*
   - (*v*) Cattle type–*M. caprae*
   - (*vi*) *M. canettii*
   - (*vii*) Pinniped type–*M. pinnipedii*
2. Atypical mycobacteria
   - (*i*) Photochromogens
   - (*ii*) Scotochromogens
   - (*iii*) Non-photochromogens
   - (*iv*) Rapid growers
3. Mycobacteria causing skin ulcers
   - (*i*) *M. ulcerans*
   - (*ii*) *M. balnei*
4. Saprophytic mycobacteria.
   - (*i*) *M. smegmatis*
   - (*ii*) *M. butyricum*
   - (*iii*) *M. stercoris*
   - (*iv*) *M. phlei*

Non-cultivable — *M. leprae*

III. MYCOBACTERIUM TUBERCULOSIS

The species contains two major types, classical and South Indian type of *M. tuberculosis*. Classical type is virulent to guinea pig but South Indian type is attenuated in this animal. South Indian type is prevalent in South India and in persons of Asian-ethnic origin living in other countries.

A. Morphology

M. tuberculosis is a slender, straight or slightly curved bacillus with rounded ends, occurring singly, in pairs or in small clumps. It measures, 1–4 µm × 0.2 – 0.8 µm (average 3 µm × 0.3 µm) in size. These bacilli are acid-fast, non-sporing, non-capsulated and non-motile. Ziehl-Neelsen staining is useful to study the morphology of these organisms. With this stain, tubercle bacilli are seen bright red (acid-fast), while the tissue cells and other organisms are stained blue (Fig. 42.1). Tubercle bacilli may also be stained with the fluorescent dyes (auramine O, rhodamine) and appear yellow luminous bacilli under the fluorescent microscope. Beaded or barred forms are frequently seen in *M. tuberculosis*. They are Gram positive but are difficult to stain with the Gram stain due to the failure of the dye to penetrate the cell wall.

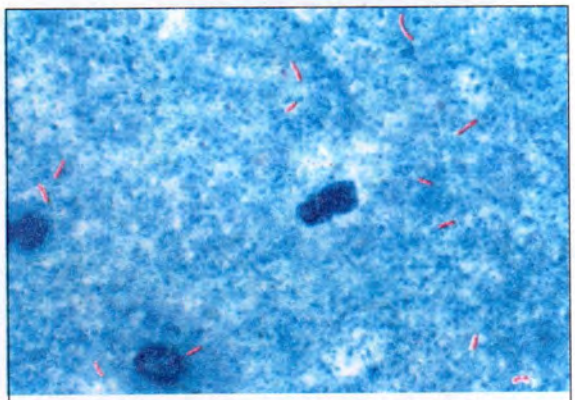


Fig. 42.1 Mycobacterium tuberculosis in Ziehl-Neelsen stained smear

Another method of staining is **Kinyoun's method** of acid fast staining. This is similar to conventional acid-fast staining (ZN staining) except that heating is not required. It is named as **cold staining** method. Carbol fuchsin used in cold staining has higher concentration of phenol which differentiation it from carbol fuchsin used in ZN staining.

M. bovis appears straighter, stouter and shorter with uniform staining (Table 42.2).

B. Culture

M. tuberculosis is an obligate aerobe whereas *M. bovis* is microaerophilic on primary isolation, becoming aerobic on subculture. The bacilli grow slowly (generation time 14–15 hours) and colonies appear only in about two weeks and sometimes it may take up to 6–8 weeks. Optimum temperature for growth is 37°C (range 30–40°C). Optimum pH is 6.4 to 7.0. Tubercle bacilli can grow on a wide range of enriched culture media but Lowenstein–Jensen (LJ) medium is most commonly used. This medium consists of beaten eggs, asparagine, mineral salts, malachite green and glycerol or sodium pyruvate It is solidified by heating (inspissation). It is one of the media which are solid without incorporation of agar. In this medium egg acts as a solidifying agent. Malachite green inhibits the growth of organisms other than mycobacteria and provides a colour to the medium. The addition of glycerol improves the growth of human type of *M. tuberculosis*, while it is without any effect or even inhibitory to *M. bovis*. Sodium pyruvate improves the growth of both *M. tuberculosis* and *M. bovis*. Colonies of *M. tuberculosis* are dry, rough, buff coloured, raised, with a wrinkled surface (Fig. 42.2). They are tenacious and not easily emulsified. In contrast, the colonies of *M. bovis* are flat, smooth, moist and white, breaking up easily when touched. *M. tuberculosis* has a luxuriant growth (*eugonic growth*) in culture as compared to sparsely grown (*dysgonic growth*) *M. bovis*.

In liquid media, the bacilli grow as surface pellicle due to hydrophobic properties of their cell wall. Diffuse

Fig. 42.2 LJ media without growth and with growth

Table 42.2 Differentiating Features of ***M. tuberculosis*** and ***M. bovis***

Test	M. tuberculosis	M. bovis
Morphology	Long, slender and usually curved	Short, stout and straight
Staining	Barred or beaded appearance	Uniform staining
Growth on LJ medium	Eugonic growth	Dysgonic growth
Presence of glycerol in medium	Enhances the growth	Inhibits the growth
Colony characters	Dry, rough, tough, raised and wrinkled, creamy white or buff coloured, difficult to emulsify	Moist, smooth, flat, white and friable
Biochemical reactions		
Niacin test	+	–
Nitrate reduction	+	–
Animal pathogenicity test		
In guinea pig	+ (progressive and fatal disease)	+ (Disease similar to that in *M. tuberculosis*)
In rabbit	– or mild lesion	+ (generalised lesion)

uniform growth can be obtained by addition of a detergent Tween 80 (polyoxyethylene sorbitan mono-oleate) in Dubo's medium. Tween 80 wets the surface and permits them to grow diffusely. Virulent strains tend to grow as *serpentine cords* in the liquid media, while avirulent strains grow in a more dispersed fashion. The cord factor by itself is not responsible for virulence. It is also present in some non-pathogenic species of mycobacteria. The cord factor consists of two mycolic acids linked to a molecule of trehalose.

Liquid media are generally used for sensitivity testing, preparation of antigens and vaccines. In automated culture methods, liquid media are used for growing mycobacteria.

Various culture media used for mycobacterial isolation include Lowenstein-Jensen (LJ) medium, Petragnani, Dorset egg medium (all the three are egg based) and Middle brook 7H10, Middle brook 7H11 (agar based media). Dubos medium and Middle brook 7H9 are two commonly used liquid media.

C. Resistance

Mycobacteria are killed at 60°C in 15–20 minutes. They are sensitive to ultraviolet rays and sunlight. Bacilli in cultures may be killed by direct sunlight exposure for two hours, but bacilli present in sputum may remain alive for 20–30 hours. They are relatively resistant to chemical disinfectants and can survive exposure to 5% phenol, 15% sulphuric acid, 5% oxalic acid and 4% sodium hydroxide. The bacilli are destroyed by tincture of iodine in five minutes and by 80% ethanol in 2–10 minutes. 80% ethanol has been recommended as disinfectant for skin and rubber gloves.

Bacilli may remain viable in droplet nuclei for 8–10 days. Cultures remain viable for 6–8 months at room temperature.

D. Biochemical Reactions

Mycobacterial species can be identified by several biochemical tests. Some of the important biochemical tests are discussed below:

1. Niacin Test

Niacin is produced as a metabolic by-product by all mycobacteria, but most species possess an enzyme that converts free niacin to niacin ribonucleotide. *M. tuberculosis*, lacks this enzyme and accumulates niacin in the culture medium. When 10% cyanogen bromide and 4% aniline in 96% ethanol are added to a suspension of bacterial culture, a canary yellow colour shows a positive reaction (Fig. 42.3). The human tubercle bacilli give a positive test, while the bovine type is negative. Positive niacin test also occurs in *M. simiae* and *M. chelonei*.

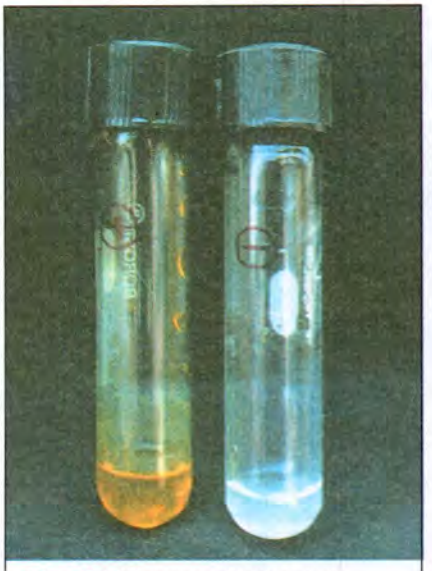


Fig. 42.3 Niacin production test

2. Arylsulphatase Test

Arylsulphatase is an enzyme formed by certain atypical mycobacteria. The organisms are grown in a medium containing 0.001 M tripotassium phenolphthalein disulphate. If arylsulphatase enzyme is produced it liberates free phenolphthalein from tripotassium phenolphthalein disulphate. This can be detected by adding 2N NaOH dropwise to the culture. A pink colour develops in a positive reaction.

3. Neutral Red Test

Virulent strains of tubercle bacilli can bind neutral red in alkaline buffer solution, whereas avirulent strains are unable to do so. Positive tests are obtained with *M. tuberculosis, M. bovis, M. avium* and *M. ulcerans*.

4. Catalase-Peroxidase Tests

Most atypical mycobacteria are strongly catalase positive and peroxidase negative. In contrast, the tubercle bacilli (*M. tuberculosis* and *M. bovis*) are peroxidase positive and weakly catalase positive. Tubercle bacilli lose catalase and peroxidase activity when they develop resistance to INH. Catalase negative tubercle bacilli are avirulent for guinea pigs.

A mixture of equal volumes of 30% H_2O_2 and 0.2% catechol in distilled water is added to a 5 ml test culture and left for few minutes. Catalase production is indicated by effervescence while browning indicates peroxidase activity.

5. Amidase Tests

Atypical mycobacteria can be differentiated by their ability to split amides. The commonly used amides include acetamide, benzamide, carbamide, nicotinamide

and pyrazinamide. A 0.00165 M solution of amide is incubated with the bacillary suspension at 37°C and to this is added 0.1 ml of $MnSO_4$. $4H_2O$, 1.0 ml of phenol solution and 0.5 ml of hypochlorite solution. The tubes are placed in boiling waterbath for 20 minutes. A blue colour indicates a positive test. *M. tuberculosis* produces nicotinamidase and pyrazinamidase, therefore, splits nicotinamide and pyrazinamide.

6. Nitrate Reduction Test

This test depends on reduction of nitrate to nitrite by an enzyme nitroreductase. This test is positive with *M. tuberculosis* and negative with *M. bovis*. Other mycobacteria, which may give positive test, are *M. kansasii, M. fortuitum* and *M. chelonei*.

The test organism is suspended in a buffer solution containing nitrate and incubated at 37°C for 2 hours. Then sulphanilamide and n-naphthyl-ethylene diamine dihydrochloride solutions are added. Development of pink or red colour indicates positive reaction (Fig. 42.4).

7. Susceptibility to Pyrazinamide

M. tuberculosis is sensitive to 50 µg/ml pyrazinamide but other mycobacteria including *M. bovis* are resistant.

8. Susceptibility to Thiophen–2–Carboxylic Acid Hydrazide (TCH)

M. bovis is usually susceptible to 10 µg/ml of TCH. *M. tuberculosis* is usually not inhibited by this chemical, however, South Indian strains are susceptible.

9. Tween 80 Hydrolysis

Certain mycobacteria possess an enzyme lipase that splits Tween 80 into oleic acid and polyoxyethylated sorbitol which modifies the optical characteristics of the test solution from a yellow to pink. A pink colour indicates hydrolysis of Tween 80. *M. kansasii* and *M. gordonae* are positive, while *M. bovis*, *M. africanum*, *M. avium* complex and *M. scrofulaceum* are negative. *M. tuberculosis* shows variable results.

Fig. 42.4 Nitrate reduction test

E. Antigenic Structure

Mycobacterial antigens are mainly of two types, *cell wall* (insoluble) and *cytoplasmic* (soluble) antigens.

1. Cell Wall Antigens

The cell wall consists of lipids, proteins and polysaccharides. The lipid content accounts for 60% of the cell wall weight. Lipids of the cell wall particularly the *mycolic acid* fraction are responsible for the acid-fastness of bacteria and the cellular tissue reactions of the body. The cell wall is made up of four distinct layers (Fig. 42.5).

(*i*) *Peptidoglycan (murein) layer*, the innermost layer which maintains the shape and rigidity of the cell.

(*ii*) *Arabinogalactan layer* lies external to the peptidoglycan layer.

(*iii*) *Mycolic acid layer* is the principal constituent of cell wall. It is a dense band on long chain α–alkyl and β–hydroxy fatty acids attached by ester bonds to the terminal arabinose units of arabinogalactan.

(*iv*) *Mycosides* (peptidoglycolipids or phenolic glycolipids) form the outermost layer of the cell wall. The agglutinogen antigens have been identified as the sugar moieties on mycosides.

The cell wall antigens include arabinomanan, arabinogalactan and lipoarabinomanan.

2. Cytoplasmic Antigens

Cytoplasmic antigens are protein antigens employed to type the mycobacteria. These include antigen 5, antigen 6, antigen 14, antigen 19, antigen 32, antigen 38 and antigen 60. All are protein in nature except antigen 60 which is a lipopolysaccharide protein complex.

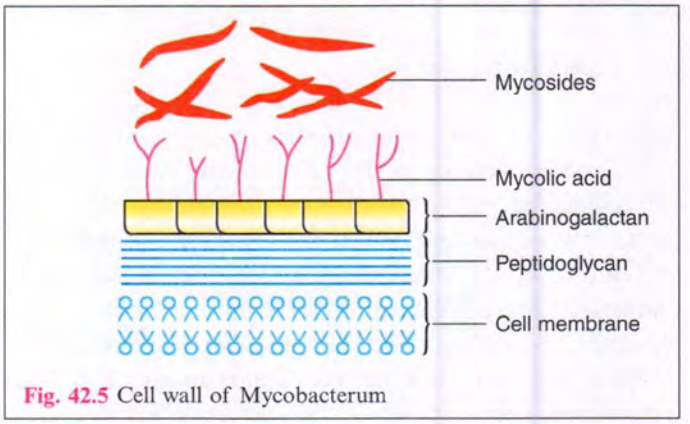


Fig. 42.5 Cell wall of Mycobacterum

F. Mycobacteriophages

Some tubercle bacilli are infected with temperate phages. Many mycobacteria infected with bacteriophage are not truly lysogenic, the phage genome appears as independent plasmid. There is no integration of phage genome into the host genome. This is called *pseudolysogeny*. Tubercle bacilli have been classified into four phage types—A, B, C and I. Type I is intermediate between A and B. Type A is worldwide in distribution and is the commonest type. Infection due to type I is more frequent in India and neighbouring countries. Type B mainly occurs in Europe and North America. Type C is rarely seen. Phage 33 D (isolated from an environmental mycobacterium) can lyse all variants of *M. tuberculosis,* but not BCG.

G. Pathogenesis

The infection is commonly acquired by inhalation of infected droplets coughed or sneezed into the air by a patient with pulmonary tuberculosis. In bovine tuberculosis, infected cows develop lesions in the udder and bacilli are excreted in the milk which can then infect people who drink it raw. In developed countries, pasteurisation of milk has virtually eradicated this organism.

Tubercle bacilli are engulfed by macrophages but they survive and multiply in macrophages. These lyse the host cell, infect other macrophages and sometimes disseminate to other parts of lung and elsewhere in the body. The cell mediated immunity (CMI) plays a major role to interact with these macrophages whereas humoral immunity appears to be irrelevant, CD4+ helper T cells secrete interferon gamma, interleukin 2, tumour necrosis factor α and others exerting different biological effects. It may result in protective immunity or delayed type hypersensitivity (DTH) reaction. Th-1 dependent cytokines activate macrophages to kill intracellular mycobacteria and thus result in protective immunity. Th-2 cytokines induce delayed type hypersensitivity (DTH), tissue destruction and progressive disease.

Human tuberculosis is divisible into *primary* and *secondary* forms.

1. Primary Tuberculosis

Inhaled tubercle bacilli are engulfed by alveolar macrophages in which they replicate to form a lesion called *Ghon focus*. It is frequently found in the lower lobe or lower part of the upper lobe. Some bacilli are transported to the hilar lymph nodes. The Ghon focus together with the enlarged hilar lymph nodes is called the *primary complex*. In case of *M. bovis* which enters through mouth, the primary complexes involve the tonsil and cervical lymph nodes or the intestine, often the ileocaecal region, and the mesenteric lymph nodes.

2. Secondary (Post-primary) Tuberculosis

It is caused by reactivation of the primary lesion (endogenous) or by exogenous reinfection. Reactivation tuberculosis is likely to occur in immunocompromised individuals. Granulomas of secondary tuberculosis most often occur in the apex of lungs. The necrotic element of the reaction causes tissue destruction and the formation of large area of caseation termed tuberculomas. Presence of caseous necrosis and cavities are two special features of secondary tuberculosis. Cavities may rupture into blood vessels, spreading mycobacteria throughout the body, and break into airways, releasing the organisms in aerosols and sputum (open tuberculosis).

H. Immunity and Hypersensitivity

Two immunological responses develop simultaneously in naturally infected host, one is antitubercular immunity and other being tuberculin hypersensitivity. Both responses are mediated by T-lymphocytes sensitised to the bacterial antigen. Humoral immunity appears to be of no relevance in tuberculosis.

Immunity (resistant to infection) and hypersensitivity (allergy) are two different manifestation of the same mechanism in tuberculosis.

1. In non-immune host, the bacilli are readily phagocytosed and multiply inside the mononuclear cells. This intracellular parasitism is associated with the development of delayed hypersensitivity and of activated macrophages which have increased ability to destroy the ingested bacilli.
2. After first infection the host acquires some resistance against reinfection. In the immune host the sensitised T-lymphocytes (developed during primary infection) proliferate and release lymphokines which make the macrophages bactericidal.

Delayed hypersensitivity can be induced by infection with virulent or avirulent tubercle bacilli. For demonstrating delayed hypersensitivity, tuberculoprotein (tuberculin) injection is commonly employed.

I. Koch's Phenomenon

The response of a tuberculous animal to reinfection was best explained by Robert Koch. When a healthy guinea pig is inoculated subcutaneously with virulent tubercle bacilli, the puncture site heals quickly and there is no immediate visible reaction. After 10–14 days, a nodule appears at the site of injection which ulcerates and the ulcer persists till the animal dies of progressive tuberculosis. The regional lymph nodes are enlarged and caseous. If on the other hand, virulent tubercle bacilli are injected in a guinea pig, which had received a prior injection of tubercle bacilli

4–6 weeks earlier, an indurated lesion appears at the site of injection in a day or two which undergoes necrosis in another day or so to form a shallow ulcer. This ulcer heals rapidly without involvement of the regional lymph nodes or tissues. This is called *Koch's phenomenon.*

Koch's phenomenon is a combination of hypersensitivity and immunity and has got three components:

1. A local reaction of induration and necrosis
2. A focal response in which there occurs acute congestion and even haemorrhage around the tuberculous foci in tissues.
3. A systemic response of fever that may sometimes be fatal.

J. Tuberculin Skin Test

1. Principle

Tuberculin skin test (TST) is delayed or type IV hypersensitivity reaction.

2. Reagents

(i) Old tuberculin (OT)

It was originally described by Robert Koch. It is a crude preparation of 6–8 week culture filtrate of tubercle bacilli, concentrated by evaporation on a heated water bath. This crude product may lead to serious complications in some patients, it is now rarely used.

(ii) Purified protein derivative (PPD)

A purified preparation of the active tuberculoprotein was prepared by *Seibert* (1941). It was prepared by growing *M. tuberculosis* in a semisynthetic medium. It is called *purified protein derivative* (PPD). The dosage of PPD is expressed in tuberculin unit (TU). One TU is equal to 0.01 ml of OT or 0.00002 mg of PPD-S. Another PPD is RT-23 with tween 80. In India PPD RT-23 of strength 1 TU and 2 TU are available. 1 TU of PPD RT-23 is equivalent of 5 TU of PPD-S.

3. Method

(i) Mantoux test

0.1 ml of PPD containing 5 IU of PPD-S is injected intradermally into flexor aspect of forearm. A PPD-S dose of 1 TU is used when extreme hypersensitivity is suspected. In India 1 TU of PPD RT-23 is recommended and not PPD-S.

(ii) Heaf test

This is done with a multiple puncture apparatus that pricks the skin. A drop of undiluted PPD is spread on the area of skin. The multiple puncture apparatus is pressed against this area of skin.

4. Result

In the Mantoux test the site of injection is examined after 48–72 hours and interpreted as follows:

(i) Positive test

In a positive reaction, there is induration (local oedema) of 10 mm diameter or more surrounded by erythema at the site of inoculation. Positive test only confirms past infection with tubercle bacilli but does not indicate presence of active stage of the disease. The test is helpful in children under five years for indication of active infection. The test becomes positive 4–6 weeks after infection or BCG vaccination.

(ii) False negative

The test may become negative in following conditions:

(*a*) miliary tuberculosis

(*b*) when anergy develops following overwhelming infection of measles, Hodgkin's disease, sarcoidosis, lepromatous leprosy, malnutrition, administration of immunosuppressive agents and corticosteroids.

(iii) False positive

This is observed in presence of related mycobacteria such as atypical mycobacteria.

5. Uses

(*i*) To measure prevalence of infection in a community.

(*ii*) To diagnose active infection in young children.

(*iii*) It is used as an indicator of successful BCG vaccination.

In recent years in vitro interferon-γ release assays (IGRA) have been introduced as a sensitive and more specific alternative to tuberculin skin test (TST). This test is used in blood specimen which contains T-lymphocytes. It uses ELISA to measure interferon-γ (IFN-γ) production by sensitised T-lymphocytes which are stimulated by *M. tuberculosis* antigens. If an individual was previously infected with *M. tuberculosis*, exposure of sensitised T-lymphocytes to *M. tuberculosis* specific antigens results in IFN-γ production.

K. Laboratory Diagnosis

Bacteriological diagnosis can be established by microscopy, culture examination or by animal inoculation test.

1. Specimen

Specimen collection depends on the site of involvement. Tuberculosis may involve lungs (pulmonary) or sites other than lungs (extrapulmonary).

(i) Pulmonary tuberculosis

Sputum is the most common specimen. It is collected in a clean wide-mouthed container. A morning specimen may be collected on three consecutive days. If sputum is scanty, a 24 hour specimen may be collected. When sputum is not available, laryngeal swab or bronchial washings are collected. In children, gastric washings may be examined as they tend to swallow sputum.

(ii) Meningitis

Cerebrospinal fluid (CSF) from tuberculous meningitis (TBM) often forms a spider web clot on standing, examination of which may be more useful than of fluid.

(iii) Renal tuberculosis

Three consecutive days morning samples of urine are examined.

(iv) Bone and joints tuberculosis

Aspirated fluid

(v) Tissue

Biopsy of tissue.

2. Direct Microscopy

Smear is made from the specimen on a new glass slide and stained by the *Ziehl-Neelsen* technique (refer to chapter 2 for method of Ziehl-Neelsen staining). It is examined under oil immersion lens. The acid-fast bacilli (AFB) appear as bright red bacilli against a blue background. To detect the bacilli microscopically, there should be at least 10,000 bacilli per ml of sputum. A negative report should not be given till at least 300 fields have been examined. Grading of smears is done according to number of bacilli seen: (Table 42.3).

Table 42.3 Grading of Ziehl-Neelsen (ZN) Smear

No. of AFB seen in oil immersion field	Report
0/300 fields	AFB not seen
1-2/300 fields	Doubtful, repeat the smear
1-9/100 fields	1+
1-9/10 fields	2+
1-9/field	3+
10 or more/field	4+

Purulent part of the sputum is used for making a smear. Microscopy is also performed on the smear which is prepared after concentration of specimen. In case of urine specimen, care must be taken to exclude commensal *M. smegmatis* bacillus which is only acid-fast and not alcohol-fast, whereas *M. tuberculosis* is both acid and alcohol fast.

If a large number of smears are to be examined, fluorescent microscopy is more convenient. Smears are stained with fluorescent dyes such as auramine 'O' or auramine rhodamine and examined under ultraviolet light. The bacilli appear as bright bacilli against dark background. WHO has recommended the use of LED (light emmiting diode) fluorescence microscopy over conventional fluorescent microscopy. Compared to conventional mercury vapour fluorescent microscopes, LED microscopes are less expensive and are able to run on batteries. LED microscopes can perform equally well without a dark room. It has more sensitivity than ZN staining.

Demonstration of AFB by microscopy provides only presumptive evidence of tuberculous infection, as saprophytic mycobacteria may present a similar appearance.

3. Concentration of Specimens

Concentration of a specimen is done to achieve:

(a) homogenisation of the specimen
(b) decontamination i.e. to kill other bacteria present in the specimen.
(c) concentration i.e. to concentrate the bacilli in a small volume without inactivation.

Such concentrate is used for culture and animal inoculation tests besides smear preparation.

Several concentration methods are in use:

(i) Petroff's method

It is a simple and widely used technique. Sputum is mixed with equal volume of 4% sodium hydroxide and is incubated at 37°C with frequent shaking for about 30 minutes. It is then centrifuged at 3,000 rpm for 30 minutes. The supernatant fluid is poured off and the deposit is neutralised by adding 8% hydrochloric acid in presence of a drop of phenol red indicator. The deposit is used for smear, culture and animal inoculation.

(ii) Other methods

Dilute acids (5% oxalic acid, 3% hydrochloric acid or 6% sulphuric acid), mucolytic agents such as N-acetyl-L-cysteine with sodium hydroxide and pancreatin are used for concentration of specimens. In urine and CSF specimens centrifugation is done to concentrate the specimen. Centrifuged deposit is used for smear and culture examination.

4. Culture (Fig. 42.6)

Culture is a very sensitive method for detection of tubercle bacilli. It may detect as few as 10 to 100 bacilli per ml. The concentrated material is inoculated on two bottles of Lowenstein-Jensen medium. In case of gastric washings, alkali is added to neutralise the acid present in the specimen and then inoculated on culture medium. Urine is centrifuged and then from deposit, culture medium is inoculated. In case of CSF, it is centrifuged and the deposit is used for culture and smear examination.

The culture media are incubated at 37°C in the dark and in the light. Cultures are examined first after 4 days (for rapid growing mycobacteria, fungi and contaminant bacteria) and thereafter weekly till 8 weeks. The tubercle bacilli usually grow in 2 to 8 weeks. Longer incubation is necessary for strains originating from patients treated with antitubercular drugs.

In a positive culture, characteristic colonies appear on culture medium. Smear is prepared from isolated colony and stained with Ziehl-Neelsen technique. When acid-fast bacillus (AFB) is slow growing, non-pigmented and niacin positive, it is regarded as *M. tuberculosis*. Confirmation is done by biochemical reactions.

In radiometric method such as BACTEC, the growth may be detected in about a week by using ^{14}C labelled substrates. Culture media contains ^{14}C-labelled palmitic acid. Mycobacteria metabolise the ^{14}C-labelled substrates and release radioactively labelled $^{14}CO_2$. The instrument measures $^{14}CO_2$ and reports in terms of 'growth index'. A growth index of ≥ 10 is considered as positive. This method can also differentiate between *M. tuberculosis* and *M. bovis*. As *M. bovis* is susceptible to TCH, incorporation of TCH in the medium inhibits the growth of *M. bovis* (i.e. growth index decreases) but not that of *M. tuberculosis*. This significant method has now been discontinued due to radioactivity.

Mycobacterial growth indicator tube (MGIT) is another rapid method for detection of mycobacterial growth. It is a non-radiometric, automated method. It consists of tubes containing liquid culture media, and a fluorescent compound is embedded on the bottom of the tube. The fluorescent compound is sensitive to dissolved oxygen in the liquid medium. Thus the dissolved oxygen in the uninoculated medium quenches any fluorescence from the compound. When mycobacteria grow they deplete the dissolved oxygen in the liquid medium and allows the compound to fluoresce brightly which can be detected by observing the tube under ultraviolet light (wood's lamp). The result are obtained in 8 to 14 days.

BacT/Alert 3D system is a non-radiometric, rapid and fully automated. It uses liquid medium vials in which specimen is inoculated. If mycobacteria are present in the specimen, carbon dioxide (CO_2) is released by actively proliferating mycobacteria. The elevated level of CO_2 lowers the pH in the medium which produces a colour change in sensor present in the liquid medium vial. This colour change is detected by the instrument.

5. Animal Inoculation

0.5 ml of the concentrated specimen is inoculated intamuscularly into the thigh of two tuberculin negative healthy guinea pigs. Inoculation by subcutaneous route is avoided as it causes local ulcer which may be infectious. The animals are weighed prior to inoculation and thereafter at weekly interval. They are tuberculin tested after 3–4 weeks. There is progressive loss of weight and

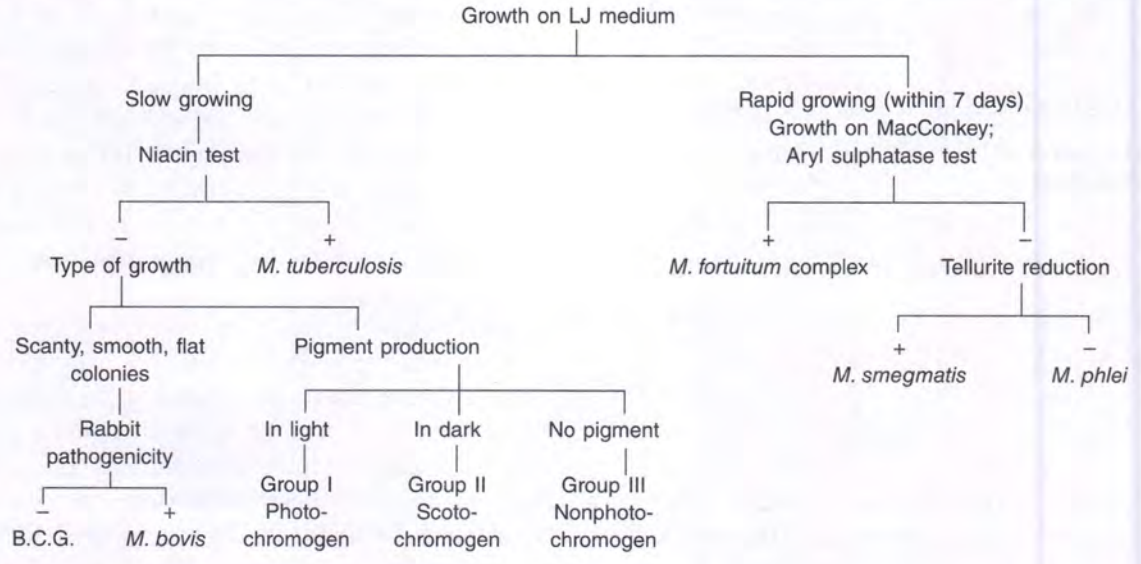


Fig. 42.6 Identification of Mycobacteria

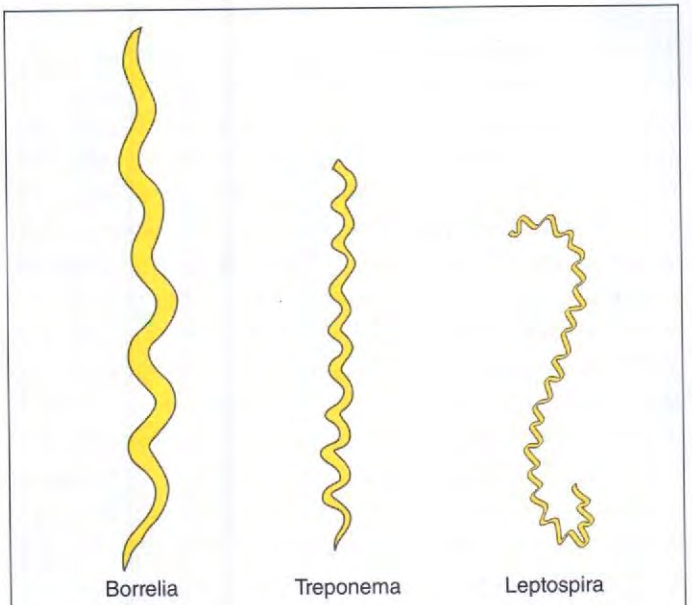


Fig. 45.2 Comparative morphology of different spirochaetes

B. parkeri and *B. turicatae*. The different members of borrelia causing relapsing fever are now considered to be strains of a single species *B. recurrentis*.

1. Morphology

Various strains of *Borrelia* causing relapsing fever are morphologically indistinguishable, but exhibit some antigenic differences. They measure 8–20 μm × 0.2–0.5 μm. They are motile, Gram negative and possess 5–8 irregular spirals at intervals of 2 μm with pointed ends (Fig. 45.3). It stains well with Giemsa or Wright stains.

2. Culture

Borrelia are microaerophilic. Optimum temperature for growth is 28–30°C. The organisms can be grown in Noguchi's medium (ascitic fluid containing rabbit kidney), on chorioallantoic membrane (CAM) of chick embryos and in peritoneal cavity of rat or mouse.

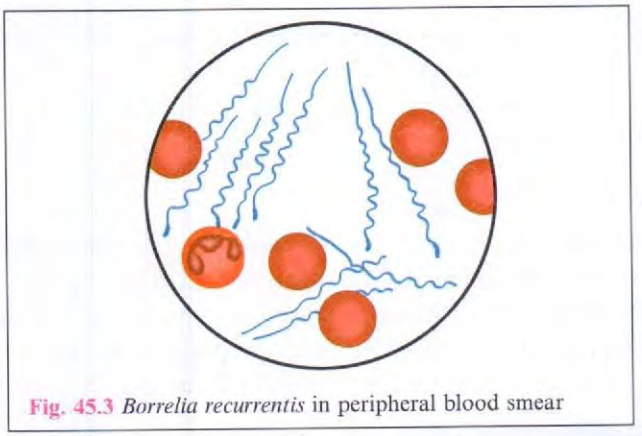


Fig. 45.3 *Borrelia recurrentis* in peripheral blood smear

3. Antigenic Structure

The borrelia readily undergoes antigenic variations *in vivo* and this may be the reason for the occurrence of relapses in the disease. Ultimate recovery occurs after a number of relapses due to development of antibodies against all the antigenic variants. Antibodies like agglutinins, complement fixing and lytic antibodies develop during infection, but their detection is not possible due to the difficulty in preparing satisfactory antigens.

4. Pathogenesis

Relapsing fever is transmitted by infected vectors: body louse (*Pediculus humanus corporis*) in *B. recurrentis* and tick (*Ornithodorus*) in *B. duttoni* and others. Incubation period varies between 2 to 10 days. Disease is characterised by febrile episode of sudden onset. The fever subsides after 3–5 days. During febrile period, borreliae are abundant in the patient's blood, while these are not demonstrable during afebrile phase. Afebrile period lasts for 4 to 10 days after which another bout of fever sets in. The borreliae reappear in blood during the relapses of fever. There are 3–10 such relapses and then the disease ultimately subsides. Splenomegaly is common. Louse-borne and tick-borne relapsing fevers are clinically indistinguishable.

5. Laboratory Diagnosis

Blood is collected during febrile period and is examined by following methods:

(*i*) *Dark ground microscopy*
A wet film of blood is made and examined under the dark ground or phase contrast microscope and borreliae can be detected by their active movements.

(*ii*) *Giemsa or Leishman stain*
Blood smears are stained with Giemsa or Leishman stain and examined for borreliae.

(*iii*) *Animal inoculation*
White mouse is inoculated intraperitoneally with 1–2 ml of patient's blood. After two days of injection, smears prepared from blood of tail vein show spirochaetes.

(*iv*) *Culture and serology*
These are too difficult and unreliable. Serological tests for syphilis are sometimes positive in patients with relapsing fever.

6. Treatment

Tetracycline, chloramphenicol, penicillin and erythromycin are effective.

7. Prophylaxis

Control of vectors (louse and tick) and maintenance of

Benzathine benzyl penicillin 24 lacs units, intramuscularly, once weekly for three weeks.

In certain cases, treatment with penicillin may lead to *Jarisch-Herxheimer reaction*. It is systemic response which consists of fever, chills, myalgia and hypotension. The reaction is believed to be due to the liberation of toxic products from the massive destruction of treponemes or due to hypersensitivity. This reaction is rapid in onset (within 2 hours).

9. Prophylaxis

1. Treatment of cases.
2. Follow up of cases and contacts.
3. Prophylactic use of sex barriers (such as condom).

B. Non-Venereal Treponematoses

Non-venereal treponemal diseases occur in communities with poor standards of hygiene. These include *Yaws*, *pinta* and *endemic syphilis*. Treponemes responsible for these diseases are virtually indistinguishable from *T. pallidum*. Like venereal syphilis, these treponemes also show reactive treponemal and non-treponemal serological tests.

1. Yaws (Framboesia)

It is an endemic disease caused by *T. pallidum* subspecies *pertenue* (still known as *T. pertenue*) occurring in the tropical areas of Africa, Asia, and America. In India, cases have been identified in Orissa, Andhra Pradesh and Madhya Pradesh. *T. pertenue* is morphologically and antigenically indistinguishable from *T. pallidum*. It cannot be cultured in artificial media but it can be cultivated in rabbit testis. The incubation period is 3 to 5 weeks.

The primary lesion is an extragenital papule which enlarges and breaks down to form an ulcerating granuloma. Like syphilis, secondary and tertiary manifestations follow. Cardiovascular or neurological involvement is rare. Infection is by direct contact with open ulcers. Flies may act as vectors. Laboratory diagnosis and treatment are similar to that of venereal syphilis.

2. Pinta

It is caused by *T. carateum* occurring mainly in Central and South America. Children of 10–15 years are most commonly affected. The disease is acquired by direct person to person contact. The skin bears the brunt of the disease. Incubation period ranges from 7–21 days. The primary lesion is non-ulcerating extragenital papule which develops into lichenoid or psoriaform patch. Secondary skin lesions are characterised by hyperpigmentation or hypopigmentation. The laboratory diagnosis and treatment are same as those of venereal syphilis.

3. Endemic Syphilis

The disease is caused by *T. pallidum* subspecies *endemicum*. Endemic syphilis is transmitted non-venereally and occurs in endemic foci. It is known as *bejel* in Middle East and *siti* in Gambia. It has also been reported from India. The disease occurs mainly in children living under poor standards of personal hygiene. It is transmitted by direct person to person contact and by sharing common contaminated utensils of eating and drinking. Venereal spread is uncommon. Primary chancre is rare but may sometimes be found on nipples of mothers infected by their children. The disease usually manifests with features of secondary syphilis such as mucous patches and skin eruptions. It progresses to tertiary lesions, particularly gummatous lesions on the skin, bone and nasopharynx. Cardiovascular and neurological involvement is rare. Congenital syphilis is also not found.

The laboratory diagnosis and treatment are same as those of venereal syphilis.

C. Non-Pathogenic Treponemes

Certain commensal treponemes may be found in buccal and genital mucosa and may cause confusion in the diagnosis of syphilis by dark-field microscopy. *T. dentium* is found in the mouth.

T. refringens and *T. gracilis* may be found as commensals in genitalia. *T. cuniculi* causes natural venereal infection in rabbit and may lead to confusion in experimental work with *T. pallidum*.

III. BORRELIA

Borreliae differ from other spirochaetes in being larger (8–30 μm × 0.2–0.5 μm) with 3–10 irregular wide and open coils (Fig. 45.2). They are motile, refractile spirochaetes, stain readily with ordinary dyes and are Gram negative. Several species of Borrelia occur as commensals on the buccal and genital mucosa.

The important pathogenic borreliae of medical importance include:

1. *B. recurrentis*—causes relapsing fever.
2. *B. vincentii*—causes vincent's angina in association with fusiform bacilli.
3. *B. burgdorferi*—the causative agent of Lyme disease

A. Borrelia recurrentis

Relapsing fever (RF) is an arthropod-borne infection, two types of which are louse-borne and tick-borne. Those strains causing epidemic or louse-borne relapsing fever are called *B. recurrentis*, while strains responsible for endemic or tick-borne relapsing fever include *B. duttoni, B. hermsii,*

pallidum. In this test gelatin particles sensitised with *T. pallidum subsp. pallidum* antigens have been used instead of erythrocytes used in microhaemagglutination assay (MHA-TP).

Table 45.3 shows the reactivity of serological tests in untreated syphilis.

Table 45.3 Reactivity of Serological Tests in Untreated Syphilis

Test	Stage of the disease, % positive		
	Primary	Secondary	Latent
VDRL/RPR	70%	100%	70%
FTA–ABS	80%	100%	65%
TPHA	65%	100%	95%

Enzyme Immunoassay (EIA)

Ultrasonicate of *T. pallidum* antigen is coated on tubes. Antibody in the patient serum is detected by enzyme immunoassay. Sensitivity and specificity has been reported as 90% and 98% respectively. It is now commercially available as the *Bio-Enza-Bead test* (Organon) which makes use of ferrous metal beads as a solid phase carrier for antigen.

INTERPRETATION OF SEROLOGICAL TESTS

1. Reagin antibodies appear in the serum only about four weeks after aquiring the infection, hence tests for these antibodies may sometimes be non-reactive in primary stage of the disease. Diagnosis of early primary syphilis can be done either by repeating a VDRL test after 1–2 weeks if the same was initially negative or by performing a FTA-ABS test. FTA-ABS test becomes reactive earlier than reagin tests.
2. With primary chancre, tests for reagin are reactive only in 70% of cases. Thereafter, antibody levels increase and become maximum in the secondary stage, when all standard tests for syphilis are invariably reactive. During the next several years, the antibody titres decline gradually and in the late syphilis, tests for reagin may become non-reactive.
3. Non-treponemal tests become negative or show decline with effective treatment, while treponemal tests usually remain positive after complete treatment. VDRL test or RPR test is more useful for the assessment of cure following treatment. If adequate treatment is given immediately after the primary lesion appears, the patient may never develop antibodies and all serological test may be negative. In late syphilis, serological tests may continue to be reactive even after complete treatment.
4. In congenital syphilis, it is necessary to distinguish between maternal antibodies passively transferred across the placenta and the antibodies produced as a result of active foetal infection. In the former repeated tests will show an increase in titre, whereas in the latter titre will fall. Demonstration of IgM antibody in the newborn is indicative of active infection, as IgM does not normally cross the placenta. Hence, FTA-ABS test to demonstrate IgM antibodies is necessary to confirm the diagnosis of congenital infection.

CHOICE OF SEROLOGICAL TESTS

1. VDRL or RPR tests are used for screening or for diagnostic purposes of large number of sera. These tests are also used for quantitative measurement of reagin titre for assessment of clinical activity of syphilis.
2. Treponemal tests (TPHA or FTA-ABS) are used to confirm the diagnosis with a positive reagin test.

Concurrent Infection of HIV and Syphilis

Syphilis is an important risk factor for HIV infection. All syphilis patients should be tested for HIV infection and vice-versa.

7. Immunity

The immune mechanisms are not well understood. A syphilitic patient is said to be refractory to reinfection as long as the infection persists (*premunition or infection immunity*). Patient is susceptible to reinfection after recovery from first infection.

Antitreponemal antibodies (IgM, IgG and IgA) appear almost with development of primary chancre. The antibody titre goes on increasing during the next several months. However, their role in resistance to infection remains uncertain. It has, therefore, been suggested that cell mediated immunity plays a significant role in resistance to syphilis.

8. Treatment

1. *Early syphilis*: Primary, secondary and latent infection of two years duration or less are included in early syphilis.
   (*i*) Benzathine benzyl penicillin 24 lacs units intramuscularly in a single dose after sensitivity test.
   (*ii*) Alternatively, doxycycline 100 mg twice a day, orally for 15 days.
2. *Late syphilis*
   Infection of more than two years duration is included in late syphilis.

strain. The test is less sensitive than the standard tests in early syphilis but is more specific and sensitive in late and latent syphilis. It detects group specific treponemal antibodies. RPCF test is rarely done nowadays.

Tests using T. pallidum (Nichol's Strain)

(i) Using live T. pallidum

Treponema pallidum immobilisation (TPI) test

This test employs live *T. pallidum*. The test serum is mixed with actively motile Nichol's strain of *T. pallidum* and incubated anaerobically. If antibodies are present, the treponemes are immobilised (rendered non-motile), when examined under dark ground illumination. The test is considered reactive if more than 50 per cent of the treponemes are immobilised. It is considered non-reactive when less than 20 per cent are immobilised and doubtful if between 50 per cent and 20 per cent are immobilised. Although TPI is the most specific test for syphilis, it is infrequently used, as live treponemes are required from infected animals and it is technically difficult to perform the test.

(ii) Using killed T. pallidum

Treponema pallidum agglutination (TPA) test

A suspension of *T. pallidum*, inactivated by formalin, is mixed with the test serum and examined under dark ground microscopy. The treponemes are found agglutinated in the presence of antibodies. The test is not very specific and false positive reactions are common.

Treponema pallidum immune adherence (TPIA) test

A suspension of inactivated *T. pallidum* is mixed with the test serum, complement and fresh heparinized whole blood from a normal individual and incubated. The treponemes will be found to adhere to the erythrocytes in the presence of antibodies. In absence of antibodies (negative test), immune adherence will not occur. Both TPA and TPIA are not used in diagnostic laboratories.

Fluorescent treponemal antibody (FTA) test

It is an indirect immunofluorescence test. Smears of killed *T. pallidum* (Nichol's strain) are prepared on slides and fixed. These slides are stored in the deep freeze so that smears can be used for several months. The patient's serum is allowed to react with the smear. The excess serum is then washed off. The antibodies that bind to the fixed smear are detected by treating the smear with fluorescein labelled antihuman immunoglobulin (conjugate). After incubation and washing off the unfixed conjugate, the smear is examined under fluorescent microscope. In a positive test, treponemes fluoresce.

As originally performed, patient's serum was used in a dilution of 1 in 5. At this dilution there were false positive reactions. This was therefore modified by using patient's serum at a dilution of 1 in 200. In this test, called FTA-200, the specificity was improved but false positive reactions were not completely eliminated. This test was further modified to fluorescent treponemal antibody absorption (FTA-ABS) test.

Fluorescent treponemal antibody-absorption (FTA-ABS) test

Patient's serum is first absorbed with an extract of non-pathogenic treponemes (Reiter treponemes) to remove reagin and the group-reactive antibody. Method of the test is same as that of FTA test. FTA-ABS test has high specificity and sensitivity. It has been found to be almost as specific as the more complicated TPI test. Like VDRL, FTA-ABS test can also be performed on cerebrospinal fluid. It is the earliest serological test to become positive in syphilis and remains positive for many years even after effective treatment of early syphilis. Another modification, the IgM-FTA-ABS test can detect IgM antibodies in congenital syphilis and helps to differentiate it from seropositivity due to passively transferred maternal antibodies which are IgG in nature. As IgM does not cross the placenta, its presence in neonatal serum confirms the diagnosis of congenital syphilis.

(iii) Using an extract of T. pallidum

Treponema pallidum haemagglutination assay (TPHA)

Tanned sheep erythrocytes are sensitised with an extract of *T. pallidum*. When these sensitised erythrocytes are mixed with patient's serum containing anti-treponemal antibodies, the erythrocytes clump together. Like FTA-ABS test, patient's serum is preabsorbed with an extract of Reiter treponemes to remove group-reactive antibody. This test can also be used to detect localised production of antibodies in cerebrospinal fluid (CSF). It is less sensitive than FTA-ABS test in primary syphilis (65%) but is as sensitive as FTA-ABS in other stages (secondary and late). Kits are available commercially and no special equipment is needed. These advantages have made TPHA a standard confirmatory test. TPHA is very simple to perform.

TPHA may be performed in microtitre plates, this is referred to as microtitre haemagglutinaton–*T. pallidum* (MHA-TP) test. Haemagglutination treponemal test for syphilis (HATTS) is an automated conversion of TPHA test. The only disadvantage with MHA-TP and HATTS is that they lack sensitivity in primary syphilis.

Treponema pallidum particle agglutination (TP-PA) test has been developed for detection of antibodies to *T.*

to inactivate it prior to the test, whereas CSF need not be heated.
2. 0.05 ml of inactivated serum (at 56°C for 30 minutes) is taken on a slide, to which one drop of freshly prepared cardiolipin antigen is added by a syringe delivering 60 drops in one ml.
3. The slide is then rotated at 180 revolutions per minute for four minutes. It can be done by VDRL rotator or manually.
4. The slide is examined under low power objective of microscope. Presence of clumps signifies positive reaction while uniformly distributed crystals indicate a negative result.
5. If the test is positive, it is quantitated by performing the test with serial dilutions (1:4, 1:8 and so on) of serum.

Results

The results of qualitative test are reported as 'reactive', 'weak reactive' or 'non reactive'. 'Reactive' means positive while 'non-reactive' is negative. For reporting of quantitative test, the reciprocal of the end point is given as the titre e.g. reactive in 1:4 dilution is reported as 'reactive 4 dilution' or R4.

VDRL test sometimes may give *false negative* reaction due to high titres of antibody in patient's serum (prozone phenomenon). In such cases the test is performed with diluted serum and it becomes positive.

(b) RPR (Rapid Plasma Reagin) test

It is almost similar to VDRL test. Finely divided carbon particles are added to cardiolipin antigen. RPR test has got the following *advantages* over VDRL test:

- Unheated serum or plasma can be used.
- A fingerprick sample of blood is sufficient.
- It does not require microscope and can be done in the field.
- It is available commercially as a kit.

The only *disadvantage* of the test is that it cannot be used with cerebrospinal fluid (CSF).

VDRL and RPR tests are useful in surveys because of their low cost. VDRL test or RPR test is positive in about 70% of primary and 100% of secondary syphilis. Automated RPR test (ART) is available for large scale tests. Similarly, an automated VDRL-ELISA test has also been developed.

Toludine red unheated serum test (TRUST), a modified RPR test, has been used. It uses paint pigment toner toludine red particles instead of carbon particles. Like RPR test, it does not require microscope for examination.

Disadvantages of standard test

The antigen (cardiolipin) is non-specific and hence may react with the sera of patients who may not have syphilis. This accounts for the *biological false positive* (BFP) reactions. These are not caused by technical faults. They represent non-treponemal cardiolipin antibody responses. These BFP reactions may occur in about one percent of normal sera. BFP antibody is usually of IgM type, while reagin antibody in syphilis is mainly IgG.

Conditions in which BFP reactions occur include:

1. Leprosy (particularly lepromatous leprosy)
2. Malaria
3. Relapsing fever
4. Infectious mononucleosis
5. Tropical eosinophilia
6. Hepatitis
7. Collagen diseases e.g. systemic lupus erythematosus (SLE) and rheumatoid arthritis.

BFP reactions are of two types

1. Acute reactions which may develop shortly after an acute febrile infectious disease and will disappear within 6 months. These are usually associated with acute infections or inflammatory conditions.
2. Chronic BFP reactions persist longer than 6 months and may persist indefinitely. These are typically seen in SLE and other collagen diseases.

To eliminate these false positive reactions, tests have been developed using treponemal antigens. These treponemal tests are described below.

Treponemal Tests

These tests may be divided as follows:

1. Tests using Reiter treponeme
   Reiter protein complement fixation (RPCF) test
2. Tests using *T. pallidum* (Nichol's strain)
   (*i*) Using live *T. pallidum*
   - Treponema pallidum immobilisation (TPI) test

   (*ii*) Using killed *T. pallidum*
   - Treponema pallidum agglutination (TPA) test
   - Treponema pallidum immune adherence (TPIA) test
   - Fluorescent treponemal antibody (FTA) test

   (*iii*) Using an extract of *T. pallidum*
   - Treponema pallidum haemagglutination assay (TPHA) test
   - Enzyme immunoassay (EIA)

Tests using Reiter's Treponemes

Reiter's protein complement fixation (RPCF) test

The principle of the test is same as that of Wassermann reaction. The antigen is prepared from cultivable Reiter

Table 45.2 Diagnostic Methods in Syphilis

Demonstration of Treponemes	1. Dark ground microscopy 2. Direct fluorescent antibody staining for *T. pallidum* (DFA–TP) 3. Treponemes in tissue (*i*) Silver impregnation method (Levaditi stain) (*ii*) Immunofluorescence staining
Serological tests	
Non-treponemal tests	1. VDRL 2. RPR
Treponemal tests	1. FTA, FTA-ABS (using killed *T. pallidum*) 2. TPHA, MHA-TP, TP-PA (using *T. pallidum* extract) 3. TPI test (using live *T. pallidum*)

be collected with extra care as the lesions are highly infectious.

Dark ground microscopy

The surface of chancre is cleaned with saline, a drop of exudate is collected on a slide by applying gentle pressure at the base of the lesion. The wet film is covered with a coverslip and examined under dark ground microscope. *Treponema pallidum*, appears as a slender, spiral organism showing rotational as well as flexion and extension movements (Fig. 45.1). Dark field examination should be repeated on three consecutive days before declaring it negative. The negative results do not exclude the diagnosis of syphilis because of its low sensitivity. A treponemal concentration of 10^4 per ml is required for the test to become positive.

Direct fluorescent-antibody staining for T. pallidum (DFA–TP)

Smear of the material to be tested is made on a glass slide. It is stained with fluorescent-labelled monoclonal antibody against *T. pallidum*. The treponemes appear distinct, sharply outlined and exhibit an apple green fluorescence. It is a better and safer method for microscopic diagnosis.

Fig. 45.1 *T. pallidum* under dark ground microscopy

Treponemes in tissues

Treponemes in tissues can be demonstrated by silver impregnation method of staining (*Levaditi's stain*) or by immunofluorescence staining.

Demonstration of treponemal antigen

T. pallidum in the lesion can also be detected by polymerase chain reaction.

SEROLOGICAL TESTS

These tests form the mainstay of laboratory diagnosis. Two types of antibodies are produced in syphilis, non-specific antibody (reagin antibody) and specific anti-treponemal antibody. Depending upon the antigen used, serological tests for syphilis are divided into *non-treponemal tests* (cardiolipin or lipoidal antigen is used) and *treponemal tests* (treponemes are used as the antigen).

NON-TREPONEMAL TESTS

In the standard tests for syphilis (STS), reagin antibodies are detected by cardiolipin antigen. Cardiolipin antigen is an alcoholic extract of beef heart tissue to which lecithin and cholesterol are added. The STS includes Venereal Diseases Research Laboratory (VDRL) test, Rapid plasma reagin (RPR), Kahn test and Wassermann reaction. All these tests are *flocculation tests* except Wassermann reaction which is a complement fixation test (CFT). The Wassermann reaction is no longer in use. Similarly Kahn test is rarely done.

Flocculation Tests

Cardiolipin antigen reacts with reagin antibody in syphilitic serum resulting in formation of visible clumps or floccules. Results can be read in a few minutes. *VDRL* and *RPR* tests are equally sensitive.

(a) VDRL (Venereal Disease Research Laboratory) test

This test was named after Venereal Disease Research Laboratory, New York, where the test was developed. It is the most widely used simple and rapid serological test. Small quantity of serum is needed. It can also be used to detect antibodies in cerebrospinal fluid (CSF). It is a slide flocculation test. The VDRL antigen (cardiolipin antigen) must be prepared fresh daily.

Method

1. The test is done in a specially prepared slide, with depressions of 14 mm diameter each. Serum is heated

polysaccharide in nature and is present in treponemal body. It is species specific. *T. pallidum* is used as antigen for detection of antibodies against this antigen. The specific *T. pallidum* tests detect these antibodies.

(ii) Non-specific antigens

A non-specific antibody (reagin) appears in the blood of syphilitic patients. The reagin antibody reacts with a hapten extracted from beef heart (antigen). This lipid hapten is chemically a diphosphatidyl glycerol and known as *cardiolipin*. It is not clear whether cardiolipin is an antigen contained in *T. pallidum* itself or a product of damaged tissue produced by infection. The reagin antibody is detected by non-specific serological tests for syphilis, such as Wassermann, Kahn and VDRL, in which cardiolipin is used as the antigen.

4. Resistance

T. pallidum is a very delicate organism. It is readily inactivated by drying or by heat (41–42°C in one hour). It is killed at 0–4°C within 3 days, so that transfusion syphilis can be prevented by storing blood for at least four days in the refrigerator before transfusion. Stored at –70°C in 10% glycerol or in liquid nitrogen, it remains viable for several years. It is inactivated by contact with distilled water, soap, common antiseptic agents, arsenicals, mercurials and antibiotics.

5. Pathogenesis

Natural infection with *T. pallidum* occurs only in human beings. Venereal syphilis is acquired by sexual contact. The treponema enters the body through minute abrasions on the skin or mucosa. Clinical disease sets in after an incubation period of about a month (range 10–90 days). There are three clinical stages of the disease in an untreated case—primary, secondary, and tertiary.

(i) Primary syphilis

A papule appears on the genital area that ulcerates forming a classical chancre of primary syphilis, called *hard chancre*. It is a painless, relatively avascular, indurated and a circumscribed lesion. It is covered by a thick exudate, very rich in spirochaetes. The regional lymph nodes are swollen, discrete, non-tender and rubbery. The spirochaetes spread from the site of entry into the lymph and bloodstream, even prior to appearance of chancre. Hence, the patient may be infectious during the late incubation period. The chancre invariably heals within 10–40 days, even without treatment, leaving a thin scar. Persistent or multiple chancres may be seen in HIV cases or other immunodeficient patients.

(ii) Secondary syphilis

After healing of primary lesion, the patient remains asymptomatic for 2 to 6 months, then secondary syphilis sets in. The secondary lesions are due to widespread multiplication of the treponemes and their dissemination through the blood. These are characterised by appearance of widespread macular rash on mucous membranes and skin which contain numerous treponemes. The patient is most infectious during the secondary stage. The rash may coalesce in intertriginous area, especially in the perianal region, producing wart-like condylomata. There may be retinitis, meningitis, periostitis and arthritis. Secondary lesions usually undergo spontaneous healing, in some instances taking as long as four or five years.

After the secondary lesions disappear, 30% of cases remain dormant (latent) for many years without any clinical symptoms but with positive serology. In many cases, natural cure occurs but in others, manifestations of tertiary syphilis appear.

(iii) Tertiary syphilis or late stage

Tertiary lesions contain few spirochaetes. There may be cardiovascular lesions including aneurysms, chronic granulomata (gummata) and meningovascular manifestations. In few cases, neurosyphilis such as tabes dorsalis or general paralysis of the insane develop. These are known as late tertiary or quatenary syphilis.

(iv) Congenital syphilis

The treponemes can cross the placental barrier. Infection in foetus usually occurs from primary and secondary infection of the mother. Antenatal screening and treatment of positive cases during pregnancy may prevent congenital syphilis.

(v) Syphilis acquired non-venereally

It may occur in doctors or nurses due to contact with patient's lesion during examination. The natural evolution is same as in venereal syphilis, except that the primary chancre is extragenital, usually on the fingers. Rarely, when syphilis is transmitted by blood transfusion, the primary chancre does not occur.

6. Laboratory Diagnosis

The diagnosis of syphilis consists of *demonstration* of treponemes and detection of antibodies by *serological tests* (Table 45.2).

DEMONSTRATION OF TREPONEMES

Demonstration of treponemes by microscopy is applicable in primary and secondary stages and in cases of cogenital syphilis with superficial lesions. Specimens should

Spirochaetes vary widely in size from 5 μm to 500 μm in length. The average size is 4–30 μm × 0.1–0.6 μm. Larger spirochaetes like *Borrelia* are Gram negative but other spirochaetes cannot be stained by routine methods. However, the spirochaetes can be seen by dark ground microscopy, silver impregnation method and immunofluorescence.

B. Diseases

Diseases caused by spirochaetes are shown in Table 45.1.

Table 45.1 Diseases caused by spirochaetes

Genus	Species	Disease
Treponema	*T. pallidum*	Syphilis
	T. pertenue	Yaws
	T. carateum	Pinta
	T. endemicum	Endemic syphilis
Borrelia	*B. recurrentis*	Relapsing fever
	B. vincentii	Vincent's angina
	B. burgdorferi	Lyme disease
Leptospira	*L. interrogans*	Leptospirosis
	L. biflexa	Saprophytes

II. TREPONEMA

Treponemes (*trepos*, meaning turn and *nema*, meaning thread) are slender spirochaetes with fine spirals having pointed ends. Some of them are pathogenic for man, while others occur as commensals in mouth and genitalia. The genus *Treponema* contains three pathogenic species.

Pathogenic treponemes

1. *T. pallidum*–venereal syphilis
2. *T. pertenue*–yaws
3. *T. carateum*–pinta
4. *T. endemicum*–endemic syphilis

These pathogenic treponemes are almost identical in their morphology, antigenic structure and other features. It has been suggested that the pathogenic treponemes represent only evolutionary variations of a single species. The species *T. pallidum* is now considered to include three subspecies—*pallidum*, *pertenue* and *endemicum*.

A. Treponema Pallidum

Treponema pallidum is the causative agent of syphilis. The name pallidum refers to its pale staining.

1. Morphology

It is a thin, delicate spirochaete with tapering ends, having about ten regular spirals. It is about 10 μm long (range 4–14 μm) and 0.1–0.2 μm wide. It is actively motile, showing rotation round the long axis, backward and forward movements and flexion of the whole body. During motility, secondary curves appear and disappear in succession, but the primary spirals are unchanged.

It does not take ordinary bacterial stains and cannot be seen under the light microscope in wet films. Because of thinness of the spirals, *T. pallidum* cannot be seen by light microscope. However, it can be made out by negative staining with India ink. Its morphology and motility can also be seen by dark ground microscopy or phase contrast microscopy. It can be stained with silver impregnation methods (*Fontana's* method is useful for staining films and *Levaditi's* for tissue sections). The treponemes reduce silver nitrate to metallic silver that is deposited on the surface enlarging the diameter of the organism.

The cytoplasm of *T. pallidum* is surrounded by a trilaminar cytoplasmic membrane, enclosed by a cell wall containing peptidoglycan. External to this is outer membrane layer which is rich in lipid. Three or four endoflagella are situated between outer membrane layer and the inner peptidoglycan layer of the cell. They are attached at each pole of the cell. They wind round the axis of the cell. Unlike the flagella of other bacteria, these endoflagella do not protrude outside the cell, but remain within the outer membrane layer.

2. Culture

Pathogenic treponemes cannot be grown in artificial culture media but are maintained by subculture in susceptible animals. Nichol's strain has been maintained in rabbit testes for several decades by serial testicular passage since its first isolation in 1912 from a case of general paralysis of the insane. The Nichol's strain is a virulent *T. pallidum* strain.

Cultivable treponemes such as *T. phagedenis* (Reiter's treponeme) and *T. refringens* (Noguchi strain) are non-pathogenic. They can be grown under strict anaerobic conditions.

3. Antigenic Structure

Treponemal antigens are poorly understood. Treponemal infections lead to production of at least three types of antibodies. On the basis of these antibodies, the treponemal antigens may be divided into specific and non-specific antigens.

(i) Specific antigens

(*a*) *Group specific antigen*: All pathogenic and non-pathogenic treponemes possess a common group antigen. Antibodies to this antigen appear in the sera of syphilitic patients. This antibody is detected by the antigen derived from the Reiter treponeme.

(*b*) *Species specific treponemal antigen*: It appears to be

Chapter 45

SPIROCHAETES

I. SPIROCHAETES

Spirochaetes (*speira*, meaning coil; and *chaite*, meaning hair) are large, motile, helical bacteria which belong to order Spirochaetales. It has two families:

1. *Spirochaetaceae*: Spirochaetes are anaerobic, facultative anaerobic or microaerophilic. They are not hooked.
2. *Leptospiraceae*: These spirochaetes are obligate aerobes and are hooked.

Family *Spirochaetaceae* has four genera:

(*i*) *Spirochaeta* (*ii*) *Christispira*
(*iii*) *Treponema* (*iv*) *Borrelia*

Family *Leptospiraceae* has three genera:

(*i*) *Leptospira*
(*ii*) *Leptonema*
(*iii*) *Turneriella*

Members of the genus *Spirochaeta* are free living organisms found in water and sewage, whereas *Christispira* are found in fresh water molluscs. The members of genus *Treponema*, *Borrelia* and *Leptospira* are pathogenic to man.

A. Morphology

The spirochaetes have Gram negative type cell wall composed of an outer membrane, a peptidoglycan layer and a cytoplasmic membrane. They are structurally more complex than other bacteria.

A characteristic feature is the presence of varying number of endoflagella. These endoflagella are situated between the outer membrane and the inner peptidoglycan layer of the cell. They are attached subterminally at each pole of the cell. The spiral shape and the motility of the spirochaetes depend on integrity of these endoflagella. Motility is of three types:

(*i*) flexion and extension of the cells
(*ii*) corkscrew-like rotatory movement about the long axis
(*iii*) translatory motion i.e. from one site to another

5. The most infectious type of leprosy is:
   (a) Lepromatous leprosy
   (b) Tuberculoid leprosy
   (c) Borderline-tuberculoid leprosy
   (d) None of the above
6. Lepromatous leprosy is observed in patients with:
   (a) Deficient cell mediated immunity
   (b) Adequate cell mediated immunity
   (c) Adequate humoral immunity
   (d) None of the above
7. Tuberculoid leprosy is observed in patients with:
   (a) Deficient cell mediated immunity
   (b) Adequate cell mediated immunity
   (c) Deficient humoral immunity
   (d) None of the above
8. Which type of hypersensitivity reaction is involved in lepromin test?
   (a) Type I
   (b) Type II
   (c) Type III
   (d) Type IV
9. Fernandez reaction in lepromin test develops in:
   (a) 1–2 hours
   (b) 3–6 hours
   (c) 8–12 hours
   (d) 24–48 hours
10. Lepromin test is negative in which type of leprosy?
   (a) Tuberculoid leprosy
   (b) Lepromatous leprosy
   (c) Both of the above
   (d) None of the above
11. Numerous acid-fast bacilli are seen in leprosy lesions in:
   (a) Tuberculoid leprosy
   (b) Lepromatous leprosy
   (c) Both of the above
   (d) None of the above

Answers (MCQs):

1. (b) 2. (b) 3. (c) 4. (d) 5. (a) 6. (a)
7. (b) 8. (d) 9. (d) 10. (b) 11. (b)

KEY POINTS

1. *Mycobacterium leprae* is a slender, slightly curved or straight bacillus. It is acid-fast, but less so than the tubercle bacillus and for which 5% sulphuric acid is used for decolourisation after staining with carbol fuchsin.
2. These bacilli are seen singly and in groups, intracellularly or lying free outside the cells. Inside the cells they are present as bundles of organisms bound together by a lipid-like substance, the glia. These masses are known as *globi*. The parallel rows of bacilli in the globi give appearance of a *cigar bundle*.
3. *M. leprae* has not yet been grown on artificial culture media or tissue culture. However, they can multiply in *foot pads of mice* and *nine-banded armadillos*.
4. *M. leprae* produces leprosy in humans and only source of infection is patient. Four types of leprosy are recognised—*lepromatous*, *tuberculoid*, *dimorphous* and *indeterminate*.
5. Bacilli are numerous in lepromatous leprosy but scanty in tuberculoid leprosy.
6. Lepromin test is a delayed type of hypersensitivity reaction. It may be used to *classify leprosy*, *assessment of prognosis* and to *assess the resistance* of an individual to leprosy.
7. Laboratory diagnosis depends on demonstration of bacilli in specimens by microscopy. The specimens from skin are obtained by *slit* and *scrape* method.
8. *Bacteriological index* (*BI*) is defined as number of total bacilli in a tissue.
9. *Morphological index* (*MI*) is defined as the percentage of live bacilli out of the total number of bacilli.
10. *Rifampicin*, *dapsone* and *clofazimine* are the drugs used in treatment of leprosy.

YOU MUST KNOW

1. Morphology of *Mycobacterium leprae*.
2. Animals used for experimental infection with *M. leprae*.
3. Different types of leprosy.
4. Differences between lepromatous leprosy and tuberculoid leprosy.
5. Ridley and Jopling's classification.
6. Lepromin test.
7. Laboratory diagnosis of leprosy.

STUDY QUESTIONS

Q. Write short notes on:
(a) Pathogenesis of leprosy
(b) Lepromin test
(c) Laboratory diagnosis of leprosy
(d) *Mycobacterium lepraemurium.*

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Who discovered *Mycobacterium leprae*?
(a) Robert Koch (b) Hansen (c) Kitasato (d) Louis Pasteur
2. What is the concentration of sulphuric acid used as decolouriser in Ziehl-Neelsen staining method for *Mycobacterium leprae*?
(a) 20% (b) 5% (c) 1% (d) 0.1%
3. What is the generation time of *Mycobacterium* leprae?
(a) 20 minutes (b) 20 hours (c) 12–13 days (d) 20 weeks
4. Which of the following animals have been used for cultivation of *Mycobacterium leprae*?
(a) Mice
(b) Nine-banded armadillos
(c) Slender loris
(d) All of the above

4. Animal Inoculation

Injection of ground tissue from lepromatous nodules or nasal scrapings from leprosy patient into the foot pad of mouse produces typical granuloma at the site of inoculation within 6 months. Nine-banded armadillo is another animal used for inoculation of material. The lesions which develop in these animals can be identified by histological examination and Ziehl-Neelsen staining. Animal experiment is useful to confirm dubious diagnosis and to test drug resistance.

5. Lepromin Test

It is not a diagnostic test but is used to assess the resistance of patient to *M. leprae* infection. The test can be used to assess the prognosis and response to treatment.

6. Serological Test

Serodiagnosis of leprosy may be carried out by detection of anti-phenolic glycolipid-1 (anti PGL-1) antibodies. Various serological tests like latex agglutination, *Mycobacterium leprae* particle agglutination (MLPA) and ELISA have been described. Anti PGL-1 antibody titres are higher in lepromatous patients but the tubercoloid patients show low titres. The antibody titres decrease following effective chemotherapy.

K. Treatment

1. Chemotherapy

Dapsone (4, 4'–diaminodiphenyl sulphone, DDS) monotherapy was the standard treatment for all types of leprosy for many years. Due to emergence of dapsone resistance, WHO recommended multiple drug therapy (MDT) for all leprosy cases. Patients with paucibacillary (TT, BT) leprosy are given rifampicin 600 mg once a month (supervised) and dapsone 100 mg daily (unsupervised) for six months. For multibacillary (BB, BL, LL) leprosy, rifampicin 600 mg once a month (supervised), dapsone 100 mg daily (unsupervised), clofazimine 300 mg once a month (supervised), and 50 mg clofazimine daily (unsupervised) are given for one year.

2. Immunotherapy

Since lepromatous patients are deficient in CMI, efforts are being made to induce effective CMI in these patients. It has been tried by following procedures:

(*i*) Intravenous injection of transfer factor, an extract of lymphocytes, from patients suffering from tuberculoid leprosy are tried. Sensitised lymphocytes secrete certain substances named lymphokines which stimulate the macrophages to ingest and kill the bacilli.

(*ii*) Administration of vaccines

L. Prevention

It includes

1. early diagnosis and treatment
2. surveillance of contacts
3. health education
4. vaccination

Long-term chemoprophylaxis has given encouraging results in child contacts of infectious leprosy cases.

At present no effective vaccine against leprosy exists but a number of vaccines have been tried. However, none of these has reached the stage of universal use. The list of various candidate vaccines tried is as follows:

(*i*) BCG
(*ii*) Armadillo-derived killed *M. leprae*
(*iii*) Combination of BCG and killed *M. leprae*
(*iv*) ICRC bacillus
(*v*) Mycobacterium 'w'

Possible second generation vaccines are natural or recombinant form of 18, 31, 65, and 70 kDa proteins.

BCG vaccine appears to give some protection against leprosy for countries where BCG has been used in prophylaxis against tuberculosis. Field trials of BCG in Burma, India, New Guinea and Uganda showed variable protection ranging from 20–80%.

Field trials in Venezuela with BCG and killed *M. leprae* provided no better protection than BCG alone. Armadillo derived vaccine protects animals against experimental infection. ICRC vaccine contains gamma radiation inactivated ICRC bacilli. With this vaccine, conversion of lepromin negative to lepromin positive was observed in majority of leprosy patients.

Mycobacterium 'w' is a fast growing, non-pathogenic, soil mycobacterium which cross- reacts with *M. leprae* antigens. High rate of lepromin conversion has been reported. Lepromin conversion from lepromin negative to lepromin positive is taken as a criterion to find out protective efficacy of vaccines.

II. MYCOBACTERIUM LEPRAEMURIUM

It is causative agent of rat leprosy, 3 to 5 μm long, curved bacillus. It is acid-fast, showing granular staining. It is Gram positive. Rat leprosy is characterised by subcutaneous indurations, lymphadenopathy, ulcerations and loss of hair. The organism has been grown in chorioallantoic membrane of fertile hen's egg and in tissue culture of rat fibroblast origin. Growth appears in 8 to 12 days. *M. leprae* and *M. lepraemurium* are not closely related by DNA studies.

dry and removing the lipids by washing with ether. It is made up in phenol saline for use.

Depot lepromin is a modification where the antigen is injected in oily media.

1. Procedure

The lepromin test is carried out by the intradermal injection of 0.1 ml of lepromin. The response to lepromin is typically biphasic consisting of early reaction of *Fernandez* and late reaction of *Mitsuda*.

(*i*) *Early reaction of Fernandez*: It consists of erythema and induration developing in 24–48 hours and usually remains for 3–5 days. Histologically, the lesion consists of serous exudate with lymphocytic infiltration.

(*ii*) *Late reaction of Mitsuda*: It appears 1–2 weeks after the injection, reaching a peak in four weeks. The reaction appears in the form of a nodule that may ulcerate. It takes few weeks to heal. Histologically, there is infiltration with lymphocytes, epitheloid cells and giant cells. Positive Mitsuda reaction indicates resistance to leprosy.

2. Uses

(*i*) *Classification of leprosy:* The test is positive in tuberculoid leprosy and negative in lepromatous leprosy.

(*ii*) *Assessment of prognosis:* A positive lepromin test indicates a good prognosis and a negative one a bad prognosis. Conversion of negative reaction to positive reaction is the evidence of improvement.

(*iii*) *Assessment of resistance:* It is done to assess the resistance of an individual to leprosy. Resistance is indicated by positive lepromin test. All field workers should be lepromin positive.

J. Laboratory Diagnosis

In lepromatous cases, bacilli are always found in large numbers but in tuberculoid cases, the bacilli are very few and found with great difficulty, or not at all.

1. Specimens

Specimens are collected from the nasal mucosa, skin lesions and ear lobules. The specimens from skin are obtained by *slit and scrape* method. Samples from skin should be obtained from the edges of the lesion. The skin is cleaned with spirit in order to remove any saprophytic acid-fast bacilli that may be present on the skin surface. The skin is pinched up tight to minimise bleeding. A cut about 5 mm long is made with a scalpel, deep enough to get into the infiltrated layers. After wiping off blood or lymph that may have exuded, the blade of the scalpel is then turned at right angle to the cut (slit). The bottom and the sides of the slit are scraped with the point of the blade so as to obtain a little tissue pulp which is smeared uniformly on a slide. About six different areas should be sampled, including the skin over the buttocks, chin, cheek, forehead and ears.

Smears from the nose are made by scraping a little material from the nasal septum with a small blade knife.

Skin biopsy is collected from active edges of the patches and nerve biopsy from thickened nerves. Histological examination of skin biopsy is useful in the diagnosis and accurate classification of leprosy lesion.

2. Acid-Fast Staining

Slit-skin smears from skin patches and ear lobes and nasal mucosal scrapings are stained by Ziehl-Neelsen method using 5% sulphuric acid as decolourising agent. Acid-fast bacilli (AFB) arranged in parallel bundles within macrophages (*Lepra-cell*) confirm the diagnosis of lepromatous leprosy. The viable bacilli stain uniformly and the dead bacilli are fragmented, irregular or granular.

The smears are graded, based on the number of bacilli as follows:

1–10 bacilli in 100 fields	=	1+
1–10 bacilli in 10 fields	=	2+
1–10 bacilli per field	=	3+
10–100 bacilli per field	=	4+
100–1,000 bacilli per field	=	5+
More than 1,000 bacilli, clumps and globi in every field	=	6+

(i) Bacteriological index (BI)

It is defined as number of total bacilli in a tissue. The bacteriological index is calculated by totalling the grades (number of pluses, +s scored in all the smears and divided by number of smears. Thus if seven smears examined have a total of fourteen pluses (14+), BI will be 2. For calculating BI, a minimum of four skin lesions, a nasal swab and both the ear lobes are to be examined.

(ii) Morphological index (MI)

It is defined as the percentage of uniformaly stained bacilli out of the total number of bacilli counted. It provides a method for assessing the progress of patients on chemotherapy.

3. Skin and Nerve Biopsy

These are required for histological confirmation of tuberculoid leprosy when acid-fast bacilli cannot be demonstrated in direct smear. Skin biopsy is also useful in the diagnosis and accurate classification of leprosy lesion.

(*ii*) Type 2 (Erythema nodosum leprosum, ENL).

Type 1 (Reversal reaction): This occurs in borderline leprosy patients who develop cell-mediated immunity and shift towards tuberculoid part of the spectrum. It may lead to permanent nerve damage. Type 1 reaction is due to delayed hypersensitivity reaction.

Type 2 (Erythema nodosum leprosum, ENL): This is generally seen in lepromatous leprosy patients undergoing chemotherapy. It is due to formation of antigen-antibody complexes. Mycobacterial antigens are released into the blood when the bacilli die during chemotherapy. The combination of these antigens with antibodies present in the serum forms the basis of ENL. Clinically crops of red nodules appear in the skin, lasting for one or two days.

F. Ridley and Jopling's Classification

On the basis of clinical, histopathological and immunological findings, *Ridley* and *Jopling* have introduced a scale for classifying the spectrum of leprosy into five groups. These are Tuberculoid (TT), Borderline tuberculoid (BT), Borderline (BB), Borderline lepromatous (BL) and Lepromatous (LL) (Table 44.2).

According to WHO, leprosy is divided into two groups, *paucibacillary* and *multibacillary* leprosy. This classification depends upon number of skin lesion and involvement of nerves. When there are ≤ 5 skin lesions and no/one nerve trunk involvement, it is called paucibacillary leprosy. In case of multibacillary leprosy, the patient has 6 or more skin lesions and more than one nerve trunk involvement. Paucibacillary includes all cases of tuberculoid types and some cases of borderline types and multibacillary includes all cases of lepromatous types and some cases of borderline type.

G. Immunity

There seems to exist a high degree of innate immunity in man, therefore, only a minority of the contacts develop clinical disease. Infection with *M. leprae* induces both humoral and cell mediated immunity (CMI). The type of leprosy in an individual is determined by the status of CMI. Patients with deficient CMI develop lepromatous type of disease and when CMI is adequate, the lesions are of tuberculoid type. Humoral antibodies do not have the major role, while cellular immune mechanisms are capable of destroying the bacilli. The patient exhibits delayed hypersensitivity to the lepra bacillus protein. The macrophages phagocytose the bacilli and destroy them.

H. Epidemiology

Leprosy is an exclusively human disease and the only source of infection is the patient. Nasal secretions and discharges from superficial lesions are the most likely infectious material. The mode of entry may be either through the respiratory tract or through the skin. Leprosy is not highly communicable. The disease develops in only about five per cent of the contacts. The incubation period is very long and averages 2–5 years. It may vary up to 30 years.

Leprosy is now confined mainly to the underdeveloped areas of the tropics and the southern hemisphere. India has the maximum prevalence, with about a third of the total world cases. Leprosy is present in all states and territories of India. Orissa and Bihar have the highest prevalence.

I. Lepromin Test

This reaction was first described by *Mitsuda* in 1919. The original antigen (lepromin) was a boiled, emulsified, lepromatous tissue rich in lepra bacilli. Nowadays, the lepromins used as antigens may be of human origin (lepromin-H) or armadillo derived (lepromin-A) and are of two types:

(*i*) Integral lepromin: Mitsuda's crude antigen is called the integral antigen. It is now increasingly being prepared from armadillo-derived *M. leprae* (lepromin-A).

(*ii*) Bacillary lepromin: This contains more of bacillary components with less of tissue. An antigen commonly employed is *Dharmendra antigen* which is prepared by floating out the bacilli from finely ground lepromatous tissue with chloroform, evaporating it

Table 44.2 Characteristics of Five Groups of Leprosy

Character	Tuberculoid (TT)	Borderline-tuberculoid (BT)	Borderline (BB)	Borderline lepromatous (BL)	Lepromatous (LL)
Bacilli in the skin	–	+/–	+	++	+++
Bacilli in nasal secretions	–	–	–	+	+++
Granuloma formation	+++	++	+	–	–
Lepromin test	+++	+	+/–	–	–
Antibodies to M. leprae	+/–	+/–	+	++	+++

immunity. Patients, infected with *M. leprae*, develop antibodies against PGL-1. The latter has been used for the serodiagnosis of leprosy.

In addition, *M. leprae* possesses a large number of protein antigens namely 18 kDa, 28 kDa, 35 kDa, 36 kDa, 65 kDa and so on, according to molecular weight of antigens in kilodaltons (kDa).

D. Resistance

Lepra bacilli can survive in warm humid environment for 9–16 days and in moist soil for 46 days. They survive exposure to ultraviolet light for 30 minutes and to direct sunlight for two hours.

E. Pathogenesis

Leprosy is a chronic granulomatous disease of humans and the only source of infection is patient. The bacilli localise primarily in the skin, peripheral nerves and nasal mucosa but any tissue or organ may be involved. Due to preference of bacilli for lower temperatures, the superficial and cooler tissues are affected.

Incubation period is very long and variable. Sources of infection are nasal discharge and skin lesions of patients. Prolonged close contact with patients is necessary for transmission of the disease. Four types of leprosy are recognised—*lepromatous, tuberculoid, dimorphous* and *indeterminate*. The type of disease is related to the immune status of the host. The lepromatous leprosy is seen when the cell mediated immunity (CMI) is either absent or deficient while patients with a high degree of CMI develop *tuberculoid* form. The *lepromatous* and *tuberculoid* types of leprosy are two extreme forms. The *indeterminate* type occupies a position in between these two forms. The type of leprosy may change with chemotherapy and alteration in immune status of the host.

1. Lepromatous Type

It is a generalised form of the disease and found in individuals with low resistance. This form is severe and prognosis is poor. It is more infectious than other types. Lepromin test is negative due to deficient cell mediated immunity. Humoral antibodies are produced in high concentration which play no protective role. Autoantibodies are also produced.

Patient develops nodular skin lesions (lepromata) on face, ear lobes, hands, feet and less commonly on trunk. There is slow and symmetric thickening of peripheral nerves and anaesthesia. As a result of loss of sensation, nodular skin lesions ulcerate with repeated trauma. The ulcerated nodules become secondarily infected which leads to distortion and mutilation of extremities. Skin lesions contain many macrophages packed with bacilli. Lepra bacilli are present in large number in skin lesions and in mucosa of nose, mouth and upper respiratory tract. These bacilli are shed in sweat, nasal and oral secretions. Continuous bacteraemia is commonly seen.

2. Tuberculoid Type

This is a localised form of the disease. It is found in patients with high degree of resistance where cell mediated immunity is intact. The skin lesions are few and consist of non-elevated hypo or hyperpigmented macular patches involving the face, limbs and trunk. The local peripheral nerves are involved in the early stage and gradually extends into bigger nerve trunks which become thickened, hard and tender. This may lead to deformities of hand and feet.

Bacilli are very few or absent in the tissues. Lepromin test is positive due to intact CMI. Antimycobacterial antibodies and autoantibodies are rarely produced. The diagnosis depends mainly on the basis of clinical and histological findings. The differentiating features of lepromatous and tuberculoid leprosy are shown in Table 44.1.

Table 44.1 Differentiating Features of Lepromatous and Tuberculoid Leprosy

Feature	Lepromatous leprosy	Tuberculoid leprosy
Lepra bacilli in lesions	Numerous	Scanty
Cell mediated immunity	Deficient/absent	Adequate
Lepromin test	Negative	Positive
Infectivity	Highly infectious	Usually non-infective
Mycobacterial antibodies	Abundant	Rarely produced

3. Dimorphous Type

The lesions clinically resemble tuberculoid leprosy but bacteriological and immunological picture is that of lepromatous type. This form may shift to any of the two extreme types depending on chemotherapy and alterations in immune status of the host.

4. Indeterminate Type

There is an early unstable tissue reaction with mild transient tissue lesion, often resembling maculo-anaesthetic patches. This is neither characteristic of tuberculoid nor lepromatous type. The lesion heals spontaneously or may progress to tuberculoid or lepromatous type.

Immune reactions in leprosy may cause additional tissue damage. These are of two types:

(*i*) Type 1 (Reversal reaction)

are arranged in clumps resembling cigarette ends. The globi are present in Virchow's *lepra cells* or *foamy cells* which are large undifferentiated histiocytes.

B. Cultivation

Lepra bacilli have not yet been grown on artificial culture media or tissue culture. There have been several reports of cultivation, but none has been confirmed. One of the best known of such reports came from the Indian Cancer Research Centre (ICRC), Bombay (1962), where an acid-fast bacillus was isolated from leprosy patients, employing human foetal spinal ganglion cell culture. This is named as ICRC bacillus. It has been adapted for growth on Lowenstein-Jensen medium but its relation to the lepra bacillus is uncertain.

There have been many attempts to transmit leprosy to different experimental animals. The first breakthrough was achieved by *Shepard* (1960) when he showed that lepra bacilli could multiply in the foot pads of mice. Following animals have been used for experimental infection with *M. leprae.*

1. Mouse (foot-pad)
2. Nine-banded armadillos
3. Chimpanzees,
4. Monkeys
5. Slender loris
6. Indian pangolin
7. Chipmunks
8. Golden hamsters
9. European hedgehog.

Among these animals, the most important are mouse and nine-banded armadillos. The generation time of *M. leprae*, from animal experiments, is found to be 12–13 days on the average.

1. Mouse

When ground tissue from lepromatous leprosy containing lepra bacilli are inoculated intradermally into the foot-pad of mouse and kept at a low temperature (20°C), a granuloma develops at the site in 1–6 months. When the cell mediated immunity of animal is suppressed by thymectomy or by administration of antilymphocytic serum, a generalised infection is produced, simulating lepromatous leprosy. Foot-pad of mouse can also be used for:

(*i*) susceptibility of organisms to chemo-therapeutic agents.
(*ii*) determining viability of *M. leprae*.
(*iii*) study of efficacy of various vaccines.

The disadvantages of the foot-pad of mouse are:

(*i*) There occurs only a limited multiplication of *M. leprae* (1×10^6 bacilli per foot pad), therefore, the yield is not sufficient for comprehensive research of *M. leprae*.
(*ii*) Due to short life span of mice and long incubation period of leprosy, it is not possible to study the pathogenesis of the disease.

2. Armadillo

The nine-banded armadillo (*Dasypus novemcinctus)* is highly susceptible to infection with *M. leprae*. Following inoculation with lepra bacilli, armadillo develops a generalised infection with extensive multiplication of the bacilli and the lesions produced resemble lepromatous leprosy. Natural infection by a mycobacterium resembling *M. leprae* has been observed in some wild armadillos, therefore, these animals should be screened for mycobacterial infection and kept in quarantine for three months before inoculation. The advantages of armadillo are:

(*i*) long life span (12–15 years).
(*ii*) low body temperature (32–35°C).
(*iii*) the yield of *M. leprae* from armadillo skin leproma is 100–1,000 times more than that of human leproma.
(*iv*) abundant source of *M. leprae*, for research and the preparation of lepromin or of a vaccine.

Disadvantages of using armadillo as an experimental model are:

(*i*) only about 40% of animals are susceptible to disseminated form of leprosy.
(*ii*) cost is very high due to availability of these animals only in limited parts of the world i.e. southern parts of USA.
(*iii*) some wild caught armadillos are naturally infected with mycobacteria resembling *M. leprae*.

3. Other Animals

Chimpanzees and mangabey monkeys of West Africa are found to suffer from natural infection with leprosy, but it is not yet known whether this has got any bearing with human infection.

C. Antigenic Structure

The cell wall of *M. leprae* is made up of four layers. The innermost is a peptidoglycan layer which gives the cell its shape and rigidity. External to this is lipoarabinomanan-B (LAM-B) layer, attached to which is mycolic acid. The outermost layer is composed of mycosides. A major component of this layer is phenolic glycolipid-1 (PGL-1).

LAM-B is highly immunogenic and is used in the serodiagnosis of leprosy. PGL-1 protects pathogens against host cell enzymes and suppresses cell mediated

Chapter 44

MYCOBACTERIUM LEPRAE

Leprosy is a chronic mycobacterial disease of ancient world and is still afflicting patients in many parts of world–mainly Asia and Africa. Leprosy was described as *Kushta* in Sushta Samhita written in 600 BC in India. It is caused by *Mycobacterium leprae* first observed by *Hansen* in 1868. Though it was the first bacterial pathogen of human to be described, it still remains one of the least understood. This is because it has not been possible to grow the bacillus in artificial culture media.

I. MYCOBACTERIUM LEPRAE

A. Morphology

M. leprae is a slender, slightly curved or straight bacillus, 1–8 μm × 0.2 – 0.5 μm and shows considerable morphological variations. It is acid-fast, but less so than the tubercle bacillus and for which 5% sulphuric acid is used for decolourisation after staining with carbol fuchsin (Fig.44.1). It is Gram positive and stains more readily. It is possible to differentiate live and dead bacilli in smears stained with Ziehl-Neelsen method. The live bacilli stain uniformly and appear solid, whereas the dead bacilli are fragmented and granular. This helps to monitor the efficacy of treatment in leprosy patients.

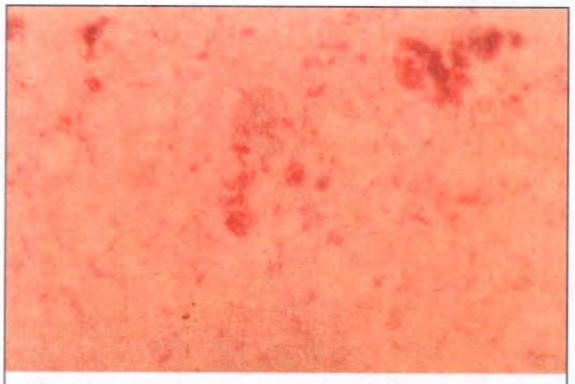


Fig. 44.1 Mycobacterium leprae stained by Ziehl-Neelsen method

The bacilli are seen singly and in groups, inracellularly or lying free outside the cells. Inside the cells they are present as bundles of organisms bound together by a lipid-like substance, the glia. These masses are known as *globi*. The parallel rows of bacilli in the globi give appearance of a *cigar bundle*. In tissue sections, the bacilli

2. Write short notes on:
   (a) Photochromogens
   (b) Scotochromogens
   (c) Mycobacterium avium-intracellulare complex
   (d) *Mycobacterium ulcerans*
   (e) Differentiation of non-tuberculous mycobacteria from tubercle bacilli.
   (f) Swimming pool granuloma.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Non-tuberculous mycobacteria can be differentiated from *M. tuberculosis* on Lowenstein-Jensen medium by:
   (a) its growth in the presence of p-nitrobenzoic acid
   (b) no growth in the presence of p-nitrobenzoic acid
   (c) Both of the above
   (d) None of the above
2. Which of the following bacteria is photochromogen?
   (a) *Mycobacterium kansasii* (b) *M. scrofulaceum*
   (c) *M. gordonae* (d) *M. szulgai*
4. Which of the following bacteria is non-photochromogen?
   (a) *Mycobacterium kansasii* (b) *M. scrofulaceum*
   (c) *M. gordonae* (d) *M. ulcerans*
5. Which of the following mycobacteria is/are saprophytic?
   (a) *Mycobacterium smegmatis* (b) *M. butyricum*
   (c) *M. phlei* (d) All of the above
6. Which of the following bacteria can cause swimming pool granuloma?
   (a) *Mycobacterium marinum* (b) *M. fortuitum*
   (c) *M. ulcerans* (d) *M. avium*
7. Which is the causative agent of buruli ulcer?
   (a) *Mycobacterium marinum* (b) *M. fortuitum*
   (c) *M. ulcerans* (d) *M. avium*
8. Which of the following mycobacteria can cause pulmonary disease?
   (a) *Mycobacterium tuberculosis* (b) *M. avium-intracellular*
   (c) *M. kansasii* (d) All of the above

Answers (MCQs):

1. (a)	2. (a)	3. (a)	4. (d)	5. (d)
6. (a)	7. (c)	8. (d)		

Table 43.4 Differentiation Between Tubercle Bacilli and Different Species of Non-Tuberculous Mycobacteria

Test	M. tuberculosis	M. bovis	M. kansasii	M. marinum	M. scrofulaceum	M. aviumintra- cellular complex	M. fortuitum	M. chelonei	M. phlei	M smegmatis
Growth within 7 days	–	–	–	–	–	–	+	+	+	+
Growth at 25°C	–	–	+	+	+	±	+	+	+	+
Growth at 45°C	–	–	–	–	–	±	–	–	+	+
Growth at 37°C	+	+	+	–	+	+	+	+	+	+
Pigment in dark	–	–	–	–	+	–	–	–	+	–
Pigment in light	–	–	+	+	+	–	–	–	+	–
Urease production	+	+	+	+	+	–	+	+	+	+
Niacin production	+	–	–	–	–	–	–	–	–	–
Nitrate reduction	+	–	+	–	–	–	+	–	+	+

KEY POINTS

1. Mycobacteria other than tubercle bacilli, that may occasionally cause human disease resembling tuberculosis, have been called *non-tuberculous mycobacteria* (*NTM*). They are also named as *atypical mycobacteria*, *opportunistic mycobacteria* or *mycobacteria other than tubercle bacilli* (MOTT).
2. They normally exist as saprophytes of soil and water. They occasionally cause opportunistic disease in man.
3. *Runyon* classified non-tuberculous mycobacteria into four groups. These include Group I—*Photochromogens*, Group II—*Scotochromogens*, Group III—*Non-Photochromogens* and Group IV—*Rapid growers*.
4. NTM grow in Lowenstein-Jensen (LJ) medium. They also grow in the presence of p-nitrobenzoic acid (PNB) 500 µg/ml. This feature differentiates NTM from *M. tuberculosis* which do not grow in the presence of PNB.
5. Photochromogens produce pigment when exposed to light. One example of photochromogens is *M. kansasii*. In contrast scotochromogens produce pigment in the dark. *M. scrofulaceum* is an example of scotochromogens. Non-photochromogens do not form pigment even on exposure to light. *M. avium* and *M. intracellulare* are non-photochromogens. Rapid growers may be photo, scoto or non-photochromogens. *M. chelonei* is an example of rapid growers which can grow rapidly (in four or five days) on culture medium.
6. Diseases caused by NTM include *lymphadenopathy*, *pulmonary disease*, *buruli ulcer*, *swimming pool granuloma*, post-injection abscesses and disseminated diseases (AIDS related and Non-AIDS related)

YOU MUST KNOW

1. Classification of non-tuberculous mycobacteria.
2. Examples of different groups of non-tuberculous mycobacteria.
3. Diseases caused by non-tuberculous mycobacteria.

STUDY QUESTIONS

1. Classify non-tuberculous mycobacteria and name the diseases caused by these bacteria.

broadly divided into three groups: *localised lymphadenitis*, *pulmonary disease* and *skin lesions* (buruli ulcer, swimming pool granuloma). Disseminated disease may occur in immunocompromised individuals especially in AIDS (Table 43.3).

Table 43.3 Diseases Caused by Non-Tuberculous Mycobacteria

Disease	Causative agent
Lymphadenopathy	*M. scrofulaceum* *M. avium-intracellulare* *M. szulgai*
Pulmonary disease	*M. avium-intracellulare* *M. kansasii* *M. xenopi* *M. simiae*
Skin lesions	
Buruli ulcer	*M. ulcerans*
Swimming pool granuloma	*M. marinum*
Post-injection abscesses	*M. chelonei* *M. fortuitum*
Disseminated disease	
AIDS related	*M. avium-intracellulare*
Non-AIDS related	*M. avium-intracellulare* *M. chelonei*

A. Localised Cervical Lymphadenitis

It usually occurs in children under five years. Cervical lymphadenitis is usually a self limiting disease. In USA, *M. scrofulaceum* is the causative agent, while in Great Britain, *M. avium–intracellulare* and *M. scrofulaceum* are responsible.

B. Pulmonary Disease

These infections resemble tuberculosis. In most but not all cases, there is some predisposing lung disease. Many species are responsible but most frequent ones are *M. avium-intracellulare* and *M. kansasii*. Diagnosis is made bacteriologically. Organism must be isolated repeatedly from the sputum to differentiate true disease from transient colonisation.

C. Skin Lesions

1. Buruli Ulcer

It is caused by *M. ulcerans*. Leg is the most common site of lesion. In the early stage, a painful, itchy, hard subcutaneous nodule is formed. The nodule enlarges and the centre becomes soft due to necrosis of subcutaneous tissue. Later on, a deep ulcer is formed. The lesion contains innumerable number of acid-fast bacilli. With the onset of healing, acid-fast bacilli disappear, cellular infiltration and granuloma formation occurs. *M. ulcerans* produces a toxin.

2. Swimming Pool Granuloma

It is caused by *M. marinum*. The disease is also called fish tank granuloma. Organism is a natural pathogen of cold blooded animals like fish. Infection occurs by use of contaminated swimming pools or fish tanks. The lesion appears as papule which breaks down to form an ulcer. The disease is self-limiting. *M. marinum* is distributed in temperate area in contrast to *M. ulcerans*, which has a tropical prevalence. Use of antibiotics (minocycline or rifampicin) hastens its resolution. The causative agent was formerly known as *M. balnei* (*balneum*, meaning bath).

3. Post-Injection Abscesses

M. chelonei and *M. fortuitum* (rapid growers) cause abscesses following injection when injectable materials are contaminated with these bacteria. Abscesses are painful and lasts for many months.

VI. LABORATORY DIAGNOSIS

A. Specimens

Sputum, pus or exudate.

B. Microscopy

Ziehl-Neelsen staining of smear shows acid-fast bacilli (AFB). Repeated smear examination is necessary.

C. Culture

They grow well on LJ medium. Several LJ media should be inoculated with the specimen. These are incubated in the dark and in the light at different temperatures for distinguishing the species.

D. Biochemical Reactions

These are important to differentiate various species of non-tuberculous mycobacteria (Table 43.4).

VII. TREATMENT

Many non-tuberculous mycobacteria are resistant to most of the anti-tuberculous drugs *in vitro*, although good clinical responses are obtained by various combinations of these drugs.

M. ulcerans is a causative agent of *Buruli ulcer*. The name Buruli ulcer is derived from Buruli district of Uganda where a large outbreak of the disease was investigated. It grows slowly at very restricted temperature range, 31°C–34°C. It produces an exotoxin which may be involved in pathogenesis of the disease. Exotoxin has been characterised as phospholipoprotein–lipopolysaccharide complex.

M. malmoense was first isolated from patients from Malmo in Sweden. It is very slow growing. On primary isolation, colonies become visible only after 10–11 weeks of incubation. It causes pulmonary disease and lymphadenitis. It is resistant to rifampicin and isoniazid and sometimes to streptomycin and ethambutol.

D. Group IV Rapid Growers

These are capable of rapid growth, colonies appear within seven days on LJ medium. Within this group, they may be photochromogenic, scotochromogenic or non-photochromogenic. All the chromogenic rapid growers are saprophytes e.g. *M. smegmatis* and *M. phlei*. The medically important species are *M. fortuitum* and *M. chelonei*. Both these species can cause chronic abscesses in man. Outbreaks of abscesses following injection of vaccines contaminated by these mycobacteria have been reported. *M. fortuitum* occasionally causes pulmonary lesions which cannot be distinguished radiologically from typical tuberculosis.

M. fortuitum and *M. chelonei* do not produce any pigment. *M. chelonei* grows better at 25°C than 37°C. *M. fortuitum* can be differentiated from *M. chelonei* in reducing nitrate and forming red colonies on MacConkey agar. Both the species are found in soil and infection usually follows some injury. These are resistant to standard anti-tuberculous drugs.

SAPROPHYTIC MYCOBACTERIA

M. phlei and *M. gordonae* are saprophytes of soil, water and plants. *M. smegmatis* is normally present in smegma and it is a frequent contaminant of urine specimens. Sometimes these organisms may be confused with pathogenic mycobacteria. These are chromogenic rapid growers.

M. smegmatis is acid-fast but not alcohol-fast, therefore, they are not seen in the smear if acid-alcohol is used as a decolouriser in the staining procedure. *M. phlei* can be differentiated from *M. smegmatis* by its ability to grow at 52°C and survive at 60°C for four hours.

The general feature to differentiate non-tuberculous mycobacteria from *M.tuberculosis* are shown in Table 43.2.

IV. EPIDEMIOLOGY

The non-tuberculous mycobacteria are widely distributed in wet soil, streams and rivers. Staining reagents prepared by contaminated water may give false positive results. Because of widespread distribution of non-tuberculous mycobacteria, human beings are regularly exposed to them through contact with water and inhalation of aerosols. Repeated contact results in subclinical infections. Due to their low virulence, the incidence of overt disease is very low. However, there is an increase in incidence of these infections in countries where tuberculosis is on decline probably due to increasing number of immunocompromised individuals, notably patients with HIV infection.

V. CLINICAL MANIFESTATIONS

Although most of them are environmental saprophytes, some of these non-tuberculous mycobacteria occasionally cause disease in man and animals. These diseases are

Table 43.2 Differentiating Characteristics of Mycobacterium Tuberculosis and Non-Tuberculous Mycobacterium

Characteristic	Mycobacterium tuberculosis	Non-tuberculous mycobacteria
Growth characters		
Rate of growth	Slow	Slow or rapid
Temperature	37°C	25 – 45°C
Growth on LJ medium	Eugonic	Dysgonic
Colony characters	Dry, rough, tough, buff coloured, difficult to emulsify	Dry, yellow, orange or creamy and easily emulsifiable
Growth in the presence of p-nitrobenzoic acid (PNB) 500 µg/ml	–	+
Biochemical reactions		
Niacin test	+	–
Nitrate reduction test	+	–

Table 43.1 Classification of Non-Tuberculous Mycobacteria

Runyon group	Name	Species
I	Photochromogens	*M. kansasii*, *M. simiae*, *M. marinum*
II	Scotochromogens	*M. scrofulaceum*, *M. gordonae*, *M. szulgai*
III	Non-photochromogens	*M. avium*, *M. intracellulare*, *M. xenopi*, *M. ulcerans*, *M. malmoense*
IV	Rapid growers	*M. chelonei*, *M. fortuitum*

presence of p-nitrobenzoic acid (PNB) 500 µg/ml. This feature differentiates NTM from *M.tuberculosis* which do not grow. Colonies appear within two to three weeks except in case of rapid growers which produce colonies in four or five days. *Photochromogens* form colonies that produce no pigment in the dark, but when colonies are exposed to light for one hour in the presence of air, and reincubated for 24 to 48 hours, a yellow orange pigment appears. Pigment is chemically *beta-carotene*. The *scotochromogens* produce pigment in the dark. Non-photochromogens do not form pigment even on exposure to light. Rapid growers may be photo, scoto or non-photochromogens.

III. DIFFERENT GROUPS

A. Group I Photochromogens

They produce pigment on exposure to light as described earlier. They are slow growing though the growth is faster than that of tubercle bacilli. This group contains, *M. kansasii, M. marinum* and *M. simiae*.

M. kansasii causes chronic pulmonary disease resembling tuberculosis, particularly in old persons with pre-existing lung disease and with impaired immune responses. Man to man transmission is not known. The bacilli have been isolated from soil, water and milk. They grow well at 37°C on LJ medium and produce yellow-orange pigment. They are usually sensitive to rifampicin and other anti-tubercular drugs.

M. simiae was originally isolated from monkeys, and like *M. kansasii*, it grows well at 37°C and principally isolated from pulmonary lesions. It synthesises niacin in significant amount and may thus be confused as *M. tuberculosis*.

M. marinum grows poorly or not at all at 37°C but grows well at 33°C. It is the causative agent of superficial granulomatous skin disease of man known as *swimming pool granuloma* or *fish tank granuloma*. Disease is acquired through water. *M. marinum* resembles *M. kansasii* in many respects but can be differentiated from *M. kansasii* by

1. its poor or no growth at 37°C.
2. its failure to reduce nitrate to nitrite.
3. its failure to produce catalase.
4. its ability to hydrolyse pyrazinamide.

B. Group II Scotochromogens

These organisms produce pigment (yellow, orange or red) in cultures incubated even in the dark. They are saprophytes, found in the environment. Only one number of this group, *M. scrofulaceum* causes scrofula (cervical lymphadenitis) in children. *M. gordonae* is often found in water and is a rare cause of pulmonary disease. It is a common contaminant of clinical specimens. *M. szulgai* is a scotochromogen at 37°C and photochromogen at 25°C. It may occasionally cause pulmonary disease and bursitis.

C. Group III Non-photochromogens

Non-photochromogens do not form pigment even on exposure to light. The medically important species include *M. intracellulare, M. avium* and *M. xenopi*.

M. intracellulare is also known as *Battey bacillus* as it was first detected at the Battey State Hospital for tuberculosis in Georgia, U.S.A. It causes chronic pulmonary disease indistinguishable from tuberculosis. The organisms occur in soil and water. Rifampicin with streptomycin is used for treatment.

M. avium causes tuberculosis in fowls and sometimes in pigs. It may cause pulmonary disease in immunocompromised patients and cervical lymphadenitis in children. It is so much similar to *M. intracellulare* that they are usually grouped together as *M. avium intracellulare complex* (MAC). *M. avium* can grow at 45°C

M. avium-intracellulare complex (MAC) possesses 28 agglutination serotypes. Types 1, 2 and 3 are regarded as *M. avium* and types 4 to 28 are *M. intracellulare*. In man, this complex causes pulmonary disease indistinguishable from tuberculosis, lymphadenitis and disseminated disease, particularly in the AIDS patients.

M. xenopi was first isolated from Xenopus toads. Like *M. avium*, it is a thermophile and grows well at 45°C. It sometimes produces chronic lung disease in man. It has been isolated from hot water taps in hospital. *M. xenopi* has a limited geographical distribution and most cases of pulmonary lesions, due to *M. xenopi*, have been reported from London.

Chapter 43

NON-TUBERCULOUS MYCOBACTERIA

Mycobacteria other than tubercle bacilli, that may occasionally cause human disease resembling tuberculosis, have been called *atypical mycobacteria*. They are also named as *anonymous* or *unclassified mycobacteria*. They normally exist as saprophytes of soil and water. They occasionally cause opportunistic disease in man and hence are also named *opportunistic mycobacteria* or *non-tuberculous mycobacteria* (NTM) or mycobacteria other than tubercle bacilli (MOTT). The name 'non-tuberculous mycobacteria' (NTM) has been accepted widely in recent years. They are non-pathogenic for guinea pigs. There is no evidence of direct transmission from man to man.

Some of them cause pulmonary disease indistinguishable from tuberculosis, while others are encountered in association with lymphadenitis, abscesses, urinary infections and cutaneous lesions. The non-tuberculous mycobacteria are the most common cause of cervical lymphadenitis in children in Britain.

The infectivity of non-tuberculous mycobacteria is much less than that of mammalian tubercle bacilli. They are resistant to antituberculous drugs, particularly streptomycin and INH.

I. CLASSIFICATION

On the basis of pigment production and rate of growth, *Runyon* (1959) classified non-tuberculous mycobacteria into four groups (Table 43.1).

These include Group I–*Photochromogens*, Group II–*Scotochromogens*, Group III–*Non-photochromogens* and Group IV–*Rapid growers*. Runyon's classification is universally accepted. Species identification is made by several additional characteristics.

II. CULTURE

Non-tuberculous mycobacteria (NTM) grow in Lowenstein-Jensen (LJ) medium. They also grow in the

7. Niacin test is positive in:
   (a) *Mycobacterium tuberculosis* (b) *M. simiae*
   (c) *M. chelonei* (d) All of the above
8. *Myobacterium tuberculosis* infection is commonly acquired by:
   (a) Inhalation (b) Ingestion (c) Skin contact (d) Parenteral
9. Which immunity plays a major role in pathogenesis of *Mycobacterium tuberculosis*?
   (a) Cell mediated immunity (b) Humoral immunity
   (c) Local immunity (d) None of the above
10. A positive tuberculin test is indicated by induration of:
   (a) 10 mm or more in diameter (b) 5–9 mm in diameter
   (c) 2–4 mm in diameter (d) None of the above
11. Culture of *Mycobacterium tuberculosis* may be positive even if number of bacteria in the specimen is:
   (a) As few as 1–5 per ml (b) As few as 6–10 per ml
   (c) As few as 10–100 per ml (d) None of the above
12. Method/s employed for antibiotic sensitivity testing of *M. tuberculosis* include/s:
   (a) Resistance ratio method (b) Absolute concentration method
   (c) Proportion method (d) All of the above
13. Multidrug resistance tuberculosis (MDR-TB) is due to *M. tuberculosis* strain:
   (a) Resistant to rifampicin only (b) Resistant to isoniazid only
   (c) Resistant to at least rifampicin and isoniazid (d) None of the above
14. Extensively drug resistant tuberculosis (XDR-TB) is due to *M.tuberculosis* strains which are resistant to:
   (a) Any fluoroquinolone
   (b) Isoniazid and rifampicin
   (c) At least one of three injectable second line drugs (capreomycin, kanamycin and amikacin)
   (d) All of the above
15. BCG vaccine is:
   (a) Live attenuated vaaccine (b) Killed vaccine
   (c) Toxoid (d) None of the above

Answers (MCQs):

1. (d)	2. (d)	3. (b)	4. (a)	5. (b)
6. (a)	7. (d)	8. (a)	9. (a)	10. (a)
11. (c)	12. (d)	13. (c)	14. (d)	15. (a)

19. BCG vaccine is used for prophylaxis of tuberculosis. It is given intradermally. It is a live attenuated vaccine.
20. Diagnosis of tuberculosis under RNTCP is mainly based on smear examination.

YOU MUST KNOW

1. Classification of mycobacteria.
2. Morphology, culture characteristics and biochemical reactions of *Mycobacterium tuberculosis*.
3. Differences between *M. tuberculosis* and *M. bovis*.
4. Koch's phenomenon.
5. Tuberculin skin test.
6. Laboratory diagnosis of tuberculosis.
7. BCG vaccine.
8. Diagnosis of pulmonary tuberculosis under RNTCP

STUDY QUESTIONS

1. Classify Mycobacteria. Discuss the laboratory diagnosis of pulmonary tuberculosis.
2. Write briefly about:
   (a) Differences between *Mycobacterium tuberculosis* and *Mycobacterium bovis*.
   (b) Antigenic structure of *M. tuberculosis*
   (c) Koch's phenomenon
   (d) Tuberculin skin test
   (e) Sensitivity testing of *M. tuberculosis*
   (f) BCG vaccine.
   (g) RNTCP

MULTIPLE CHOICE QUESTIONS (MCQs)

1. *Mycobacterium tuberculosis* complex includes:
   (a) *Mycobacterium tuberculosis* (b) *M. bovis*
   (c) *M. microti* (d) All of the above
2. Which of the following bacteria can cause tuberculosis?
   (a) *Mycobacterium tuberculosis* (b) *M. bovis*
   (c) *M. africanum* (d) All of the above
3. Which of the following characteristics differentiate classical type of *M. tuberculosis* from South Indian type of *M. tuberculosis*?
   (a) Sensitivity to pyrazinamide
   (b) Resistance to Thiophen-2 carboxylic acid hydrazide (10 μg/ml)
   (c) Sensitivity to cycloserine
   (d) Niacin production
4. Eugonic growth on Lowenstein-Jensen medium is a characteristic of:
   (a) *Mycobacterium tuberculosis* (b) *M. bovis*
   (c) Both of the above (d) None of the above
5. Which of the following bacteria require/s microaerophilic environment for primary isolation?
   (a) *Mycobacterium tuberculosis* (b) *M. bovis*
   (c) Both of the above (d) None of the above
6. Liquid medium for isolation of *Mycobacterium tuberculosis* is:
   (a) Middle brook 7H9 (b) Middle brook 7H10
   (c) Middle brook 7H11 (d) Lownstein-Jensen medium

If both smears are negative, diagnosis is done on X-ray chest findings. In case of X-ray chest suggestive of tuberculosis, patient is diagnosed as sputum negative pulmonary tuberculosis. Antitubercular treatment is started. Patient is declared not suffering from tuberculosis when there is no finding suggestive of tuberculosis on X-ray chest.

DOTS treatment is given for tuberculosis as described earlier. However in MDR-TB cases, treatment is given with second line drugs under DOTS plus. There are five categories (category I to V) of patients under RNTCP, from newly diagnosed cases to MDR-TB and XDR-TB patients. MDR-TB patients are included in Category IV and XDR-TB patients in Category V. Details of RNTCP are beyond the scope of this book, therefore only diagnostic aspects are described. Diagnosis in the form of a flow chart is shown in Fig. 42.7.

KEY POINTS

1. Mycobacteria are slender bacilli that are difficult to stain, but once stained, resist decolourisation with dilute mineral acids and are therefore called *acid-fast bacilli* or *AFB*. *Lipids* of the cell wall particularly the *mycolic acid* fraction are responsible for the acid-fastness of bacteria. These organisms are aerobic, non-motile, non-capsulated and non-sporing. Growth is generally slow.
2. Ziehl-Neelsen (ZN) staining is useful to study the morphology of these organisms. With this stain, tubercle bacilli are seen bright *red* (acid-fast). Tubercle bacilli may also be stained with the fluorescent dyes (auramine O, rhodamine) and appear yellow luminous bacilli under the fluorescent microscope.
3. Lowenstein-Jensen (LJ) medium is the most commonly used medium to grow *M. tuberculosis*. This medium consists of beaten eggs, asparagine, mineral salts, malachite green and glycerol or sodium pyruvate. It is solidified by heating (*inspissation*). It is one of the media which are solid without incorporation of agar. In this medium egg acts as a solidifying agent.
4. On LJ medium colonies of *M. tuberculosis* are dry, rough, buff coloured, raised with a wrinkled surface. *M. tuberculosis* has a luxuriant growth (*eugonic growth*) as compared to sparsely grown (*dysgonic growth*) *M. bovis*. The tubercle bacilli usually grow in 2 to 8 weeks.
5. *Niacin test* and *Nitrate reduction* test are two important tests for identification of *M. tuberculosis*.
6. *M. tuberculosis* may involve lungs (*pulmonary tuberculosis*) or sites other than lungs (*extrapulmonary tuberculosis*).
7. Tuberculin skin test is *delayed* or *type IV hypersensitivity reaction*. In a positive reaction, there is induration (local oedema) of 10 mm diameter or more surrounded by erythema at the site of inoculation. Positive test only confirms past infection with tubercle bacilli but does not indicate presence of active state of the disease.
8. Bacteriological diagnosis of tuberculosis can be established by direct microscopy, culture examination or by animal inoculation test.
9. Petroff's method is a simple and widely used concentration technique for sputum specimens.
10. Guinea pigs are used for animal inoculation test in case of *M. tuberculosis*.
11. ELISA was the most commonly used method for detection of antimycobacterial antibodies in patient serum.
12. PCR is a rapid method in diagnosis of tuberculosis.
13. HPLC is very specific and rapid method for identification of mycobacterial species. It is chromatographic method for the analysis of mycobacterial lipids.
14. Resistance ratio method, absolute concentration method and proportion method are used to determine the sensitivity testing of *M. tuberculosis*.
15. Other methods for sensitivity testing include *BACTEC radiometric method*, MGIT, BacT/Alert 3D system, *chemiluminescence* and *Epsilometer test* (*E-test*).
16. Drug resistance in *M. tuberculosis* is due to mutation.
17. Emergence of *multidrug resistance tuberculosis* (MDR-TB) is a serious problem. The term multidrug resistance refers to resistance to *rifampicin* and *isoniazid*, with or without resistance to one or more other drugs.
18. Another serious condition *extensively drug resistant-tuberculosis* (XDR-TB) has emerged recently. XDR-TB is due to *M. tuberculosis* strains which are resistant to any fluoroquinolone and at least one of three injectable second line drugs (capreomycin, kanamycin and amikacin) in addition to isoniazid and rifampicin.

2. *Chemoprophylaxis*

It is advocated only in some risk groups.

Risk groups

(*i*) Individuals who are in unavoidable contact with a patient with open tuberculosis e.g. baby born to a mother with the disease.

(*ii*) Tuberculin positive but radiologically clear unvaccinated children.

(*iii*) Adults with the radiological evidence suggestive of inactive disease.

These individuals are given isoniazid alone with an assumption that the bacillary load is small and the chance of emergence of a drug-resistant mutant is remote.

O. Revised National Tuberculosis Control Programme (RNTCP)

Under RNTCP, any patient with cough for 2 weeks or more is included for diagnosis of pulmonary tuberculosis. Diagnosis is mainly based on good quality microscopy. Two sputum samples are collected from the patient. One early morning specimen and other is collected on spot when patient visits the chest clinic.

Both the sputum specimens are stained with Ziehl-Neelsen staining and observed for acid-fast bacilli (AFB). If one or both smears are positive then patient is diagnosed as sputum positive pulmonary tuberculosis and antitubercular treatment is started. If both the sputum smears are negative for AFB, a course of antibiotic is given for 10-14 days. If cough persists after antibiotic treatment, again two sputum specimens are collected and examined for AFB by ZN staining. Antitubercular treatment is started if one or both smears are positive and it is declared as smear positive pulmonary tuberculosis.

Grading of smears is done under RNTCP as shown in Table 42.5.

Table 42.5 Grading of Ziehl-Neelsen (ZN) Smear according to RNTCP

No. of AFB seen in oil immersion field*	Number of fields examined	Report
>10 AFB/field	20	Positive 3+
1–10 AFB/field	50	Positive 2+
10–99 AFB/100 fields	100	Positive 1+
1–9 AFB/100 fields	100	Doubtful positive
No AFB/100 fields	100	Negative, repeat

* 10X eye piece should be used along with oil immersion objective

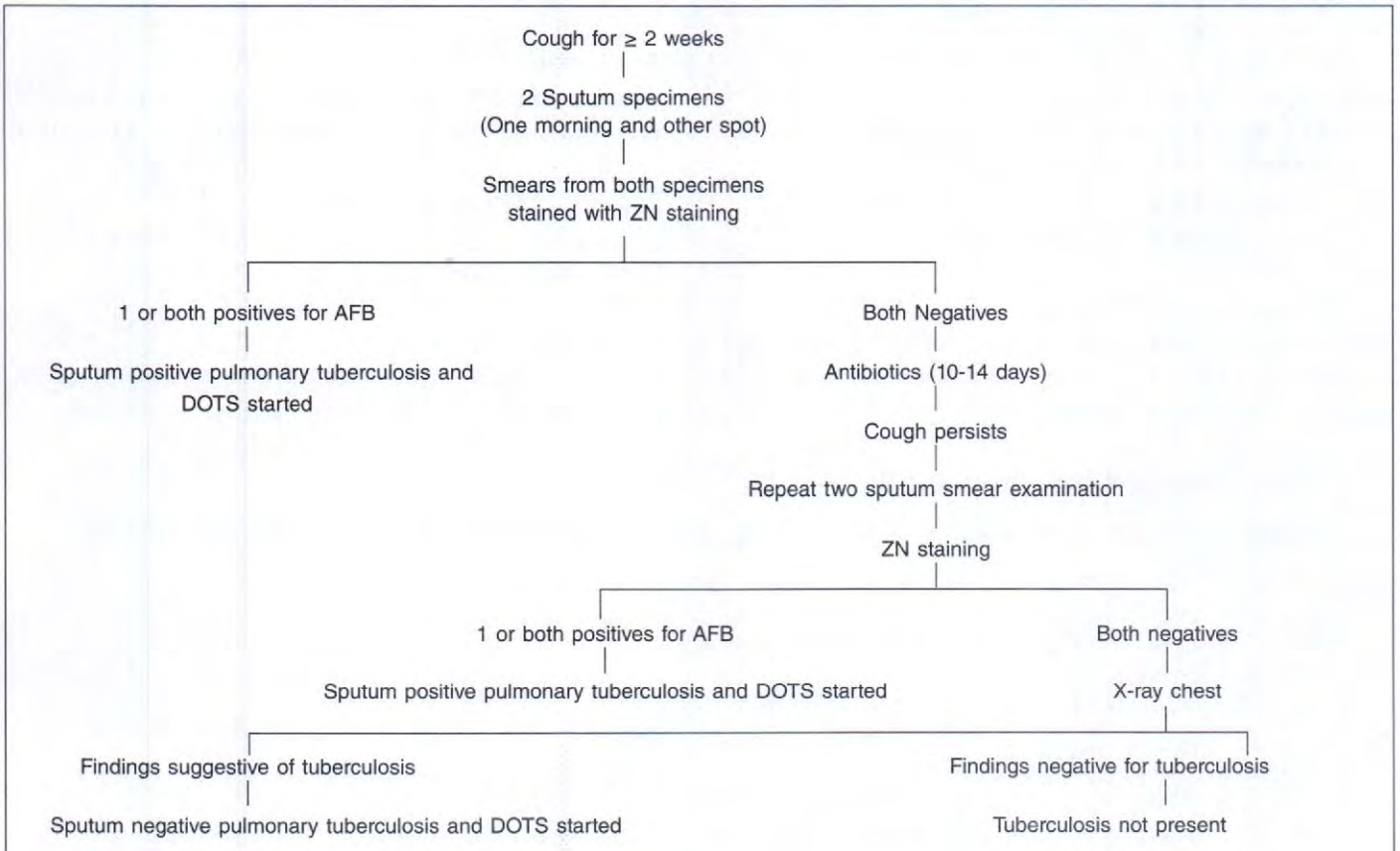


Fig. 42.7 Diagnosis of pulmonary tuberculosis under RNTCP.

Italy. In 2009, Iran was the second country to report 15 cases of XXDR-TB or TDR-TB. Recently in 2011, 12 such cases have been reported from Mumbai, India. However, XXDR-TB and TDR-TB terms are not recoganised by WHO. According to WHO these cases are considered as XDR-TB. These new terms are still to be accepted at International level.

N. Prophylaxis

Protection from tuberculosis may be done by public health measures, BCG vaccination and by chemoprophylaxis. General measures such as nutrition and health education are also important.

1. BCG Vaccine

Calmette and Guerin (1921) prepared an attenuated strain of *M. bovis* by growing it on potato medium. The strain was attenuated by repeated subcultures, every three weeks, for 239 times in glycerine potato medium over a period of 13 years (1908–1921). When the strain became incapable of producing tuberculosis in the susceptible guinea pig, it was named *Bacille Calmette Guerin* (BCG). The species *M. bovis* was selected for vaccine preparation because of the false assumption that bovine tubercle bacilli were of limited virulence in man. The four widely used BCG strains for vaccine preparation include *Pasteur 1173 P2, Tokyo–172, Copenhagen 1331* and *Glaxo–1077*.

The strains of the BCG although derived from *M. bovis* differ in several aspects from *M. bovis*:

- BCG strains grow well on glycerol containing medium.
- They are aerobic.
- They are resistant to cycloserine.

(i) Dose and administration

BCG vaccine is available in liquid form and freeze-dried (*lyophilised*) form. The freeze-dried form is commonly used. The freeze-dried vaccine supplied by BCG laboratory, Chennai, is reconstituted by sterile physiological saline to make a final concentration of 0.1 mg (moist weight) in 0.1 ml of the vaccine. After reconstitution, vaccine should be utilised within 3–6 hours. Vaccine is given intradermally in a dose of 0.1 ml. BCG vaccine should be given soon after birth failing which it may be administered at any time during the first year of life.

A small nodule develops at the site of vaccination 2–3 weeks after injection. It increases slowly in size and attains a diameter of 4 to 8 mm after about 5 weeks. It then subsides or breaks into a shallow ulcer, which heals spontaneously leaving a 4 to 8 mm diameter permanent round scar. Such individuals become tuberculin positive after 4–6 weeks.

(ii) Protective efficacy

A number of BCG vaccine trials were undertaken and the results varied from 0 to 80% (Table 42.4). The immunity has been reported to last for about 10 years. The general opinion at present is that BCG does protect from tuberculosis. Even if disease occurs, it runs a milder course in vaccinated children. It is also believed to prevent serious forms of tuberculosis such as meningeal, skeletal and miliary form of the disease. BCG vaccine stimulates T lymphocytes, therefore, it may offer some protection against leprosy and leukaemia.

(iii) Complications

Complications are rare. Occasional complications include:

1. Local—(a) abscess (b) keloid formation (c) indolent ulcer
2. Regional—lymphadenitis
3. Systemic—(a) fever (b) mediastinal adenitis (c) erythema nodosum

(iv) Contraindications

BCG vaccination is contraindicated in patients of AIDS, eczema, pertussis, measles and patients on steroids.

Table 42.4 Results of Field Trials with BCG Vaccine

Period	Region	Age of vaccinated population (years)	Efficacy of BCG (%)
1935–38	North American Indians	0–20	80
1937–48	Chicago, USA	Neonates	74
1947	Georgia, USA	6–17	0
1949–51	Puerto Rico	1–18	31
1950	Georgia and Alabama, USA	> 5	14
1950–52	Britain	14–15	79
1950–55	Madanapalle, South India (Rural)	All ages	60
1968–71	Chingleput, South India (Rural)	All ages	0

3. Proportion Method

In this method, the number of colonies growing from a standard inoculum on a medium containing drug is compared with the number of colonies from same sized inoculum on a medium without drug. When more than 1% of the mycobacteria grow in the presence of drug, it is regarded as a resistant strain.

4. Radiometric Method

BACTEC radiometric method was most commonly used. For each antituberculous drug tested, a standardised inoculum is inoculated into the liquid media, one containing drug and other without drug. The liquid medium contains ^{14}C-labelled substrate. The rate and amount of $^{14}CO_2$ produced in the absence or presence of drug is measured by the special instrument and is then compared. As described earlier due to radioactivity this method has now been discontinued.

5. Non-Radiometric Method

Mycobacterial growth indicator tube (MGIT) can also be used for sensitivity testing of *M. tuberculosis*.

6. BacT/Alert 3D System

Liquid medium vials with and without antitubercular drugs are inoculated with growth of mycobacteria. Vials without drugs act as controls. *M. tuberculosis* isolate is susceptible when the drug containing vial remains negative for growth or becomes positive later than the control vial. The strain is resistant when the drug containing vial becomes positive prior to or on the same day as that of control vial.

7. Chemiluminescence

Luciferase reporter mycobacteriophage (containing the firefly luciferase gene) has been used for susceptibility testing of *M. tuberculosis*. Only viable mycobacteria can be infected with and replicate this mycobacteriophage; dead tubercle bacilli cannot. The isolate of *M. tuberculosis* to be tested is grown in the presence and absence of drug and the reporter mycobacteriophage is added. Following infection, a substrate of luciferase, luciferin is added. If bacteria are viable, the luciferin is broken down and light is emitted which can be measured. This method is named as chemiluminescence. If the isolate is resistant to drug, light will be emitted, while bacteria susceptible to the drug will not emit any light. The amount of light emitted is directly proportional to the number of viable bacteria.

8. Epsilometer Test

Epsilometer test (E-test) has also been applied for susceptibility testing of *M. tuberculosis*.

9. Mutation

Demonstration of mutation in specific genes for different drugs is a useful indicator of drug resistance.

M. Treatment

The antitubercular drugs include bactericidal agents such as rifampicin (R), isoniazid (H), pyrazinamide (Z), streptomycin and bacteriostatic agents include ethambutol (E), thiacetazone, ethionamide, para-aminosalicylic acid (PAS) and cycloserine. Short course regimens of 6–7 months are used. A combination of four drugs (HRZE) given three times a week during an initial intensive phase for 2 months, followed with only two drugs (HR) three times a week during continuing phase of 4–5 months. As resistant strains emerge readily by mutation and selection, combinations of two or more drugs are used. If patient is treated with only one antitubercular drug, initially the bacilli die in large numbers but soon resistant mutants emerge and multiply unchecked. The bacterial resistance may be primary (prior to start of treatment) or secondary which emerges during the course of treatment.

A serious consequence of unchecked drug resistance has been the emergence of *multidrug resistance tuberculosis* (MDR-TB). The term multidrug resistance refers to resistance to rifampicin and isoniazid, with or without resistance to one or more other drugs. MDR-TB is a global problem especially in HIV infected persons. When first line drugs are ineffective, second line drugs are to be used. Quinolones, aminoglycosides, para amino salicylic acid (PAS), ethambutol, thiacetazone, cycloserine and capreomycin are being used. The directly observed therapy under supervision (DOTS) is being used to prevent deterioration of resistance problem by ensuring the patient's compliance.

When the patient does not show improvement, antibiotic sensitivity test is done to detect resistant strains.

Another serious condition *extensively drug resistant tuberculosis (XDR-TB)* has emerged recently. XDR-TB is due to *M. tuberculosis* strains which are resistant to any fluoroquinolone and at least one of three injectable second line drugs (capreomycin, kanamycin and amikacin), in addition to isoniazid and rifampicin.

In recent years, two new terms *extremely drug resistant tuberculosis (XXDR-TB)* and *totally drug resistant tuberculosis (TDR-TB)* have been described by different researchers. These terms have been used for *M. tuberculosis* strains which are resistant to all first line drug (isoniazid, ethambutol, pyrazinamide and streptomycin) and second line drugs (ofloxacin, moxifloxacin, kanamycin, amikacin, capreomycin, para-aminosalicylic acid and ethionamide). In 2007, two cases of TDR-TB were first reported from

tuberculin test becomes positive in animals that develop tuberculosis. Animal is killed after six weeks.

Autopsy shows:

1. Caseous lesion at the site of inoculation.
2. Enlarged caseous inguinal lymph nodes. The infection may spread to other lymph nodes such as lumbar, portal, mediastinal and cervical lymph nodes.
3. Tubercles may be seen in spleen, lungs, liver or peritoneum.
4. Kidneys are unaffected.

The identity of the bacteria is then confirmed by demonstration of acid-fast bacilli (AFB) from the lesions. Infections with *Y. pseudotuberculosis*, *Brucella, Salmonella* and several fungi may produce lesions which macroscopically simulate tubercles.

M. tuberculosis is highly pathogenic to guinea pigs and hamsters and virtually non-pathogenic for rabbits, while *M. bovis* is highly pathogenic for both rabbits and guinea pigs. Catalase-negative and INH-resistant strains and most of the Asian strains isolated from South India have low virulence for guinea pigs. Sometimes these organisms produce only enlarged lymph node and the animals may have to be observed for 12 weeks. Guinea pig inoculation is rarely used nowadays for laboratory diagnosis.

6. Molecular Methods

Polymerase chain reaction (PCR) is a rapid method in diagnosis of tuberculosis. It is based on DNA amplification and has been used to detect *M. tuberculosis* directly in clinical specimens. The restriction fragment length polymorphism (RFLP) is used to type different strains for epidemiological purposes. The principle of this technique is that restriction endonuclease treatment yields nucleic acid fragments of different lengths, the patterns of which are strain specific.

Gene Xpert MTB/RIF

Gene Xpert MTB/RIF is an automated diagnostic test. It detects DNA sequences specific for *M. tuberculosis* and rifampicin resistance by PCR. Results can be obtained from unprocessed sputum specimen within 2 hours. WHO has endorsed this test for use in TB endemic countries.

DNA probes have been used to identify mycobacterial species isolated on solid culture media or from broth culture.

Ligase chain reaction (LCR) and Trancription mediated amplification (TMA) are other molecular methods used for detection of *M. tuberculosis*. TMA is based on amplification of ribosomal RNA as compared to DNA amplification in PCR.

7. Chromatographic Methods

The analysis of mycobacterial lipids by chromatographic methods, such as thin-layer chromatography, gas-liquid chromatography (GLC) and high performance liquid chromatography (HPLC), has been used to identify mycobacteria. HPLC is very specific and rapid method for identification of species.

8. Serology

Serology includes detection of antimycobacterial antibodies in patient serum. Various methods such as enzyme linked immunosorbent assay (ELISA), radio immunoassay (RIA), latex agglutination assay have been employed. Several antigens like BCG, antigens 5 and 6, 64 kDa, antigen 60 and 32 kDa protein have been tried for detection of antibody against them. Diagnostic utility of these antibodies is equivocal. WHO has recommended that these tests should not be used for diagnosis of active tuberculosis.

L. Sensitivity Testing

Drug-resistant mutants continuously arise at a low rate in any mycobacterial population. With the emergence of multidrug resistance in mycobacteria, it is essential to determine the sensitivity testing. The isolated tubercle-bacilli are tested in LJ media after incorporating different concentrations of antitubercular drugs in the media before inoculation. Following techniques for sensitivity testing are used.

1. Resistance Ratio Method

This is the most important method. Bottles of LJ medium incorporating doubling concentrations of drugs are inoculated with a test strain and a known sensitive strain (H37Rv strain of *M. tuberculosis*). After 3 weeks of incubation at 37°C, culture media are examined for growth. The medium with the lowest concentration of drug showing no more than 20 colonies is taken as the minimum inhibitory concentration (MIC). The result is expressed as resistance ratio.

Interpretation

Resistance ratio of 1 or 2 = sensitive strain
Resistance ratio of 4 = Doubtfully resistant strain, test should be repeated.
Resistance ratio of 8 = Unequivocally resistant strain

2. Absolute Concentration Method

The MIC of the drug against the test strain only is determined. Known sensitive strain is not tested for MIC, therefore, this method is inferior to resistance ratio method.

good personal hygiene are effective measures. No vaccine is available.

B. Borrelia vincentii (Treponema vincentii)

It is a motile spirochaete, about 5–20 μm long and 0.2–0.6 μm wide, with 3–8 coils of variable size. It is easily stained with dilute carbol fuchsin, methyl violet and is Gram negative. It is a normal mouth commensal but a potential pathogen. Under predisposing conditions such as malnutrition or viral infections, it gives rise to ulcerative gingivostomatitis or oropharyngitis (Vincent's angina). In these cases, *B. vincentii* is often associated with fusiform bacilli (*Fusobacterium fusiforme*) (Fig. 45.4). This symbiotic infection is known as *fusospirochetosis*. *B. vincentii* grows in media containing ascitic fluid and serum. Laboratory diagnosis may be made by demonstrating spirochaetes and fusiform bacilli in stained smears of exudates from the lesions. Cultivation is difficult but can be done in enriched media anaerobically. Penicillin and metronidazole are effective in treatment.

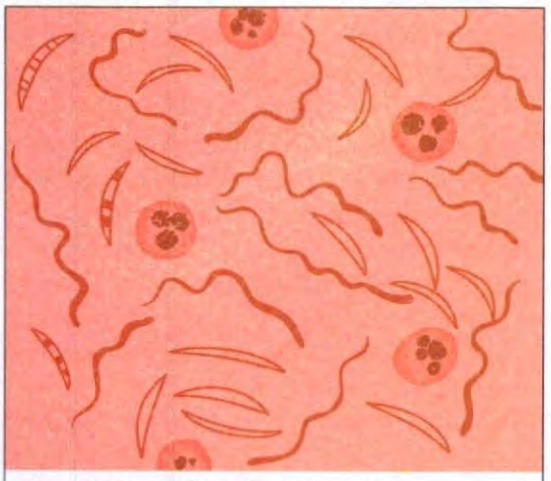


Fig. 45.4 Smear from Vincent's angina

C. Borrelia burgdorferi

Borrelia burgdorferi was first observed in 1975 in Lyme, USA. It causes Lyme disease. The disease is widespread in the USA. It has been reported from other parts of world also.

1. Morphology

It measures 4–30 μm × 0.2–0.25 μm. It is flexible, helical and Gram negative,

2. Culture

It is a microaerophilic spirochaete. It is fastidious bacterium and can be grown in a modified Kelley's (BSK) medium, after incubation for two weeks or more. Optimum temperature for growth is 33–37°C.

3. Pathogenesis

Rodents, deer and other mammals are the natural reservoir hosts. Ixodid ticks are the vectors. The borrelia grows mainly in the midgut of the tick and transmission to humans occurs by regurgitation of the gut contents during biting.

B. burgdorferi produces Lyme borreliosis (Lyme disease) and incubation period is about 3–30 days. Lyme borreliosis occurs in three stages. The first stage of 'localised infection' appears as a small red macule or papule at the site of bite (*erythema migrans* or EM). A few weeks later, the second stage of 'disseminated infection' develops with headache, fever, myalgia and lymphadenopathy. The third stage of 'persistent infection' develops in months or years later with chronic arthritis, polyneuropathy and encephalopathy.

4. Laboratory Diagnosis

Laboratory diagnosis can be made by isolation of the organism or by serology. The borrelia has been isolated from skin lesions, CSF and the blood of patients. The culture is too slow and difficult for routine use. For the serological diagnosis, ELISA and IF are commonly used. Antibodies take 1–2 months to appear. Lyme borreliosis patients may give a positive FTA-ABS test but the VDRL test is negative.

5. Treatment

Tetracycline, penicillin and erythromycin have been used for treatment of Lyme borreliosis.

IV. LEPTOSPIRA

Members of the genus *Leptospira* are actively motile, delicate spirochaetes possessing numerous closely wound spirals and characteristic hooked ends. They cannot be seen under the light microscope due to its thinness (*leptos*, meaning fine or thin). They do not stain readily. They may be observed by dark ground illumination.

A. Classification

The genus *Leptospira* is now classified into two species

1. L. interrogans

It includes pathogenic leptospires of man and animals. The species *L. interrogans* is classified into 22 serogroups (Icterohaemorrhagiae, Canicola, Australis, Hebdomadis, Andamana etc.). Each serogroup has one or more serovars (serotypes) e.g. the serogroup Icterohaemorrhagiae contains the serovars *icterohaemorrhagiae, copenhageni, smithi, budapest* etc. About 200 serovars have been identified among 22 serogroups.

2. *L. biflexa*

It contains saprophytic leptospires found in surface waters.

B. Leptospira interrogans

1. Morphology

These are spiral bacteria, 5–20 μm × 0.1 μm with numerous closely set coils and hooked ends (Fig. 45.5). They are actively motile. They stain poorly with aniline dyes but can be observed by fluorescent antibody and silver impregnation techniques. Because of narrow diameter, they are best observed by dark ground, phase-contrast or electron microscopy. Leptospires rotate rapidly about their long axis and bending or flexing sharply.

2. Culture

They are aerobic and microaerophilic. Optimum temperature for their growth is 28–32°C and optimum pH 7.2–7.5. They can be grown in media enriched with rabbit serum. Several media, such as Korthof's, Stuart's and Fletcher's media, have been described. Semisynthetic medium, such as, EMJH (Ellinghausen, McCullough, Johnson, Harris) is now commonly used. In semisolid media, growth occurs a few millimetres below the surface. Growth is detected usually after 6–14 days of incubation. The generation time of leptospires in laboratory media is 12–16 hours and 4–8 hours in inoculated animals.

Leptospires may be grown on chorioallantoic membrane (CAM) of chick embryos. They can also be grown in guinea pigs.

3. Resistance

They are killed at 50°C in ten minutes and in 10 seconds at 60°C. They are readily destroyed by most antiseptics and disinfectants. Their survival in water depends on temperature, acidity and amount of pollution.

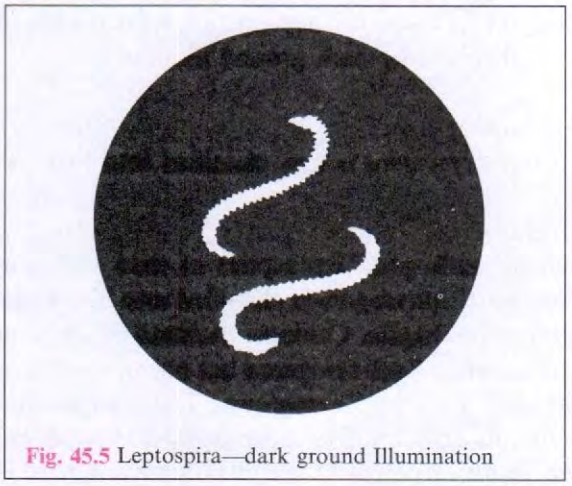


Fig. 45.5 Leptospira—dark ground Illumination

At 26°C and pH 7.0, they die within 30 days in polluted river water, 12–14 hours in sewage and three minutes in water containing 1 ppm chlorine.

4. Antigenic Structure

Leptospires show considerable antigenic cross reactions. A genus specific somatic antigen is present in all members of the genus. Serogroups differentiation is based on surface antigens. Based on more specific antigens, many different serovars are distinguished within each serogroup.

5. Pathogenesis

L. interrogans causes a zoonotic disease named leptospirosis in rodents and sometimes in domestic animals. It is transmitted to humans by direct or indirect contact with water contaminated by urine of carrier animals. Leptospires enter the body through cuts or abrasions on the skin, or through the mucous membranes of the mouth, nose or conjunctiva. After an incubation period of 6–8 days, there is onset of febrile illness with leptospires in blood (septicaemic phase) which lasts for 3–7 days. The organisms disappear from the blood but enter into the liver, kidney, spleen and meninges producing meningeal irritation (headache, vomiting). Leptospirosis is an established cause of aseptic meningitis. They persist in the internal organs, and most abundantly in the kidney and therefore may be demonstrated in the urine in the later stages of the disease. Severe leptospirosis (*Weil's disease*) associated with fever, conjunctivitis, albuminuria, jaundice and haemorrhage is usually caused by *L. icterohaemorrhagiae* serogroup. It is a fatal illness with hepatorenal damage.

Three important serogroups of *L. interrogans*—*L. canicola, L. hebdomadis* and *L. icterohaemorrhagiae* are responsible for most of the human cases of leptospirosis. Aseptic meningitis is common in *canicola* infection.

6. Laboratory Diagnosis

The diagnosis depends on demonstration of the leptospires microscopically in blood or urine, by isolating them in culture or by animal inoculation or by serological tests.

(i) Demonstration of leptospires in the blood or urine

Leptospires can be observed in the blood by dark-ground microscopy. Blood examination is useful in first week as leptospires disappear from blood after 8 days.

Leptospires may be present in the urine in the second week of the disease and intermittently thereafter up to six weeks. Centrifuged deposit of urine may be examined by dark-ground microscopy for leptospires.

(ii) Culture

Specimen of blood during first week and urine in second week (up to six weeks) can be cultured in modified Korthof's medium or Fletcher's semisolid medium. Centrifuged deposit of urine is cultured. Media are incubated at 28–32°C aerobically and examined by dark ground microscopy every third day up to six weeks before discarding it as negative.

(iii) Animal inoculation

The blood or urine from the patient is inoculated intraperitoneally into young guinea pigs. From the third day after inoculation, the peritoneal fluid is examined daily for leptospires by dark ground illumination. Heart blood of animal is inoculated into the culture media for isolation of organisms. The identification of serovar of the isolate is done by agglutination.

(iv) Serological tests

It is very useful method of diagnosis. Antibodies begin to appear at the end of first week and continue to rise till the fourth week and then begin to decline. Two types of serological tests are in use, *screening tests* and the *serotype specific tests*.

(a) Screening tests

These tests are genus-specific and are done by using a broadly reactive genus specific antigen usually the non-pathogenic *L. bifexa* Patoc I strain. These tests include complement fixation test, haemagglutination test, enzyme–linked immunosorbent assay (ELISA), sensitised erythrocyte lysis (SEL), agglutination test and indirect immunofluorescence. These tests are capable of detecting IgM and IgG leptospiral antibodies. A rapid dip-stick assay has been developed for detection of leptospira-specific IgM antibody.

(b) Serotype specific tests

These serotype specific tests identify the infecting serovar by demonstrating specific antibodies. Microscopic and macroscopic agglutination tests are used for this purpose. Commercially available, formalin-killed suspensions of a number of reference strains are tested with the serial dilutions of the test serum in macroscopic agglutination test. The microscopic agglutination test (MAT) uses formalised or living suspensions of well grown cultures and agglutination is observed under the dark field microscope.

Due to the presence of some cross reactions between different serovars, agglutinin absorption tests may sometimes be necessary for accurate diagnosis.

7. Epidemiology

Leptospirosis is a zoonosis. Pathogenic leptospires survive for long periods in the kidneys in natural hosts, multiply and are shed in the urine. The infected urine contaminates the water and leptospires survive for weeks. When human beings come into contact with such water, the organisms enter the body through abraded skin or mucosa. Man is the 'end' host and there is no evidence that human patients infect others.

Several animals act as carriers. Rats are particularly important as they carry the most pathogenic serotype *icterohaemorrhagiae*. Dogs carry *canicola*; field mouse *hebdomadis* and pigs *pomona* serotypes.

Leptospirosis is worldwide in distribution. The information on the prevalence of the disease is very incomplete due to lack of facilities for diagnosis of leptospirosis.

8. Treatment

Leptospires are sensitive to penicillin, tetracycline and erythromycin.

9. Prophylaxis

Preventive measures include:

(*i*) rodent control
(*ii*) disinfection of water
(*iii*) the wearing of protective clothing.

Vaccine has been attempted in dogs, cattle and pigs and persons at high risk such as agricultural workers. A serovar specific immunity develops following natural infection.

KEY POINTS

1. *Spirochaetes* are large, motile, helical bacteria. They are motile because of endoflagella.
2. The members of genera *Treponema*, *Borrelia* and *Leptospira* are pathogenic to man.
3. *Treponema pallidum* is the causative agent of syphilis. It is a sexually transmitted disease (STD).
4. The laboratory diagnosis of syphilis consists of *demonstration* of *treponemes* and detection of antibodies by *serological* tests.
5. Demonstration of treponemes by microscopy is applicable in primary and secondary states and in cases of congenital syphilis with superficial lesions. *Dark ground microscopy* and *direct fluorescent antibody staining* for *T. pallidum* (DFA-TP) are used for demonstration of treponemes in specimens.
6. Serological tests form the mainstay of laboratory diagnosis. Two types of antibodies are produced in syphilis, *non-specific* antibody (reagin antibody) and *specific* anti-treponemal antibody.
7. Depending upon the antigen used, serological tests for syphilis are divided into *non-treponemal tests* (cardiolipin or lipoidal antigen is used) and *treponemal tests* (treponemes are used as the antigen).
8. In the standard tests for syphilis (STS), reagin antibodies are detected by cardiolipin antigen. The STS includes Venereal Diseases Research Laboratory (VDRL) test and rapid plasma reagin (RPR). These are *flocculation tests*. *Biological false positive* (*BFP*) reactions may occur in certain conditions because of use of non-specific antigen (cardiolipin) in STS. BFP are not caused by technical faults.
9. *Fluorescent treponemal antibody absorption* (*FTA-ABS*) *test* and *Treponema pallidum hemagglutination assay* (*TPHA*) are serological tests for detection of specific anti-treponemal antibody.
10. *IgM-FTA-ABS* test, a modification of FTA-ABS, can detect IgM antibodies in *congenital syphilis* and helps to differentiate it from seropositivity due to passively transferred maternal antibodies.
11. VDRL and FTA-ABS tests can also be performed in cerebrospinal fluid.
12. VDRL or RPR tests are used for screening or for diagnostic purposes of large number of sera. These tests are also more useful for the assessment of cure following treatment.
13. *Yaws* is caused by *T. pallidum* sub-species *pertenue*. *Pinta* is caused by *T. carateum* and *endemic syphilis* by *T. pallidum* sub-species *endemicum*.
14. The important pathogenic borreliae of medical importance include *B. recurrentis*, *B. vincentii* and *B. burgdorferi*. *B. recurrentis* is the causative agent of *relapsing fever* while *B. vincentii* causes *vincent's angina*. *Lyme borreliosis* is caused by *B. burgdorferi*.
15. *Leptospira interrogans* causes a zoonotic disease named *leptospirosis* in rodents and sometimes in domestic animals. It is transmitted to humans by direct or indirect contact with water contaminated by urine of carrier animals.

YOU MUST KNOW

1. Diseases caused by different spirochaetes.
2. List of pathogenic treponemes.
3. Morphology of *Treponema pallidum*.
4. Diseases caused by *T. pallidum* and their laboratory diagnosis.
5. Serological diagnosis of syphilis.
6. FTA-ABS test.
7. TPHA.
8. Biological false positive (BFP) reactions.
9. Yaws.
10. Pinta.
11. *Borrelia recurrentis*.
12. *Borrelia vincentii*.

13. Lyme borreliosis.
14. Leptospirosis.

STUDY QUESTIONS

1. Name different spirochaetes and diseases caused by them. Describe the laboratory diagnosis of syphilis.
2. Write short notes on
   (a) VDRL test
   (b) Rapid plasma reagin (RPR) test
   (c) Standard tests for syphilis (STS)
   (d) TPHA
   (e) FTA-ABS test
   (f) Non-venereal treponematoses
   (g) *Borrelia recurrentis*
   (h) *Borrelia vincentii*
   (i) Lyme borreliosis
   (j) Leptospirosis.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. All of the following genera belong to family *Spirochaetaceae* except:
   (a) *Treponema*
   (b) *Borrelia*
   (c) *Christispira*
   (d) *Leptospira*
2. Which of the following genera does not belong to family *Spirochaetaceae*?
   (a) *Treponema*
   (b) *Borrelia*
   (c) *Christispira*
   (d) *Leptonema*
3. Motility of spirochaetes may be due to:
   (a) Flexion and extension
   (b) Corkscrew-like rotatory movement
   (c) Translatory motion
   (d) All of the above
4. Which of the following species of Treponema is non-pathogenic?
   (a) *Treponema pallidum*
   (b) *T. pertenue*
   (c) *T. carateum*
   (d) *T. phagedenis*
5. Which of the following methods can be used to demonstrate *Treponema pallidum*?
   (a) Dark ground microscopy
   (b) Silver impregnation method
   (c) Immunofluorescence staining
   (d) All of the above
6. Hard chancre is characteristic of:
   (a) Primary syphilis
   (b) Secondary syphilis
   (c) Tertiary syphilis
   (d) None of the above
7. Which of the following serological test is commonly employed for diagnosis of syphilis?
   (a) Venereal disease research laboratory (VDRL) test
   (b) Kahn test
   (c) Wassermann reaction
   (d) Reiter protein complement fixation (RPCF) test
8. Which of the following serological tests is employed for diagnosis of congenital syphilis?
   (a) FTA-ABS test
   (b) IgM FTA-ABS test
   (c) TPHA test
   (d) Reiter protein complement fixation test
9. The causative agent of yaws is:
   (a) *Treponema pallidum* subspecies *pertenue*
   (b) *T. pallidum* subspecies *endemicum*
   (c) *T. carateum*
   (d) None of the above
10. The causative agent of Pinta is:
    (a) *Treponema pallidum* subspecies *pertenue*
    (b) *T. pallidum* subspecies *endemicum*
    (c) *T. carateum*
    (d) None of the above
11. Endemic syphilis is caused by:
    (a) *Treponema pallidum* subspecies *pertenue*
    (b) *T. pallidum* subspecies *endemicum*
    (c) *T. carateum*
    (d) None of the above

12. Which of the following disease/s is/are transmitted non-venereally?
    (a) Yaws (b) Pinta
    (c) Endemic syphilis (d) All of the above
13. The causative agent of louse-borne relapsing fever is:
    (a) *Borrelia recurrentis* (b) *B. duttoni*
    (c) *B. vincentii* (d) *B. burgdroferi*
14. The causative agent of lyme disease is:
    (a) *Borrelia recurrentis* (b) *B. duttoni*
    (c) *B. vincentii* (d) *B. burgdroferi*
15. Which serogroup of *Leptospira interrogans* is responsible for causing Weil's disease?
    (a) *Icterohaemorrhagiae* (b) *Hebdomadis*
    (c) *Australis* (d) *Canicola*
16. Which of the following serological tests can be employed for diagnosis of leptospirosis?
    (a) Microscopic agglutination test (b) Macroscopic agglutination test
    (c) Both of the above (d) None of the above

Answers (MCQs):

1. (d) 2. (d) 3. (d) 4. (d) 5. (d) 6. (a) 7. (a) 8. (b) 9. (a) 10. (c)
11. (b) 12. (d) 13. (a) 14. (d) 15. (a) 16. (c)

Chapter 46

MYCOPLASMA AND UREAPLASMA

Nocard and Roux (1898) first isolated a filterable and highly pleomorphic microorganism from bovine pleuropneumonia. Subsequently, several such morphologically similar organisms, some pathogens and other commensals or saprophytes, were isolated from animals, man, soil and sewage. Because of their resemblance to the organism causing bovine pleuropneumonia, they were called *pleuropneumonia like organisms* or PPLO. These organisms are now named *Mycoplasma*, which refers to the fungus like form of branching filaments.

I. CLASSIFICATION

Mycoplasmas are classified in the class *Mollicutes* (*mollis*, soft; *kutis*, skin) and four orders *Mycoplasmatales, Entoplasmatales, Acholeplasmatales* and *Anaeroplasmatales*. The order *Mycoplasmatales* contains one family which contains two genera, *Mycoplasma* and *Ureaplasma*. They require cholesterol or other sterols as essential growth factor.

Other three orders contain four families *Entoplasmataceae, Spiroplasmataceae* (both families in order *Entoplasmatales*), *Acholeplasmataceae* (order *Acholeplasmatales*) and *Anaeroplasmataceae* (order *Anaeroplasmatales*)

The term mycoplasmas is often used for any organism of the class *Mollicutes*, irrespective of whether they belong to the genus *Mycoplasma* or other genera. There are approximately 100 *Mycoplasma* species in the class *Mollicutes*. Ten species are associated with human diseases but only four are more frequently encountered which include *M. pneumoniae, M. hominis, M. genitalium* and *U. urealyticum*.

II. MYCOPLASMA

A. General Characters

1. Mycoplasmas differ from other bacteria in that they lack a rigid cell wall resulting in the plasticity of the organisms.

2. The cytoplasm is surrounded by a single triple layered membrane that, unlike other bacteria, contains cholesterol.
3. They cannot synthesise their own cholesterol which is required for their growth. However, *Acholeplasma* synthesises carotenol as a substitute for cholesterol and therefore can grow without incorporation of sterol in culture medium.
4. Due to lack of rigid cell wall, they are extremely pleomorphic.
5. They are resistant to cell wall active antibiotics such as penicillins and cephalosporins. This is again due to absence of cell wall.
6. They differ from viruses in containing both RNA and DNA and can reproduce in cell-free media.
7. The members of the genus *Ureaplasma* can hydrolyse urea while other genera cannot do so. This is a differentiating feature of *Ureaplasma*.

B. Morphology

Mycoplasmas are the smallest free living microorganisms. They can pass through bacterial filters. They are most pleomorphic and may present as small spherical shapes (125–250 nm in diameter) to longer branching filaments (500–1000 nm in size). They lack cell wall but have a triple layered cell membrane which is rich in cholesterol and other lipids. They are Gram negative, but are better stained by Giemsa stain.

The method of reproduction is not fully understood but it appears to be by binary fission and budding. They are non-sporing and non-flagellated. They are usually non-motile, though a gliding motility is described in some species.

C. Culture

Mycoplasmas are generally facultative anaerobes, growth being better aerobically. Optimum temperature for growth is 35–37°C. All mycoplasmas, except *Acholeplasma*, require sterol for growth. They grow on enriched media containing 20% horse or human serum and yeast extract. High concentration of serum provides not only cholesterol but also saturated and unsaturated fatty acids for membrane synthesis. A medium widely used for the isolation of mycoplasmas is PPLO broth which contains bovine heart infusion broth enriched with 20% horse serum and 10% fresh yeast extract along with glucose, and phenol red as an indicator. This medium can be made solid by the addition of agar. Penicillin, ampicillin and polymyxin B may be added in the medium to inhibit contaminating bacteria and amphotericin B to inhibit fungi.

Colonies usually appear after incubation for 48–72 hours. Typical tiny *fried egg* colonies appear in the medium (Fig. 46.1). Colony consists of a central opaque granular area of growth, surrounded by a flat, translucent peripheral zone. Colony size varies from 200–500 μm for 'large colony' mycoplasmas and 15–60 μm for the ureaplasmas. Colonies can be examined by hand lens but are best studied after staining by *Dienes method*. For this, a block of agar containing the colony is cut and placed on a microscopic slide. It is covered with a coverslip on which an alcoholic solution of methylene blue and azure has been dried.

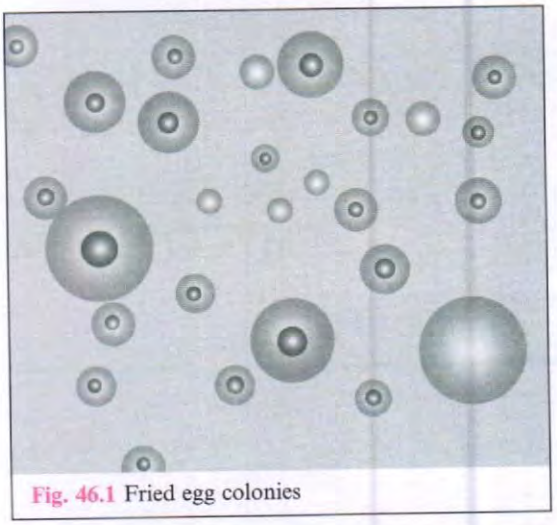


Fig. 46.1 Fried egg colonies

As the colonies are too small, they can not be picked with platinum loops. Subcultures are done by cutting out an agar block with colonies and rubbing it on fresh culture plates. Most of the mycoplasma colonies are haemolytic.

D. Biochemical Reactions

Mycoplasmas are mainly fermentative. Most species utilise glucose or arginine as the main source of energy. Urea is not hydrolysed, except by ureaplasmas. They are generally not proteolytic.

E. Resistance

They are killed by heating at 56°C for 30 minutes. They are very sensitive to lysis by surface active agents and lipolytic agents. They are resistant to penicillins and cephalosporins as well as to lysozymes that act on bacterial cell walls but are sensitive to tetracycline and erythromycin. Antiseptic solutions such as cetrimide and chlorhexidine inhibit mycoplasmas.

F. Antigenic Properties

They possess cell membrane bound glycolipids and proteins which act as haptens. Glycolipids induce antibodies that react in serological tests such as complement fixation test.

G. Mycoplasmas and L-forms

Kleineberger (1935) found pleuropneumonia-like forms in a culture of *Streptobacillus moniliformis* and named them L-forms, after Lister Institute, London, where this observation was made. Subsequently, it was shown that many bacteria, either spontaneously or induced by substances like penicillin, lost part or all of their cell wall and developed into L-forms. Both mycoplasmas and cell wall deficient forms (L-forms, protoplasts, spheroplasts) of bacteria show extreme pleomorphism. L-forms also produce 'fried egg' colonies like mycoplasma, but they differ in the following respects:

1. Unstable L-forms revert to their normal morphology. The stable L-forms continue in the cell wall deficient state permanently but resemble parent bacteria both biochemically and antigenically.
2. Though L-forms lack cell walls, the remnants of cell wall components can be demonstrated.
3. L-forms of bacteria do not require sterol for growth.
4. L-forms are not filterable.
5. L-forms may not initiate disease but they play an important role in persistence of chronic infection during antibiotic therapy.

As agglutinins to *Streptococcus* MG are frequently detected following infection with *M. pneumoniae*, it is thought that the later is an L-form for the former, but all available evidences are against this hypothesis.

H. Pathogenesis

Mycoplasma causes two type of diseases in man — pneumonia and genital infections.

1. *Mycoplasma pneumoniae*

M. pneumoniae causes primary atypical pneumonia (mycoplasmal pneumonia). *Eaton* (1944) first isolated the organism and considered it to be a virus (*Eaton agent*). Primary atypical pneumonia is self limiting and recovery occurs within two weeks. Transmission is by droplets of nasopharyngeal secretions. Spread occurs by close contact, as in families and among military recruits. The mycoplasma may remain in the throat for two or more months after recovery.

2. *Mycoplasma hominis*

It may be isolated from 20–25% cases with non-gonococcal urethritis (NGU). This has also been incriminated in postpartum sepsis, proctitis, acute salpingitis, pelvic inflammatory disease, cervicitis and vaginitis. It is transmitted by sexual contact.

I. Laboratory diagnosis

Laboratory diagnosis may be carried out by isolation of the organism or by serological tests.

1. *Isolation*

The mycoplasmas can be recovered from throat swab, nasopharyngeal swab, respiratory secretions, sputum (*M. pneumoniae*) or urethral secretions, prostatic secretions, cervical swabs, urine (*M. hominis* and *Ureaplasma urealyticum*).

Culture media should be inoculated immediately after collection of specimen. If inoculation is not possible immediately then specimen may be kept at 4°C up to 24 hours. In case of delay more than 24 hours, the specimen should be frozen at –70°C. *Mycoplasma* broth medium containing penicillin, polymyxin B, amphotericin B glucose and phenol red (indicator) is inoculated with the specimen and incubated at 37°C. If specimen contains *M. pneumoniae*, growth is detected by turbidity and a colour change (red to yellow) of phenol red indicator, due to fermentation of glucose. Ureaplasma and some other mycloplasmas which do not ferment glucose show only turbidity. It is then subcultured on solid medium containing agar and incubated at 37°C for 5–7 days. Typical *fried egg* colonies can be seen. Colonies of *M. pneumoniae* are beta-haemolytic. These colonies may be identified by:

(*i*) *Haemadsorption test*: Colonies of *M. pneumoniae* adsorb guinea pig erythrocytes to their surface.

(*ii*) *Tetrazolium reduction test*: Colonies of *M. pneumoniae* appear red when these are flooded with solution of tetrazolium compound which is colourless. *M. pneumoniae* reduces tetrazolium (colour-less) to red coloured compound.

(*iii*) *Serological techniques*: Growth is inhibited by specific antiserum. This includes inhibition of colony development around discs impregnated with specific antiserum.

2. *Serological tests*

These are of two types: (i) detection of antigen or nucleic acids (ii) detection of antibody.

(i) *Detection of antigen or nucleic acids*

(a) Antigen can be detected by direct immunofluorescence test, counter–immunoelectrophoresis (CIEP) and enzyme immunoassay (EIA).

(*b*) Specific DNA can be detected by hybridisation technique and PCR in respiratory secretions.

(ii) Detection of antibody

It can be done by using specific mycoplasmal antigens or by non-specific methods. Among the former are immunofluorescence, haemagglutination inhibition, complement fixation, enzyme immunoassay (EIA) and indirect haemagglutination assays (IHA). The non-specific tests are *Streptococcus* MG and *cold agglutination* tests.

(*a*) *Streptococcus MG agglutination test*: Serial dilution of unheated patient's serum is mixed with heat killed suspension of *Streptococus* MG and incubated at 37°C for overnight. The agglutination reaction is observed. A titre of 1:20 or more is considered suggestive of *M. pneumoniae* infection.

(*b*) *Cold agglutination test*: Cold agglutinins (macroglobulin antibodies) appear in the blood of primary atypical pneumonia. These cold agglutinins have a property to agglutinate human group O erythrocytes at low temperature. The blood sample should not be refrigerated before separation of the serum because cold agglutinins are readily adsorbed by homologous erythrocytes at low temperature. Serial dilutions of patient's serum are mixed with washed human O group erythrocytes and kept at 4°C overnight. The clumping is dissociated at 37°C. Clumping is observed and a titre of 1:32 or more is suggestive but a rising titre in a paired serum sample is more reliable. Cold agglutinins are occasionally induced in other infections such as rubella, adenovirus infections, infectious mononucleosis etc.

(c) *Complement fixation test*: This is the most widely used test for detecting antibody against *M. pneumoniae*. The antigen used is a glycolipid. A recent infection is indicated by a single titre of 1:64 or more or four fold rise in paired sera.

(d) *Enzyme immunoassay (EIA)*: This is more sensitive test than complement fixation test. It can be used to detect specific IgM, IgG and IgA antibodies against *M. pneumoniae*.

J. Treatment

Tetracyclines and erythromycins are the drugs of choice.

III. UREAPLASMA UREALYTICUM

Some strains of mycoplasma form very tiny colonies generally 15–50 μm in size. They are named T strains or T-form mycoplasmas (T for tiny). Now, they are classified as *Ureaplasma urealyticum*. They are peculiar in their ability to hydrolyse urea, which is an essential growth factor in addition to sterol. They are implicated in non-specific urethritis or genital infections. They are transmitted by sexual contact and may cause urethritis, proctitis and Reiter's syndrome in males. In females, they may cause acute salpingitis, pelvic inflammatory disease (PID), cervicitis and vaginitis. They have also been associated with infertility, abortion, postpartum fever and low birth weights of infants. All these genital infections may also be caused by *M. hominis*, another mycoplasma responsible for genital infections. They are highly susceptible to erythromycin. They are more susceptible to thallium acetate than the other mycoplasmas.

IV. MYCOPLASMA AND HIV INFECTION

Mycoplasmas may cause more severe and prolonged infections in HIV infected and other immunodeficient states.

V. MYCOPLASMA AS CELL CULTURE CONTAMINANTS

Mycoplasmas are common contaminants of continuous cell cultures. The contamination may derive from the mouth of those handling the cells, from animal sera or trypsin used in cell culture. Contamination may interfere with growth of viruses in such cell cultures. These contaminant mycoplasmas have often been mistaken for viruses. Their eradication from infected cell cultures is difficult.

KEY POINTS

1. Mycoplasmas differ from other bacteria in that they *lack a rigid cell wall*. Due to lack of rigid cell wall, they are extremely pleomorphic.
2. They are resistant to cell wall antibiotics such as penicillins and cephalosporins. This is again due to absence of cell wall.
3. The members of the genus *Ureaplasma* can hydrolyse urea while other genera cannot do so. This is a differentiating feature of *Ureaplasma*.
4. Mycoplasmas are the *smallest* free living microorganisms. They can pass through bacterial filters. They are Gram negative, but are better stained by Giemsa stain. They were previously named as *pleuropneumonia like organisms* or *PPLO*.

5. All mycoplasmas except *Acholeplasma*, require sterol for their growth.
6. A medium widely used for the isolation of mycoplasmas is PPLO broth. This medium can be made solid by the addition of agar. Typical tiny *fried egg* colonies appear in the medium.
7. Mycoplasma causes two types of diseases in man—*pneumonia* and *genital infections*.
8. *Mycoplasma pneumoniae* causes *primary atypical pneumonia* (*mycoplasmal pneumonia*).
9. *Mycoplasma hominis* causes *non-gonococcal urethritis* (*NGU*). This has also been incriminated in postpartum sepsis, proctitis, acute salpingitis, pelvic inflammatory disease, cervicitis and vaginitis. It is transmitted by *sexual contact*.
10. Laboratory diagnosis of mycoplasmal infections may be carried out by isolation of the organism or by serological tests.
11. *Streptococcus MG* and *cold agglutination tests* are non-specific test used for detection of antibody in serum.
12. *Complement fixation test* and *enzyme immunoassay* (*EIA*) are specific tests to detect antibody against *M. pneumoniae*.
13. Tetracyclines and erythromycins are the drugs of choice.
14. Mycoplasmas may cause more severe and prolonged infections in HIV infected and other immunodeficient states.

YOU MUST KNOW

1. General characters of mycoplasma.
2. Morphology and culture characteristics of *Mycoplasma sp*.
3. Mycoplasmas and L-forms.
4. Diseases caused by different species of mycoplasma.
5. Serological tests used for diagnosis of mycoplasmal infections.
6. *Ureaplasma urealyticum*.

STUDY QUESTIONS

1. Classify mycoplasmas. Discuss the laboratory diagnosis of *Mycoplasma* infections.
2. Write short notes on:
   (a) General characters of *Mycoplasma*
   (b) L-forms
   (c) *Mycoplasma pneumoniae*
   (d) Streptococcus MG agglutination test
   (e) Cold agglutination test for *Mycoplasma* infection
   (f) *Ureaplasma urealyticum*
   (g) NGU.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Which of the following species of *Mycoplasma* is/are associated with human diseases?
   (a) *Mycoplasma pneumoniae* (b) *M. hominis*
   (c) *M. genitalium* (d) All of the above
2. Which of the following bacteria lack cell wall?
   (a) Helicobacter (b) Burkholderia
   (c) Mycoplasma (d) All of the above
3. Which of the following bacteria can synthesise carotenol?
   (a) Mycoplasma (b) Acholeplasma
   (c) Ureaplasma (d) Actinomycetes

4. Which of the following media is/are used for isolation of *Mycoplasma*?
   (a) PPLO broth
   (b) PPLO agar
   (c) Both of the above
   (d) None of the above
5. Which of the following bacteria may show fried egg colonies on culture medium?
   (a) *Mycoplasma*
   (b) *Helicobacter*
   (c) *Burkholderia*
   (d) *Bordetella*
6. Which of the following can hydrolyse urea?
   (a) *Mycoplasma*
   (b) *Acholeplasma*
   (c) *Ureaplasma*
   (d) None of the above
7. Dienes method is used to examine colonies of:
   (a) *Mycoplasma*
   (b) *Burkholderia*
   (c) *Helicobacter*
   (d) *Bordetella*
8. Which is the causative agent of primary atypical pneumonia?
   (a) *Mycoplasma pneumoniae*
   (b) *Streptococcus pneumoniae*
   (c) *Haemophilus influenzae*
   (d) *Influenza virus*
9. Which of the following bacteria is/are associated with non-gonococcal urethritis?
   (a) *Mycoplasma hominis*
   (b) *Ureaplasma urealyticum*
   (c) *Chlamydia trachomatis*
   (d) All of the above
10. Which of the following tests can be used to identify *Mycoplasma pneumoniae*?
    (a) Haemadsorption test
    (b) Tetrazolium reduction test
    (c) Inhibition of growth by specific antiserum
    (d) All of the above
11. Which of the following serological tests can be used for diagnosis of mycoplasma infections?
    (a) Streptococcus MG agglutination test
    (b) Cold agglutination test
    (c) Complement fixation test
    (d) All of the above
12. Which of the following bacteria is/are also named T strains?
    (a) *Ureaplasma urealyticum*
    (b) *Mycoplasma hominis*
    (c) *Mycoplasma pneumoniae*
    (d) *Acholeplasma*
13. Which of the following bacteria was named as 'Eaton agent'?
    (a) *Ureaplasma urealyticum*
    (b) *Mycoplasma hominis*
    (c) *Mycoplasma pneumoniae*
    (d) *Acholeplasma*
14. Which of the following antibiotics is most suitable for treatment of *Mycoplasma infections*?
    (a) Tetracyclines
    (b) Cephalosporins
    (c) Penicillins
    (d) Norfloxacin

Answers (MCQs):

1. (d)	2. (c)	3. (b)	4. (c)	5. (a)
6. (c)	7. (a)	8. (a)	9. (d)	10. (d)
11. (d)	12. (a)	13. (c)	14. (a)	

◆◆◆

Chapter 47

ACTINOMYCETES

Actinomycetes are Gram positive, filamentous bacteria intermediate in properties between true bacteria and fungi. Like bacteria, they possess cell wall containing muramic acid, prokaryotic nuclei and are susceptible to antibacterial antibiotics, whereas like fungi they form a mycelial network of branching filaments. Hence, the actinomycetes are true bacteria with a superficial resemblance to fungi. They are related to corynebacterium and mycobacterium. They are non-motile, non-sporing, non-capsulated filaments that break up into small bacterial fragments and live freely in nature, particularly in the soil.

The family *Actinomycetes* contains three major medically important genera, *Actinomyces, Nocardia* and *Actinomadura*. Another genus, *Streptomyces* rarely causes disease in man, but its medical importance lies in the production of antibiotics by its several species. In addition, inhalation of some thermophilic actinomycetes such as *Micropolyspora faeni* and *Thermoactinomyces sp.* may cause allergic alveolitis (farmer's lung and bagassosis). *Actinomyces* is anaerobic or microaerophilic and non-acid-fast, while *Nocardia* is acid-fast and aerobic. *Streptomyces* and *Actinomadura* are non-acid-fast and aerobic.

I. ACTINOMYCES

The actinomyces refers to ray like appearance (*Actinomyces*, meaning ray fungus) of the organism in the granules that characterise the lesions. Most of the *Actinomyces* are soil saprophytes and others are commensals of mouth of both man and animals. The *Actinomyces* causes a disease known as actinomycosis. In humans, it is usually caused by *A. israelii*.

A. Morphology

Actinomyces is a Gram positive, non-motile, non-sporing, non-acid-fast organism. They often grow in mycelial forms and break up into coccal and bacillary forms. Most of them show true branching (Fig. 47.1).

In tissue, the organisms look different from that of culture. The organisms appear in the pus as *granules*. When these granules are crushed between two slides and examined by Gram's staining, they consist of a central filamentous Gram positive mycelium surrounded by a peripheral zone of swollen radiating club shaped structures, presenting a *sun-ray appearance* (Fig. 47.2).

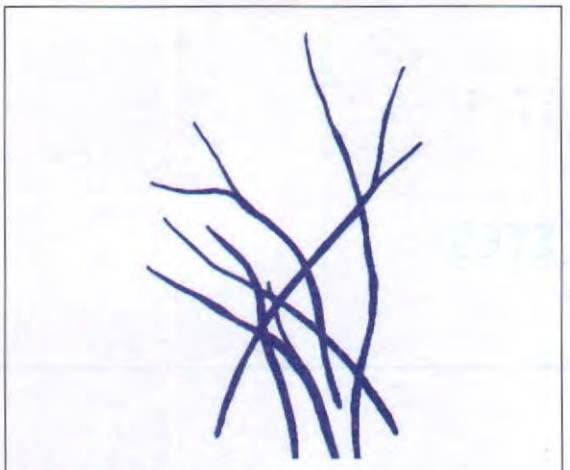


Fig. 47.1 Actinomyces

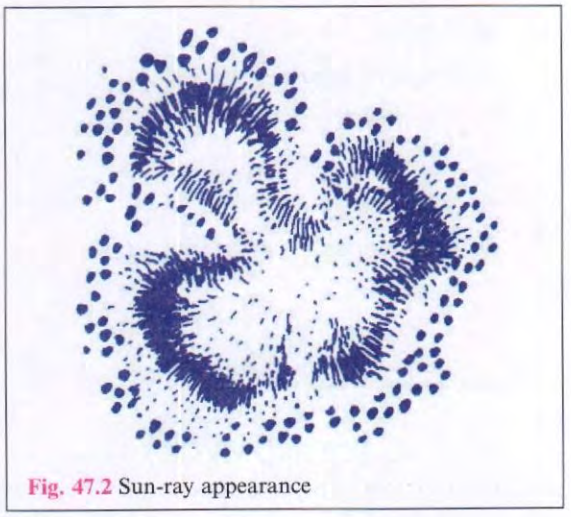


Fig. 47.2 Sun-ray appearance

These clubs are Gram negative, acid-fast and are of host origin.

The sulphur granules are white or yellowish in colour and found only in tissues. Their size varies from minute specks to about 5 mm.

B. Culture

They grow best under anaerobic or microaerophilic conditions at the optimum temperature of 37°C under 5–10% CO_2. They can be grown on brain heart infusion agar, blood agar or thioglycollate broth. Most species show good growth after 2–4 days, however, *A. israelii* may take 7–14 days. On solid media, *A. israelii* forms *spidery colonies* in 48–72 hours that become heaped up. In liquid medium, it grows as small fluffy balls below the surface of medium.

C. Pathogenesis

The actinomyces causes the disease known as *actinomycosis*. It is a chronic granulomatous disease characterised by multiple abscesses, tissue destruction, fibrosis and formation of multiple sinuses. *A. israelii* causes actinomycosis in man. The other species *A. naeslundii, A. meyeri, A. odontolyticum* and *A. viscosus* are very rare causes of actinomycosis and are associated with formation of dental plaque. Actinomyces has also been incriminated to cause gingivitis and periodontitis (inflammatory diseases of gums).

A. israelii may occur as commensals in mouth of normal individuals mainly around the teeth. The infection is mostly endogenous and may result from trauma e.g. dental extraction. Actinomycosis occurs in four clinical forms:

1. *Cervicofacial*: This is the commonest type and it occurs mainly in cheek and submaxillary regions.
2. *Thoracic*: It involves lungs.
3. *Abdominal*: It occurs usually in the ileocaecal region.
4. *Pelvic*: Pelvic actinomycosis has been reported in association with the use of intrauterine devices.

Macroscopically, it is a painless indurated swelling with multiple discharging sinuses. The pus contains usually yellow coloured sulphur granules. Actinomycosis may also present as mycetoma.

D. Epidemiology

The disease is not communicable. It is mostly endogenous infection. The disease is more frequently seen in rural areas. About 70% of actinomycosis are cervicofacial and only 20% are abdominal.

E. Laboratory Diagnosis

1. Specimens

- Pus from lesion or sinuses
- Discharge from fistula
- Sputum in pulmonary disease
- Tissue or biopsy

2. Microscopy

Pus is shaken along with some saline in a test tube and the mixture is allowed to settle. The sulphur granules sediment is withdrawn with a capillary pipette. Granules are crushed between two slides and smears are prepared. One smear is stained by Gram stain and other by acid-fast stain (decolourisation with 1% sulphuric acid). Gram staining shows a dense network of thin Gram positive filaments, surrounded by a peripheral radiating Gram negative 'clubs' presenting a *sunray appearance* (Fig. 47.2). Acid fast staining shows central part as non-acid-fast surrounded by acid-fast 'clubs'. In absence of sulphur

granules, Gram's staining of pus shows Gram positive branching filaments.

In tissue sections, sulphur granules and mycelia are detected by using fluorescein-conjugated specific antisera.

3. Culture

The sulphur granules or pus containing 'Actinomycetes' are washed and inoculated into thioglycollate broth and streaked on brain heart infusion agar (BHI agar) and blood agar. Cultures are incubated anaerobically and aerobically with 5% CO_2 at 37°C for at least 2 weeks. *A. israelii* produces *spider colonies* that resemble molar teeth. The isolate is identified by microscopy, biochemical reactions and fluorescent antibody methods.

4. Biopsy

Haematoxylin-eosin stained section shows mycelial mass surrounded by pus cells and chronic inflammatory cells.

F. Treatment

Surgical removal of affected tissue along with penicillin therapy is effective.

II. NOCARDIA

Nocardiae resemble Actinomyces morphologically but are strictly aerobic. They are non-motile, Gram positive bacteria. They are acid-fast when decolourised with 1% sulphuric acid. Majority of the species of Nocardia are environmental saprophytes; only a few are pathogenic to humans.

A. Species

The important species include:

1. *N. asteroides* so named due to its star shaped colonies.
2. *N. brasiliensis.*
3. *N. caviae.*

N. madurae is now known as *Actinomadura madurae.* It is non-acid-fast.

B. Morphology

Nocardiae are Gram positive bacteria and form a mycelium, that fragments into rod shaped and coccoid elements. *Nocardia* resembles *Actinomyces*, but some species are acid-fast, and a few are non-acid-fast. Differentiating features of *Actinomyces* and *Nacardia* are shown in Table 47.1.

Table 47.1 Differentiating Features of Actinomyces and Nocardia

Property	Actinomyces spp.	Nocardia spp.
O_2 requirement	Anaerobic or microaerophilic	Strict aerobe
Temperature range for growth	35–37°C	Wide range
Habitat	Oral commensals	Saprophytes of environment
Acid-fastness	Non-acid-fast	Weakly acid-fast
Mode of infection	Endogenous	Exogenous

C. Culture

Nocardiae readily grow in ordinary media. They are strict aerobes. They are slow growing (require 5–14 days). They can be grown on nutrient agar, Sabouraud dextrose agar (SDA) and brain heart infusion agar (BHI agar). The culture plates should be incubated at 36°C for up to 3 weeks. They can grow at wide range of temperature. Nocardiae form dry, granular, wrinkled colonies with pigmentation (white, yellow, pink or red).

D. Pathogenesis

Nocardiae produce opportunistic pulmonary disease known as *nocardiosis* in immunocompromised individuals including those with AIDS. Soil is known to be natural habitat of *Nocardia*. It is, therefore, believed that man acquires infection by inhalation of the bacteria from environmental sources. It causes systemic nocardiosis which manifests primarily as pulmonary disease, pneumonia, lung abscess or other lesions resembling tuberculosis. Metastasis may involve the brain, kidneys and other organs. *N. asteroides* and *N. brasiliensis* are the main opportunistic agents.

It may also cause mycetoma. It is a chronic granulomatous lesion involving the subcutaneous and deeper tissues mainly localised in the region of foot. The disease is worldwide but common in tropical countries (Sudan, North Africa and West-Coast of India). The disease occurs among bare footed persons who are prone to contamination by soil derived organisms. It presents as a tumour with multiple sinuses. The pus contains granules which are larger than those of actinomycosis. It came to be known as *Madura foot* or *Madura disease*. It is mainly caused by *Actinomadura madurae* but *N. brasiliensis* is also responsible for some cases.

E. Laboratory Diagnosis

1. Specimens

Pus or purulent sputum

2. *Microscopy*

The smears are stained with Gram staining and Ziehl-Neelsen (ZN) technique using decolourisation with 1% sulphuric acid. Gram positive filamentous bacteria can be seen on Gram staining. Acid-fast bacilli are detected on ZN technique though some species are non-acid-fast.

3. *Culture*

The specimens are inoculated on nutrient agar, SDA and BHI agar and incubated at 36°C for 3 weeks. Colony morphology is seen and bacteria are identified by staining. Nocardia can also be isolated by paraffin bait technique. The specimen is homogenised and 2 ml of it is inoculated into carbon-free broth containing paraffin coated glass rod. The organisms grow on the rod at the air-liquid surface which may be subcultured onto solid media for identification.

F. Treatment

Cotrimoxazole is given for 3 months or more. Nocardiae are also susceptible to nalidixic acid, amikacin, tobramycin and vancomycin.

III. MYCETOMA

Mycetoma is a localised, chronic granulomatous disease of the subcutaneous and deeper tissues affecting commonly the foot and presenting as a tumour with multiple discharging sinuses. This clinical syndrome was first described from Madura, India and came to be known as *Maduramycosis*. Mycetomas may be of fungal origin or may be caused by bacteria. Bacterial mycetomas are usually caused by *Actinomyces* (*A. israelii, A. bovis*), *Nocardia* (*N. asteroides, N. brasiliensis, N. caviae*), *Actinomadura* (*A. madurae, A. pelletierii*) *Streptomyces* (*S. somaliensis*).

Filamentous fungi responsible for mycotic mycetoma are *Madurella mycetomi* and *Madurella griseus*. Mycetoma like lesion (*botryomycosis*) is occasionally produced by *Staph. aureus*.

Diagnosis of mycetomas is important in choosing appropriate treatment. The colour of granules gives some clue to diagnosis. The granules are white to yellow in actinomycotic mycetoma, while in mycotic mycetomas the granules are generally black. In actinomycotic mycetoma the filaments are thin (about 1 μm) but in the mycotic mycetoma they are stout (4–5 μm). Isolation of organism in culture establishes the diagnosis.

KEY POINTS

1. Actinomycetes are Gram positive, filamentous bacteria intermediate in properties between true bacteria and fungi.
2. The family *Actinomycetes* contains three major medically important genera, *Actinomyces*, *Nocardia* and *Actinomadura*.
3. Actinomyces is a Gram positive, non-motile, non-sporing, non-acid-fast organism. They often grow in *mycelial forms*. Most of them show true branching.
4. In the pus, the organisms appear as *granules*. When these granules are crushed between two slides and examined by Gram's staining, they present as a *sun-ray appcarance*.
5. The *Actinomyces* causes a disease known as actinomycosis. The disease occurs in four clinical forms : *cervicofacial*, *thoracic*, *abdominal* and *pelvic* actinomycosis.
6. *Nocardiae* resemble actinomyces morphologically but are acid-fast when decolourised with 1% sulphuric acid. They produce opportunistic pulmonary disease known as *nocardiosis* in immunocompromised individuals. It may also cause *mycetoma*.
7. Mycetoma is a localised chronic granulomatous disease of the subcutaneous and deeper tissues affecting commonly the foot and presenting as a tumour with *multiple discharging sinuses*.

YOU MUST KNOW

1. Morphology of *Actinomyces*.
2. Diseases caused by *Actinomyces* and their laboratory diagnosis.
3. Different species of *Nocardia*.
4. Differences between *Actinomyces sp.* and *Nocardia sp.*
5. Mycetoma.

STUDY QUESTIONS

1. Write short notes on:
   (a) Actinomycosis
   (b) Laboratory diagnosis of actinomycosis
   (c) Nocardia
   (d) Mycetoma.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. 'Sun-ray appearance' morphology is characteristic of:
   (a) *Actinomyces*
   (b) *Nocardia*
   (c) *Streptomyces*
   (d) *Actinomadura*
2. Actinomycosis may involve:
   (a) Cervicofacial region
   (b) Thoracic region
   (c) Abdominal region
   (d) All of the above
3. Which of the following bacteria is acid-fast?
   (a) *Actinomyces*
   (b) *Actinomadura*
   (c) *Streptomyces*
   (d) *Nocardia*
4. Which of the following organs is the most commonly involved in nocardiosis?
   (a) Lung
   (b) Skin
   (c) Spleen
   (d) Bone

Answers (MCQs):

1. (a) 2. (d) 3. (d) 4. (a)

Chapter 48

MISCELLANEOUS BACTERIA

I. LISTERIA

A. Morphology

The organisms of the genus *Listeria* are small, Gram positive coccobacilli or rod shaped measuring 1–3 μm × 0.5 μm. They are motile at 25°C showing tumbling motility but are non-motile at 37°C. They produce peritrichous flagella optimally at 20–30°C, but at 37°C they produce scanty or no flagella at all. They are non-sporing, non-capsulated and non-acid-fast. They may resemble the morphology of diptheroids.

B. Culture

Listeriae can grow at wide range of temperature, 4°C–45°C, but the optimum temperature for the growth is 37°C. They are aerobic or microaerophilic but better growth is obtained when cultures are incubated in presence of 5–10% CO_2. They grow on ordinary media but growth is improved by the addition of glucose, blood or liver extract. Colonies are 1–2 mm in diameter, round, smooth, translucent, easily emulsifiable and non-pigmented. On blood agar, colonies are surrounded by a narrow zone of β–haemolysis.

Instillation of bacteria into eyes of rabbits produces keratoconjunctivitis (*Anton test*).

C. Biochemical Reactions

Listeriae produce acid from glucose, salicin and aesculin. They are catalase positive but oxidase, urease and indole negative. H_2S is not produced by these organisms.

D. Various Species

Seven species of Listeria are recognised, which are differentiated by a number of biochemical tests (Table 48.1). Two major tests of differentiation of various species include D–xylose fermentation and CAMP test with *Staph. aureus* and *Rhodococcus equi*. *L. monocytogenes* gives a positive CAMP test with *Staph. aureus*.

E. Pathogenesis

All the seven species may cause disease in animals. Only *L. monocytogenes* and very rarely *L. ivanovii* are likely to be associated with human disease. Experimental inoculation in rabbits causes a marked monocytosis (hence the name *monocytogenes*). *L. monocytogenes* has been isolated from uncooked meat, milk and cheese. The bacteria are able to grow in refrigerated foods. Human infections result from contact with infected animals, inhalation of contaminated dust or ingestion of contaminated milk or food. Hospital-acquired infections have also been reported. The haemolysin produced by *L. monocytogenes* is known as *listeriolysin*. It is antigenically similar to streptolysin-O. It is an important virulence factor of *L. monocytogenes*. Infections caused by *L. monocytogenes* can be divided into following groups:

1. Neonatal Infections

Infection from pregnant woman to foetus is transmitted either transplacentally or intrapartum. It may lead to abortion or stillbirth or neonatal disease. Meningitis or septicaemia may occur in neonates.

2. Adult Infections

Meningitis accounts for the most cases of listeriosis. Other forms of listeriosis include encephalitis, brain abscess, spinal abscess, septicaemia and endocarditis. These infections are more common in immunocompromised individuals and in persons with damaged valves. Butchers, poultry workers and veterinarians may develop cutaneous infections due to the direct contact with infected animals or birds.

Table 48.1 Differentiation of the Species of Listeria

Species	Haemo–lysis	CAMP test with		Nitrate reduction	Acid from			
		Staph. aureus	Rhodococcus equi		D-xylose	D-mannitol	α-methyl-D mannoside	L-rhamnose
L. monocytogenes	+	+	–	–	–	–	+	+
L. ivanovii	+	–	+	–	+	–	–	–
L. innocua	–	–	–	–	–	–	+	V
L. welshimeri	–	–	–	–	+	–	+	V
L. seeligeri	+	+	–	–	+	–	V	–
L. grayi	–	–	–	–	–	+	NK	–
L. marthii	–	NK	NK	–	–	–	NK	–

+, positive reaction; –, negative reaction; V, Variable; NK, Not known.

3. Mild or Inapparent Infections

Asymptomatic carriage of listeria is common in general population. Carriage rates of *Listeria* in faeces and genital tract varies from 5–10%. Asymptomatic infection of the female genital tract may cause infertility.

F. Laboratory Diagnosis

1. Specimens

(*i*) Blood
(*ii*) CSF
(*iii*) Amniotic fluid
(*vi*) Pus
(*v*) Swabs from cervical and vaginal secretions
(*vi*) Meconium
(*vii*) Cord blood

2. Direct Microscopy

In Gram stained smears of CSF sediments, the organisms may be seen as Gram positive coccobacilli.

Leucocytes in CSF are raised, of which 40–60% may be lymphocytes. Protein content of CSF is raised and glucose is slightly lowered.

3. Culture

Specimens should be inoculated on

(*i*) blood agar
(*ii*) chocolate agar.

These specimens may also be added to nutrient broth and incubated at 35–37°C for 5 days followed by subculture on solid media.

Blood agar shows small colonies surrounded by a narrow zone of β-haemolysis. The bacteria are actively motile when grown at 25°C. The isolate is identified by its morphology and biochemical tests (Table 48.1).

G. Treatment

Ampicillin or amoxycillin and gentamicin are drugs of choice. Co-trimoxazole may also be used.

II. ERYSIPELOTHRIX RHUSIOPATHIAE

A. Morphology

Erysipelothrix rhusiopathiae is a slender, Gram positive, non-motile, non-sporing, non-capsulated bacillus with tendency towards formation of long filaments.

B. Culture

It is aerobic and facultative anaerobe but growth is improved with 5–10% CO_2. It can grow on ordinary media. Addition of glucose, serum or blood in media improves the growth. The optimum temperature for growth is 30–37°C. Colonies on blood agar are small, circular, convex, translucent and surrounded by a zone of α–haemolysis.

C. Biochemical Reactions

It ferments glucose, lactose, fructose, maltose and galactose with the production of acid only. It is catalase, nitrate reduction, indole production and urease negative. It produces H_2S.

D. Pathogenesis

E. rhusiopathiae causes erysipelas in animals especially swine and sheep. In humans, it causes a cutaneous inflammatory disease known as *erysipeloid*. It is an occupational hazard of meat and fish handlers and veterinary surgeons. The organisms are believed to enter the skin through minor abrasions. Rarely septicaemia and endocarditis may develop.

E. Laboratory Diagnosis

1. Specimen

Skin biopsy from the advancing edge of a lesion is the material of choice.

2. Culture

Specimen is inoculated into glucose broth and incubated aerobically under 5–10% CO_2 at 37°C. Subcultures are made on blood agar. Blood culture should be done in suspected cases of endocarditis and septicaemia. Colony morphology, staining and biochemical reactions confirm the identification.

F. Treatment

Penicillin G is the drug of choice. It is also highly susceptible to ampicillin, ciprofloxacin, piperacillin, methicillin and clindamycin.

III. STREPTOBACILLUS MONILIFORMIS

A. Morphology

Streptobacillus moniliformis is Gram negative, non-motile, non-capsulated and highly pleomorphic short bacillus 1–3 μm in length, forming chains appearing as filaments with club-shaped (moniliform) swellings. It may lose its cell wall and exist as an L-form. In fact, it was during the study of this bacillus, that L-forms were originally discovered.

B. Habitat

S. moniliformis is a normal inhabitant of the nasopharynx of rats.

C. Culture

It is aerobic and facultative anaerobic. Growth is enhanced by addition of 5–10% CO_2. Optimum temperature for growth is 37°C. It can grow on culture media containing blood, serum or ascitic fluid. It grows well on Loeffler's serum slope.

Colonies appear on the surface after 48 hour incubation and are discrete granular and greyish yellow. L phase variants show minute colonies (0.1–0.2 mm diameter) with a *fried egg* appearance.

L phase variants have little or no virulence for laboratory animals.

D. Biochemical Reactions

The organism is biochemically inactive except for acid from glucose.

E. Pathogenesis

S. moniliformis causes rat-bite fever in man (the other variety of rat-bite fever is caused by a spiral organism, *Spirillum minus*). In man the organisms enter the body through the wound caused by the bite of rat. When the infection is acquired by ingestion of food, milk or water contaminated with rat excreta, it is known as *Haverhill fever*, which is characterised by fever, polyarthritis and petechial rash.

F. Laboratory Diagnosis

1. Specimens

(i) Blood—During acute phase of disease.
(ii) Joint fluid—When arthritis develops.

2. Culture

Specimen is inoculated on blood agar or Loeffler's serum slope.

3. Animal Pathogenicity Test

When specimen or culture is inoculated intraperitoneally in a mouse, the animal develops a rapidly fatal generalised condition or a progressive disease with swelling of feet and legs.

4. Serology

Agglutination, complement fixation and fluorescent antibody tests are used for detection of antibody in serum.

G. Treatment

Penicillin is effective for most of the cases, the L phase variants are penicillin resistant but sensitive to tetracycline. *S. moniliformis* is also sensitive to erythromycin, clindamycin, tetracycline and aminoglycosides.

IV. SPIRILLUM MINUS

Rat bite fever is caused by *Streptobacillus moniliformis* and *Spirillum minus*. *S. minus* is of uncertain taxonomic position.

A. Morphology

S. minus is a spiral, Gram negative organism measuring 2–5 μm in length and 0.2 μm in diameter. It is very actively motile showing darting motility. The motility is due to polar flagella (1 to 7 flagella at each pole). The organisms can be demonstrated with the dark ground microscopy or by staining with Leishman or Giemsa stain.

B. Culture

S. minus has not been grown in artificial media and is best isolated by intraperitoneal inoculation of specimens (infected tissue or blood) into mice and guinea pigs.

C. Pathogenesis

S. minus is a natural parasite of wild rats and other rodents. The organism is inoculated into humans through the bite of a rat. It causes regional lymph node swellings near the site of bite, skin rashes and fever of relapsing type. Rat-bite fever in Japan is known as *Soduku*.

D. Laboratory Diagnosis

S. minus can be isolated by intraperitoneal inoculation of blood or material from lymph node into mice and guinea pigs. After 1–3 weeks, small number of spirilla may be demonstrated by dark field microscopy in blood and peritoneal fluid of animal. Occasionally, *S. minus* may be demonstrated directly by dark ground microscopy of material from the local lesion, regional lymph node or blood of the patient.

E. Treatment

Penicillin and tetracyclines are effective drugs.

V. CHROMOBACTERIUM VIOLACEUM

Chromobacterium violaceum is a Gram negative, non-sporing bacillus, motile by means of polar and lateral flagella. It occurs singly or in pairs. It is an aerobe and facultative anaerobe. It can grow on ordinary media at 37°C, producing a violet pigment soluble in ethanol and insoluble in chloroform and water. It is catalase and oxidase positive. It is a saprophyte found in water and soil. It may cause septicaemic illness with pneumonia, skin lesions with pyaemia and multiple abscesses. It is sensitive to erythromycin, tetracycline, co-trimoxazole and carbenicillin.

VI. FLAVOBACTERIUM MENINGOSEPTICUM

Flavobacterium meningosepticum is a Gram negative, non-motile, non-sporing small bacillus. It can grow on nutrient agar at 37°C. It forms smooth, circular colonies measuring 1–2 mm in diameter. It forms a yellow non-diffusible pigment. It grows poorly or not at all on MacConkey agar. It is catalase and oxidase positive. It is ubiquitous saprophyte capable of causing opportunistic infections. It may cause neonatal meningitis. In adults, it may cause pneumonia in immunosuppressed patients. Cotrimoxazole, rifampicin, novobiocin and clindamycin are effective in treatment.

VII. KLEBSIELLA GRANULOMATIS

(Calymmatobacterium granulomatis, Donovania granulomatis)

A. Morphology

Klebsiella granulomatis is a small, capsulated, Gram negative, non-motile, coccobacillus measuring 1–2 μm × 0.5 – 1.5 μm. It has a bipolar condensation of chromatin giving rise to *safety pin* appearance. It is non-sporing, non-flagellate and non-acid-fast. It was formerly named as *Calymmatobacterium granulomatis* It is found to be morphologically and antigenically related to klebsiellae. Hence, it has been designated as *Klebsiella granulomatis* under *genus Klebsiella*. In the tissues, it can be best stained by Wright's stain appearing as blue bacillus surrounded by pink capsule. They are present within the phagosomes of large macrophages and are known as *Donovan bodies*. These are sometimes seen inside polymorphs or lying free outside the cells.

B. Culture

It can be grown on egg-yolk medium and in the yolk-sac of embryonated hen's eggs.

C. Pathogenesis

Pathogenicity is limited to man. The incubation period ranges from a few days to three months. It causes a chronic granulomatous disease known as *granuloma inguinale*, *granulomatous venereum* or *donovanosis*. Transmission is by sexual contact. The initial subcutaneous nodule breaks down to give painless genital ulcers unlike ulcers of chancroid which are painful.

D. Treatment

Donovanosis can be treated with tetracycline, chloramphenicol, erythromycin, streptomycin or co-trimoxazole.

VIII. EIKENELLA CORRODENS

A. Morphology

Eikenella corrodens is a fastidious, small Gram negative bacillus. It is non-sporing, non-capsulated and non-acid fast. It lacks flagella and shows *twitching* motility which is due to contractile fimbria-like filamentous appendages.

B. Culture

It is an aerobe and facultative anaerobe. It can grow on blood agar or chocolate agar. Optimum temperature for growth is 37°C. Growth is improved by 5–10% CO_2 and moist atmosphere. Colonies are small (0.5–1 mm in diameter) with characteristic pitting or corroding of blood agar, hence the name *corrodens*.

C. Biochemical Reactions

It is oxidase positive and catalase negative. It does not produce acid from carbohydrates. It is indole and urease negative but lysine and ornithine decarboxylase positive.

D. Pathogenesis

Eikenella corrodens is a normal inhabitant of the mouth, upper respiratory tract and gastrointestinal tract of humans. It occasionally causes opportunistic infections such as dental and periodontal infections, otitis media, sinusitis, pneumonia, lung abscess, meningitis, endocarditis and osteomyelitis.

E. Treatment

It is sensitive to ampicillin, tetracycline, chloramphenicol and rifampicin.

IX. CARDIOBACTERIUM HOMINIS

Cardiobacterium hominis is a commensal in the human nose and throat and is a rare cause of endocarditis, particularly in those with preexisting cardiovascular disease. It is a Gram negative, non-motile, pleomorphic bacillus. It is fastidious aerobe and facultative anaerobe. It grows on blood agar under 3–5% CO_2 and high humidity. It is oxidase positive and catalase negative. It ferments a wide range of sugars, forms indole and is nitrate reduction test negative. It is sensitive to penicillin, chloramphenicol, erythromycin and streptomycin.

X. ALCALIGENES FAECALIS

These are Gram negative, short, non-sporing bacilli, which are strict aerobes and do not ferment sugars. They are motile by means of peritrichous flagella. They are catalase and oxidase positive. They can grow on MacConkey agar.

Alc. faecalis is a saprophyte found in water and soil. They are also commensals in the intestines of humans. They have been considered responsible for urinary tract infections, infantile gastroenteritis and suppurative lesions.

XI. ACINETOBACTER

(Mima polymorpha; Bacterium anitratum)

These bacilli are strict aerobes and grow well on ordinary media. They are Gram negative encapsulated, pleomorphic rods. They are oxidase negative. They were previously named 'Mimeae' due to their mimicking neisseriae in appearing as Gram negative diplococci and causing urethritis and conjunctivitis.

The two most common species of *Acinetobacter* include *A. baumannii* (formerly known as *A. calcoaceticus var anitratus*) and *A. lwoffi* (formerly known as *A. calcoaceticus var lwoffi*). Both species are saprophyte of soil and sewage. They are also commensal of skin of groin and axilla of man.

A. baumannii: On MacConkey's medium, they form pinkish colonies. Acid without gas is produced from glucose, xylose and arabinose. A characteristic reaction is the production of acid in 10%, but not in 1% lactose.

A. lwoffi: They form yellow colonies on MacConkey's medium. They do not produce acid from sugars. Some strains are oxidase positive.

Acinetobacter species are opportunistic pathogens and are often found in hospital infections, particularly in iatrogenic meningitis. They may also cause pneumonia and infections of respiratory tract. All strains are resistant to penicillin. Penicillinase is probably produced. Most strains are sensitive to broad spectrum antibiotics.

XII. KINGELLA

Members of the genus *Kingella* are Gram negative bacilli with a tendency to occur as coccobacillary forms. They are non-motile but may demonstrate a 'twitching' motility. They are oxidase positive, catalase negative and ferments glucose and other sugars. They are part of normal oral and genital flora of humans. *K. kingae* has been associated with endocarditis, bacteraemia and infections of bone and joints.

XIII. AGGREGATIBACTER ACTINOMYCETEMCOMITANS

Actinobacillus actinomycetemcomitans has now been included under new genus *Aggregatibacter* as *Aggregatibacter actinomycetemcomitans*. It is a small, Gram negative, non-motile coccobacillus. It is a part of normal flora of oral cavity. It can grow on blood agar and chocolate agar but requires increased CO_2 for its growth. It does not grow on MacConkey agar. It is catalase positive and ferments glucose but not lactose and sucrose.

It may cause subacute bacterial endocarditis, periodontal disease, abscesses and osteomyelitis. It is sensitive to aminoglycosides, quinolones, rifampicin and tetracycline.

Two former *Haemophilus* species *H. aphrophilus* and *H. paraphrophilus* have been reclassified together as one species *A. aphrophilus* in this new genus *Aggregatibacter*.

KEY POINTS

1. The organisms of the genus *Listeria* are small, Gram positive coccobacilli or rod shaped. They are motile at 25 °C showing *tumbling motility* but are *non-motile* at *37 °C*. They may resemble the morphology of diphtheroids.
2. *Listeria monocytogenes* is the important species for human diseases. Infections caused by this species include *neonatal infections, meningitis, encephalitis, brain abscess, septicaemia* and *endocarditis*. Asymptomatic infection of the female genital tract may cause infertility.
3. *Streptobacillus moniliformis* causes rat-bite fever in man (the other variety of rat-bite fever is caused by a spiral organism, *Spirillum minus*).
4. *Calymmatobacterium granulomatis* has been designated as *Klebsiella granulomatis* under genus *Klebsiella*. It causes a chronic granulomatous disease known as *granuloma inguinale, granulomatous venereum* or *donovanosis*. Transmission of the disease is by *sexual contact*.

YOU MUST KNOW

1. Morphology and culture characteristics of *Listeria*.
2. Infections caused by *Listeria monocytogenes* and their laboratory diagnosis.

3. Rat-bite fever.
4. *Klebsiella granulomatis* and disease caused by it.
5. *Acinetobacter sp.*

STUDY QUESTIONS

Q. Write briefly about:
(a) *Listeria monocytogenes*
(g) *Acinetobacter.*
(f) *Alcaligenes faecalis*
(d) *Calymmatobacterium granulomatis*
(c) Rat-bite fever
(e) *Eikenella*
(b) *Erysipelothrix rhusiopathiae*

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Tumbling motility is characteristic of:
(a) *Listeria monocytogenes*
(b) *Proteus vulgaris*
(c) *Enterobacter cloacae*
(d) *Hafnia alvei*

2. Anton test is associated with:
(a) *Listeria monocytogenes*
(b) *Proteus vulgaris*
(c) *Enterobacter cloacae*
(d) *Hafnia alvei*

3. In adults, listeriosis may lead to:
(a) Meningitis
(b) Encephalitis
(c) Brain abscess
(d) All of the above

4. Which of the following bacteria can cause rat bite fever?
(a) *Streptobacillus moniliformis*
(b) *Listeria monocytogenes*
(c) *Chromobacterium violaceum*
(d) *Flavobacterium meningosepticum*

5. Rat bite fever may be caused by:
(a) *Streptobacillus moniliformis*
(b) *Spirillum minus*
(c) Both of the above
(d) None of the above

6. What is the causative agent of donovanosis?
(a) *Klebsiella pneumoniae*
(b) *Klebsiella oxytoca*
(c) *Klebsiella granulomatis*
(d) None of the above

7. Which of the following bacteria does not produce pigment?
(a) *Staphylococcus aureus*
(b) *Chromobacterium violaceum*
(c) *Flavobacterium meningosepticum*
(d) *Listeria monocytogenes*

Answers (MCQs):

1. (a) 2. (a) 3. (d) 4. (a) 5. (c)
6. (c) 7. (d)

Chapter 49

RICKETTSIA, ORIENTIA, COXIELLA, EHRLICHIA, BARTONELLA

I. INTRODUCTION

The family *Rickettsiaceae* contains small coccobacillary organisms which cause typhus fever and related diseases. The *Rickettsiae* were formerly considered closely related to viruses because of their obligate intracellular parasitism and inability to grow in cell free cultures. It is now clear that they are true bacteria adapted to obligate intracellular parasitism.

Characters Similar to Bacteria

1. They are Gram negative pleomorphic rods dividing by binary fission.
2. Cell wall contains muramic acid.
3. They contain metabolic enzymes independent of the host cell.
4. They have both DNA and RNA.
5. They are large enough to be seen under the light microscope.
6. They are susceptible to antibiotics.
7. They are held back by bacterial filters.

The *Rickettsiae* are primarily parasitic in arthropods such as fleas, lice, ticks and mites. The name rickettsia has been given in honour of *Ricketts* who first observed the organism in Rocky Mountain spotted fever (1906). He died of typhus fever contracted during the course of his work.

II. CLASSIFICATION

The family *Rickettsiaceae* consists of two genera, *Rickettsia* and *Orientia* (Table 49.1).

Rickettsia contains the agents causing typhus fevers and spotted fevers. Scrub typhus is caused by *Orientia tsutsugamushi*. Coxiella and Ehrlichia are now included in family *Coxiellaceae* and family *Anaplasmataceae* respectively. Previously both these genera belonged to family *Rickettsiaceae*.

III. RICKETTSIA

A. Morphology

Rickettsiae are pleomorphic, coccobacilli, 0.3–0.6 μm × 0.8–2.0 μm in size, non-motile, non-capsulated and Gram negative, though they do not take the stain well. The organisms stain bluish purple with Giemsa and Castaneda stains and deep red with Machiavello and Gimenez stains.

Electron microscopy of the rickettsiae has revealed a three-layered cell wall and trilaminar plasma membrane thus resembling Gram negative bacteria. *R. prowazekii* has an amorphous capsule.

B. Culture

Rickettsiae are unable to grow in cell free media They are cultivated in yolk sac of chick embryo. The optimum temperature for growth is 32–35°C. They can also grow on HeLa, mouse fibroblast, HEp 2, Detroit 6 and other continuous cell lines. The tissue cultures are not satisfactory for primary isolation from patient. Growth generally occurs in the cytoplasm of infected cells, but in the case of spotted fever rickettsiae, growth may take place in nucleus as well. They may also be propagated in arthropods.

C. Animal Inoculation

Laboratory animals like mice and guinea pigs are useful for primary isolation of rickettsiae from clinical materials.

D. Resistance

Rickettsiae are destroyed at 56°C and at room temperature when separated from host components, unless preserved in appropriate medium (skimmed milk). They become non-infectious with 1% lysol, 2% formaldehyde, 5% hydrogen peroxide, 70% ethanol and hypochlorite solution. They are susceptible to tetracycline and chloramphenicol.

E. Antigenic Structure

Rickettsiae possess at least three types of antigens:

1. *Group specific soluble antigen*: It is present on the surface of rickettsiae.
2. *Species specific antigen*: It is associated with the bodies of rickettsiae. In case of scrub typhus, it is strain specific.
3. *Alkali-stable polysaccharide antigen*: It is found in some rickettsiae and is shared by certain strains of *Proteus*. The sharing of antigens between rickettsiae and *Proteus* forms the basis of *Weil-Felix reaction* used for the diagnosis of rickettsial infections.

F. Pathogenesis

Rickettsiae are generally transmitted to humans by the bite or by the faeces of an infected arthropod vector. On entry into the human body, they multiply locally and enter the blood stream. The organisms become localised in the vascular endothelial cells, which enlarge, degenerate and cause thrombosis of the vessels leading to rupture and necrosis.

Rickettsiae may have two types of toxicity. They possess an endotoxin which kills mice and this endotoxic activity is specifically neutralised by antiserum. The second toxicity is haemolysis of sheep and rabbit red blood cells. However, the role of endotoxin and haemolytic activity in human disease remains unclear.

Diseases caused by various species of the genus *Rickettsia* include typhus fevers and spotted fevers.

Table 49.1 Organisms of the Family Rickettsiaceae

Genus	Species	Disease	Insect vector
Rickettsia	*R. prowazekii*	Epidemic typhus, Brill-Zinsser disease	Human body louse
	R. typhi (R. mooseri)	Endemic typus	Rat flea
	R. rickettsii	Rocky-Mountain spotted fever	Ixodid ticks
	R. conori	Boutonneuse fever	Ixodid ticks
	R. australis	Australian tick typhus	Ixodid ticks
	R. siberica	Siberian tick typhus	Ixodid ticks
	R. akari	Rickettsial pox	Mites
Orientia	*O. tsutsugamushi*	Scrub typhus	Trombiculid mites

1. Typhus Fever Group

This group consists of epidemic typhus and its recrudescent infection (Brill-Zinsser disease) and endemic (murine) typhus.

(i) Epidemic typhus (Classical typhus)

The louse-borne epidemic typhus is the most important of all rickettsial infections. The causative organism is *R. prowazekii*. Human body louse (*Pediculus humanus corporis*) is the vector. The head louse (*Pediculus humanus capitis*) may also transmit the infection, but not the pubic louse. The lice become infected by ingesting the blood from a rickettsiaemic patient. The organisms multiply in its gut and appear in the faeces after 3 to 5 days. Lice succumb to the infection within 2–4 weeks, remaining infective till they die. Lice defecate while feeding and scratches by the host produce minute abrasions which act as portal of entry for the rickettsiae present in the louse faeces. The incubation period ranges from 5–21 days.

Patient develops severe headache, chills, generalised myalgia, high fever (39–41°C) and vomiting. A macular rash appears 4 to 7 days after the onset of illness, first on the trunk and then spreads to the limbs. During the second week of illness, the patient develops cloudy state of consciousness (*typhos*, meaning smoke or cloud). The disease occurs mainly in Eastern Europe, Northern and Eastern Africa. In India, the disease occurs in Kashmir. Mortality rate varies from 20 to 30% in untreated cases.

(ii) Brill-Zinsser disease (Recrudescent typhus)

In some who recover from epidemic typhus, the rickettsiae may remain latent in the lymphatic tissues or organs for years. Such latent infections may be reactivated leading to recrudescent typhus (Brill-Zinsser disease). *Brill* (1898) noticed the disease and *Zinsser* (1934) isolated *R. prowazekii* from such cases and proved that they were recrudescences of infections acquired many years ago. Lice that feed on such patients can become infective and if conditions are favourable for louse-human-louse transmission, epidemics of typhus fever may occur. It explains the manner in which the rickettsia is able to survive without extrahuman reservoirs.

Brill-Zinsser disease is a milder illness and the duration of the disease is shorter. Case fatality is lower.

(iii) Endemic typhus (Murine typhus)

The disease is caused by *R. mooseri* (*R. typhi*). It is a milder disease than epidemic typhus. Rat acts as a reservoir. Vector is rat flea (*Xenopsylla cheopis*). Infection is transmitted in rats by rat flea. The rickettsiae multiply in the gut of rat flea and are shed in its faeces. The flea is unaffected, but remains infectious for the rest of its life. Unlike epidemic typhus, man is not in the normal cycle of the disease as the disease occurs in rats. Man acquires the disease accidentally through the bite of infected fleas or by recently contaminated food with infected rat urine or flea faeces. Human infection is a dead end as man to man transmission does not occur. The disease is world-wide in distribution but is significant in Mexico. In Kashmir and China, lice have been known to transmit murine typhus in man. *R. mooseri* infection gives immunity to both endemic and epidemic typhus.

R. mooseri can be differentiated from *R. prowazekii* by the following tests:

(1) *Neil-Mooser or tunica reaction*: When male guinea pigs are injected intraperitoneally with blood from a case of endemic typhus or with a culture of *R. mooseri*, they develop fever and a characteristic scrotal inflammation. The scrotum becomes enlarged and the testes cannot be pushed back into the abdomen because of the adhesions due to inflammation between the layers of tunica vaginalis. This is known as *Neil-Mooser* or *tunica reaction*. This reaction is negative in epidemic typhus (*R. prowazekii*).

(2) The two rickettsiae can also be differentiated by IFA, ELISA and PCR.

2. Spotted Fever Group

Rickettsiae of this group possess a common soluble antigen and multiply in both the nucleus and the cytoplasm of host cells. They are all transmitted by ticks, except *R. akari*, in which mite is the vector. Six species have been recognised in this group. The diseases of this group include Rocky Mountain spotted fever (RMSF), boutonneuse fever (Indian, Mediterranean, Kenyan and South African tick typhus), Australian tick typhus (Queensland tick typhus), Siberian tick typhus and rickettsial pox. *R. rickettsii, R. conori, R. australis, R. siberica* and *R. akari* are responsible for these diseases. Another species *R. parkeri* causes infection in guinea pigs, but has not been identified as a human pathogen.

(i) Rocky Mountain spotted fever (RMSF)

It is the most serious type of spotted fevers. Causative agent is *R. rickettsii*. The incubation period is about one week. Clinical picture is similar to that of typhus fever but the rash appears earlier and is more pronounced.

(ii) Other tick borne diseases

The Siberian tick typhus is mild rickettsial disease caused by *R. siberica*. Boutonneuse fever is caused by *R. conori* and Australian tick typhus by *R. australis*. All these

three rickettsiae are maintained in nature in ixodid ticks. Humans accidentally enter the natural cycle. Diseases produced by these rickettsiae resemble RMSF but are of milder form. A black spot having a necrotic centre (eschar) is present at the site of tick bite.

(iii) Rickettsial pox

This is a benign febrile disease carried from mouse to man by a mite and produces a vesicular rash. It is caused by *R. akari* (*akari* meaning mite). The reservoir of infection is the domestic mouse (*Mus musculus*) and the vector is the mite, *Liponyssoides* (*Allodermanyssus*) *sanguineus*, in which transovarian transmission occurs. The disease is self limiting, non-fatal and resembles chickenpox.

G. Laboratory Diagnosis

Laboratory diagnosis of rickettsial diseases may be carried out by

1. Isolation of rickettsiae
2. Serology

1. Isolation of Rickettsiae

As rickettsiae are highly infectious, their isolation should be attempted only in laboratories equipped with appropriate safety provisions. Rickettsiae have caused several serious and fatal infections among laboratory workers. Blood clot ground in skimmed milk is inoculated intraperitoneally in male guinea pigs or mice. The inoculated animals are to be observed for 3 to 4 weeks. Temperature of animals are recorded daily. The response of animals to different rickettsial infections vary.

(*i*) In Rocky Mountain spotted fever, guinea pigs develop fever, scrotal necrosis and may even die due to overwhelming infection.

(*ii*) In *R. mooseri, R. conori* and *R. akari* infection, the guinea pigs develop fever and inflammation of tunica.

(*iii*) In *R. prowazekii* infection, the animals develop fever without any testicular inflammation.

For demonstration of rickettsiae, smears from peritoneum, tunica and spleen of infected animals may be stained by Giemsa or Gimenez methods.

Rickettsiae can also be grown in the yolk sac of chick embryo and tissue cultures but these are not suitable for primary isolation. Rickettsiae grow well on Vero and MRC5 cell cultures and can be identified by immunofluorescence test using group and strain specific monoclonal antibodies.

2. Serology

Serological diagnosis may be done by (*i*) Weil-Felix reaction or by (*ii*) specific tests using rickettsial antigens.

(i) Weil-Felix reaction

It is a heterophile agglutination test which detects anti-rickettsial antibodies that cross react with certain non-motile proteus strains OX 19, OX 2 and OX K. The basis of the test is the sharing of an alkali stable carbohydrate antigen of some rickettsiae with non-motile strains of proteus, *P. vulgaris* OX 19, and OX 2 and *P. mirabilis* OX K. The proteus organisms are used as antigens.

Sera from patients with epidemic and endemic typhus strongly agglutinates OX 19 and sometimes OX 2 also. In Brill-Zinsser disease the test is negative or weakly positive. In spotted fevers both OX 19 and OX 2 are agglutinated. OX K agglutinins are found only in scrub typhus. The test is of no value in diagnosis of rickettsial pox (Table 49.2).

Table 49.2 Weil–Felix Reaction in Diagnosis of Rickettsial Diseases

Disease	Agglutination with Proteus strain		
	OX19	OX2	OXK
Epidemic typhus	++++	±	–
Brill-Zinsser disease	$\overline{+}$	–	–
Endemic typhus	+++	±	–
Spotted fever group	++	++	–
Scrub typhus	–	–	+++
Rickettsial pox	–	–	–

The agglutinins usually appear as early as 5 to 7 days and reach peak titres of up to 1 : 1,000 or 1 : 5,000 by the end of second week and decline rapidly during convalescence. The test does not differentiate between epidemic and endemic typhus. It may be negative in 50 percent of scrub typhus cases. False positive reaction may also occur in infections by *Proteus* and *S. typhi*. Hence, a rise in titre of antibodies is significant for diagnosis of rickettsial infections.

(ii) Specific tests using rickettsial antigens

Serological methods using rickettsial antigens are specific, which include complement fixation test, latex agglutination test and enzyme immuno assay.

(*a*) *Complement fixation test* (CFT): It is the most frequently employed serological method using rickettsial antigens. The test is done by using the group specific soluble antigen or the type specific washed rickettsial antigen. The CFT using type specific antigen helps to differentiate between epidemic and endemic typhus.

(*b*) *Other serological tests*: Latex agglutination test is available for Rocky Mountain spotted fever. It is positive only during an acute infection. Enzyme immunoassay with particulate or extracted antigens has been used.

H. Treatment

Tetracyclines or chloramphenicol are used for treatment of rickettsial infections.

I. Prophylaxis

1. General Measures

General measures such as control of vectors and animal reservoirs are useful to prevent rickettsial diseases.

2. Vaccination

There is no safe, effective vaccine for any of the rickettsial diseases.

(*i*) A live vaccine containing attenuated *E strain* of *R. prowazekii* grown in yolk sac have been found to be effective but a proportion of vaccinees develop mild disease.

(*ii*) Formalin inactivated *R. rickettsii* has been used but does not prevent the disease completely.

(*iii*) In earlier days, phenolised intestinal contents of lice infected per rectum with *R. prowazekii* (Weigl's vaccine) was developed. It was too complicated for mass production.

IV. ORIENTIA

Scrub typhus is caused by *O. tsutsugamushi*. It occurs in Japan, China, Sumatra, Australia and islands along the Western Pacific coast. The disease is transmitted by the larval forms of the mite, *Trombicula deliensis*. Human infection occurs by an infected mite. The incubation period is 7–10 days. The clinical features are similar to those of epidemic typhus. A necrotic lesion (eschar) occurs at the site of bite in the skin. The patient develops severe headache, chills, fever, conjunctivitis and maculopapular rash. Spleen and lymph nodes are enlarged. Case fatality rate ranges from 10 to 60 per cent.

Laboratory diagnosis is same as that of rickettsial diseases (refer page 400). For isolation of *O. tsutsugamushi*, mice are preferred over guinea pigs. Infected animals become ill and develop ascites. Weil-Felix reaction is also useful for the diagnosis.

V. COXIELLA

Coxiella is named after Cox who first isolated the agent and introduced the technique of yolk sac inoculation for its cultivation. It differs from rickettsiae in being smaller, more resistant to physical and chemical agents and transmissible without arthropod vectors. It belongs to family *Coxiellaceae* (Table 49.3).

Table 49.3 Family Coxiellaceae

Genus	Species	Disease	Insect vector
Coxiella	*C. burnetii*	Q fever	1. Extrahuman-Tick 2. Human-Nil

Q fever is caused by *Coxiella burnetii* (after the names of *Cox* and *Burnet* who identified the same agent independently). When the aetiology of the disease was unknown, it was referred to as 'Query' or Q fever.

C. burnetii is an obligate intracellular pathogen. It is pleomorophic, occurring as small bacilli, 0.2 – 0.4 μm × 0.4 – 1.0 μm in size. It is Gram negative and relatively resistant to physical and chemical agents. It is filterable. It is not inactivated at 60°C or by 1% phenol, in one hour. In milk it may survive pasteurisation by the holder method, but the flash method is effective. It can remain infectious for months in milk, water, soil and on wool. It can survive in dust and aerosols, therefore, can be transmitted as an airborne infection. It can be inactivated by 2% formaldehyde, 5% hydrogen peroxide and 1% lysol.

Q fever is primarily a zoonosis. Wild animals such as bandicoot may constitute the primary reservoir. Infection is transmitted among them by ixodid ticks. Ticks transmit the disease to domestic animals (cattle, sheep and poultry). The organisms are shed in the milk of infected cattle. Infected domestic animals shed large numbers of organisms in their urine, faeces and particularly in their placental product. Infectious organisms in placental products become aerosolised after parturition which contaminate the surrounding area. Human infections have been traced to (*i*) consumption of infected milk (*ii*) handling of infected wool (*iii*) contaminated soil (*iv*) contaminated clothing. Man to man transmission can occur but is rare. Ticks do not play any significant role in transmission of the disease to man. The incubation period is 2–4 weeks.

Patient develops headache, chills, myalgia, pneumonia, endocarditis, hepatitis and meningoencephalitis. In contrast to other rickettsial diseases, no skin rash occurs in Q fever. The organisms may remain latent in tissues of patients for years.

C. burnetii appears to be widely distributed in India though very few human cases have been reported.

Laboratory diagnosis is mainly by serology using complement fixation test or indirect immunofluorescence assay (IFA). IFA is the method of choice. ELISA kits are commercially available and have sensitivities and specificities similar to those of IFA test. Weil-Felix test is negative in Q fever. Isolation of organisms from clinical

specimens is possible, but is not recommended due to the hazard of laboratory infection.

For the prevention of Q fever, vaccines have been prepared from formalin killed whole cells, attenuated strains and trichloroacetic acid extracts. They are not in general use. Care in the handling of animals and their products will be helpful in prophylaxis of Q fever.

VI. EHRLICHIA, ANAPLASMA, NEORICKETTSIA

Ehrlichiae are now included in family *Anaplasmataceae*. This family contains genera *Ehrlichia*, *Anaplasma* and *Neorickettsia* (Table 49.4). Ehrlichia species infect predominantly leucocytes of humans and other mammals while *Anaplasma* species infect bone marrow derived cells. *Neorickettsia* species infect predominantly mononuclear phagocytes.

Table 49.4: Organisms of Family Anaplasmataceae

Genus	Species	Disease	Insect vector
Ehrlichia	*E. chaffensis*	Human monocytic ehrlichiosis	Tick
	E. ewingii	Human monocytic ehrlichiosis like	Tick
Anaplasma	*A. phagocytophilum*	Human granulocytic anaplasmosis	Tick
Neorickettsia	*N. sennetsu*	Sennetsu ehrlichiosis	—

Enrlichiae are small Gram negative bacilli which are obligately intracellular and have an affinity towards blood cells. They grow as mulberry-like clusters called *morula* in the cytoplasm of infected phagocytic cells. Two species have been reported to cause human infection.

A. Species of Ehrlichia, Anaplasma and Neorickettsia

1. Ehrlichia chaffensis

It is transmitted by ticks. It infects human monocytes and produces febrile illness with leucopaenia and thrombocytopaenia. The infection is named as 'human monocytic ehrlichiosis'. It occurs in USA, Europe and Africa.

2. Ehrlichia ewingii

It was found in the blood of patients with human monocytic ehrlichiosis like disease.

3. Anaplasma phagocytophilum

It infects human granulocytic cells. It produces febrile illness with leucopaenia and thrombocytopaenia and is transmitted by ticks. The infection is named as 'human granulocytic anaplasmosis'. It was previously known as human granulocytic ehrlichioisis caused by *Ehrlichia phagocytophila*.

4. Neorickettsia sennetsu

It was previously known as *Ehrlichia sennetsu*. It causes sennetsu ehrlichiosis, an illness resembling glandular fever (*sennetsu* meaning glandular fever). Infection occurs with ingestion of raw fish. No arthropod vector has been identified. It occurs in Japan and Malaysia.

B. Laboratory Diagnosis

1. Intracytoplasmic inclusions may be seen in Giemsa-stained peripheral blood smear.
2. Specific antibodies can be demonstrated by indirect immunofluorescent methods.

VII. BARTONELLA

Family *Bartonellaceae* contains two genera: *Bartonella* and *Grahamella*. Members of genus *Grahamella* do not infect humans. The genus *Bartonella* contains *B. bacilliformis*, *B. quintana* and *B. henselae* which cause Oroya fever, trench fever and cat-scratch disease in man respectively (Table 49.5).

Table 49.5: Organisms of Family Bartonellaceae causing human infections

Genus	Species	Disease	Insect vector
Bartonella	*B. bacilliformis*	Oroya fever, Veruga peruana	Sandfly
	B. quintana	Trench fever	Body louse
	B. henselae	Cat-scratch disease	—

A. Bartonella bacilliformis

It is a pleomorphic, Gram negative, motile, small bacillus (0.3 – 1.5 μm × 0.2 – 0.5 μm). It is found inside the erythrocytes of infected persons. It is strict aerobe and optimum temperature for growth is 25–28°C. It can grow in semisolid nutrient agar with 10% rabbit serum and 0.5% haemoglobin. Growth is slow and takes about 10 days.

1. Pathogenesis

B. bacilliformis causes *Oroya fever* transmitted by sandflies. The incubation period is 3 weeks to 3 months. Patient develops fever, severe headache and chills, followed by severe anaemia due to destruction of erythrocytes

by the organism. Several weeks after recovery, patient may develop nodular lesions on exposed part of the body. These nodules may become secondarily infected producing ulcers, this condition is known as *Verruga peruana*. Oroya fever and Verruga peruana are also called *Carrion's disease*.

2. Laboratory Diagnosis

(*i*) Organisms can be demonstrated in blood smears stained by Giemsa stain. They are seen in the cytoplasm as well as adhering to cell surfaces.
(*ii*) It can be grown on nutrient agar containing rabbit serum and haemoglobin.
(*iii*) Guinea pig inoculation leads to verruga peruana but not Oroya fever.

3. Treatment and Prophylaxis

It is susceptible to penicillin, streptomycin, tetracycline and chloramphenicol. For prevention, insecticides such as DDT should be used to eliminate the sandfly.

B. Bartonella quintana

B. quintana is a small Gram negative bacillus measuring 0.3 – 0.5 μm × 1.0 – 1.7 μm. It does not possess flagella. It may show twitching movement caused by fimbriae. It grows on rabbit or sheep blood agar. Optimum temperature for growth is 35°C in 5% CO_2. Colonies appear after 14 days in primary culture. It was formerly called *Rochalimaea quintana*. It causes trench fever.

1. Pathogenesis

Trench fever is an exclusively human disease and no animal reservoir is known. It is transmitted by body louse. It is also known as five day fever (*quintana*, meaning fifth). Trench fever broke out in epidemic form during the First World War amongst soldiers fighting in the trenches in Europe, that is why the name *trench fever*.

The lice after acquiring infection remains infective throughout their lives. Vertical transmission does not occur in lice. The clinical picture of trench fever is mild. It leads to chronic rickettsiaemia and relapses have been observed even 20 years after primary disease. The chronic nature of disease and relapses help to maintain the organism in the environment, even in the absence of animal reservoirs.

Rochalimaea differs from rickettsiae in the following respects:

(*i*) It occurs extracellularly in the arthropod host.
(*ii*) It grows poorly in the yolk sac of chick embryo.
(*iii*) It can be grown on blood agar.
(*iv*) Convalescent sera from patients do not react with rickettsial or *Proteus* antigens (Weil-Felix reaction).
(*v*) It does not cause experimental infection in any of the common laboratory animals. Only monkeys can be infected besides man and the louse.

2. Laboratory Diagnosis

B. quintana can be isolated by allowing healthy lice to feed upon the patient and the organisms may be detected in the gut of these lice. It can also be isolated from patient's blood, by culture on rabbit or sheep blood agar. Weil-Felix reaction in trench fever is negative. PCR has also been used for detection of *B. quintana* in the tissues.

C. Bartonella henselae

It causes cat-scratch disease. It is a small, slightly curved, Gram negative bacillus. It displays twitching motility. It grows on chocolate agar, columbia agar with 5% sheep blood and tryptic-soy agar.

Optimum temperature for growth is 35–37°C in 5% CO_2. Growth is slow and takes 5–15 days. Colonies are white, dry, cauliflower-like and embedded in the agar.

1. Pathogenesis

The disease occurs by contact, scratch or bite of an infected cat and is named cat-scratch disease. It is a severe condition of regional lymphadenopathy and fever. It occurs throughout the world. It may also cause endocarditis. In AIDS patients, it may lead to bacillary angiomatosis, a condition resembling Kaposi's sarcoma.

2. Laboratory Diagnosis

(*i*) Lymph node biopsies stained with the *Warthin-Starry silver impregnation* stain show clusters of bacilli.
(*ii*) It can be grown by culture of clinical specimens on chocolate agar or columbia agar with 5% sheep blood.

3. Treatment

Cat-scratch disease is usually self limiting and requires no treatment.

KEY POINTS

1. The family *Rickettsiaceae* consist of two genera, *Rickettsia* and *Orientia*.
2. *Coxiella* and *Ehrlichia* are now included in family *Coxiellaceae* and family *Anaplasmataceae* respectively.
3. Rickettsia contains the agents causing typhus fever and spotted fevers. Scrub typhus is caused by *Orientia tsutsugamushi* and *Coxiella* is the causative agent of 'Q' fever.
4. Rickettsiae are pleomorphic, coccobacilli, non-motile, non-capsulated and Gram negative, though they do not take the stain well. The organisms stain bluish purple with Giemsa stain and deep red with Machiavello and Gimenez stains.
5. Rickettsiae are unable to grow in cell free media. They are cultivated in *yolk sac* of chick embryo. They can also grow on *Hela, mouse fibroblast, HEp2, Detroit 6* and other continuous cell lines.
6. *R. prowazekii* causes epidemic typhus and its recrudescent infection (Brill-Zinsser disease). Human body louse (*Pediculus humanus corporis*) is the vector.
7. Endemic typhus is caused by *R. mooseri* (*R. typhi*). Vector is rat flea (*Xenopsyella cheopis*).
8. *R. mooseri* can be differentiated from *R. prowazekii* by *Neil-Mooser* or *tunica reaction*.
9. *R. rickettsii* and *R. conori* are responsible for Rocky-Mountain spotted fever and boutonneuse fever respectively. Both are transmitted by ixodid ticks.
10. *Orientia tsutsugamushi* is the causative agent of *scrub typhus*. The disease is transmitted by trombiculid mite.
11. Q fever is caused by *Coxiella burnetii*.
12. Laboratory diagnosis of rickettsial diseases may be carried out by *isolation of rickettsiae* and *serology*.
13. *Weil-Felix reaction* is a heterophile agglutination test which detects anti-rickettsial antibodies that cross react with certain non-motile proteus strains OX19, OX2 and OXK. These proteus organisms are used as antigens in Weil-Felix reaction.
14. Specific serological tests using rickettsial antigens include complement fixation test, latex agglutination test and enzyme immunoassay.
15. The genus Bartonella contains *B. bacilliformis, B. quintana* and *B. henselae* which cause Oroya fever, trench fever and cat-scratch disease in man respectively.

YOU MUST KNOW

1. Diseases caused by different rickettsiae.
2. Brill-Zinsser disease.
3. Laboratory diagnosis of rickettsial infections.
4. Weil-Felix reaction.
5. Q fever.
6. *Bartonella bacilliformis.*
7. Trench fever.

STUDY QUESTIONS

1. Discuss the laboratory diagnosis of rickettsial diseases.
2. Write short notes on:

(a) Typhus fevers	(b) Cat-scratch disease	(c) Scrub typhus	(d) Trench fever
(e) Q fever	(f) Brill-Zinsser disease	(g) Neil-Mooser reaction	(h) Weil-Felix reaction
(i) Ehrlichia	(j) *Bartonella bacilliformis*	(k) Rocky-Mountain spotted fever.	

MULTIPLE CHOICE QUESTIONS (MCQs)

1. What is the causative agent of epidemic typhus?
   (a) *Rickettsia prowazekii* (b) *R. typhi* (c) *R. conori* (d) *R. australis*
2. Endemic typhus is caused by:
   (a) *Rickettsia prowazekii* (b) *R. typhi* (c) *R. conori* (d) *R. australis*
3. What is the causative agent of Rocky-Mountain spotted fever?
   (a) *Rickettsia prowazekii* (b) *R. typhi* (c) *R. rickettsii* (d) *R. conori*
4. Insect vector for epidemic typhus is:
   (a) Human body louse (b) Rat flea (c) Tick (d) Mite
5. Rat flea is responsible for transmission of which of the following diseases?
   (a) Epidemic typhus (b) Endemic typhus
   (c) Rocky-Mountain spotted fever (d) Rickettsial pox
6. What is the causative agent of rickettsial pox?
   (a) *Rickettsia prowazekii* (b) *R. typhi* (c) *R. akari* (d) *R. conori*
7. Insect vector for rickettsial pox is:
   (a) Human body louse (b) Rat flea (c) Tick (d) Mite
8. Scrub typhus is caused by:
   (a) *Rickettsia prowazekii* (b) *Ehrlichia sennetsu* (c) *Coxiella burnetii* (d) *Orientia tsutsugamushi*
9. Q fever is caused by:
   (a) *Rickettsia prowazekii* (b) *Ehrlichia sennetsu* (c) *Coxiella burnetii* (d) *Orientia tsutsugamushi*
10. Brill-Zinsser disease is associated with:
    (a) Epidemic typhus (b) Endemic typhus (c) Rickettsial pox (d) Australian tick typhus
11. Weil-Felix reaction is negative in:
    (a) Epidemic typhus (b) Scrub typhus (c) Q fever (d) Spotted fever group
12. In scrub typhus, Weil-Felix reaction shows positive agglutination with:
    (a) OX19 antigen (b) OX2 antigen (c) OXK antigen (d) None of the above
13. Q fever in humans can be acquired by:
    (a) Consumption of infected milk (b) Handling of infected wool
    (c) Contaminated soil (d) All of the above
14. Oroya fever is caused by:
    (a) *Bartonella bacilliformis* (b) *B. quintana* (c) *B. henselae* (d) All of the above
15. Trench fever is caused by:
    (a) *Bartonella bacilliformis* (b) *B. quintana* (c) *B. henselae* (d) All of the above
16. Cat-scratch disease is caused by:
    (a) *Bartonella bacilliformis* (b) *B. quintana* (c) *B. henselae* (d) All of the above
17. Which of the following bacteria can be grown on blood agar?
    (a) *Bartonella quintana* (b) *Coxiella burnetii* (c) *Rickettsia typhi* (d) *Rickettsia conori*

Answers (MCQs):

1. (a)	2. (b)	3. (c)	4. (a)	5. (b)
6. (c)	7. (d)	8. (b)	9. (c)	10. (a)
11. (c)	12. (c)	13. (d)	14. (a)	15. (b)
16. (c)	17. (a)			

Chapter 50

CHLAMYDIA AND CHLAMYDOPHILA

I. INTRODUCTION

Chlamydiae are obligate intracellular parasites which are small, non-motile and Gram negative. They cause psittacosis, lymphogranuloma venerum (LGV) and trachoma in man and diverse diseases in birds and mammals. They were called psittacosis-lymphogranuloma-trachoma (PLT) or TRIC (trachoma-inclusion conjunctivitis) organisms. In recognition of the work of *Sir Samuel Bedson*, the name Bedsoniae was proposed for this group. However, the official term for this group now is Chlamydia. The name Chlamydia is derived from the characteristic appearance of inclusion body by these agents. The inclusion bodies enclose the nuclei of infected cells as a cloak or mantle (*chlamys*, meaning mantle).

Differences between Chlamydiae and Viruses

Since the chlamydiae are obligate intracellular parasites, they were previously thought to be viruses. They lack the ability to produce their own ATP, therefore, they use ATP from host cells. They differ from viruses in following properties:

1. They possess both DNA and RNA, like bacteria.
2. They have rigid cell walls and ribosomes similar to bacteria.
3. They multiply by binary fission.
4. They are susceptible to antibiotics and chemotherapeutic agents.
5. They do not have an 'eclipse phase' following cellular infection.

II. CHLAMYDIAE

A. Classification

Chlamydiae are classified in the order *Chlamydiales* which contains only one family *Chlamydiaceae*. The family previously contained a single genus *Chlamydia* having four species, *C. trachomatis, C. psittaci, C. pneumoniae* and *C. pecorum*. *C. trachomatis* is classified into two biovars, namely, TRIC (causing trachoma, inclusion conjunctivitis) and LGV (causing lymphogranuloma venereum).

The use of ribosomal sequence data has prompted a revision of this classification. The family *Chlamydiaceae* has been divided into two genera *Chlamydia* and *Chlamydophila*. *Chlamydia psittaci*, *Chlamydia pneumoniae* and *Chlamydia pecorum* are placed into new genus

Chlamydophila. However, simultaneous use of the former and the new nomenclature is going on in the literature. *Chlamydophila abortus* is a new species which causes abortion and foetal loss in sheep. There are some reports of pregnant women who have had spontaneous abortion following exposure to animals which were infected with *C. abortus*.

1. *Chlamydophila psittaci*

It causes psittacosis and ornithosis and has many serotypes.

2. *Chlamydia trachomatis*

C. trachomatis strains have been classified by neutralisation and immunofluorescence tests into 20 serotypes namely A, B, Ba, C, D, Da, E, F, G, H, I, Ia, J, Ja, K, L1, L2, L2a, L2b and L3.

(*a*) Serotypes A, B, Ba, C – Cause hyperendemic trachoma.

(*b*) Serotypes D, Da, E, F, G, H, I, Ia, J, Ja, K – Cause inclusion conjunctivitis, non-gonococcal urethritis, salpingitis, cervicitis, epididymitis, proctitis, pneumonia of newborns.

(*c*) Serotypes L1, L2, L2a, L2b, L3 – Cause lymphogranuloma venereum (LGV).

3. *Chlamydophila pneumoniae*

It was formerly known as TWAR strain. It is an exclusive human pathogen with no animal host. It causes acute respiratory disease in humans. It has only one serotype.

Diseases caused by three species of chlamydia are shown in Table 50.1.

B. Morphology and Development Cycle

Chlamydiae exist in two forms the elementary body and the reticulate (initial) body.

1. Elementary Body (EB)

It is a spherical particle measuring 200–300 nm in diameter with an electron dense nucleoid. It is the extracellular infective form.

2. Reticulate (Initial) Body (RB)

Reticulate body is non-infectious in nature. It is the intracellular growing and replicative form.

The infectious particle (EB) enters the host cell by phagocytosis. It enlarges to form reticulate body, 500–1000 nm in size. This is the reproductive form which grows in size and divides repeatedly by binary fission to produce a large number of elementary bodies. The newly formed infectious particles (EB) on liberation from host cell, may infect new cells and cycle is repeated (Fig. 50.1). The duration of development cycle is about 24–28 hours.

Chlamydiae are Gram negative but they stain better with other methods like Castaneda, Machiavello or Gimenez stains. The inclusion bodies are basophilic and are present in cytoplasm. The inclusion bodies of *C. trachomatis* can be stained with Lugol's iodine because of the presence of glycogen matrix. Chlamydiae can also be demonstrated by immunofluorescent technique using antibody tagged with fluorescin isothiocyanate (FITC).

C. Resistance

Chlamydiae are heat labile and are inactivated at 56°C within minutes. They are susceptible to ethanol and dilute solutions of formalin and phenol. They can be preserved at –70°C or by lyophilisation.

D. Antigenic Structure

Chlamydiae possess the following antigens:

1. Complement Fixing Genus Specific Antigen

It is a lipopolysaccharide similar to the LPS present in the Gram negative bacteria. It can be extracted by deoxycholate, ether, chloroform or methanol. It is heat

Table 50.1 Diseases Caused by Chlamydia and Chlamydophila

Species	*Serotype*	*Disease*
C. psittaci	Many serotypes	Psittacosis and ornithosis
C. trachomatis	A, B, Ba, C	Hyperendemic trachoma
C. trachomatis	D, Da, E, F, G, H, I, Ia, J, Ja, K	Inclusion conjunctivitis (neonatal and adult), Non-gonococcal urethritis, salpingitis, cervicitis, pelvic inflammatory disease (PID), epididymitis, proctitis, pneumonia of newborns
C. *trachomatis*	L1, L2, L2a, L2b, L3	Lymphogranuloma venereum
C. pneumoniae	One serotype	Acute respiratory disease (ARD)

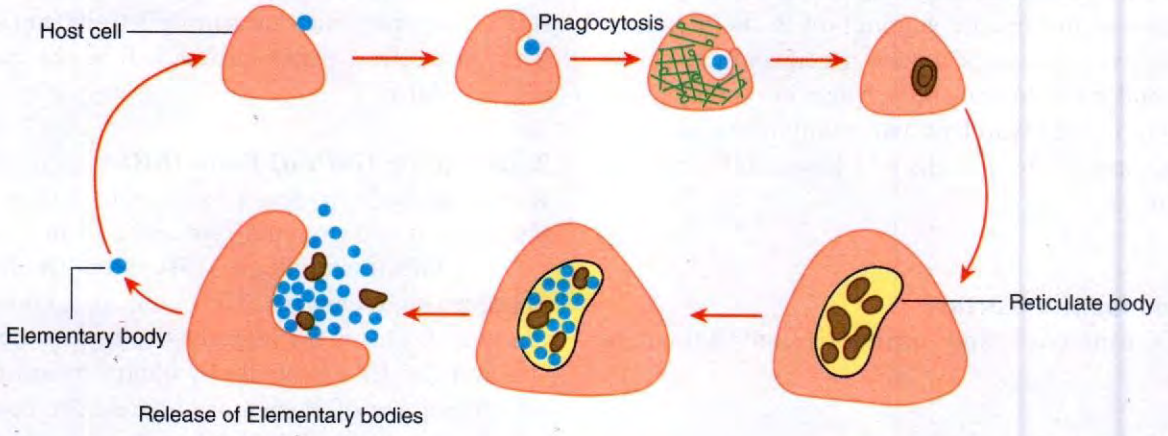


Fig. 50.1 Development cycle of Chlamydiae

stable. It is a complement fixing antigen and present in all chlamydiae

2. Species Specific Protein Antigens

These are present at the envelope surface. They help in classifying chlamydiae into different species.

3. Serotype Specific Antigens

They help in intraspecies typing. They are located on the major outer membrane proteins (MOMP). These antigens can be demonstrated by microimmunofluorescence. Within each species, a number of serotypes can be defined. *C. trachomatis* has 20 serotypes (A to K and L1 to L3) as described earlier.

E. Culture

Chlamydiae can be isolated by

1. Animal inoculation
2. Yolk sac inoculation
3. Tissue culture

1. Animal Inoculation

Mice are inoculated by intranasal, intraperitoneal or intracerebral inoculation. The mice die within 10 days and smears from various tissues (lung, peritoneal exudate, spleen or brain) show the elementary bodies.

2. Yolk Sac Inoculation

Yolk sac of chick embryo is inoculated and the organisms can be detected in impression smears stained by the Giemsa or Gimenez methods.

3. Tissue Culture

Cell lines that have been irradiated or treated with a metabolic inhibitor are used for isolation of chlamydiae. McCoy cells treated with cycloheximide are the most commonly used cell line. Mouse fibroblast cell lines, HeLa 229 or monkey kidney cells can also be used for isolation of chlamydiae. *C. pneumoniae* grows better in HEp-2 or monkey kidney cells. The organisms in the tissue culture can be detected by staining for inclusions or elementary bodies.

F. Pathogenesis

Chlamydial infections in man occur in three forms:

1. Ocular infections
2. Genital infections
3. Respiratory infections

1. Ocular Infections

(i) Trachoma

It is a chronic keratoconjunctivitis. It is one of the major causes of blindness. It is characterised by follicles, papillary hyperplasia, pannus formation and in the late stages, cicatrization. *C. trachomatis* serotypes A, B, Ba and C are the causative agents of trachoma. The infection is transmitted from eye to eye by fingers or contaminated towels and clothing. Incubation period of the disease is 3–10 days. Children below 9 years of age are the most frequently affected in endemic areas. Trachoma has been classified into four stages i.e., I to IV. Infectivity is maximum in early stages.

(ii) Inclusion conjunctivitis

It is caused by *C. trachomatis* serotypes D to K. It is most prevalent in sexually active young people and is spread from genital secretions to the eye by hand contact. This was formerly known as *swimming pool conjunctivitis* as it was associated with bathing in contaminated water of swimming pool. It is also known as *paratrachoma*.

(iii) Ophthalmia neonatorum (Inclusion blennorrhoea)

It is the neonatal form of inclusion conjunctivitis. The infant acquires infection during passage through the infected birth canal. It usually becomes apparent between 5–12 days after birth. About 20–50% infants of infected mothers develop the infection.

2. Genital Infections

Chlamydia trachomatis causes 'genital chlamy-diasis' and LGV. Both are sexually transmitted.

(i) Genital chlamydiasis

C. trachomatis serotypes D to K are responsible for about 40% of cases of non-gonococcal urethritis (NGU). It is a sexually transmitted disease (STD). In addition to urethritis, it may cause epididymitis and proctitis. In females, it may cause urethritis, cervicitis, salpingitis and pelvic inflammatory disease (PID). The infection may be symptomatic or may remain asymptomatic. The symptoms include dysuria, non-purulent discharge and frequency of urination.

(ii) Lymphogranuloma venereum

Lymphogranuloma venereum (LGV) is a sexually transmitted disease caused by *C. trachomatis* serotypes L1–L3. The primary lesion consists of a small painless papule or vesicle on the external genitalia. It may ulcerate or heal spontaneously in a few days. The regional lymph nodes (inguinal in males and intrapelvic and pararectal in females) are enlarged, tender, and may break open with the formation of sinuses. The enlarged inguinal lymph nodes are named *bubos*.

3. Respiratory Infections

(i) Chlamydophila pneumoniae

In 1965, a strain of chlamydia TW-183 was isolated from the eye of a patient of Taiwan. In 1983, a strain of chlamydia AR-39 was isolated from throat swab of an American student. These two organisms differed from *C. trachomatis* and *C. psittaci* and were then called TWAR (from Taiwan Acute Respiratory) strains. TWAR agents are now classified as *Chlamydia pneumoniae*. It is the third most common cause of pneumonia following *Str. pneumoniae* and *H. influenzae*. Serological studies show a prevalence rate of 40–50% *C. pneumoniae* in many countries.

C. pneumoniae has been implicated as an important risk factor in cardiovascular disease, where it has been isolated from coronary artery atheromatous plaques. However, more studies are required to ascertain its role.

(ii) Chlamydophila psittaci

It causes psittacosis and ornithosis in birds and man. Human infection occurs by inhalation of infected dried faeces. *Psittacosis* (*psittacos,* meaning parrot) is a disease of parrots. The disease acquired by contact with non-psittacine birds was known as ornithosis (*ornithos*, meaning birds). Both conditions (psittacosis and ornithosis) are now called psittacosis. Incubation period is 1–2 weeks. The disease may vary from a mild 'influenza like' syndrome to severe illness with pneumonia, septicaemia and meningoencephalitis.

G. Laboratory Diagnosis

Laboratory diagnosis depends on

1. Direct detection of antigens
2. Isolation
3. Serology for antibody detection
4. Skin test.

Specimens

Ocular, urethral, vaginal and cervical specimens are best collected by scraping the mucosa. In addition, blood, sputum, respiratory secretions, and other tissues can also be collected. In case of LGV, pus or discharge from bubo should be collected.

1. Direct Detection of Antigens

The following methods may be used for detection of chlamydial antigens in specimens.

(i) Light microscopy

C. trachomatis infections of conjunctiva, urethra and cervix may be diagnosed by demonstrating inclusion bodies in the smears stained with Giemsa, Castanaeda or Machiavello methods. The inclusion bodies of trachoma and inclusion conjunctivitis are named *Halberstaedter Prowazek* or HP bodies whereas the elementary bodies of *C. psittaci* are called *Levinthal Cole Lillie* or LCL bodies. The inclusion bodies of *C. trachomatis* contain a glycogen matrix, therefore, these may also be stained with iodine solution. This technique has a low sensitivity.

(ii) Immunofluorescence (IF)

It is a rapid test. Smears are stained with FITC-labelled monoclonal antibodies and fluorescence can be detected in positive smears. This method cannot only identify inclusions but also extracellular elementary bodies.

(iii) ELISA

It detects soluble genus-specific antigen. Sensitivity and specificity of ELISA is similar to that of

immunofluorescence test. It is useful diagnostic test in *C. pneumoniae* as its isolation is very difficult.

(iv) DNA probes

DNA hybridisation can be used for detection of DNA of *C. trachomatis* in conjunctival and cervical smears. Specific DNA probes are used.

(v) Chemiluminescence assay

An acridium-ester-labelled DNA probe, complementary to RNA of *C.trachomatis*, is used. The labelled DNA-RNA hybrid is detected by measuring the light emitted by acridium ester label.

(vi) Polymerase chain reaction (PCR)

DNA is amplified and detected by PCR. This method is more sensitive than culture.

2. Isolation

Chlamydiae may be isolated by inoculation into mice, yolk sac of chick embryo or in tissue cultures.

(i) Inoculation in mice

Mice are inoculated by intranasal, intraperitoneal or intracerebral route. The mice die within 10 days and smears from various tissues show the inclusion bodies. It is more useful in isolation of *C. psittaci* and lymphogranuloma venereum serotypes. Mice inoculation is no longer in use for isolation of chlamydia.

(ii) Yolk sac

The chlamydiae may be grown in the yolk sac of 6–8 days old chick embryo. The specimen is treated with streptomycin or polymyxin B before inoculation. The eggs are incubated at 35°C in a humid atmosphere. The group specific antigen as well as the inclusion bodies and the elementary bodies can be demonstrated in the yolk sac. Isolation by egg inoculation is tedious and relatively insensitive, therefore, it has been replaced by tissue cultures.

(iii) Tissue culture

McCoy cells rendered non-replicating by treatment with antimetabolites (cycloheximide) or irradiation are used. Pretreated cell lines enhance chlamydial replication and facilitate detection of inclusion bodies. HeLa cells treated with DEAE dextran is another cell line used for isolation. In both the cell lines, the inoculum has to be be driven into the cells by centrifugation up to 15,000 g to get a good growth.

The organisms in the tissue culture can be detected by staining for inclusions. Immunofluorescence is a better method to identify these inclusions.

3. Serology for Antibody Detection

(i) Complement fixation test (CFT)

Complement fixing antibodies appear in the serum but these are group specific. The CFT is used mainly in psittacosis and LGV. A four fold rise in titres is diagnostic. A single titre is not diagnostic unless the titre is high—1:64 or greater. CFT is of little value in TRIC infections, in which microimmunofluorescence is more useful.

(ii) Microimmunofluorescence (MIF)

Antibodies against *C. trachomatis* and *C. pneumoniae* can be detected. Type specific antibodies are demonstrated by this method. It can detect IgG and IgM separately.

(iii) Immunoperoxidase test

(iv) ELISA test

The diagnostic criteria for serology are fourfold rise in IgG titre or presence of IgM antibody. In neonatal chlamydial pneumonia, detection of IgM is diagnostic.

4. Skin Test

Frei's test is available for diagnosis of LGV. It has been described with laboratory diagnosis of LGV.

H. Treatment

1. *Trachoma*: Tetracycline is given topically as well as systemically for several weeks. In young children, erythromycin is given.
2. *Inclusion conjunctivitis*: Tetracycline or erythromycin may be given orally for 10–14 days.
3. *Ophthalmia neonatorum*: Erythromycin may be given.
4. *Genital infections*: Tetracyclines are administered.
5. *LGV*: Both sulphonamides and tetracycline should be given at least for 3 weeks.
6. *C. psittaci and C. pneumoniae infections*: Tetracycline and erythromycin can be used.

I. Prophylaxis

The control of trachoma depends on the improvement of living standards. Psittacosis can be controlled by checking the import of birds. Person to person spread can be prevented by isolation and proper disposal of sputum. No successful vaccine is available.

III. LYNMPHOGRANULOMA VENEREUM (LGV)

It is a sexually transmitted disease caused by *C. trachomatis* serotypes L1–L3. Incubation period ranges from three days to five weeks. The primary lesion is a

small painless papule or vesicle on the external genitalia. This primary lesion may ulcerate or heal spontaneously. The regional lymph nodes (inguinal in males and intrapelvic and pararectal in females) are enlarged and tender. The bacilli multiply in lymph nodes. The enlarged inguinal lymph nodes are known as *bubos*. The lymph nodes suppurate and finally discharge pus forming multiple sinuses. Metastatic complications may sometimes occur involving joints, eyes and meninges. In the late stage, fibrotic change may lead to rectal strictures and elephantiasis of the vulva.

Laboratory Diagnosis

1. Smear Examination

Smears from bubos (inguinal adenitis) or scrapping of urethra, cervix or vagina are stained by Giemsa stain. Elementary bodies (Miyagawa's granulocorpuscles) may be detected in positive cases. Staining of smears by fluorescent antibodies may also show elementary bodies. DNA probes can be used for antigen detection.

2. Isolation

It is difficult. The material is inoculated into the yolk sac of chick embryo, mice or tissue culture. Details of these methods have already been described in laboratory diagnosis of chlamydial infections.

3. Serology

Complement fixation test becomes positive 2 weeks after infection. A titre of 1:64 or more is significant.,

4. Skin Test

The method previously employed for the diagnosis of LGV is the intradermal test first described by *Frei* (1925).

Frei's test

A heat inactivated LGV (0.1 ml) grown in yolk sac of embryonated egg is injected intradermally on the forearm and a material prepared from non-infected yolk sac on the other forearm (control). The test is read 48–72 hours after injection. An inflammatory nodule appears at the test site in two days and reaching a maximum in 4–5 days measuring at least 7 mm in diameter. This skin test becomes positive 2–6 weeks after infection and remains positive for several years. The test is now not in use due to the frequent occurrence of false positive reactions.

KEY POINTS

1. Chlamydiae are obligate intracellular parasites which are small, non-motile and Gram negative.
2. The family *Chlamydiaceae* previously contained a single genus *Chlamydia* having four species *C. trachomatis, C. psittaci, C. pneumoniae* and *C. pecorum*. According to newer classification the family *Chlamydiaceae* has been divided into two genera *Chlamydia* and *Chlamydophila*. *Chlamydia psittaci, Chlamydia pneumoniae* and *Chlamydia pecorum* are placed into new genus *Chlamydophila*.
3. *C. trachomatis* causes *hyperendemic trachoma, inclusion conjunctivitis, non-gonococcal urethritis, salpingitis, cervicitis* and *lymphogranuloma venereum* (*LGV*). *C. pneumoniae* is responsible for *acute respiratory disease* (ARD).
4. *C. trachomatis* has serotypes A to K and L1 to L3.
5. Laboratory diagnosis of chlamydial infections depends on direct detection of antigens, isolation of organism and serology for antibody detection.
6. *Frei's test* is a skin test previously employed for diagnosis of LGV.
7. Tetracycline and erythromycin are used for treatment of chlamydial infections.

YOU MUST KNOW

1. Differences between chlamydiae and viruses.
2. Serotypes of various chlamydiae and diseases produces by these.
3. Morphology and development cycle of chlamydiae.
4. Laboratory diagnosis of chlamydial infections.
5. Lymphogranuloma venereum (LGV).
6. Frei's test.

STUDY QUESTIONS

1. Discuss the laboratory diagnosis of chlamydial infections.
2. Write short notes on:
   (a) Developmental cycle of chlamydiae (b) TRIC agents
   (c) LGV (d) Frei's test
   (e) *Chlamydophila pneumoniae* (f) NGU.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Which of the following genera belong to family *Chlamydiaceae*?
   (a) *Chlamydia* (b) *Chlamydophila* (c) Both of the above (d) None of the above
2. Which of the following serotypes of *Chlamydia trachomatis* cause hyperendemic trachoma?
   (a) A, B, Ba, C (b) D to K (c) L1 to L3 (d) None of the above
3. Which of the following serotypes of *chlamydia trachomatis* cause non-gonococcal urethritis?
   (a) A, B, Ba, C (b) D to K (c) Both of the above (d) None of the above
4. Which of the following serotypes of *Chlamydia trachomatis* cause lymphogranuloma venereum?
   (a) A, B, Ba, C (b) D to K (c) L1 to L3 (d) None of the above
5. Which of the following bacteria was formerly known as TWAR strain?
   (a) *Chlamydia trachomatis* (b) *Chlamydophila pneumoniae*
   (c) *Chlamydophila psittaci* (d) *Chlamydophila pecorum*
6. Which of the following is infective form of *Chlamydia*?
   (a) Elementary body (b) Reticulate body (c) Both of the above (d) None of the above
7. Which of the following methods can be used for detection of chlamydial antigens in specimens?
   (a) Light microscopy (b) Immunofluorescence (c) ELISA (d) All of the above
8. Which of the following methods can be used for isolation of chlamydiae in specimens?
   (a) Inoculation in mice (b) Inoculation in yolk sac of chick embryo
   (c) Inoculation in tissue cultures (d) All of the above
9. Which of the following methods can be used for detection of chlamydial antibody in specimens?
   (a) Complement fixation test (b) Microimmunofluorescence
   (c) ELISA (d) All of the above
10. Which of the following tests is associated with lymphogranuloma venereum?
    (a) Frei's test (b) Neil-Mooser reaction
    (c) Weil-Felix reaction (d) All of the above

Answers (MCQs):

1. (c)	2. (a)	3. (b)	4. (c)	5. (b)
6. (a)	7. (d)	8. (d)	9. (d)	10. (a)

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UNIT IV

VIROLOGY

Chapter 51

GENERAL PROPERTIES OF VIRUSES

Viruses are the smallest obligate intracellular infective agents containing only one type of nucleic acid (DNA or RNA) as their genome. They have no metabolic activity outside the living cells. They do not possess a cellular organisation and lack the enzymes necessary for protein and nucleic acid synthesis. Viral genome (nucleic acid) diverts the host's metabolism to synthesise a number of virus specific macromolecules required for the production of virus progeny. They multiply by a complex process and not by binary fission. They do not grow in inanimate media. They are resistant to antibiotics. The major differences between prokaryotes and viruses are shown in Table 51.1.

I. MORPHOLOGY OF VIRUSES

A. Size

Viruses are much smaller than other organisms. The extracellular infectious virus particle is called the *virion*.

Table 51.1 Differentiating Features of Prokaryotes and Viruses

Properties	Bacteria except chlamydiae	Chlamydiae	Viruses
Cell wall	+	+	–
Ribosomes and cellular enzymes	+	+	–
DNA and RNA	Both present	Both present	Only one present
Binary fission	+	+	–
Growth on inanimate media	+	–	–
Sensitivity to antibacterial antibiotics	+	+	–
Sensitivity to interferon	–	+	+

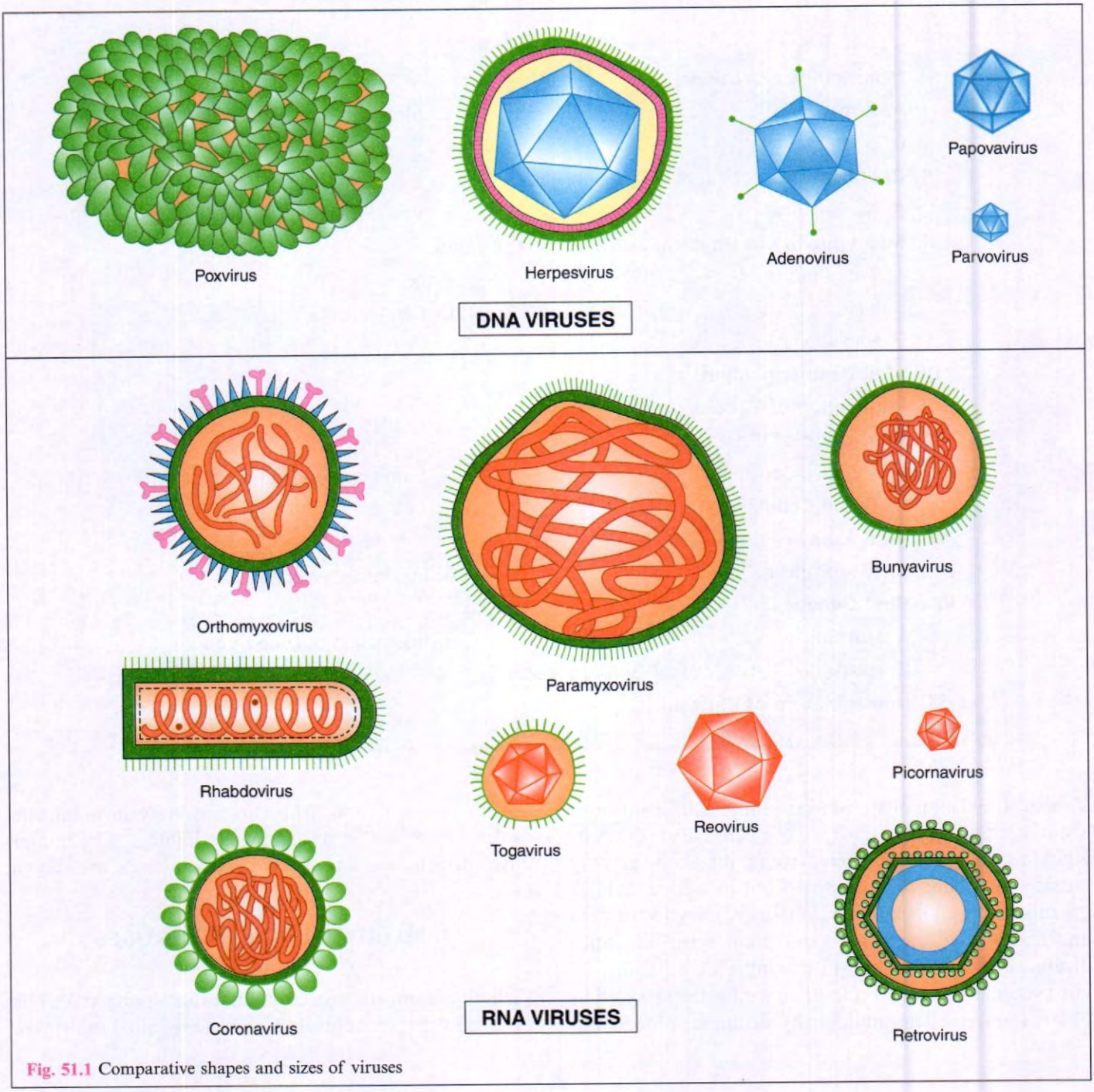


Fig. 51.1 Comparative shapes and sizes of viruses

The size of viruses ranges from 20 to 300 nm in diameter. The largest virus is the smallpox virus (300 nm) and the smallest is the parvovirus (20 nm) (Fig. 51.1). In earlier days the virus particles were measured by passing them through the collodion membrane filters of different pore sizes (gradocol membranes). With the development of ultracentrifuge, the virus size could be calculated from the rate of sedimentation of virus in the ultracentrifuge. The latest and the most direct method for measuring virus size is electron microscopy. By this method, both size and the shape of viruses can be made out.

B. Structure and Symmetry

1. Structure

The virion consists of a nucleic acid core (genome) surrounded by a protein coat, the *capsid*. The capsid together with the enclosed nucleic acid is known as the *nucleocapsid*. The capsid is composed of a large number of protein subunits (polypeptides) which are known as *capsomers* (Fig. 51.2). Two major functions of capsid are, forming an impenetrable shell around the nucleic acid core and to introduce viral genome into the host cells by adsorbing readily to cell surfaces. Certain viruses also contain envelope that surrounds the nucleic acid. The envelope is acquired by the progeny virus during release by budding through the host cell membrane. It is lipoprotein in nature. The lipid is largely of host cell origin while the protein is virus coded. Protein subunits are exposed as projectile spikes on the surface of the envelope. These structures are called *peplomers* (from *peplos* meaning envelope). Enveloped viruses are susceptible to the action of lipid solvents like ether and chloroform. Envelopes confer antigenic, biological and chemical properties on viruses.

2. Symmetry

Three types of symmetry are determined by the arrangement of capsid around the nucleic acid core.

(*i*) *Icosahedral (cubical) symmetry:* An icosahedron is a polygon with 12 vertices or corners and 20 facets in the shape of equilateral triangular faces (Fig. 51.2). Icosahedral symmetry has a rigid structure. This type of symmetry is found in papova, picorna, adenoviruses (all naked or non-enveloped) and herpes, togaviruses (enveloped).

(*ii*) *Helical symmetry:* The nucleic acid and the capsomers are wound together to form a helical or spiral tube (Fig. 51.2). Most of the helical viruses are enveloped and all are RNA viruses.

(*iii*) *Complex symmetry:* Some viruses do not show either icosahedral or helical symmetry due to the complexity of their structures. These are referred to have complex symmetry e.g. poxvirus.

C. Shape

The overall shape of virus particles varies in different groups (Fig. 51.1). Pox virus is brick-shaped, rabies virus is bullet-shaped and tobacco mosaic virus is rod-shaped. Some are irregular and pleomorphic in shape.

II. CHEMICAL PROPERTIES

A. Nucleic Acid

Viruses contain only one kind of nucleic acid, either single or double stranded DNA or RNA. Viral nucleic acid may be extracted by treatment with detergents or phenol. In some viruses (for example picornavirus, papovavirus), extracted viral nucleic acid is capable of initiating infection when introduced into the host cells.

B. Viral Protein and Lipids

Viruses contain protein which makes up the capsid. Viral protein, besides protecting the nucleic acid, also determines the antigenic specificity of the virus. In case of enveloped viruses, they contain lipids (present in the envelope) derived from the host cell membrane.

III. SUSCEPTIBILITY TO PHYSICAL AND CHEMICAL AGENTS

A. Temperature

Most viruses are heat labile and are inactivated within seconds at 56°C, minutes at 37°C and days at 4°C. However, hepatitis 'B' virus resists heating at 60°C for one hour and some strains of scrapie (slow virus) resist autoclaving at 121°C for one hour. They are stable at low temperatures. For long term storage, they are kept frozen

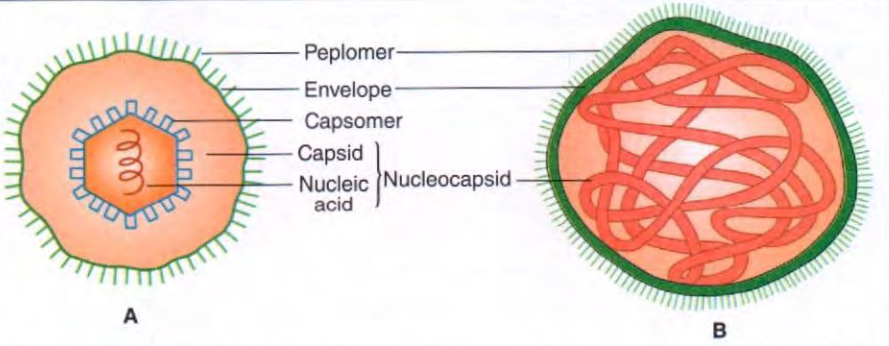


Fig. 51.2 Virus particles A. Enveloped virus with icosahedral symmetry B. Virus with helical symmetry.

at –70°C. Another better method for prolonged storage is lyophilisation or freeze drying (drying the frozen virus under vacuum). Lyophilised virus can be reconstituted by addition of water. Some viruses (e.g. poliovirus) do not withstand freeze drying.

B. pH

The viruses remain viable in a pH range of 5-9, but are killed by extreme acidity and alkalinity. Enteroviruses are very resistant to acidic pH while rhinoviruses are very susceptible.

C. Lipid Solvents

Chloroform, ether and detergents destroy all enveloped viruses which contain lipoprotein envelopes. Naked viruses are resistant to these agents.

D. Disinfectants

Most viruses are destroyed by oxidising agents such as H_2O_2, hypochlorite, and iodine compounds. Formaldehyde and β-propiolactone (BPL) are actively virucidal and are commonly used for the preparation of killed viral vaccines. However, most viruses are resistant to phenol. Chlorination of drinking water kills most viruses but hepatitis A and polioviruses are relatively resistant to chlorination, particularly if present with organic or faecal material.

E. Radiations

Viruses are inactivated by sunlight, ultraviolet rays and ionising radiations.

IV. VIRAL HAEMAGGLUTININ

A large number of viruses contain *haemagglutinin* spikes (peplomers) on the capsid or envelope which can agglutinate erythrocytes of different species. Haemagglutination of influenza virus has been studied extensively. The viral haemagglutinin (glycoprotein) has special affinity for a different glycoprotein located in the 'receptor areas' on the surface of erythrocyte. When erythrocytes are added to serial dilutions of viral suspension, the virus and ertythrocytes collide in the suspension and adhere to each other resulting in haemagglutination. This test provides a simple and rapid method for detection of viruses in egg or tissue culture fluid. The haemagglutination reaction is specifically inhibited by the antibody to the virus. The haemagglutination inhibition test (HI) is routinely used for detecting antiviral antibody in diagnosis and research.

Some viruses, particularly influenza and parainfluenza viruses also carry on their surface another peplomer, the enzyme *neuraminidase* which acts on the receptors on erythrocytes and destroys them. It is known as *receptor destroying enzyme* (RDE). It is also produced by many other bacteria including *Vibrio cholerae*. Destruction of surface receptors results in the reversal of haemagglutination and the release of viruses from the surface of erythrocyte. This process is known as *elution*. After elution, the receptors are irreversibly damaged and erythrocytes are no longer agglutinable by that particular virus. The free viruses are, however, unharmed.

Procedure of Viral Haemagglutination Test

Haemagglutination test can be carried out in test tubes or special plastic trays. When erythrocytes are added to serial dilutions of a viral suspension, the highest dilution that produces haemagglutination provides the titre. The haemagglutinaton titre is defined in the form of HA units. Erythrocytes which are not agglutinated settle at the bottom in the form of a 'button', while the agglutinated erythrocytes are seen spread into a shield-like pattern (Fig. 51.3). As inactivated virus can also haemagglutinate, the test is also used to titrate killed influenza vaccine.

Use of erythrocytes in haemagglutination

1. The influenza, parainfluenza and mumps viruses haemagglutinate erythrocytes of fowl, man and guinea pig and elution occurs at 37°C.
2. Toga, rubella and rabies viruses haemagglutinate goose erythrocytes at 4°C.
3. Enteroviruses and reovirus haemagglutinate human erythrocytes at 37°C.

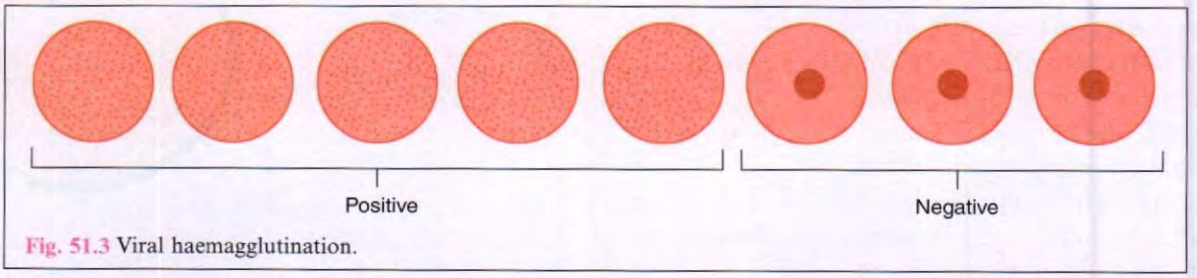


Fig. 51.3 Viral haemagglutination.

V. REPLICATION OF VIRUSES

Due to lack of biosynthetic enzymes, viruses replicate by taking over the biochemical machinery of the host cell to synthesise virus specific macromolecules required for the production of virus progeny. The genetic information necessary for viral replication is contained in the viral nucleic acid. The replicative cycle can be divided into six sequential phases.

1. Adsorption
2. Penetration
3. Uncoating
4. Biosynthesis
5. Maturation and
6. Release.

1. Adsorption or Attachment

The viruses come in contact with the cells by random collision but adsorption or attachment is mediated by the binding of virus surface structures, known as *ligands*, to the receptors on cell surface. In case of influenza virus, the haemagglutinin (a surface glycoprotein) binds specifically to sialic acid residue of glycoprotein receptor sites on the surface of respiratory epithelium. With the human Immunodeficiency virus (HIV), attachment is between the viral surface glycoprotein gp 120 and the CD4 receptor on host cells.

2. Penetration

After attachment, the virus particles may be engulfed by a mechanism resembling phagocytosis, a process known as *viropexis*. Alternatively, in case of the enveloped viruses, the envelope may fuse with the plasma membrane of the host cell releasing the nucleocapsid into the cytoplasm.

3. Uncoating

This is the process of stripping the virus of its outer layers and capsid to release the nucleic acid into the cell. With most viruses, uncoating is affected by the action of lysosomal enzymes of the host cells.

4. Biosynthesis

After uncoating, the viral genome directs the biosynthetic machinery of the host cell to shut down the normal cellular metabolism and direct the sequential production of viral components. In general, the nucleic acid genome of most DNA viruses is synthesised in the host cell nucleus. However, the poxviruses synthesise all their components in the cytoplasm. Nucleic acid genome of most RNA viruses is synthesised in the cytoplasm. The exceptions are orthomyxoviruses, some paramyxoviruses and retroviruses which are synthesised partly in the nucleus of the host cell. Viral protein is synthesised only in the cytoplasm.

Biosynthesis consists of the following steps:

(*i*) Transcription of messenger RNA (mRNA) from viral nucleic acid.

(*ii*) Translation of the mRNA into 'early proteins' or 'nonstructural proteins'. These are enzymes which initiate and maintain synthesis of virus components. They may also induce shutdown of host protein and nucleic acid synthesis.

(*iii*) Replication of viral nucleic acid.

(*iv*) Synthesis of 'late proteins' or 'structural proteins' which constitute daughter virion capsids.

The mechanisms of nucleic acid synthesis differ in the different type of viruses.

(a) Replication of single stranded DNA viruses

In case of these viruses (for example parvovirus), a complementary strand is first synthesised, producing double stranded 'replicative forms'. This double stranded viral DNA acts as a template for its replication, and also for transcribing into mRNA which are translated into viral proteins.

(b) Replication of double stranded DNA viruses

Initially only a part of the viral DNA is transcribed into early mRNA. This encodes for synthesis of early proteins which are required for DNA replication. Late proteins are synthesised after viral DNA replication has commenced.

(c) Replication of RNA viruses

- In many single stranded RNA viruses (e.g. poliovirus), the viral RNA can act directly as mRNA. These are named as *positive strand* (*plus strand, positive sense*) RNA viruses. The single stranded parental RNA (positive strand) acts as the template for the production of a complementary strand (negative strand), which acts as the template for progeny viral RNA.
- In some other single stranded RNA viruses (e.g. influenza and parainfluenza viruses), they carry their own RNA polymerases for mRNA transcription. These are named as *negative strand* (*minus sense*) RNA viruses. Parental RNA produces complementary negative strands which act both as mRNA and as template for the synthesis of progeny viral RNA.
- In the double stranded RNA viruses (e.g. reoviruses), the viral RNA is transcribed to mRNA by viral polymerases.
- Retroviruses exhibits a unique replicative cycle. Virus genome (single stranded RNA) is converted into

an RNA : DNA hybrid by the viral enzyme, RNA directed DNA polymerase (reverse transcriptase). Double stranded DNA is synthesised from the hybrid (RNA : DNA). The double stranded DNA form of the virus (*provirus*) integrates into the host cell genome. The provirus acts as the template for the synthesis of progeny viral RNA. The integration of the provirus into the host cell genome may lead to transformation of the cell and development of neoplasia.

5. Maturation

The viral nucleic acid and capsid polypeptide assemble together to form the daughter virions. The assembly takes place in either the nucleus (herpes and adenoviruses) or cytoplasm (picorna and pox viruses). In case of enveloped viruses, the envelope is derived from the nuclear membrane (herpes virus) and from plasma membrane when the assembly occurs in the cytoplasm of host cell (orthomyxoviruses and paramyxoviruses).

6. Release

Enveloped viruses are released by a process of budding from the cell membrane over a period of time. The host cell is usually not affected but there are exceptions e.g. polioviruses not only damage host cell but may also be released by the lysis of the host cell. In case of bacterial viruses (e.g. bacteriophages), they are usually released by lysis of the infected bacterium.

ECLIPSE PHASE

From the stage of penetration of virus into the host cell till the appearance of first infectious virus progeny particle, the virus cannot be demonstrated inside the host cell. This period is known as *eclipse phase*. The duration of eclipse phase is about 15 to 30 minutes for bacteriophages and 15–30 hours for animal viruses.

Abnormal Replicative Cycles

(i) Incomplete Viruses

A proportion of daughter virions that are produced may not be infective. This is the result of defective assembly. One example of such defective assembly is influenza virus. They will have a high haemagglutinin titre but low infectivity. This is known as *Von Magnus phenomenon*.

(ii) Pseudovirions

The capsid occasionally encloses host cell nucleic acid instead of viral nucleic acid. They are non-infective and do not replicate. These are called *pseudovirions*.

(iii) Abortive Infection

This occurs due to wrong selection of host cells by the virus. The viral components may be synthesised but the maturation is defective. The virus progeny either is not released or is non-infectious. Here, the defect is in the host cell and not in the parental viruses.

(iv) Defective Viruses

Some viruses are genetically defective and they are unable to give rise to fully formed progeny. Yield of progeny virions occurs only in the presence of helper virus, which can supplement the genetic deficiency. Examples of defective viruses are hepatitis D virus and adeno-associated satellite viruses which replicate only in the presence of hepatitis B and adenoviruses (both act as helper viruses) respectively.

VI. CULTIVATION OF VIRUSES

As viruses multiply only in living cells, they cannot be grown on any of the inanimate culture medium. Three methods are employed for the cultivation of viruses:

A. Animal inoculation
B. Embryonated egg inoculation
C. Tissue culture

A. Animal Inoculation

Animal inoculation is used for:

1. Primary isolation of certain viruses
2. To study pathogenesis of viral diseases
3. To study viral oncogenesis

Infant (suckling) mice are used in the isolation of arboviruses and coxsackie viruses, many of which do not grow in any other system. Animals may be inoculated by several routes— intracerebral, subcutaneous, intraperitoneal or intranasal. After inoculation, animals are observed for signs of disease or death. Later on, they are sacrificed and tissues are tested for the presence of virus. The viruses are identified by neutralisation test using antiviral sera. In some viruses, inclusion bodies may be observed in stained smear.

Besides mice, other animals such as guinea pigs, rabbits and ferrets are also used in some situations.

B. Embryonated Egg Inoculation

Goodpasture (1931) first used embryonated hen's egg for cultivation of viruses. Embryonated hen's eggs (7 to 12 days old) are inoculated by one of the several routes (Fig. 51.4) such as chorioallantoic membrane (CAM), allantoic cavity, amniotic sac and yolk sac. After inoculation, eggs are incubated for 2-9 days.

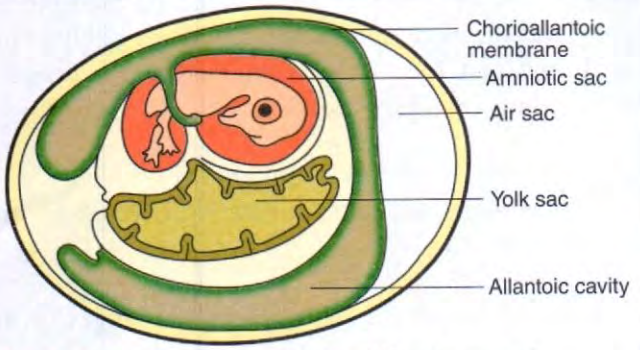


Fig. 51.4 Embryonated egg showing different routes of inoculation.

1. Chorioallantoic Membrane (CAM)

CAM is inoculated mainly for growing poxviruses. It produces visible lesions (*pocks*). Each pock is derived from a single virion. Pock counting, therefore, indicates the number of viruses present in the inoculum. Pocks produced by different viruses have different morphology (Fig. 51.5).

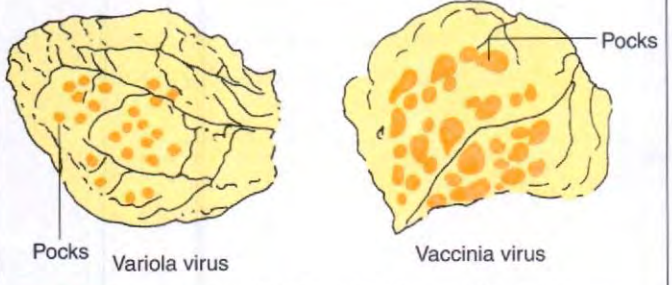


Fig. 51.5 Pocks produced by Variola virus and Vaccinia virus.

2. Allantoic Cavity

Allantoic inoculation is employed for growing influenza virus for vaccine production. Other chick embryo vaccines include yellow fever (17D strain) and rabies (flury strain) vaccines. Duck's eggs being bigger, provide a better yield of rabies virus and were used for the preparation of the inactivated non-neural rabies vaccine.

3. Amniotic Sac

Inoculation into the amniotic sac is mainly used for the primary isolation of the influenza virus.

4. Yolk sac Inoculation

It is inoculated for the cultivation of some viruses and certain bacteria (chlamydia and rickettsiae).

C. Tissue Culture

Three types of tissue cultures are available:

1. Organ culture
2. Explant culture
3. Cell culture

1. Organ Culture

Small bits of organs are maintained in tissue culture growth medium. Organ cultures are useful for the isolation of highly specialised parasites of certain organs e.g. tracheal ring culture for the isolation of the coronavirus, a respiratory pathogen.

2. Explant Culture

Fragments of minced tissue can be grown as 'explants' which was originally known as tissue culture. This method is rarely done nowadays.

3. Cell Culture

This is the type of culture routinely employed for diagnostic virology. Tissues are dissociated into the component cells by the action of proteolytic enzymes such as trypsin. The dissociated cells are washed, counted and suspended in a growth medium. The cell suspension is distributed in glass or plastic bottles, tubes or petri dishes. On incubation, the cells adhere to glass or plastic surface (wall of test tube) and divide to form a confluent monolayer sheet of cells within a period of one week.

Cell cultures are classified into three different types on the basis of their origin, chromosomal characters and the number of generations through which they can be maintained (Table 51.2):

(*i*) Primary cell culture
(*ii*) Diploid cell strains
(*iii*) Continuous cell lines.

Table 51.2 Cell Cultures in Common Use

Type	Name of the cell culture
Primary cell cultures	Rhesus monkey kidney cell culture Human amnion cell culture Chick embryo fibroblast cell culture
Diploid cell strains	WI–38 (Human embryonic lung cell strain) HL–8 (Rhesus embryo cell strain)
Continuous cell lines	HeLa (Human carcinoma of cervix cell line) HEP–2 (Human epithelioma of larynx cell line) Vero (Vervet monkey kidney cell line) McCoy (Human synovial carcinoma cell line) BHK–21 (Baby Hamster kidney cell line) KB (Human carcinoma of nasopharynx cell line)

(i) Primary cell cultures

These are normal cells freshly taken from the organs of animal or human being and cultured. They are capable of very limited growth in culture perhaps 5-10 divisions at the most. They are commonly employed for the primary

isolation of viruses and their cultivation for vaccine production. Common examples of primary cell culture include monkey kidney cell, human amnion cell and chick embryo cell cultures.

(ii) Diploid cell strains

These are cells of a single type that contain the same number of chromosomes as the parent cells and are diploid. They can be subcultured for a limited number. After about 50 serial subcultures they undergo 'senescence' and the cell strain is lost. Diploid cells developed from human fibroblasts are susceptible to a number of human viruses. They are useful for the isolation of some fastidious pathogens. They are also employed for the production of viral vaccines e.g. rabies vaccine production in WI–38 human embryonic lung cell strain.

(iii) Continuous cell lines

These are cells of a single type that are capable of indefinite growth in vitro. They are usually derived from cancerous tissue. These cells grow faster and their chromosomes are haploid. They can be serially cultivated indefinitely, therefore, they are termed *continuous cell lines*. These cell lines may be maintained by serial subcultures or stored in the cold (–70°C) for use when necessary. HeLa, HEp-2 and KB cell lines have been used in the virus laboratories throughout the world for many years. Some cell lines are now being used for vaccine manufacture, for example Vero cell line for rabies vaccine.

Detection of virus growth in cell cultures

Virus growth in cell cultures can be detected by the following methods:

1. *Cytopathic effect*

Many viruses cause morphological changes in the cultured cells in which they grow. These morphological changes are known as *cytopathic effects* (CPE). The viruses causing CPE are called *cytopathogenic viruses*. CPE induced by viruses are of the following types (Fig. 51.6).

(*i*) *Syncytium formation*: Some viruses (measles, respiratory syncytial virus) lead to syncytium formation in which infected cells fuse with neighbouring cells to form multinucleated giant cells.

(*ii*) *Cell necrosis and lysis*: Enteroviruses produce rapid CPE with crenation of cells and degeneration of the entire cell sheet.

(*iii*) *Cellular clumping:* Adenoviruses produce large clumps resembling clusters of grapes.

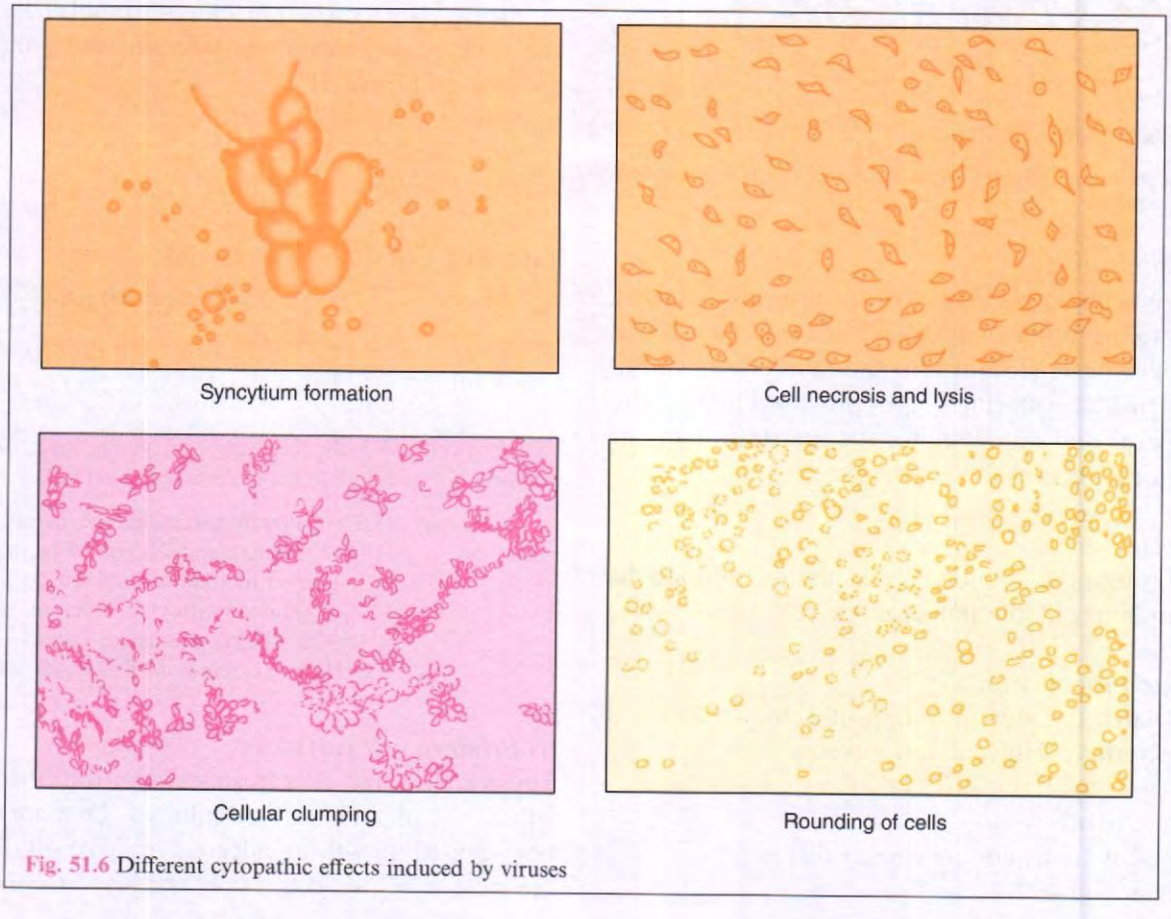


Fig. 51.6 Different cytopathic effects induced by viruses

(*iv*) *Rounding of cells*: Viral replication may lead to nuclear pyknosis, rounding, refractility and degeneration. This is seen in picornaviruses.

(*v*) *Discrete focal degeneration*: Herpes virus produces discrete focal degeneration.

2. *Haemadsorption*

When haemagglutinating viruses (such as orthomyxo and paramyxo viruses) grow in cell cultures, their presence can be detected by the addition of guinea pig erythrocytes to the cell cultures. If the viruses are multiplying in the culture, the erythrocytes will adhere to the infected cells. This is known as *haemadsorption*. Specific antiserum against the virus blocks haemadsorption.

3. *Interference*

The growth of a noncytopathgenic virus in a cell culture can be detected by the subsequent challenge with a known cytopathogenic virus. The growth of first virus will inhibit the infection by the second virus by interference. Example is rubella virus which do not produce cytopathic changes although they multiply within the cell. A known cytopathogenic challenge virus is then introduced into the cells. No CPE will be seen in the cell culture as replication of challenge virus will be prevented because of interference by rubella virus.

4. *Transformation*

Tumour forming (oncogenic) viruses induce cell *transformation* and loss of contact inhibition and the cell growth appears in a piled up fashion producing 'microtumours'. Some herpesviruses, adenoviruses and retroviruses (human T lymphotropic virus type 1) can transform cells.

5. *Immunofluorescence*

Viruses can be detected in infected cells by staining with fluorescent conjugated antiserum and examined under the fluorescent microscope for the presence of virus antigen. This gives positive results earlier than other methods and, therefore, it is used to identify many viral isolates.

6. *Electron microscopy*

Viruses can be detected by electron microscopy of ultra thin sections of infected cells.

7. *Detection of enzymes*

The virus isolates can be identified by detection of viral enzymes in the culture fluid. Reverse transcriptase enzyme can be detected in retroviruses.

VII. VIRAL ASSAYS

The content of virus particles in a specimen can be assayed in two ways: either by *total virus particle count* or *infectious virions assay*.

A. Total Virus Particle Count

Two methods employed are *electron microscopy* and *haemagglutination*.

1. Electron Microscopy

The virus particles in a negatively stained suspension can be counted directly under the electron microscope. The virus suspension is mixed with a known concentration of latex particles. The number of virus particles in the suspension is calculated from the ratio between the virus and latex particles under the electron microscope.

2. Haemagglutination

With haemagglutinating viruses, quantitation is done by the determination of haemagglutination titres. It is not a very sensitive method but because of its simplicity, haemagglutination becomes a very convenient method of virus assay. Approximately 10^7 influenza virions are required to produce macroscopic agglutination.

B. Infectious Virions Assay

The infectivity of viruses can be measured in two ways *quantal* and *quantitative* assays.

1. Quantal Assay

The quantal assays do not enumerate the infectious particles but only indicate the presence or absence of infectious viruses. Serial dilutions of the virus suspension are inoculated into animals, embryonated eggs or tissue cultures. Endpoints used for infectivity titration are the death of the animal, haemagglutination in allantoic fluid or the appearance of CPE in cell cultures. The virus titre is usually expressed as the '50 per cent infectious dose' (ID 50) per ml, which represents the highest dilution of the virus suspension that would produce an effect in 50 per cent of inoculated animals, embryonated eggs or tissue cultures.

2. Quantitative Infectivity Assay

This assay measures the number of viable infectious virus particles in a suspension and is similar to the estimation of viable counts of bacteria by colony counting. Two methods are employed—*plaque assay* in monolayer cell culture and *pock assay* on chick embryo CAM.

(i) Plaque assay

A viral suspension is inoculated into confluent monolayer of cultured cells in a bottle or petri dish. After allowing time for adsorption, the monolayer is covered with agar gel, to ensure that the spread of progeny virions is confined to immediate vicinity of infected cells. Each infectious virus particle gives rise to a localised focus of infected cells, called a *plaque*. Plaques can be seen with the naked eye. Each plaque indicates an infectious virus, therefore, the plaque titre is the infectivity titre.

(ii) Pock assay

Each pock on CAM arises from a single virus particle. Viruses that form pocks on CAM can be assayed by counting the number of pocks which corresponds with the number of viruses present in the inoculum. This is known as *pock assay*.

VIII. VIRAL GENETICS

Like other living beings, viruses also obey the laws of genetics. Several properties of viruses (e.g. virulence and antigenicity) are under genetic control. Genetic modifications may occur by different mechanisms which are as follows:

A. Mutation

The frequency of mutation is about 10^{-4} to 10^{-8}, which is almost the same as in bacteria. Mutations, therefore, occur during every viral infection. Most mutations are lethal, because the mutant virus is unable to replicate. A mutant becomes evident only if the mutation confers some readily observable property or survival advantage. Mutants occur spontaneously or may be induced by chemical agents such as nitrous oxide, hydroxylamine or 5-fluorouracil and physical agents such as UV light or irradiation.

Mutations may affect virulence, antigenicity, plaque or pock morphology and resistance.

Types of Mutants

1. Conditional lethal mutants

Conditional lethal mutants are of great importance in laboratory studies. They can grow only under certain conditions (*permissive conditions*) but cannot grow under certain other specified conditions (called *non-permissive* or *restrictive conditions*). The majority of conditional lethal mutants are temperature sensitive (*ts*) mutants. They can grow at a low (permissive) temperature (28-31°C), but not at high (restrictive) temperature (37°C). Because of their low virulence, *ts* mutants have been used to produce attenuated live virus vaccine.

2. Host dependent mutants

The wild type rabbit pox virus grows very well in pig kidney (PK) cells but the PK-negative mutants fail to grow.

B. Interaction Between Viruses

1. Recombination

Genetic recombination may occur between two different but related viruses infecting a cell simultaneously. The two viruses exchange segments of nucleic acid between them so that a hybrid (*recombinant virus*) results. It possesses genes from both parents. Such recombinants are genetically stable and breed progeny like itself after replication. This type of recombination arises mainly from DNA viruses and at a low frequency with some RNA viruses.

2. Reassortment

This type of recombination is found in those viruses which have segmented genomes e.g. influenza, bunya and arena viruses. In a single cell infected with two related viruses, there is an exchange of segments with the production of reassortants. It is probably one of the ways by which the pandemic strains of the influenza virus originate in nature.

3. Reactivation

When a cell is infected with an active virus and a different but related inactive virus, recombination may occur between an active virus and inactive virus. This process is called *cross reactivation* or *marker rescue*. As a result of this the progeny possessing one or more genetic traits of inactive virus may be produced.

New epidemic strains of influenza virus often do not grow well in eggs as compared to well established laboratory strains. When such an epidemic strain (A2) is grown along with a standard strain (A0) that is inactivated by UV irradiation, a progeny may be produced which has antigenic characters of A2 but growth characters of A0. This finds application in the production of the influenza virus vaccines during epidemics.

C. Interaction Between Viral Gene Products

1. Phenotypic Mixing

When two different viruses infect the same cell, some "mix up" may take place during assembly so that progeny genome of one virus may be surrounded by a capsid belonging partially or entirely to the other virus. This is known as *phenotypic mixing*. This altered phenotype is not a stable variation. Upon subsequent passage, the phenotypically mixed parent will yield progeny containing

the original type only. In phenotypic mixing, when the genome of one virus is surrounded by the entire capsid of the other virus, it is called *transcapsidation*.

2. Genotypic Mixing

It occurs when more than one complete genomes are accidentally surrounded by a single virus capsid. It is not a stable genetic change. Since there is no recombination between the different genomes, the two kinds of viral progeny are formed on passage.

3. Complementation

In complementation, one virus provides the gene products (proteins specified by genes) to the second virus in which the latter is defective, this allows the second virus to replicate. For example, adenovirus is defective and cannot grow in simian cells but it can be rescued by SV 40. The latter grows in monkey cells and provides a gene product that allows adenovirus to grow.

IX. NOMENCLATURE OF VIRUSES

Viruses are classified on the basis of biological, physical and chemical properties. They are divided into families based on nucleic acid of the genome, size, shape, structure and replicative cycle. Families are subdivided into genera on the basis of physiochemical or serological differences. Each genus has got a number of species. Sometimes family has also got subfamily. The families, subfamilies and genera of viruses are named with suffix *viridae, virinae* and *virus* respectively. Viruses are broadly classified into DNA and RNA viruses and then further divided into families, subfamilies, genera and species (Table 51.3 and 51.4). Viruses can also be differentiated according to their genomes and presence or absence of envelope (Table 51.5).

VIROIDS

Viroids are single-stranded circular RNA molecules that lack a protein coat. They are plant pathogens.

PRIONS

Prions are infectious proteins without any detectable nucleic acid. They are highly resistant to physical and chemical agents. They are resistant to heat (90°C for three minutes), UV rays and nucleases and sensitive to proteases. They produce slow infections with long incubation period (in years). Diseases caused by them include scrapie of sheep and goats, mink encephalopathy, bovine spongiform encephalopathy, Kuru and Creutzfeldt-Jakob disease. For more details, refer 'Slow viral diseases' in Chapter 64. Also refer Chapter 4 for 'Sterilisation of Prions'.

Table 51.3 Taxonomy of DNA Viruses

Family	Subfamily	Genus	Species
Poxviridae	*Chordopoxvirinae*	*Orthopoxvirus*	Variola, Vaccinia, Cowpox, Monkeypox
		Parapoxvirus	Orf virus, milker's node virus
		Molluscipox virus	Molluscum contagiosum virus
		Yatapox virus	Tanapox, Yabapox virus
Herpesviridae	*Alphaherpesvirinae*	*Simplexvirus*	Herpes simplex virus type 1 and 2
		Varicellovirus	Varicella-zoster virus
	Betaherpesvirinae	*Cytomegalovirus*	Human cytomegalovirus
		Roseolovirus	Human herpesvirus type 6
			Human herpesvirus type 7
	Gammaherpesvirinae	*Lymphocryptovirus*	Epstein-Barr Virus
		Rhadinovirus	Human herpesvirus type 8
Adenoviridae	—	*Mastadenovirus*	Adenovirus
Papillomaviridae	—	*Alphapapillomavirus*	Human papillomavirus 32
		Betapapillomavirus	Human papillomavirus 5
		Gammapapillomavirus	Human papillomavirus 4
		Mupapapillomavirus	Human papillomavirus 1
		Nupapapillomavirus	Human papillomavirus 41
Polyomoviridae	—	*Polyomavirus*	Polyoma viruses, Simian virus 40 (SV 40)
Hepadnaviridae	—	*Orthohepadnavirus*	Hepatitis B virus, Woodchuck virus
Parvoviridae	*Parvovirinae*	*Bocavirus*	Not classified (human bocavirus)
		Dependovirus	Adeno-associated viruses (AAV)
		Erythrovirus	Parvovirus B19

Table 51.4 Taxonomy of RNA Viruses

Family	Subfamily	Genus	Species
Orthomyxoviridae	—	*Influenzavirus* A and B	Influenza virus A and B
		Influenzavirus C	Influenza C virus
Paramyxoviridae	*Paramyxovirinae*	*Respirovirus*	Human parainfluenza viruses types 1 and 3
		Rubulavirus	Human parainfluenza viruses type 2, 4a, and 4b, mumps virus
			Measles virus
		Morbillivirus	Hendra virus, Nipah virus
		Henipavirus	Human respiratory syncytial virus,
	Pneumovirinae	*Pneumovirus*	Human metapneumovirus
		Metapneumovirus	
Picornaviridae	—	*Enterovirus*	Human enteroviruses A to D
			Human rhinovirus A to C
		Parechovirus	Parechovirus types 1 and 2
		Hepatovirus	Hepatitis A virus
		Aphthovirus	Foot-and-mouth disease virus
Rhabdoviridae	—	*Vesiculovirus*	Vesicular stomatitis virus
		Lyssavirus	Rabies virus
Filoviridae	—	*Marburg virus*	Lake victoria marburg virus
		Ebola virus	Sudan ebola virus, Zaire ebola virus
Bornaviridae		*Bornavirus*	Bornadisease virus
Caliciviridae	—	*Norovirus*	Norwalk virus
		Sapovirus	Sapporo virus
Reoviridae	*Sedoreovirinae*	*Rotavirus*	Rotaviruses
		Orbivirus	Kemerovo viruses
		Seadornavirus	Banna virus
	Spinareovirinae	*Coltivirus*	Colorado tick fever virus
		Orthoreovirus	Orthoreovirses
Picobirnaviridae	—	*Picobirnavirus*	Human picobirnavirus
Togaviridae	—	*Alphavirus*	Eastern, Western and Venezuelan equine encephalitis viruses, Chikungunya virus
		Rubivirus	Rubella virus
Flavivirdiae	—	*Flavivirus*	Yellow fever, dengue, Japanese encephalitis virus
		Hepacivirus	Hepatitis C virus
Hepeviridae		*Hepevirus*	Hepatitis E virus
Bunyaviridae	—	*Phlebovirus*	Sandfly fever virus
		Orthobunyavirus	California encephalitis virus
		Nairovirus	Crimean-Congo haemorrhagic fever virus
		Hanta virus	Hantaan virus, Sin nombre virus
Coronaviridae	*Coronavirinae*	*Alphacoronavirus*	Human coronavirus 229 E, Human coronavirus NL 63
		Betacoronavirus	Betacoronavirus 1, severe acute respiratory syndrome related coronavirus
	Torovirinae	*Torovirus*	Human torovirus
Arenaviridae	—	*Arenavirus*	Lymphocytic choriomeningitis virus, Lassa viruses
Astroviridae	—	*Mamastrovirus*	Astroviruses
Retroviridae	*Orthoretrovirinae*	*Deltaretrovirus*	HTLV-1 and HTLV-2
		Lentivirus	HIV-1 and HIV-2
	Spumaretrovirinae	*Spumavirus*	Human foamy virus
—	—	*Deltavirus*	Hepatitis D virus

Table 51.5 Viruses According to Their Genomes and Envelopes

Genome	Envelope	Viruses
ds DNA	Yes	Herpes simplex viruses (type 1 and 2), Varicella-zoster virus, Cytomegalovirus (human herpesvirus 5), Human herpesvirus 4, Vaccinia, Variola, Molluscum contagiosum virus, Hepatitis B virus (HBV)
	No	Human adenovirus, Human papillomavirus, JC virus, BK virus
ss DNA	No	Parvovirus B 19
ds RNA	No	Human reovirus, Human rotavirus Colarado tick fever virus
ss RNA	Yes	Yellow fever virus, Dengue virus, Japanese encephalitis, Hepatitis C virus, Human corona virus, Sindbis virus, Semliki forest virus, Ross River virus, Eastern equine encephalitis virus, Western equine encephalitis virus, Rubella virus, HTLV, HIV type 1, HIV type 2, Simian immunodeficiency virus, Influenza A virus, Influenza B virus, Influenza C virus, Ebola virus, Marburg virus, Rabies virus, Lassa virus, Hantaan virus
	No	Norwalk virus, Hepatitis E virus, Human poliovirus, Coxsackie A virus, Coxsackie B virus, Echovirus, Enterovirus, Human rhinovirus, Hepatitis A virus

ds – double stranded; ss – single stranded

KEY POINTS

1. Viruses are the smallest obligate intracellular infective agents containing only one type of nucleic acid (DNA or RNA) as their genome.
2. They do not grow in inanimate media. They are resistant to antibiotics.
3. Viruses may have *icosahedral* (*cubical*) *symmetry*, *helical symmetry*, or *complex symmetry*.
4. The viruses multiply only in living cells. Three methods are employed for cultivation of viruses namely *animal inoculation*, *embryonated egg inoculation* and *tissue culture*.
5. Embryonated egg inoculation is done by one of the several routes such as *chorioallantoic membrane* (*CAM*), *allantoic cavity*, *amniotic sac* and *yolk sac*.
6. Three types of tissue cultures are available; *organ culture*, *explant culture*, *cell culture*. Cell cultures are of three types such as *primary cell cultures*, *diploid cell strains*, and *continuous cell lines*.
7. *Prions* are infectious proteins without any detectable nucleic acids. They produce *slow infections* with long incubation period (in years). Diseases caused by them include Kuru and *Creutzfeldt-Jakob disease*.

YOU MUST KNOW

1. Size, shape and symmetry of viruses.
2. Cultivation of viruses.
3. List of various cell cultures.
4. Detection of virus growth in cell cultures.
5. List of DNA viruses.
6. List of RNA viruses.
7. Prions.

STUDY QUESTIONS

1. Discuss the various methods for isolation of viruses in the laboratory.
2. Write short notes on:
   (a) Morphology of viruses
   (b) Viral haemagglutination
   (c) Replication of viruses
   (d) Determination of size of viruses
   (e) *ts* mutants
   (f) Recombination in viruses
   (g) Prions
   (h) Nomenclature of viruses.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Which of the following characteristics are true for viruses?
   (a) Obligate intracellular infective agents
   (b) Contain either DNA or RNA
   (c) Do not multiply by binary fission
   (d) All of the above
2. Which of the following is the largest virus?
   (a) Smallpox virus
   (b) Parvovirus
   (c) Coronavirus
   (d) Adenovirus
3. Which of the following is the smallest virus?
   (a) Papovavirus
   (b) Parvovirus
   (c) Adenovirus
   (d) Reovirus
4. Enveloped viruses are susceptible to:
   (a) Ether
   (b) Chloroform
   (c) Both of the above
   (d) None of the above
5. Poxviruses show:
   (a) Icosahedral symmetry
   (b) Helical symmetry
   (c) Complex symmetry
   (d) None of the above
6. Which of the following viruses is/are non-enveloped?
   (a) Papovaviruses
   (b) Picornaviruses
   (c) Adenoviruses
   (d) All of the above
7. Which of the following viruses is/are enveloped?
   (a) Herpesviruses
   (b) Adenoviruses
   (c) Papovaviruses
   (d) None of the above
8. Which of the following is the continuous cell line used in diagnostic virology?
   (a) Rhesus monkey kidney cell culture
   (b) Human amnion cell culture
   (c) Human embryonic lung cell stain
   (d) Human carcinoma of cervix cell line
9. Which of the following is a diploid cell line?
   (a) Hela
   (b) WI-38
   (c) McCoy
   (d) BHK-21
10. Which of the following routes of inoculating embryonated hen's egg is/are used for cultivation of viruses?
    (a) Chorioallantoic membrane
    (b) Allantoic cavity
    (c) Amniotic cavity
    (d) All of the above

Answers (MCQs):

1. (d)	2. (a)	3. (b)	4. (c)	5. (c)
6. (d)	7. (a)	8. (d)	9. (b)	10. (d)

Chapter 52

VIRUS-HOST INTERACTIONS

I. INTRODUCTION

Virus-host interaction may cause different effects, ranging from no apparent cellular damage to rapid cell destruction. Some viruses (e.g. poliovirus) cause cell death (cytocidal infection). Others may cause cellular proliferation or malignant transformation (oncogenic viruses). In some instances, viruses remain as latent infections (herpes simplex virus) whereas others produce some morphological change in cells to form inclusion bodies (rabies virus).

Cytocidal Infection

Viruses like enteroviruses and reoviruses kill host cells by inhibition of protein, RNA and DNA synthesis.

Cell Transformation

Infection with hepatitis B virus (HBV), hepatitis C virus (HCV), Epstein-Barr virus (EB virus) and several papilloma viruses does not result in cell death, but leads to cell transformation. The transformed cells divide unrestrictedly leading to tumour production and these cells express certain antigens called *tumour-associated antigens*.

Latent Infections

Latent infections are of different types. Herpes simplex and varicella-zoster viruses remain latent in the nerve root ganglia, to be reactivated periodically in some individuals causing recurrent lesions. Some viruses like hepatitis B virus (HBV) may cause chronic infections which may remain inapparent for many years. However, it may lead to serious consequences, such as cirrhosis or hepatocellular carcinoma. Another type of latent infection is slow progressive infection. An important example of this type of infection is subacute sclerosing panencephalitis (SSPE). This disease develops between 1-10 years after recovery from measles virus infection. Other slowly progressive infections in man are due to human immunodeficiency virus (HIV), Kuru agent and Creutzfeldt-Jakob agent. Yet another class of latent infections is infection by oncogenic viruses.

Inclusion Bodies

Inclusion bodies are virus-specific intracellular globular masses which are produced during replication of virus in host cells. They can be demonstrated in virus infected cells under the light microscope after fixation and staining. They may be present in the cytoplasm (e.g. rabies virus), nucleus (e.g. herpesviruses) or both (e.g. measles virus). In general, viruses that are assembled in the nucleus (usually DNA viruses) produce intranuclear inclusions, whereas assembly in the cytoplasm (mainly RNA viruses) yields cytoplasmic inclusions. Intracytoplasmic inclusions are found in cells infected with rabies virus (Negri bodies), vaccinia (Guarnieri bodies), molluscum contagiosum (molluscum bodies) and fowlpox (Bollinger bodies). Molluscum bodies are very large (20-30 mm). Intranuclear inclusions were classified into two types by *Cowdry* (1934). Cowdry type A includes inclusion bodies of variable size and granular appearance (e.g. herpes virus, yellow fever virus) and type B inclusions are more circumscribed and multiple (e.g. adenovirus, poliovirus). They are generally acidophilic and can be seen as pink coloured bodies when stained with Giemsa or eosin-methylene blue stains. However, inclusions of some viruses (e.g. adenovirus) are basophilic (stained by haematoxylin). Demonstration of inclusion bodies helps in the diagnosis of some viral infections. Intracytoplasmic eosinophilic inclusions (Negri bodies) in the brain cells of animals are useful for presumptive diagnosis of rabies.

Damage to the Chromosomes

Certain viruses (measles, mumps, cytomegalovirus, adenovirus) cause damage to the chromosomes of host cells. Chromatid gaps and breaks in chromosome 17 occur frequently with adenovirus types 12 and 31.

Alteration in Infected Cells

Respiratory syncytial virus (RSV) causes fusion of adjacent cell membranes leading to syncytium formation. Sometimes virus coded antigens may appear on the surface of infected cells. These antigens may confer new properties on the host cells. For example, viral haemagglutinin appears on the cells infected with influenza virus and leads to adsorption of erythrocytes to the cell surface (haemadsorption).

II. PATHOGENESIS OF VIRAL INFECTIONS

Viruses enter the body through the following routes (Table 52.1).

1. Respiratory tract
2. Alimentary tract
3. Skin
4. Genital tract
5. Conjunctiva
6. Congenital

Table 52.1 Routes of Transmission of Viral Infections

Route of transmission	Viruses
Respiratory tract	Influenza A, B and C Parainfluenza Respiratory syncytial virus (RSV) Measles Mumps Rubella Rhinovirus Adenovirus Coronavirus Coxsackie virus 'A' Varicella-zoster Cytomegalovirus (CMV) Epstein-Barr virus (EBV)
Alimentary tract	Poliovirus Adenoviruses Coxsackieviruses Echovirus Hepatitis A Virus Hepatitis E Virus Rotavirus Norwalk virus
Skin	Herpes simplex Papilloma viruses (Human warts) Molluscum contagiosum Rabies virus (animal bite) Arboviruses (insect bite) HBV, HCV, HIV, HTLV (through injections via blood)
Genital tract	Herpes simplex viruses Hepatitis B virus (HBV) Hepatitis C virus (HCV) Human immunodeficiency virus (HIV) Papillomaviruses
Conjunctiva	Some adenoviruses Few enteroviruses

1. Respiratory Tract

The respiratory tract offers the most important portal of entry for viruses. Viruses enter the body through droplets expelled from the nose or mouth of infected persons during talking, coughing or sneezing. Some viruses, such as influenza and rhinoviruses are restricted to the respiratory tract where they multiply and produce local disease. These are known as respiratory viruses. Other viruses, such as measles, rubella, varicella-zoster and cytomegalovirus (CMV) multiply locally to initiate a silent local infection which is followed by lymphatic or haematogenous spread to other sites where more extensive

multiplication takes place before producing generalised disease.

2. Alimentary Tract

Next to the respiratory tract, the alimentary tract is the most important route of entry of viruses. The viruses that initiate infection via the alimentary tract are many enteroviruses (e.g. poliovirus, hepatitis A virus), adenoviruses and viruses causing gastroenteritis such as rotavirus, Norwalk virus. Some of these (e.g. gastroenteritis viruses) remain localised to the gut while the others (e.g. poliovirus) are transported to other sites for further multiplication and subsequent spread to the target organs.

3. Skin

Of the viruses that enter through the skin, only a few produce local lesions. Viruses may enter the skin, through abrasions (e.g. papillomaviruses, molluscum contagiosum), insect bites (e.g. arboviruses), animal bites (e.g. rabies virus) or injections (e.g. hepatitis B virus, human immunodeficiency virus). Some viruses such as papillomaviruses, vaccinia, cowpox, molluscum contagiosum produce only local lesions in the skin at the site of entry. Systemic spread of viruses occurs through lymphatic or blood. However, rabies virus travels along the nerves to the spinal cord or brain.

4. Genital Tract

Papillomaviruses and herpes simplex viruses are sexually transmitted and produce local lesions on the genitalia and perineum. HIV, HBV and HCV are also sexually transmitted but do not produce local lesions.

5. Conjunctiva

It may also act as a portal of entry for some adenoviruses (local disease) and a few enteroviruses.

6. Congenital

Congenital infections may occur at any stage from the development of the ovum up to birth. In acute systemic infections, these usually lead to foetal death and abortion. Rubella and cytomegalovirus may produce maldevelopment or severe neonatal disease.

SPREAD OF VIRUS

After entry of the virus, it multiplies initially and proceeds along the lymphatics to the local lymph nodes. After multiplication in the nodes, the virus enters the bloodstream (*primary viraemia*). It is then transported to the spleen and liver which act as the 'central foci' for viral multiplication. After extensive multiplication in the spleen and liver, there occurs a massive spillover of the virus into the blood stream (*secondary viraemia*). This heralds the onset of clinical symptoms. The virus reaches the target organ through the bloodstream. Multiplication in the target sites lead to distinctive lesions.

SIGNIFICANCE OF INCUBATION PERIOD

The incubation period is the time taken for the virus to spread from the site of entry to the target organs for the production of lesions. Its duration is therefore influenced by the relation between the site of entry, multiplication and lesion. When the site of entry and site of lesion are the same (e.g. respiratory viral infections), the incubation period is short—one to three days. In systemic diseases where the virus enters through the alimentary tract and produces lesions in remote target site (e.g. poliomyelitis), the incubation period is longer—10-20 days. There are, however, exceptions to this rule. In dengue fever, the incubation period may be shorter (5-6 days), probably because the virus is introduced directly into the bloodstream by the insect vector. The incubation period in HBV may be 2–6 months and in slow viruses, many years.

III. HOST RESPONSES TO VIRUS INFECTIONS

The outcome of a virus infection depends on the virulence of the infecting strain and resistance offered by the host. The mechanism of host resistance may be non-specific or immunological.

A. Non-Specific Responses

1. Age

Most of the viral infections tend to be most serious at extremes of life. Rotaviruses cause severe disease only in infants.

2. Hormones

Corticosteroids administration enhances most viral infections. Injudicious use of steroids in the treatment of herpetoconjunctivitis may cause blindness. Normally mild infections such as varicella and vaccinia may be lethal in patients on cortisone. The deleterious effect of cortisone may be due to its depression of the immune response and inhibition of interferon synthesis.

3. Malnutrition

Malnutrition interferes with the humoral and cell-mediated immune responses, therefore, it can exacerbate viral infections.

4. Body Temperature

Most of the viral infections are accompanied by fever. Fever may act as a natural defence mechanism against viral infections as most viruses are inhibited by temperature above 39°C.

5. Phagocytosis

Macrophages phagocytose viruses and are important in clearing viruses from blood-stream. Polymorphonuclear leucocytes do not play any significant role.

6. Interferons

Interferons (IFNs) are a family of glycoproteins produced by cells on induction by viral or non-viral microorganisms. These interferons have antiviral activity by inhibiting protein synthesis. There is little or no viral specificity, hence, IFN induced by one virus can confer protection against infection by the same or unrelated viruses. However, some IFNs have a certain degree of host species specificity e.g. mouse interferons are ineffective in humans and vice versa.

Types of interferons

They are classified into three types:

(*i*) *IFN–α* : It is induced by virus infection and produced by leucocytes. It has antiviral activity.

(*ii*) *IFN–β* : It is also induced by virus infection but produced by fibroblasts and epithelial cells. It has antiviral effect.

(*iii*) *IFN–γ* : It is produced by T-lymphocytes and NK cells, on stimulation by antigens or mitogens. It is a lymphokine with immunoregulatory functions. It enhances MHC antigens and activates cytotoxic T-lymphocytes, macrophages and NK cells.

Mechanism of action

Interferons α and β are potent antiviral agents. These are synthesised when a cell becomes infected with a virus. IFN α and β induce the production of three enzymes namely synthetase, RNase L and protein kinase. This leads to inhibition of viral protein synthesis but does not affect host protein synthesis.

B. Immunological Responses

Viruses in general are good antigens and induce both antibody mediated and cell-mediated immunity (CMI). In some viral infections such as poxviruses, measles, herpes simplex virus and CMV infections, CMI appears to be the main immunospecific defence, for others, such as entero and arbovirus infections, antibody production appears to play a major role.

1. Antibody-Mediated Immunity

IgG, IgM and IgA antibodies are produced in response to virus infection. IgG and IgM play a major role in blood and tissue spaces while IgA is more important in mucosal surfaces. IgA is important in resistance to infection of the respiratory, intestinal and urogenital tracts. Antibodies may act in the following ways:

(*i*) Neutralisation of virus which prevents attachment, penetration or subsequent events.

(*ii*) Antibody may attach to viral antigens on the surface of infected cells, rendering these cells prone to lysis by complement or destruction by phagocytes or killer (K) lymphocytes.

(*iii*) Immune opsonisation of virus for phagocytosis and destruction of virus by macrophages.

2. Cell-Mediated Immunity (CMI)

CMI prevents infection of target organs and promotes recovery from disease by destroying virus and virus-infected cells. The different mechanisms involved for virus destruction are as follows:

(*i*) Cytolysis by cytotoxic T-cells and Natural-killer (NK) cells.

(*ii*) Antibody-dependent cell-mediated cytotoxicity (ADCC).

(*iii*) Antibody-complement-mediated cytotoxicity.

IV. LABORATORY DIAGNOSIS OF VIRAL INFECTIONS

Following are the indications for laboratory diagnosis of viral infections:

1. *For proper management of certain diseases,* for example:
   - If rubella is diagnosed in the first trimester of pregnancy, abortion is recommended.
   - If a baby is borne of an HBsAg positive mother, immunisation at birth is mandatory.
2. *Diagnosis of diseases caused by viruses* for which antiviral chemotherapy is available (herpes viruses).
3. *Screening of blood donors* for HIV and hepatitis B virus helps to prevent spread of these viruses.
4. *Early detection of epidemics* like influenza, poliomyelitis, encephalitis etc. to initiate appropriate control measures.

In the laboratory, the following methods are commonly employed:

A. Direct demonstration of virus and its components

B. Isolation of virus

C. Detection of the specific antibodies

It is necessary that the appropriate specimens should be collected, preserved and transported to the laboratory in

the proper manner along with clinical and epidemiological information.

A. Direct Demonstration of Virus and its Components

1. Electron Microscopy

The detection of virus by electron microscopy (EM) is being used increasingly especially for viruses that are difficult to culture. Clinical applications of electron microscopy include detection of rotavirus and hepatitis A virus in faecal specimens. Many other viruses can also be detected in different specimens by electron microscopy (Table 52.2).

Table 52.2 Detection of viruses in specimens by electron microscopy

Specimen	Viruses
Faeces	Rotavirus, hepatitis A virus, adenovirus, Norwalk virus, astrovirus
Vesicular fluid	Herpes simplex, Varicella-zoster
CSF	Enterovirus, Varicella-zoster
Urine	Cytomegalovirus (CMV)

2. Immunoelectron Microscopy

The sensitivity of electron microscopy can be increased by adding specific antibody to the specimen to aggregate the virus particles. These aggregates can be observed under electron microscopy.

3. Fluorescent Microscopy

Direct or indirect fluorescent antibody technique can be used to detect viruses or viral antigens in vesicle fluid, in cell cultures or in frozen tissue sections. Fluorescent microscopy is a very useful method for diagnosis of rabies in brain of animals suspected to be rabid. The method is also useful for rapid diagnosis of respiratory infections caused by paramyxoviruses, orthomyxoviruses, adenoviruses and herpesviruses.

4. Light Microscopy

Inclusion bodies in tissue sections may be detected by light microscopy. Demonstration of inclusion bodies helps in diagnosis of some viral infections. Negri bodies (inclusion bodies) demonstration in the brain cells of animals is a useful method for presumptive diagnosis of rabies.

5. Viral Antigens

These may be detected by enzyme linked immunosorbent assay (ELISA), radioimmunoassay and latex agglutination.

6. Nucleic Acid Probes

Enzyme-labelled or radiolabelled nucleic acid sequence complementary to unique regions in nucleic acid sequence of a virus is known as nucleic acid probe. Such nucleic acid probes for most viruses are now manufactured commercially.

Two strands of the target DNA molecule in the clinical specimens are first separated and then allowed to hybridise with a labelled single stranded DNA or RNA probe. After hybridisation, this hybridised labelled probe can be detected by different methods depending on the type of label attached to the probe. For example, hybridised enzyme labelled probes can be detected by colour detection using appropriate substrate. Cytomegalovirus, papillomavirus and Epstein-Barr virus have been identified by use of nucleic acid probes.

7. Polymerase Chain Reaction (PCR)

With a PCR technique, a target DNA sequence can be amplified to the point where it can readily be identified using labelled probes in a hybridisation assay. Thus viral DNA extracted from a very small number of virions or infected cells can be detected. The technique can be used for the diagnosis of infections caused by HIV-1, HIV-2, human papillomaviruses, herpes simplex virus, hepatitis B virus, hepatitis C virus, enteroviruses, coxsackieviruses, rotavirus, rubella virus and Epstein-Barr virus.

B. Isolation of Virus

This is the commonest method used in the diagnosis of virus infections. The specimen should be collected properly and transported with least delay to the laboratory. Most viruses are heat labile, therefore, refrigeration is essential during transport. The methods used for isolation depend on the virus sought. In general, the viruses can be grown by inoculation into animals, eggs or cell cultures, after the specimen is processed to remove bacterial contaminants. The isolates are identified by neutralisation or other suitable serological techniques. Since many viruses (e.g. adenoviruses, enteroviruses) are frequently found in normal individuals, therefore, the results of isolation should always be correlated with clinical data. The techniques for the isolation of virus are described in detail in Chapter 51.

C. Detection of Specific Antibodies

The demonstration of a rise in titre of antiviral antibodies during the course of a disease is strong evidence that it is the aetiological agent. For this, paired sera should be collected from the patient, the *acute* sample collected early in the course of the disease and the *convalescent*

sample collected ten to fourteen days later. Examination of a single sample of serum is meaningful when IgM specific antibodies are detected. The serological techniques employed would depend on the virus, but those in general use are neutralisation, ELISA, haemagglutination inhibition, complement fixation test, immunofluorescence and latex agglutination tests.

V. IMMUNOPROPHYLAXIS

A. Active immunisation
B. Passive immunisation

A. Active Immunisation

Viral vaccines are used for active immunisation.

Viral Vaccines

Viral vaccines confer solid protection and are, in general, more effective than bacterial vaccines. Viral vaccines may be live or killed (Table 52.3).

LIVE VIRAL VACCINES

These are prepared from

- attenuated strain (e.g. yellow fever vaccines)
- temperature sensitive (*ts*) mutants (e.g. influenza)
- live recombinant viruses (e.g. influenza)

Advantages

1. A single dose of live vaccine is usually sufficient because they multiply in the host and provide continuous antigenic stimulation resulting in more lasting immunity than killed vaccines.
2. They may be administered by the route of natural infection so that local immunity is induced.
3. They induce a wide spectrum of immunoglobulins against the whole range of viral antigens.
4. They also induce cell mediated immunity.
5. They can, in general, be prepared more economically and administered more conveniently for mass immunisation.

Disadvantages

1. There is a risk, however remote, of reversion of virulence.
2. The vaccine may be contaminated with potentially dangerous viruses e.g. oncogenic viruses.
3. Live viral vaccines are heat-labile and they have to be kept under strict refrigeration.
4. Interference by preexisting viruses may sometimes prevent a good immunological response following vaccination.
5. The virus may spread from the vaccinees to contact and this is a serious danger in some situations (as in rubella, if the vaccine strain is teratogenic). In other cases, this may even be an advantage (as in poliomyelitis where the vaccination is extended to the community by the natural spread of the vaccine virus among children and adults).
6. Some live viral vaccines may cause local but remote complications.

Table 52.3 Commonly used Viral Vaccines

Type of vaccine	Mode of preparation
1. Live viral vaccines	
(a) Measles	(a) Attenuated virus grown in cell culture
(b) Mumps	(b) Attenuated virus grown in chick embryo fibroblast culture
(c) Rubella	(c) Attenuated virus grown in cell culture
(d) Poliomyelitis (Sabin type)	(d) Avirulent strain grown in monkey kidney cell culture
(e) Influenza	(e) Prepared by
(*i*) Live (attenuated)	(*i*) Virus attenuated by serial passage in eggs
(*ii*) Live (mutant)	(*ii*) Use of *ts* mutant which are avirulent
(*iii*) Live (recombinant)	(*iii*) Recombinants with surface antigens of new strains and growth characters of established strains
(f) Yellow fever (17D)	(f) Attenuated virus grown in chick embryo
2. Killed viral vaccines	
(a) Hepatitis B	(a) HBs Ag from human carrier sera
(b) Rabies	(b) Prepared by
(*i*) Cell culture vaccine	(*i*) Virus grown in cell culture and inactivated
(*ii*) Semple type vaccine	(*ii*) Fixed virus grown in sheep brain and inactivated by phenol or beta propiolactone (BPL)
(c) Japanese encephalitis	(c) Virus grown in mouse brain and inactivated by formalin
(d) Influenza (subunit vaccine)	(d) Virus disintegrated with sodium deoxycholate
(e) Poliomyelitis (Salk type)	(e) Virulent strain grown in monkey kidney cell culture

Oral polio, measles, mumps, rubella, yellow fever, varicella and measles-mumps-rubella (MMR) are currently available live viral vaccines.

KILLED VIRAL VACCINES

Killed vaccines are prepared by inactivating viruses with heat, phenol, beta-propiolactone and formaldehyde. To reduce the adverse reactions, subunit vaccines have been used. In subunit vaccines, the virus is split by detergents or other chemicals and only the relevant antigens incorporated in the vaccine. These vaccines include purified viral proteins and synthetic peptides. Vaccine production by cloning the desired antigen in bacteria or yeast may be used e.g. hepatitis B vaccine.

Advantages

1. Safety and stability
2. They can be given in combination as polyvalent vaccines.
3. There is no danger of spread of virus from the vaccinee.

Disadvantages

1. Multiple injections are needed. Booster doses may be required at intervals to revive waning immunity.
2. These vaccines have to be given by injection, therefore, local (IgA immunoglobulins) immunity fails to develop.
3. Cell mediated immunity is not induced.

Some of the currently available killed viral vaccines include rabies, influenza, hepatitis B, Japanese B encephalitis and inactivated polio vaccine.

B. Passive Immnunisation

This is indicated for non-immune individuals who are at special risk and are exposed to infection. Passive immunisation with human gammaglobulins or specific antiserum gives temporary protection against diseases such as measles, mumps and infectious hepatitis. Combination of active and passive immunisation is an established method for the prevention of rabies.

VI. CHEMOPROPHYLAXIS AND CHEMOTHERAPY OF VIRAL DISEASES

As viruses are strict intracellular parasites, they are absolutely dependent on the biosynthetic mechanisms of the host cell for their replication. Antiviral agents block the replication of viruses by various mechanisms. Viral replication may be checked at the level of attachment, penetration, transcription of viral nucleic acid, translation of viral mRNA and inhibition of viral DNA polymerase and reverse transcriptase and replication of viral nucleic acid (Table 52.4).

Antiviral chemoprophylaxis and therapy is at present very limited. *Amantadine* which inhibits the penetration of the influenza virus into cells has been found useful in prophylaxis. A derivative *rimantadine* is less toxic and equally effective. Acyclovir has emerged as a very useful drug in treatment of herpesvirus infections. *Zidovudine* (*azidothymidine, AZT*) is used widely against HIV infections (Table 52.4). Interferons are effective against many viruses.

Table 52.4 Antiviral Agents and their Modes of Action

Modes of action	Antiviral agents	Active against
1. Inhibits viral DNA polymerase	(a) Acyclovir (b) Ganciclovir (c) Ribavirin (d) Trisodium phosphonoformate	(a) Herpes simplex, varicella zoster (b) Cytomegalovirus (c) Respiratory syncytial virus (RSV), Lassa virus (d) Herpes viruses, hepatitis B virus
2. Inhibits reverse transcriptase	(a) Zidovudine (azidothymidine) (b) Dideoxycytidine (ddc) (c) Dideoxyinosine (ddI) (d) Trisodium phosphonoformate	Human immunodeficiency virus (HIV)
3. Inhibits proteases	(a) Indinavir (b) Nelfinavir (c) Ritonavir (d) Saquinavir	Human immunodeficiency virus (HIV)
4. Blocks penetration of virus into cells	Amantadine	Influenza virus
5. Inhibits protein synthesis	Interferons	Many viruses

KEY POINTS

1. Virus-host interaction may cause different effects, ranging from no apparent cellular damage to rapid cell destruction.
2. Some viruses (e.g. poliovirus) cause cell death (*cytocidal infection*). Others may cause cellular proliferation or malignant transformation (*oncogenic viruses*). In some instances, viruses remain as *latent infections* (herpes simplex virus) whereas others produce some morphological change in cells to form *inclusion bodies* (rabies virus).
3. Inclusion bodies are virus-specific intracellular globular masses which are produced during replication of virus in host cells.
4. Viruses may enter the body through *respiratory tract*, *alimentary tract*, *skin*, *genital tract*, *conjunctiva*, or *congenitally*.
5. *Interferons* (IFNs) are a family of glycoproteins produced by cells on induction of viral or non-viral microorganisms. These interferons have antiviral activity. They are classified into three types namely *IFN-α*, *IFN-β* and *IFN-γ*.
6. Laboratory diagnosis of viral infections depends on *direct demonstration* of virus and its components, *isolation* of virus, and detection of the *specific antibodies*.
7. Viral vaccines are used for active immunisation. These may be *live* or *killed*.
8. Passive immunisation is indicated for non-immune individuals who are at special risk and are exposed to infection. Human gammaglobulins or specific antiserum may be used for passive immunisation.

YOU MUST KNOW

1. Inclusion bodies.
2. Routes of transmission of viral infections.
3. Laboratory diagnosis of viral infections.
4. List of live viral vaccines.
5. List of killed viral vaccines.

STUDY QUESTIONS

1. Discuss the laboratory diagnosis of viral infections.
2. Discuss the pathogenesis of viral infections.
3. Write short notes on:
   (a) Latent infections (b) Inclusion bodies (c) Interferons
   (d) Live viral vaccines (e) Chemotherapy of viral diseases.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Intracytoplasmic inclusion bodies are found in cells infected with:
   (a) Rabies virus (b) Vaccinia virus
   (c) Molluscum contagiosum virus (d) All of the above
2. All of the following viruses are transmitted by the respiratory route except:
   (a) Coronovirus (b) Measles virus
   (c) Rubella virus (d) Norwalk virus
3. All of the following viruses may be transmitted through genital tract except:
   (a) Herpes simplex virus (b) Hepatitis C virus
   (c) Papillomavirus (d) Coronavirus
4. Electron microscopy can be used for the laboratory diagnosis of:
   (a) Rotavirus infections (b) Hepatitis A virus infections
   (c) Adenovirus infections (d) All of the above

5. All of the following vaccines are live except:
   (a) Measles vaccine
   (b) Rubella vaccine
   (c) Yellow fever vaccine
   (d) All of the above
6. Which of the following vaccines is killed vaccine?
   (a) Measles vaccine
   (b) Rubella vaccine
   (c) Rabies vaccine
   (d) Mumps vaccine
7. Acyclovir is used in the treatment of:
   (a) Herpes simplex infections
   (b) Hepatitis B infections
   (c) HIV infections
   (d) influenza
8. Amantidine is used in the treatment of:
   (a) Herpes simplex infections
   (b) Hepatitis B infections
   (c) HIV infections
   (d) Influenza
9. Which of the following drugs is/are active against HIV?
   (a) Zidovudine
   (b) Indinavir
   (c) Dideoxycytidine
   (d) All of the above

Answers (MCQs):

1. (d)	2. (d)	3. (d)	4. (d)	5. (d)
6. (c)	7. (a)	8. (d)	9. (d)	

Chapter 53

BACTERIOPHAGE

Bacteriophage

A. Morphology B. Life Cycle C. Significance of Phages

BACTERIOPHAGE

Bacteriophages are viruses that infect and parasitise bacteria. *Twort* (1915) described an infectious agent that distorted the appearance of staphylococcal colonies. *d' Herelle* (1917) observed that the filtrates of faeces cultures from dysentery patients induced transmissible lysis of a broth culture of a dysentery bacillus. He suggested that the lytic agent was a virus and gave it the name bacteriophage (Greek *phagein*, to eat, bacteriophage meaning bacteria-eater). These are now commonly abbreviated as *phages*. Phages occur widely in the environment such as sewage, faeces, soil and other natural sources of mixed bacterial growth. They may transmit genetic information from one bacterium to another by the process named *transduction*. Bacteriophages are highly host specific and on the basis of phages, typing of bacteria can be done.

A. Morphology

Bacteriophages that infect *Esch. coli*, called the T-even phages (T2, T4, T6), have been extensively studied. T-even phages serve as the prototypes in describing the properties of bacteriophages.

T even phages are tadpole-shaped, possess a head and a tail.

1. *Head*: It is hexagonal in shape and consists of a tightly packed core of nucleic acid (double stranded DNA) enclosed by a protein coat, called a *capsid*. The size of the head varies in different phages from 28 nm to 100 nm. The head of phage T4 has a diameter of 65 nm and is 100 nm long.
2. *Tail*: It is composed of a hollow core surrounded by a contractile sheath, and a terminal base plate which has attached to it prongs, tail fibres (usually six in number) or both (Fig. 53.1). The tail of phage T4 is 100 nm in length and 25 nm in diameter. In contrast, phages T1, T5 and lambda of *Esch. coli* do not possess a contractile sheath.

Though most bacteriophages have the morphology as described above, phages that are spherical or filamentous have been identified. Most of the phages possess double stranded DNA, but single stranded DNA or RNA is also present in some phages.

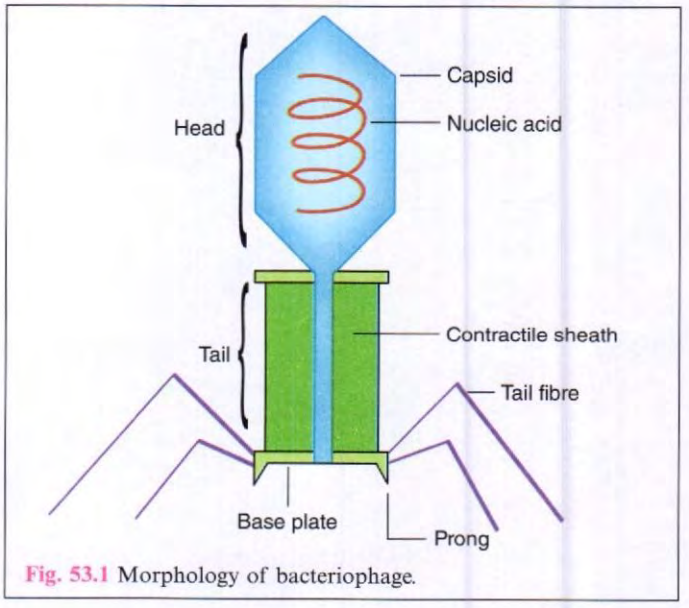


Fig. 53.1 Morphology of bacteriophage.

B. Life Cycle

Phages exhibit two different types of life cycle. In the *virulent* or *lytic cycle*, there is intracellular multiplication of phages producing lysis of infected cells and the release of progeny virions. In the *temperate* or *lysogenic cycle* the phage DNA either becomes integrated with the bacterial genome or exists as a free plasmid in the bacterial cell and replicates synchronously with it causing no harm to the host cell (Fig. 53.2).

Due to the presence of phage specific receptors on the bacterial surface, phages are usually specific for a few bacterial strains. This constitutes the basis of bacteriophage typing, by which bacteria can be identified and typed.

1. Lytic Cycle

Replication of a virulent phage can be divided into five stages—adsorption, penetration, synthesis of phage components, maturation and release of progeny phages.

(i) Adsorption

The phage particles come into contact with bacterial cells by random collision and a phage attaches to a specific receptor site by means of tail fibres. Adsorption is a specific process and depends on the presence of complementary chemical groups on the receptor sites of the bacterium and on the terminal base plate of the phage. Under optimal conditions, adsorption occurs within minutes. Infection cannot occur in

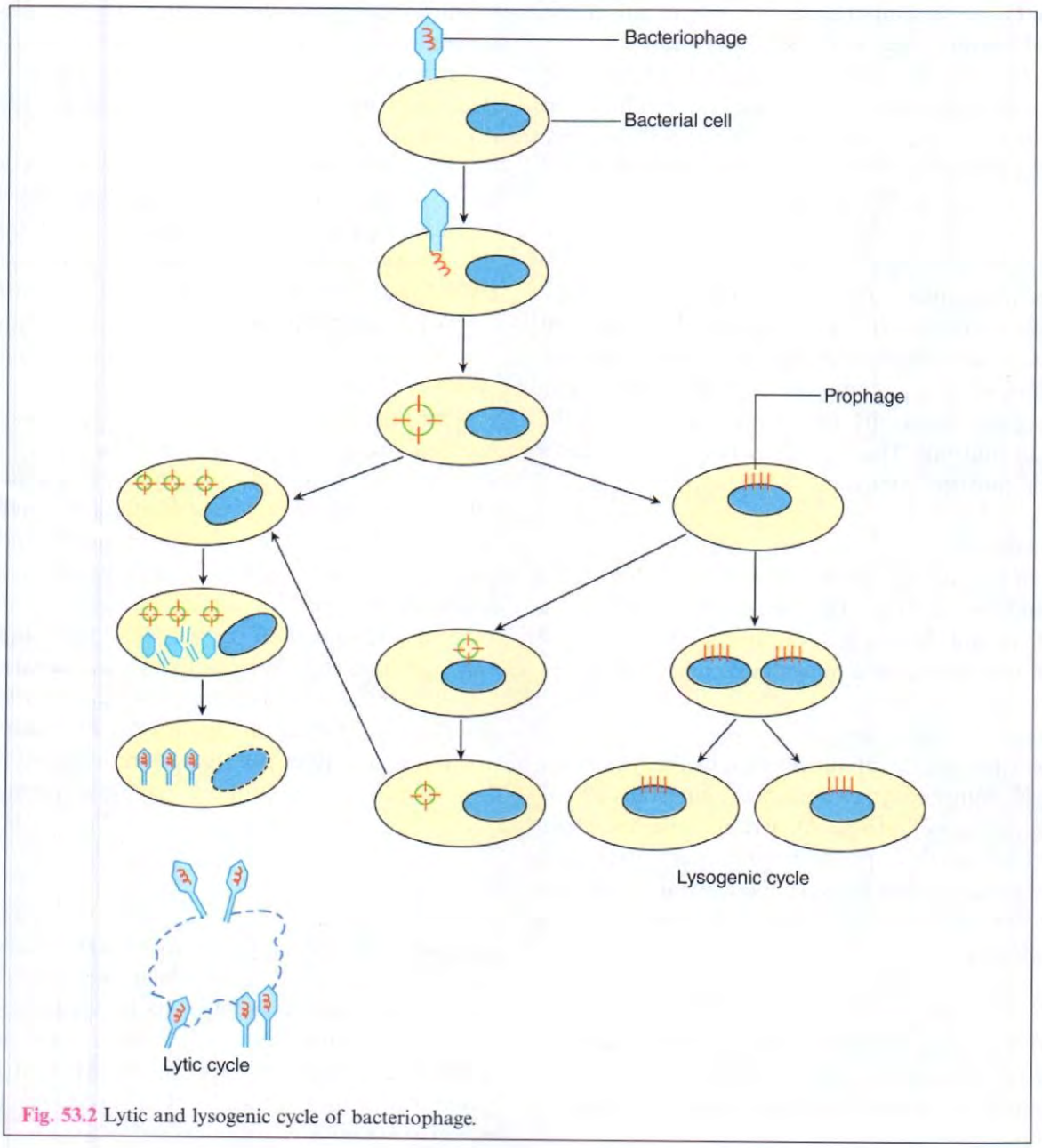


Fig. 53.2 Lytic and lysogenic cycle of bacteriophage.

the absence of adsorption. However, experimental infection by direct injection of phage DNA can be achieved even in bacterial strains that cannot be infected by the whole phage. The infection of a bacterium by the naked phage nucleic acid is known as *transfection*.

(ii) Penetration

Following attachment the base plate and tail fibres are held firmly against the bacterial cell causing the hollow core to pierce through the cell wall. The phage nucleic acid is injected into the bacterial body through the hollow core. Penetration may be facilitated by the presence on the phage tail of lysozyme which produces a hole on the bacterial cell wall for the entry of phage core. The process of penetration resembles injection through a syringe. After penetration, the empty head (capsid) and tail remain outside the bacterial cell as the shell or 'ghost'.

When bacteria are mixed with very large number of phages per bacterial cell, multiple holes are produced on the cell with the consequent leakage of the cell contents. Thus bacterial lysis occurs without viral multiplication, this is known as *lysis from without*.

(iii) Synthesis of phage components

Immediately after penetration, the synthesis of the phage components is initiated. The first products to be synthesised are specific enzymes (called *early proteins*) necessary for synthesis of phage components. Subsequently, *late proteins* appear, which are the protein subunits of the phage head and tail. During this period, the synthesis of bacterial protein, DNA and RNA ceases.

(iv) Maturation

Maturation consists of assembly of phage DNA, head protein and tail protein. The phage DNA acquires a protein coat and finally the tail structures are added forming a virion (infective phage particle).

(v) Release of progeny phages

The progeny phages are rapidly released by the lysis of the bacterial cell. Phage enzymes weaken the bacterial cell wall during replication of phage. As a result, the bacterium assumes a spherical shape. Enzyme concentration rises in the late stages of growth cycle and acts on the already damaged cell wall causing lysis of cell with release of progeny phages.

ECLIPSE PHASE

The interval between the entry of phage nucleic acid into the bacterial cell and the appearance of first infectious intracellular phage particle is called *eclipse phase* because the viruses cannot be detected within the host cell during this period. It represents the time required for the synthesis of the phage components and their assembly. The interval between the infection of a bacterial cell and the first release of phage particles is known as the *latent period.* The duration of eclipse phase is about 15 to 30 minutes in phages.

2. Lysogenic Cycle

In lysogenic cycle, the bacteriophage nucleic acid becomes inserted into the bacterial chromosome. The integrated phage genome in this state is known as the *prophage*. The prophage behaves like a segment of the host chromosome and replicates synchronously with it. This phenomenon is called *lysogeny*. The bacterium that carries a prophage is known as a *lysogenic bacterium*. The prophage confers certain new properties on the lysogenic bacterium. This is known as *lysogenic conversion* or *phage conversion*. Bacteriophage that parasitise a bacterium without lysing it is known as *temperate phage*.

Exotoxin production in *C. diphtheriae* is determined by the presence in it of the prophage *beta*. The elimination of this phage abolishes the toxigenicity of the bacillus. *C. botulinum* types C and D produce toxin only if these are infected with phage CE β and DE β respectively.

A lysogenic bacterium is resistant to reinfection by the same or related phages. It is known as *superinfection immunity*.

Occasionally integrated prophage may become 'excised' from bacterial DNA. The excised prophage initiates lytic cycle and the daughter phage particles are released, which infect other bacteria and render them lysogenic. This is known as 'spontaneous induction of prophage.' It is a rare event but all lysogenic bacteria in a population can be induced to shift to the lytic cycle by exposure to certain physical (UV rays) and chemical (nitrogen mustard, hydrogen peroxide) agents.

If a bacterium simultaneously adsorbs two related but slightly different phage particles, both can infect and reproduce. On lysis, both types are released. When this occurs many of the progeny may be recombinants.

C. Significance of Phages

1. Virulent Phage

(i) Phage typing

It is used as epidemiological marker to discriminate between bacterial strains that are biochemically and serologically indistinguishable. The strain to be typed is inoculated on a nutrient agar plate to produce a lawn culture. The phages are applied on a lawn culture in a fixed dose (*routine test dose*). Routine test dose (RTD) is the highest

dilution of the phage preparation that just produces confluent lysis. After overnight incubation, the culture will be lysed by some phages but not by others. The phage type of the strain is expressed by designation of phage/phages that lyse it. For example, if a bacterium is lysed by phages 52, 52A and 80, then phage typing is expressed as 52/52A/80. The area of lysis caused by a phage is known as *plaque* (Fig. 53.3).

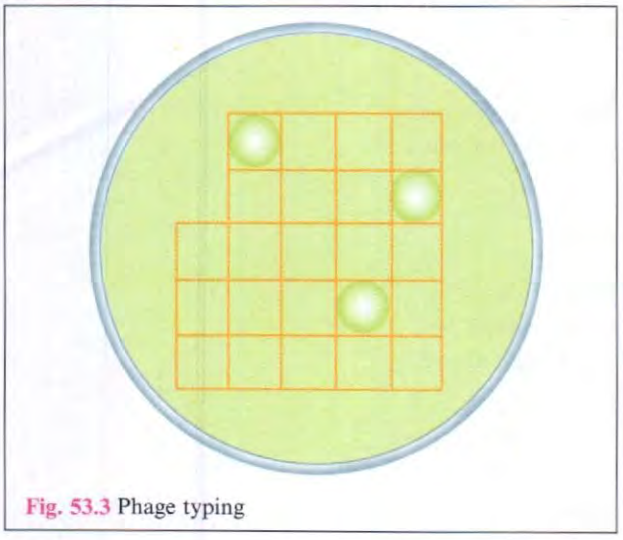


Fig. 53.3 Phage typing

Phages are available that lyse all members of a bacterial genus (e.g. genus-specific phages for *Salmonella*), all members of a species (e.g. phages for *B. anthracis*), and all members of a biotype (e.g. Mukerjee's phages for *V. cholerae*). The most important application of phage typing is for intraspecies typing of bacteria (e.g. phage typing of *Staph. aureus*).

(ii) Phage assay

When phages are applied on a lawn culture of a susceptible bacterium, areas of clearing or lysis occur after incubation. These zones of lysis are known as 'plaques'. Under optimum conditions, a single particle is capable of producing one plaque, plaque assay can be employed for titrating the number of viable phages in a preparation.

2. Temperate Phage

(i) Toxin production

Toxins production in *C. diphtheriae* and *Clostridia* are determined by genes carried in prophage DNA.

(ii) Antigenic property

The temperate phages of *Salmonella* can modify the antigenic properties of somatic O antigen. The antigenic formula of *S. anatum* is 3, 10 : e, h: 1, 6 but when it is lysogenised by a phage, it changes to 3, 15: e, h: 1, 6 which is an antigenic formula of *S. newington*.

(iii) Transduction

Bacteriophages may act as carriers of genes from one bacterium to another. This process is known as *transduction*. Plasmid mediated drug resistance in staphylococci is an example of a property that is transmitted by transduction. This has been discussed in the Chapter 9.

(iv) Cloning vector

Bacteriophages have been used as *cloning vectors* in genetic manipulations.

KEY POINTS

1. Bacteriophages are viruses that infect and parasitise bacteria. They may transmit genetic information from one bacterium to another by the process named *transduction*.
2. Bacteriophages that infect *Esch. coli*, called the T even phages have been extentisvely studied. T even phages are tadpole-shaped, possess a *head* and a *tail*.
3. Bacteriophages exhibit two different type of life cycle. In the *virulent* or *lytic* cycle, there is intracellular multiplication of phages producing lysis of infected cells and the release of progeny virions. In the *temperate* or *lysogenic cycle* the phage DNA either becomes integrated with the bacterial genome or exists as a free plasmid in the bacterial cell and replicates synchronously with it causing no harm to the host cell.
4. Bacteriophage typing is used as epidemiological marker to discriminate between bacterial strains that are biochemically and serologically indistinguishable.
5. There is a role of bacteriophage (Beta phage) in production of *toxin* by *C. diphtheriae*.
6. Bacteriophages have also been used as *cloning vectors* in genetic manipulations.

YOU MUST KNOW

1. Morphology of bacteriophage.
2. Lytic and lysogenic cycle of bacteriophage.
3. Phage typing.

STUDY QUESTIONS

1. Describe the life cycle of a bacteriophage.
2. Write short notes on :
   (a) Morphology of T-even phage
   (b) Lysogenic conversion
   (c) Phage typing
   (d) Significance of phages.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Bacteriophage that parasitises a bacterium without lysing it, is known as:
   (a) Temperate phage
   (b) Virulent phage
   (c) Both of the above
   (d) None of the above
2. Which of the following bacteria can be typed by using phages?
   (a) *Salmonella typhi*
   (b) *Staphylococcus aureus*
   (c) *Bacillus anthracis*
   (d) All of the above
3. The area of lysis on a bacterial growth caused by a phage is known as:
   (a) Plaque
   (b) Pock
   (c) Prophage
   (d) None of the above
4. In which of the following processes, bacteriophage may act as carrier of genes from one bacterium to another?
   (a) Transformation
   (b) Transduction
   (c) Conjugation
   (d) Transposition
5. Which of the following character/s is/are due to lysogenic conversion?
   (a) Production of toxin in *Corynebacterium diphtheriae*
   (b) Production of toxin in *Clostridium botulinum* type C
   (c) Antigenic variation in *Salmonella anatum*
   (d) All of the above

Answers (MCQs):

1. (a) 2. (d) 3. (a) 4. (b) 5. (d)

Chapter 54

POXVIRUSES

Poxviruses

A. Morphology
B. Resistance
C. Antigenic Structure
D. Cultivation
E. Viruses Causing Human Infections
F. Prophylaxis

POXVIRUSES

Poxviruses belong to the family *Poxviridae* and cause a number of human diseases. The family poxviridae is divided into two subfamilies, *Chordopoxvirinae* (the poxviruses of vertebrates) and *Entomopoxvirinae* (the poxviruses of insects). Chordopoxvirinae have eight genera but members of only four genera are related to human infections (Table 54.1). They are the largest and the most complex of all viruses. They can be seen under the light microscope. They replicate in the cytoplasm unlike other DNA viruses.

A. Morphology

The Poxviruses are brick-shaped. They are the largest animal viruses measuring 300 × 200 × 100 nm in size and can be seen under the light microscope. Nucleocapsids of these viruses do not show any discernible symmetry and hence are known as *complex viruses*. In vertical section, poxviruses have a biconcave double stranded DNA core, surrounded by a double layered membrane. The envelope is the outermost layer and it surrounds the outer membrane. On either side of the DNA core is a lens shaped structure called the lateral body (Fig. 54.1).

B. Resistance

Poxviruses, if protected from sunlight, may remain viable for months at room temperature. In the cold or in freeze dried form, they survive for years. They are susceptible to UV light and other irradiations. They are resistant to 1% phenol but are readily inactivated by formalin.

Table 54.1 Poxviruses Causing Human Diseases

Genus	Virus	Primary host	Diseases
Orthopoxvirus	Variola	Man	Smallpox (now eradicated)
	Vaccinia	Man	Vaccinia
	Monkey pox	Monkeys	Human generalised infections (rare)
	Cow pox	Cows	Human localised ulcerating lesions (rare)
Parapoxvirus	Milker's node	Cows	Human localised infections (rare)
	Orf	Sheep	
Molluscipoxvirus	Molluscum contagiosum	Man	Benign skin lesions
Yatapoxvirus	Tanapox	Monkeys	Human localised infections (rare)
	Yabapox	Monkeys	Human localised infections (very rare)

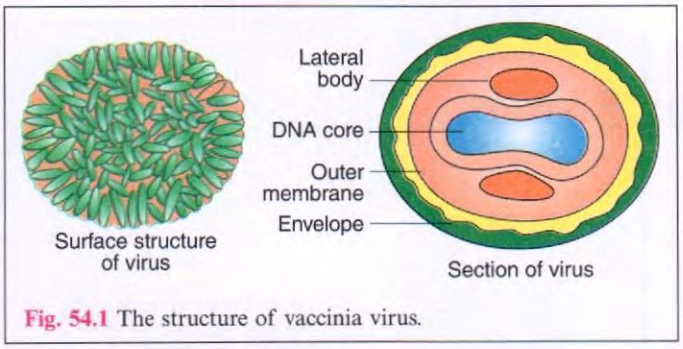


Fig. 54.1 The structure of vaccinia virus.

Though poxviruses are enveloped, they are not inactivated by ether.

C. Antigenic Structure

All poxviruses share a common nucleoprotein (NP) antigen. Other antigens include LS antigen (a complex of the heat labile L and the heat stable S antigens), agglutinogen and haemagglutinin.

D. Cultivation

They grow in chorioallantoic membrane (CAM) of chick embryo and in tissue culture. Both variola and vaccinia viruses produce pocks on the CAM in 48-72 hours. Variola pocks are small, shiny, white, convex, non-necrotic and non-haemorrhagic. In contrast, vaccinia pocks are larger, irregular, greyish, flat, necrotic and some of these are haemorrhagic (Fig. 54.2).

Tissue cultures of monkey kidney, HeLa and chick embryo cells can be used to grow these viruses. Cytopathic effects are produced by vaccinia in 24-48 hours but variola takes longer to produce these changes. Eosinophilic inclusion bodies (*Guarnieri bodies*) can be demonstrated in the stained preparations. Vaccinia can produce plaques in chick embryo tissue cultures but variola virus cannot.

E. Viruses Causing Human Infections

1. *Variola*

Variola virus causes smallpox and has a narrow host range i.e. humans and monkeys only. In past, smallpox epidemics appeared in two forms *florid* (fatal disease) occurred in India and *alastrim* (non-fatal disease) seen in Latin America. The name *variola major* was given to the virus causing classical smallpox and *variola minor* to the other virus causing alastrim. Two viruses are antigenically similar but differ in certain biological properties.

Smallpox has been eradicated and routine vaccination is now stopped. The world's last naturally occurring case of smallpox was recorded in Somalia, in October 1977. On May 8, 1980, the WHO announced the global eradication of small pox. Eradication could be achieved because of

- no subclinical infection or carrier state,
- an effective vaccine (originally discovered by Jenner in 1796),
- no animal reservoir, and
- aggressive surveillance-containment measures.

Although smallpox has been eradicated,two laboratories still hold stocks of variola virus. These two laboratories are the WHO Collaboration Centre in Atlanta, USA and Koltsovo, Russian Federation.

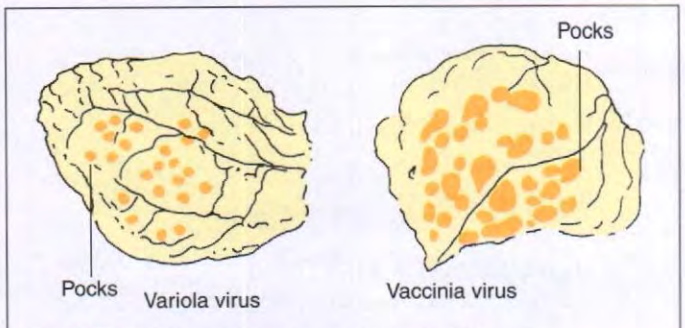


Fig. 54.2 Variola and Vaccinia pocks on CAM.

2. *Vaccinia*

Origin of vaccinia virus is not known. In the past, the vaccinia virus was used for small pox vaccination. It may have evolved from cowpox or smallpox virus. It is an 'artificial virus' and does not occur in nature as such. It causes a localised skin infection and has a broad host range including rabbit and mice. Vaccinia virus is being used as a vector for incorporating genes for protective antigens for several different pathogens. Many genes have been inserted, including those coding for the antigens of HIV, hepatitis B virus and rabies. Vaccinia virus is being employed as a vector for the development of recombinant vaccines. Vaccinia and variola viruses are similar in their properties. However, vaccinia virus has been studied in greater detail than variola, as it is safer to work with.

3. *Cowpox*

In cows, it produces ulcers on the teats and udders and human infection is acquired from cows by the process of milking. The lesions appear on the hands of man. These are localised lesions and undergo changes from macules to pustules as in smallpox. Rodents are the reservoir hosts of cowpox virus.

4. *Human Monkeypox*

Infection is probably acquired by handling infected animals. Human monkeypox resembles mild smallpox.

5. *Milker's Node*

It is transmitted to humans from teats and udder of

cattle. It is similar to that of cowpox, but rarely becomes pustular.

6. Orf

It is a contagious pustular dermatitis of sheep and goats. It is acquired by man after contact with an infected animal. In man, the disease occurs as a single lesion on finger or hand or occasionally on the face.

7. Molluscum Contagiosum

It is a benign epidermal tumour-like lesion, that occurs only in humans. It mainly involves the arms, legs, buttocks and genital area. It is a contagious disease. The virus is also transmitted sexually in adults. Nodules show hyaline acidophilic inclusion bodies (molluscum bodies) within the proliferated epidermal cells. Molluscum contagiosum virus has not yet been cultured.

8. Tanapox

It is probably acquired by insect bite. Reservoirs are some wild animals. It produces scanty vesicular lesions of the skin. Epidemics have been reported in East Africa.

9. Yabapox

It produces large benign tumours in monkeys. Similar lesions have been reported in a laboratory person handling affected monkeys.

F. Prophylaxis

Both vaccinia and variola viruses can be grown on the CAM of chick embryo. The two viruses differ from each other by only a single antigen. A natural infection of smallpox gives complete protection against reinfection. Vaccination with vaccinia induces protection against smallpox for about five years.

KEY POINTS

1. Poxviruses are the *largest* and the most complex of all viruses. They are brick-shaped.
2. They are DNA viruses.
3. They can be seen under the *light microscope*.
4. They grow in *chorioallantoic membrane* (*CAM*) of chick embryo and in tissue culture.
5. *Variola virus* is the causative agent of smallpox.
6. Smallpox has been eradicated and routine vaccination is now stopped.
7. Vaccinia virus is being employed as a vector in recombinant technology.
8. Molluscum contagiosum is a benign epidermal tumour like lesion in humans. It is a contagious disease. It is caused by *Molluscum contagiosum virus*. The virus is also transmitted sexually.

YOU MUST KNOW

1. Structure of vaccinia virus.
2. Molluscum contagiosum.

STUDY QUESTION

1. Write short notes on:
   (a) Vaccinia virus
   (b) Molluscum contagiosum.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Which is the largest virus among following viruses?
   (a) Adenovirus
   (b) Poxvirus
   (c) Papovavirus
   (d) Togavirus

2. The symmetry of nucleocapsid of poxviruses is:
   (a) Icosahedral (b) Helical
   (c) Complex (d) None of the above
3. On which day, the WHO announced the global eradication of small pox?
   (a) May 8, 1970 (b) May 8, 1975
   (c) May 8, 1980 (d) May 8, 1985
4. Which of the following viruses produces pocks on chorioallantoic membrane of embryonated hen's egg?
   (a) Vaccinia virus (b) Variola virus
   (c) Both of the above (d) None of the above
5. Which of the following viruses was used for small pox vaccination?
   (a) Vaccinia virus (b) Variola virus
   (c) Cowpox virus (d) Tanapox virus

Answers (MCQs):

1. (b) 2. (c) 3. (c) 4. (c) 5. (a)

Chapter 55

HERPESVIRUSES

I. CLASSIFICATION AND MORPHOLOGY

Herpesviruses are included in the family *Herpesviridae* which has been divided into three subfamilies (Table 55.1). This family consists of a number of DNA viruses, characterised by their ability to establish life-long latent infections which enable them to undergo periodic reactivation.

Morphology

Herpesvirus is 100–200 nm in diameter, contains an icosahedral capsid composed of 162 capsomers, containing linear double stranded DNA genome and is surrounded by a lipid envelope containing peplomers. Between capsid and envelope is 'tegument' (Fig. 55.1). They multiply in the nuclei of infected cells and produce Cowdry type A intranuclear inclusion bodies.

II. HERPES SIMPLEX VIRUS (HSV)

Man is the only natural host. There are two types of the herpes simplex virus, type 1 and type 2. *Herpes simplex* virus type 1 is usually associated with oral and ocular

Table 55.1 Classification of Human Herpesviruses

Subfamily	Scientific name	Common name
Alphaherpesvirinae	Human herpesvirus 1	Herpes simplex virus type 1
	Human herpesvirus 2	Herpes simplex virus type 2
	Human herpesvirus 3	Varicella-zoster virus
	Cercopithecine herpesvirus 1	Herpes B virus or herpesvirus simiae
Betaherpesvirinae	Human herpesvirus 5	Cytomegalovirus
	Human herpesvirus 6a	—
	Human herpesvirus 6b	—
	Human herpesvirus 7	—
Gammaherpesvirinae	Human herpesvirus 4	Epstein-Barr (EB) virus
	Human herpesvirus 8	Kaposi's sarcoma associated virus

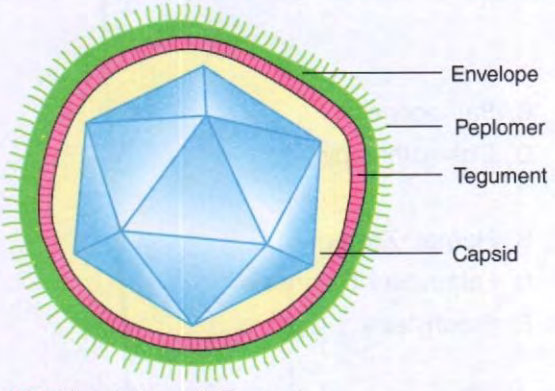


Fig. 55.1 Morphology of Herpesvirus.

lesions, while type 2 is responsible for the majority of genital infections.

A. Infections

HSV-1:

1. acute gingivostomatitis
2. herpes labialis
3. keratoconjunctivitis
4. eczema herpeticum
5. encephalitis
6. dendritic keratitis

HSV-2:

1. genital herpes (penis, urethra, cervix, vulva, vagina)
2. neonatal herpes
3. aseptic meningitis

Besides above mentioned primary infections, herpesviruses may present as latent infection, reactivation and recrudescence.

HSV-1 and HSV-2 cross react serologically. They can be differentiated by the following features:

(*i*) By using specific monoclonal antibodies.

(*ii*) HSV-2 forms larger pocks (resembling variola) on chick embryo CAM.

(*iii*) HSV-2 replicates well in chick embryo fibroblast cells, while HSV-1 does so poorly.

(*iv*) HSV-2 is more neurovirulent in laboratory animals than HSV-1.

(*v*) The infectivity of HSV-2 is more temperature sensitive than that of HSV-1.

(*vi*) HSV-2 is more resistant to antiviral agents like cytarabine and IUDR in culture.

(*vii*) Restriction endonuclease analysis of viral DNA can differentiate HSV-1 and HSV-2. By this method, strains within the same type can also be differentiated.

B. Pathogenesis

The primary infection occurs through the skin, oral mucous membranes or eyes resulting in a vesicle formation. The virus spreads to draining lymph nodes producing lymphadenitis. Lesions usually heal with residual scarring.

After primary infection the virus travels by retrograde intraaxonal flow to sensory root ganglia. They settle within the neurons in the ganglia, most common being trigeminal (HSV-1) and sacral (HSV-2) ganglia. The *Herpesvirus* DNA gets integrated into the host cell genome. These get reactivated when provoked by various stimuli such as common cold, fever, pneumonia, stress, exposure to sunlight etc.

C. Laboratory Diagnosis

1. Specimens

Specimens include vesicle fluid, skin swab, saliva, corneal scrapings, brain biopsy and CSF, according to the site of involvement.

2. Direct Examination

Smears prepared from scrapings from the base of vesicles are stained with toludine blue. Multinucleated giant cells with faceted nuclei and homogeneously stained 'ground glass' chromatin (*Tzanck cells*) are present in a positive smear. *Cowdry type A* intranuclear inclusion bodies may be seen in Giemsa stained smears.

Herpes virions may be demonstrated in specimens by electron microscopy. Viral antigens can also be demonstrated in the scrapings from the base of the lesions and tissue preparations, stained by immunofluorescent staining. The fluorescent antibody test on brain biopsy specimens provides reliable and early diagnosis in cases of encephalitis.

3. Tissue Culture

Virus can be isolated on human fibroblasts, HEp-2 cells, Vero cells and chorioallantoic membrane. Swollen, rounded cells may appear within 1-5 days. Some virus strains (particularly HSV-2 strains) may give rise to syncytium (fusion of infected cells) formation. Diagnosis can be confirmed by immunofluorescent staining of infected cell culture. HSV-1 and HSV-2 can be differentiated by use of monoclonal antibodies in immunofluorescent staining or by neutralisation test with specific antiserum.

4. Serology

Primary infections can be diagnosed by detection of virus specific IgM antibody or by a rising titre of antibody. Various tests like complement fixation test (CFT), neutralisation, immunofluorescence, ELISA and RIA have been employed for antibody detection. However, serology is not widely used.

5. Polymerase Chain Reaction (PCR)

PCR can be used for detection of HSV DNA in CSF.

D. Chemotherapy

HSV infection can be treated with acyclovir (acyclo-guanosine). It acts by interfering with viral DNA synthesis by inhibiting DNA-dependent DNA polymerase. It may be used in the form of ointment for the treatment of ocular lesions. Type 2 infection is more resistant to treatment than type 1. Valaciclovir and famciclovir are more effective oral agents. When resistance to these drugs develop, drugs like trisodium phosphonoformate (Foscarnet) may be useful.

III. VARICELLA-ZOSTER

Varicella (chickenpox) and herpes zoster (shingles) are caused by a single virus, for which it is named Varicella-zoster virus (VZV). Chickenpox follows primary infection in a non-immune individual, whereas herpes zoster is a reactivation of the latent virus when immunity falls to ineffective level. Thus, contact with either chickenpox or zoster may lead only to chickenpox, but not zoster.

VZV is similar to the herpes simplex virus in its morphology. It can be grown in human fibroblasts, human amnion or HeLa cells. The cytopathic effects are similar to but less marked than those produced by the herpes simplex virus. Only one antigenic type of VZV is known. VZV can be distinguished from HSV-1 and HSV-2 using specific antisera.

A. Varicella

Chickenpox is one of the commonest childhood exanthemata. The virus enters through respiratory route. The source of infection is a chickenpox or herpes-zoster patient. Incubation period is usually 7-23 days. It is a highly infectious disease characterised by vesicular rash mostly on the trunk. The rash progresses through macule, papule, vesicle, pustule and scab. The rash is centripetal in distribution.

When varicella occurs in an adult, the disease is much more intense than in children. Varicella pneumonia is more common in adults, and is usually fatal when it occurs in the elderly.

Chickenpox is usually an uneventful disease and recovery is the rule. One attack confers life long immunity. Varicella may cross the placenta following viraemia in the pregnant woman and may infect the foetus. It may result in congenital malformations.

Pneumonia is a relatively common complication. Rarely post viral encephalitis and haemorrhagic (fulminating) varicella may occur.

B. Herpes zoster

The name is derived from *Herpein*, meaning to creep and *Zoster*, meaning girdle.

While chickenpox is typically a disease of childhood, herpes zoster is a disease of old age and occurs usually in persons who had chickenpox several years earlier. The virus remains latent in the sensory ganglia. The virus is usually held in check by the residual immunity. Years after the initial infection, when the immunity has fallen to ineffective levels, the virus may be reactivated and triggered by some precipitating stimulus. The reactivation is associated with the inflammation of the nerve which leads to neuritic pain that often precedes the skin lesions. The accompanying pain is often very severe and persists for weeks or months. The vesicles are usually confined to the area supplied by a single sensory ganglion. The vesicles are like those of varicella. These are usually unilateral. Thoracic nerves are more frequently involved.

Occasionally ophthalmic branch of trigeminal nerve is affected, causing *Ophthalmic zoster*.

The *Ramsay Hunt syndrome* is a rare form of zoster affecting the facial nerve, with a rash on the tympanic membrane and the external auditory canal, and often a facial palsy. In immunocompromised and cancer patients, disseminated zoster may sometimes be seen.

C. Immunity

Although the single virus is responsible for both the clinical entities, one attack of chickenpox confers life long immunity, but antibody fails to eliminate the virus from dorsal root ganglia. Hence, zoster occurs in persons who have immunity to varicella.

D. Laboratory Diagnosis

Diagnosis is usually clinical. Laboratory diagnosis includes:

1. Direct Microscopy

Stained smears from the base of early vesicles show multinucleated giant cells and type A intranuclear inclusion bodies under light microscope. Toludine blue or Giemsa stains are used for staining the smears. Herpes particles can also be detected by electron microscopy. Fluorescent antibody technique using monoclonal antibody is another rapid method for diagnosis. These methods may be used directly in the vesicle fluid.

2. Virus Isolation

Virus can be isolated in human fibroblast cells, human amnion, HeLa or Vero cells. Cytopathic effect is focal with refracticle ballooned cells. However, varicella zoster antigen can be demonstrated in nuclear inclusions by immunofluorescence using monoclonal antibody.

3. Polymerase Chain Reaction (PCR)

DNA can be extracted from specimen and amplified by PCR. It is useful for detection of varicella zoster virus (VZV) in CSF and other body fluids.

4. Serology

Varicella-zoster specific IgM antibody in patient's serum can be detected by ELISA. Other methods used for detecting antibody are CFT, neutralisation test and immunofluorescence.

E. Treatment

Acyclovir and vidarabine are effective in the treatment of severe varicella and zoster.

F. Prophylaxis

1. Varicella-zoster virus immunoglobulin prepared from patients convalescing from zoster gives passive protection in immunocompromised children exposed to infection. It is not useful in treatment.
2. A live attenuated varicella vaccine (*Oka* strain) has been developed by serial passage in tissue culture by Japanese workers. It is given by subcutaneous injection and is reported to provide protection lasting for several years. It was very labile and had to be stored frozen. A modified lyophilised form of the vaccine is now available. It can be stored between 2°C and 8°C. It is administered subcutaneously. Single dose is recommended for children 1-12 years old, and for those older as two doses 6-10 weeks apart. It is safe and effective. It is not considered safe in pregnancy.

IV. CYTOMEGALOVIRUS

Cytomegalovirus (CMV) is the largest virus in the herpesvirus family, being 150-200 nm in size. The virus exhibits strict host-specificity. Human cytomegalovirus can be grown in human fibroblast cultures. Cultures have to be incubated for prolonged periods, as the cytopathic effects are slow in appearance.

Cytomegalovirus infections are almost always inapparent, leading to prolonged latency, with occasional reactivation. An individual infected with CMV carries the virus for life. The virus is shed in urine, saliva, semen, cervical secretions, tears and breast milk. About 10% of healthy women may carry the virus in the cervix. Cytomegalovirus disease is rare but infection with the virus is extremely common.

A. Pathogenesis

Cytomegalovirus can be transmitted transplacentally from a mother with latent infection to the foetus. Congenital infection may remain inapparent (asymptomatic) at birth or may lead to cytomegalic inclusion disease which is often fatal. The disease is characterised by hepatosplenomegaly, jaundice, thrombocytopenic purpura, microcephaly and chorioretinitis. The virus is excreted in urine and saliva.

Postnatal infections are usually inapparent. This may be acquired by sexual intercourse, blood transfusion and organ transplantation. Clinical disease in adults resembles infectious mononucleosis. It may also cause hepatitis or pneumonitis. CMV may cause disseminated infection in immunocompromised individuals and can even lead to fatal infections. CMV is an important pathogen in AIDS.

B. Laboratory Diagnosis

1. Specimens

CMV can be isolated from urine, saliva, breast milk, semen, cervical secretions and blood leucocytes.

2. Demonstration of Cytomegalic Cells

Enlarged cells with large intranuclear "owl's eye" appearance inclusions (*cytomegalic cells*) can be demonstrated in the centrifuged deposits from urine or saliva. It is a simpler but less reliable technique.

3. Isolation of Virus

Virus can be grown in human fibroblast cultures. The virus replicates very slowly, therefore, cytopathic effects (swollen refractile cells with cytoplasmic granules) may take 2-3 weeks to appear. For precise identification, these cultures may be stained by immunofluorescence or immunoperoxidase technique using monoclonal antibody.

4. Antigen Detection

CMV antigen can be detected from blood leucocytes using monoclonal antibodies.

5. Polymerase Chain Reaction (PCR)

PCR is the most widely used molecular method for detection of CMV DNA.

6. Serology

CMV-specific IgM can be detected in the serum by ELISA.

C. Treatment

For treatment of severe CMV infections, ganciclovir is the drug of choice.

D. Prophylaxis

It is indicated only in high risk cases such as organ transplants, premature infants and immunodeficient persons. Screening of blood and organ donors and 'administration' of CMV immunoglobulins have been employed. Acyclovir is also useful in prophylaxis. No vaccine is available.

V. EPSTEIN-BARR VIRUS (EBV)

The virus is named after the discoverers *Epstein* and *Barr* who isolated it from Burkitt's lymphoma. EBV has affinity for lymphoid tissue. The B lymphocytes of human beings have receptors (CD21 molecules) for EBV, therefore, the virus specially affects these cells. EBV infected B lymphocytes are transformed in such a way that they multiply continuously. The transformed cells contain many EBV genomes.

A. Clinical Manifestations

EBV is ubiquitous in all human populations. 80 to 90% of children acquire EBV infection by the age of three years. Most EBV infections are inapparent. Once infected, the virus is present in the individual for life. The source of infection is usually the saliva of infected persons who shed the virus in oropharyngeal secretions. The virus is not highly contagious and droplets and aerosols are not efficient in transmitting infection. Intimate oral contact, as in kissing, appears to be the main mode of transmission. EBV has oncogenic properties and may cause Burkitt's lymphoma and nasopharyngeal carcinoma.

The following clinical manifestations may result from EBV infection:

1. Infectious mononucleosis or Glandular fever
2. Infections in immunocompromised hosts
3. EBV-associated malignancies

1. Infectious Mononucleosis (Glandular Fever)

It is an acute self limiting disease of children and young adults characterised by fever, sore throat, lymphadenopathy and the presence of abnormal lymphocytes in peripheral blood. There is often associated subclinical hepatitis.

The incubation period is 4 to 7 weeks and infection is believed to occur through respiratory route by close contact with patients. The disease is also believed to be transmitted by kissing (*kissing disease*) which supports the prevalence of the disease among adolescents and young adults. The disease usually lasts for 2-3 weeks.

EBV activates B lymphocytes and leads to secretion of immunoglobulin. IgM producing B lymphocytes predominate that produce high levels of IgM.

2. Infections in Immunocompromised Hosts

EBV may cause progressive lymphoproliferative disease in immunodeficient children, transplant recipients and AIDS patients.

3. EBV-Associated Malignancies

(i) Burkitt's lymphoma

It is a malignant neoplasm of B-lymphocytes (tumour of jaw) which occurs in regions of Africa and New Guinea. The disease occurs in endemic or sporadic type. The endemic or African type is almost always associated with EBV infection, and the sporadic type in which only 20 per cent show evidence of EBV infection. Cells of Burkitt's lymphoma carry multiple copies of EBV genome.

(ii) Nasopharyngeal carcinoma

Undifferentiated nasopharyngeal carcinoma is found in males of Chinese origin in South-East Asia and East Africa. EB viral DNA is regularly present in the malignant epithelial cells of the tumour.

(iii) B-cell lymphoma

Recipients of transplants and HIV infected patients may develop EBV associated B cell lymphoma.

B. Pathogenesis

The virus enters the pharyngeal epithelial cell through CD21 (or CR2) receptors. It multiplies locally, enters the bloodstream and infects B lymphocytes. In most cases, the virus remains latent inside the lymphocytes, which become transformed. These transformed or *immortalised* cells are capable of indefinite growth in vitro. They are polyclonally activated and produce many types of immunoglobulins. Some of the infected B lymphocytes show lytic infection, with cell death and release of progeny virions.

EB virus antigens are expressed on the surface of infected B lymphocytes. T lymphocytes undergo blast transformation in response to such neoantigens. These T lymphocytes are seen as *atypical lymphocytes* in blood smears of infectious mononucleosis patients.

Reactivation of the latent virus leads to clonal proliferation of infected B lymphocytes. In immunocompetent individuals, this is kept in check by T lymphocytes. In the immunodeficient persons, lymphomas may occur because of unchecked replication of B lymphocyte clones. Nearly half the lymphomas in immunodeficient persons contain EB virus DNA. Hyperendemic malaria in Africa is believed to be responsible for the immune impairment in children with Burkitt's lymphoma.

Genetic and environmental factors are important in the nasopharyngeal carcinoma in males of Chinese origin. EB virus DNA is regularly found in tumour cells. These patients have high levels of antibodies against EB virus.

C. Laboratory Diagnosis

1. White Blood Cell Count

During the initial phase, patient develops leucopenia due to a drop in the number of polymorphs. Later there is leucocytosis with a predominance of abnormal or atypical lymphocytes. These atypical cells are lymphoblasts derived from T cells reactive to the virus infection. These cells are important for diagnosis and constitute about 30-90% of total leucocytes.

2. Paul-Bunnell Test

During infectious mononucleosis, heterophile antibodies appear in the serum of the patient. These are IgM antibodies elicited by EBV infection. These antibodies appear in 85-90% of patients sera during the acute phase of illness. Their titre decreases rapidly after fourth week and are not detectable after 3 months. These antibodies agglutinate sheep erythrocytes.

(i) Procedure

Inactivated serum (56°C for 30 minutes) in doubling dilutions is mixed with equal volumes of 1% sheep erythrocytes suspension. These tubes are incubated at 37°C for four hours and examined for agglutination. A titre of 100 or above is suggestive of infectious mononucleosis.

(ii) Confirmation

Similar type of antibodies may occur after injections of sera and even sometimes in normal individuals. To confirm these antibodies, absorption of agglutinins with guinea pig kidney and ox red cells is necessary. Forssman antibody induced by injection of horse serum is removed by treatment with guinea pig kidney and ox red cells. Infectious mononucleosis antibody is removed by ox red cells whereas normally occurring agglutinins are removed by guinea pig kidney (Table 55.2).

Table 55.2 Absorption test for Paul-Bunnell Antibody

	Absorption with	
	Guinea pig kidney	Ox red cells
Normal serum	Absorbed	Not absorbed
Antibody after serum injections	Absorbed	Absorbed
Infectious mononucleosis	Not absorbed	Absorbed

3. EBV–Specific Antibodies

These are specific antibodies against EBV viral capsid antigen (VCA). The IgM antibody to VCA appears soon after primary infection and disappears in 1–2 weeks. It indicates primary infection. The IgG antibody to VCA persists throughout life and is an indication of past or recent infection. These can be demonstrated by indirect immunofluorescence or ELISA. The new appearance of antibody to the EB nuclear antigen (EBNA) is also a reliable marker for primary infection.

Antibodies to early antigens (EA) can be demonstrated in EB-associated lymphomas.

4. Antigen Detection

EBV antigen can be detected by immunofluorescence using monoclonal antibodies. EBNA 1 is very important antigen.

5. Nucleic Acid Hybridisation

It is the most sensitive method for detection of EB virus in patient material. Direct demonstration of viral antigen can be done in lymphoid tissues and peripheral blood cells.

6. Virus Isolation

Saliva or throat washings and peripheral blood cells can be inoculated onto lymphocytes. If specimen contains EBV, it produces a lymphoblastoid cell line. Viral culture is beyond the capabilities of most clinical laboratories.

7. Polymerase Chain Reaction (PCR)

EBV DNA can be detected by PCR.

VI. HUMAN HERPESVIRUS 6

Human herpesvirus 6 (HHV-6) infects dividing CD4+ T lymphocytes. Macrophages are also infected. Saliva is the main route of transmission. Most HHV-6 infections appear to be asymptomatic. They may, however, cause exanthem subitum or roseola infantum and mononucleosis with cervical lymphadenopathy.

Laboratory Diagnosis

HHV-6 can be isolated from peripheral blood mononuclear cells in early febrile stage of the illness by co-cultivation with lymphocytes. Virus antigen can be detected by immunofluorescence using monoclonal antibodies. Both antigen and antibodies can be detected in patient serum by ELISA.

VII. HUMAN HERPESVIRUS 7

Like HHV-6, it may also cause roseola infantum. Both HHV-6 and HHV-7 infect T lymphocytes using same CD4 receptors.

VIII. HUMAN HERPESVIRUS 8

It has been associated in causation of Kaposi's sarcoma, which is the commonest tumour in HIV infected individuals. HHV-8 can be detected by PCR.

IX. CERCOPITHECINE HERPESVIRUS 1 OR HERPESVIRUS SIMIAE (B VIRUS)

This virus was isolated from the brain of a laboratory worker who developed fatal ascending myelitis after being bitten by an apparently healthy monkey. It came to be known as 'B' virus from the initials of this patient. Herpesvirus simiae is similar to herpes simplex virus. The two are antigenically related but herpes simplex virus antibody does not protect against herpesvirus simiae infection.

Herpesvirus simiae infects monkeys in the same manner that herpes simplex infects man. Human cases occur by a bite or from handling infected animals. The typical lesions produced are vesicles on the buccal mucosa, which ulcerate shedding the virus and infecting contacts. The disease in man is usually fatal.

Laboratory Diagnosis

1. Electron microscopy of vesicle fluid.
2. Isolation of virus from blood, vesicle fluid and CSF.
3. DNA amplification by PCR.

KEY POINTS

1. Herpesviruses are DNA viruses, characterised by their ability to establish life-long *latent infections* which enable them to undergo periodic reactivation.
2. Herpesvirus contains a double stranded DNA genome and is surrounded by a lipid envelope containing peplomers. It has got icosahedral symmetry.
3. Human herpesviruses include human herpesvirus 1 (HHV1) to human herpesvirus 8 (HHV-8). HHV 3, HHV 4 and HHV 5 are *varicella-zoster virus*, *Epstein-Barr* (*EB*) and *cytomegalovirus* (*CMV*) respectively. Herpes simplex virus type 1 and 2 are designated as HHV 1 and HHV 2.
4. *Herpes simplex* virus type 1 is usually associated with *oral* and *ocular* lesions, while type 2 is responsible for the majority of *genital* infections.
5. Varicella (chickenpox) and herpes zoster (shingles) are caused by a single virus, for which it is named Varicella-zoster virus (VZV). Chickenpox follows primary infection in a non-immune individual, whereas herpes zoster is a reactivation of the latent virus when immunity falls to ineffective level.
6. Cytomegalovirus (CMV) infections are almost always inapparent, leading to prolonged latency, with occasional reactivation. An individual infected with CMV carries the virus for life. The virus is shed in urine, saliva, semen,

cervical secretions, tears and breast milk. Cytomegalovirus disease is rare but infection with the virus is extremely common.

7. CMV can be transmitted transplacentally from a mother with latent infection to the foetus.
8. Postnatal infections are usually inapparent. This may be acquired by sexual intercourse, blood transfusion and organ transplantation. CMV is an important pathogen in AIDS
9. CMV can be isolated from urine, saliva, breast milk, semen, cervical secretions and blood leucocytes.
10. Enlarged cells with large intranuclear "owl's eye" appearance inclusions (*cytomegalic cells*) can be demonstrated in the centrifuged deposits from urine or saliva. It is simpler but less reliable technique.
11. Epstein-Barr virus (EBV) has affinity for lymphoid tissue. The virus especially affects *B lymphocytes*. EBV infected B lymphocytes are transformed in such a way that they multiply continuously.
12. EBV is ubiquitous in all human populations. Most EBV infections are inapparent. Once infected, the virus is present in the individual for life.
13. The source of infection is usually the saliva of infected persons. Intimate oral contact, as in kissing, appears to be the main mode of transmission.
14. EBV has *oncogenic* properties and may cause *Burkitt's lymphoma* and *nasopharyngeal carcinoma*.
15. The other clinical manifestations include *infectious mononucleosis* and infections in immunocompromised hosts.
16. *Atypical lymphocytes* are seen in blood smears of infectious mononucleosis patients. These atypical cells are lymphocytes derived from T cells reactive to the virus infection.
17. *Paul-Bunnell* test is done to detect heterophile antibodies in the serum of patients with infectious mononucleosis.
18. Human herpesvirus 8 (HHV-8) has been associated in causation of *Kaposi's sarcoma*, which is the commonest tumour in HIV infected individuals.

YOU MUST KNOW

1. Morphology of Herpesvirus.
2. Classification of human herpesviruses.
3. Infections caused by *Herpes simplex* virus type 1 and *Herpes simplex* virus type 2.
4. Varicella-zoster virus.
5. Cytomegalovirus.
6. Clinical manifestations of Epstein-Barr virus (EBV).
7. Paul-Bunnell test.

STUDY QUESTIONS

1. Classify human herpesviruses. Discuss the laboratory diagnosis of infections caused by herpes simplex virus.
2. Write short notes on:
   (a) Varicella-zoster virus (b) Cytomegalovirus (c) Epstein-Barr virus (EBV)
   (d) Human herpesvirus 6 (e) Cercopithecine herpesvirus 1.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Which of the following infections is caused by human herpesvirus 1?
   (a) Acute gingivostomatitis (b) Infectious mononucleosis
   (c) Kaposi sarcoma (d) None of the above
2. What is the common name for human herpesvirus 4?
   (a) Cytomegalovirus (b) Epstein-Barr virus
   (c) Varicella-zoster virus (d) Herpesvirus simiae

3. What is the common name for human herpesvirus 5?
   (a) Cytomegalovirus (b) Epstein-Barr virus
   (c) Varicella-zoster virus (d) Herpesvirus simiae
4. Which of the following drugs is effective against herpes simplex virus infections?
   (a) Acyclovir (b) Ribavirin
   (c) Indinavir (d) Amantadine
5. Ramsay-Hunt syndrome can be caused by:
   (a) Herpes-zoster virus (b) Herpes simplex virus
   (c) Cytomegalovirus (d) Epstein-Barr virus
6. Which of the following tests can be used for laboratory diagnosis of Cytomegalovirus infections?
   (a) Demonstration of cytomegalic cells (b) Isolation of virus
   (c) Antigen detection (d) All of the above
7. 'Owl's eye' appearance inclusions can be demonstrated in laboratory diagnosis of:
   (a) Cytomegalovirus infections (b) Herpes simplex virus infections
   (c) Epstein-Barr virus infections (d) None of the above
8. Which of the following malignancy/ies is/are associated with Epstein-Barr virus?
   (a) Burkitt's lymphoma (b) Nasopharyngeal carcinoma
   (c) B-cell lymphoma (d) All of the above
9. Which of the following diseases can be caused by Epstein-Barr virus?
   (a) Infectious mononucleosis (b) Burkitt's lymphoma
   (c) Nasopharyngeal carcinoma (d) All of the above
10. Which of the following serological tests is employed for laboratory diagnosis of infectious mononucleosis?
    (a) Paul-Bunnel test (b) Widal test
    (c) Weil-Felix reaction (d) None of the above
11. Which of the following viruses is associated in causation of Kaposi's sarcoma?
    (a) Herpes simplex virus (b) Human herpesvirus 6
    (c) Human herpesvirus 8 (d) Herpesvirus simiae
12. Which of the following viruses may cause 'roseola infantum'?
    (a) Human herpesvirus 6 (b) Human herpesvirus 8
    (c) Herpes simplex virus (d) Herpesvirus simiae

Answers (MCQs):

1. (a)	2. (b)	3. (a)	4. (a)	5. (a)
6. (d)	7. (a)	8. (d)	9. (d)	10. (a)
11. (c)	12. (a)			

Chapter 56

ADENOVIRUSES

Adenoviruses were first detected from human adenoid tissue (*adeno*, from adenoid). They belong to family *adenoviridae*. These are non-enveloped, icosahedral, DNA viruses that replicate in the nucleus of infected cell.

I. ADENOVIRUSES

A. Classification

Adenoviruses are grouped into two genera, *Aviadenovirus* and *Mastadenovirus*. They possess avian and mammalian adenoviruses respectively. Human adenoviruses are divided into 7 species (previously named as subgenera)— A to G. Species B has been divided into two subspecies B1 and B2. These species contain about 52 human adenovirus serotypes (Table 56.1). All adenoviruses share a common complement fixing antigen. Type specific antigenic determinants are present on the pentons and fibres.

Table 56.1 Classification of Human Adenoviruses

Species	Serotype	Haemagglutination with red cells of	Oncogenicity in hamsters
A	12, 18, 31	Rat (partial)	High
B1	3, 7, 16, 21, 50	Monkey (complete)	Weak
B2	11, 14, 34, 35	Monkey (complete)	Weak
C	1, 2, 5, 6	Rat (partial)	None or low
D	8-10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, 42-49, 51	Rat (complete)	None but can transform cells
E	4	Rat (partial)	None or low
F	40, 41	Rat (partial)	None
G	52	Not determined	Not known
	Total serotypes 1 to 52		

Note: Haemagglutination is either complete or partial.

B. Morphology

Adenovirus is 70-75 nm in diameter with an icosahedral capsid containing double stranded DNA. Each capsid is made of 252 capsomers, arranged as icosahedron with 20 triangular facets and 12 vertices. Of the 252 capsomers, 240 hexons make up the 20 traingular facets of icosahedron and 12 pentons form the vertices. From each penton projects an apical fibre that serves to bind specifically to receptor sites on the host cell. Each penton unit consists of a penton base anchored in the capsid and an apical fibre consisting of a rod like portion with a knob attached at the distal end. This makes the appearance of the virion as a space vehicle (Fig. 56.1).

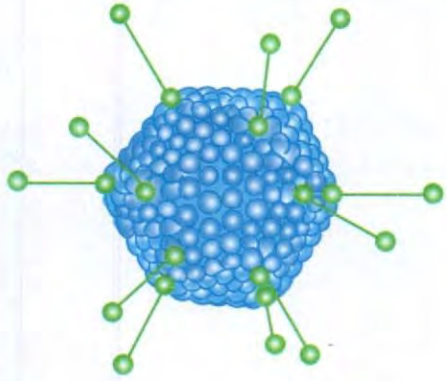


Fig. 56.1 Morphology of adenovirus

C. Resistance

Adenoviruses remain viable for about a week at 37°C but are readily inactivated at 50°C. They resist ether and bile salts.

D. Pathogenesis

Adenoviruses cause infections of the respiratory tract, eye, gastrointestinal tract and urinary tract (Table 56.2). Infection occurs through conjunctiva or nasal mucosa. Faeco-oral spread, particularly among children can also occur. Incubation period ranges from 5-7 days. They multiply initially in the conjunctiva, pharynx or small intestine and spread to draining lymph nodes. Serotypes 1 to 8 account for most illnesses associated with adenoviruses. Acute febrile pharyngitis is mainly caused by subgenus C viruses and acute respiratory diseases by subgenus B viruses. Serotype 40 and 41 may cause infantile gastroenteritis, serotype 8, 19 and 37 lead to eye infections while 19 and 37 may also cause genital infections. Serotypes 3, 4 and 11 are responsible for acute follicular conjunctivitis. The observations that some adenoviruses produce sarcoma in baby hamsters, had led to studies on the possible role of these viruses in malignancy. However, there is no evidence at all relating adenoviruses to human cancer.

Table 56.2 Diseases Associated with Various Serotypes of Adenoviruses

Serotypes	Disease
1, 2, 3, 4, 5, 6, 7	Acute febrile pharyngitis
4, 7, 14, 21	Acute respiratory disease
1, 2, 3, 7	Pneumonia
3, 7	Pharyngoconjunctival fever
3, 4, 11	Acute follicular conjunctivitis
8, 19, 37	Epidemic keratoconjunctivitis
40, 41	Diarrhoea and vomiting
1, 2, 5	Intussusception
11, 21	Haemorrhagic cystitis
19, 37	Genital infections
5, 34, 35, 43-47	Disseminated infection

E. Immunity

Adenoviruses induce long-lasting immunity. Maternal antibodies protect infants from adenoviruses.

F. Laboratory Diagnosis

1. Specimens

Throat swab, nasopharyngeal aspirate, bronchial lavage, conjunctival swab, corneal scraping, urine, anal swab, genital secretions and biopsy.

2. Microscopy

Viral particles may be seen directly in stool by electron microscopy. Viral antigens in the cells from respiratory tract, eye, urine and infected cell cultures may be demonstrated by immunofluorescence using monoclonal antibodies.

3. Isolation

Virus may be isolated from the throat, eye, urine or faeces by inoculating the clinical specimen in tissue cultures such as HeLa, Hep-2, KB and human embryo kidney cells. The cytopathic effects include rounding and clustering of swollen cells into grape-like clusters. Identification is done by immunofluorescence, CFT and haemagglutination tests. Typing is done by neutralisation tests.

4. Latex Agglutination Test

Enteric adenoviruses may be detected by using latex particles coated with specific antibody.

5. Polymerase Chain Reaction

It is the most sensitive technique for antigen detection. It is a rapid method for detecting all the human serotypes.

6. Serology

Rise in titre of antibodies should be demonstrated in paired sera. Examination of a single specimen of serum

is inconclusive as adenovirus antibodies are so common in the population.

G. Prophylaxis

Because of the existence of several serotypes and mild nature of adenovirus infections, vaccines are not practicable.

II. ADENOVIRUS-ASSOCIATED VIRUSES (AAV)

Small icosahedral viral particles (20-25 nm in diameter) have been found in several adenovirus preparations by electron microscopy. These particles are unable to multiply except in cells simultaneously infected with adenoviruses. These are named as *adeno-associated viruses* (AAV) or *adenosatellite viruses*. They can be detected by electron microscopy and CFT or immunofluorescence with specific antisera. Types 1, 2 and 3 are of human origin, while type 4 is of simian origin. Their pathogenic role is uncertain. They have been classified in the *Parvovirus* family.

KEY POINTS

1. Adenoviruses are non-enveloped, icosahedral, DNA viruses.
2. The appearance of adenovirus is like a *space vehicle*.
3. They cause infections of the respiratory tract, eye, gastrointestinal tract and urinary tract.
4. Adenovirus-associated viruses (AAV) are unable to multiply except in cells simultaneously infected with adenoviruses.

YOU MUST KNOW

1. Morphology of adenovirus.
2. Diseases associated with adenoviruses.
3. Adenovirus–associated viruses (AAV).

STUDY QUESTIONS

1. Discuss the laboratory diagnosis of infections caused by adenoviruses.
2. Write short notes on :
   (a) Classification of adenoviruses
   (b) Adenovirus-associated viruses (AAV).

MULTIPLE CHOICE QUESTIONS (MCQs)

1. What is the symmetry of capsid of adenoviruses?
   (a) Icosahedral (b) Helical (c) Complex (d) None of the above
2. Which of the following disease/s is/are associated with adenoviruses?
   (a) Acute febrile pharyngitis
   (b) Epidemic keratoconjuctivitis
   (c) Infantile gastroenteritis
   (d) All of the above
3. Which of the following cell lines may be used for isolation of adenoviruses from clinical specimens?
   (a) Hela cell line (b) Hep-2 cell line (c) KB cell line (d) All of the above
4. Which of the following viruses morphology appear as a space vehicle?
   (a) Herpes simplex virus (b) Adenoviruses (c) Cytomegalovirus (d) Rabies virus

Answers (MCQs):

1. (a) 2. (d) 3. (d) 4. (b)

Chapter 57

PICORNAVIRUSES

The picornavirus group comprises of a large number of very small (*pico*, small; *rna*, RNA) viruses containing single stranded RNA.

I. CLASSIFICATION

Picornaviruses belong to family *picornaviridae* which consists of six genera namely:

1. *Enterovirus* — viruses of enteric tract. It also contains rhinoviruses.
2. *Parechovirus* — Echoviruses 22 and 23 are classified in a separate genus as parechovirus types 1 and 2.
3. *Hepatovirus* — Hepatitis A virus is included in this genus.
4. *Aphthovirus* — causes foot and mouth disease.
5. *Cardiovirus* — produces encephalitis in mice.

Enteroviruses have been classified into four species, i.e., human enteroviruses A to D. Polioviruses are classified into human enterovirus C.

Picornaviruses of medical importance are shown in Table 57.1.

II. GENERAL CHARACTERISTICS OF PICORNAVIRUSES

1. Size– 22-30 nm in diameter
2. Capsid–60 capsomers in icosahedral (cubic) symmetry
3. Envelope–Non-enveloped
4. Genome–Positive sense RNA genome
5. Culture–Most members can be grown in human or simian cell cultures except some coxsackie group A serotypes for which inoculation into suckling mice may be necessary for their detection.

Important properties of these viruses are summarised in Table 57.2.

III. ENTEROVIRUSES

Enteroviruses are among the most stable viruses. Being non-enveloped they are insensitive to ether, ethanol and various detergents. They can be inactivated by ultraviolet light, formaldehyde (0.3%) or free residual chlorine (0.3–0.5 ppm).

A. Polioviruses

Polioviruses have now been classified under human enterovirus C group.

These viruses have affinity for nervous tissue. Poliovirus is the causative agent of poliomyelitis. Poliomyelitis is a

Table 57.1 Classification of Picornaviruses of Medical Importance

Genus	Species	Antigenic types	Number of serotypes
Enterovirus			
	Human enterovirus A (17 serotypes)		
	Human coxsackie virus A	2-8, 10, 12, 14, 16	11
	Enterovirus	71, 76, 89-92	6
	Human enterovirus B (56 serotypes)		
	Human coxsackie virus A	9	1
	Human coxsackie virus B	1-6	6
	Human echovirus	1-7, 9, 11-21, 24-27 and 29-33	28
	Enterovirus	69, 73-75, 77-88, 93, 97, 98, 100, 101	21
	Human enterovirus C (16 serotype)		
	Human coxsackie virus A	1, 11, 13, 17, 19-22 and 24	9
	Poliovirus	1, 2, 3	3
	Enterovirus	95, 96, 99, 102	4
	Human enterovirus D (3 serotype)		
	Enterovirus	68, 70, 94	3
	Human rhinovirus A, B, C (100 serotypes)	1-100	100
Parechovirus	Parechovirus	1-14	14
Hepatovirus	Hepatitis A virus	Previously classified as enterovirus 72	1

* Coxsackie A–type 23 is now classified as Echo 9 and coxsackie A type 4 and 6 are not yet assigned to a species
** Echo 10 is classified as Reovirus-1 and Echo 28 as Rhino-1, while Echo 22, 23 are classified as parechovirus types 1 and 2.

Table 57.2 Properties of Picornaviruses

Property	Enteroviruses	Rhinoviruses
1. Size	22-30 nm	30 nm
2. Capsid		
Symmetry	Icosahedral	Icosahedral
Genome	RNA, single stranded, positive sense	RNA, single stranded, positive sense
Polypeptides in shell	VP1, VP2, VP3, VP4	VP1, VP2, VP3, VP4
3. Optimum temperature for growth	37°C	33°C
4. Effect of acid	Stable (pH 3-9)	Labile (pH 3-5)

major problem in the developing countries. The poliovirus strains have been classified into three types: 1, 2 and 3.

1. Morphology

(*i*) Size : 27-30 nm diameter
(*ii*) Genome : RNA
(*iii*) Capsid : Composed of 60 capsomers arranged in icosahedral symmetry. Each capsomer is made of one molecule each of the four virion proteins VP1, VP2, VP3 and VP4.

2. Resistance

(*i*) It is resistant to ether, chloroform, bile, proteolytic enzymes of the intestinal contents and detergents. In faeces, it can survive for months at 4°C and years at –20°C.
(*ii*) Formaldehyde and oxidising disinfectants destroy poliovirus.
(*iii*) It is inactivated by heat (55°C for 30 minutes), but molar $MgCl_2$ protects the virus against heat inactivation.
(*iv*) The virus survives at 4°C for months and for years at –20°C.
(*v*) Chlorination destroys the virus in water, but much higher concentration of chlorine is necessary to destroy the virus in the presence of organic matter.
(*vi*) Poliovirus does not survive lyophilisation well.

3. Antigenic Properties

On the basis of neutralisation test, the poliovirus strains have been classified into three types 1, 2

and 3. Type 1 is the most common and causes most epidemics. Each type contains two type specific antigens C and D (C–coreless or capsid, D–dense). C antigens of all types are cross reactive but D antigen is type specific. The D antigen is also called the Native or N antigen. One attack of poliomyelitis gives life long immunity only against the type causing the infection.

4. Pathogenesis

Natural infection occurs only in man. Children are the most susceptible. The virus enters the body by ingestion. It multiplies in both the oropharynx and intestine, therefore, the virus can also spread by pharyngeal secretions (droplet infection) during first week of illness. No intermediate host is known.

Virus → Mouth → Multiplies in lymphatic tissues of oropharynx and intestine
↓
Bloodstream
↓
Spinal cord and brain → Destroys the anterior horn cells of spinal cord

5. Cultivation

Following cell cultures are used to grow poliovirus:

(*i*) Primary monkey kidney tissue cultures

(*ii*) Vero, HeLa and Hep-2

Cytopathogenic effect (CPE) produced by poliovirus is characterised by cellular disorganisation consisting of separation, swelling, increased refractivity and lysis.

6. Clinical Features

The incubation period is on an average about 10 days, but it may range from 4 days to 4 weeks. The earliest manifestations consist of fever, malaise, headache, drowsiness, constipation and sore throat lasting 1–5 days. There are four types of poliovirus infection:

(*i*) *Inapparent infection* (90-95%): These patients do not have any symptom but the virus may be isolated from stool or throat or both.

(*ii*) *Minor illness* (4–8%): Patient develops mild 'influenza-like' illness.

(*iii*) *Non-paralytic poliomyelitis* (1-2%): Patient develops headache, neck stiffness and back pain that may indicate some degree of aseptic meningitis.

(iv) *Paralytic poliomyelitis* (0.1-2%): Patient develops flaccid paralysis on the basis of the site of involvement, the paralysis may be classified as spinal, bulbar or bulbospinal.

7. Laboratory Diagnosis

(i) Specimens

Virus can be isolated from the faeces (throughout the course of disease), the pharyngeal washings (first 3-5 days) and autopsy specimen (spinal cord and brain). Virus usually cannot be recovered from CSF.

(ii) Direct demonstration of virus

Virus can be demonstrated in stool by direct electron microscopy or by immune electron- microscopy.

(iii) Isolation of virus

Specimens are inoculated into tissue culture. Primary monkey kidney cells, HeLa and HEp-2 are usually employed. The virus growth is indicated by typical cytopathic effect seen in cells within 2-3 days. The identification of serotype is made by neutralisation tests with pooled and specific antisera. Mere isolation of poliovirus from the faeces does not constitute a diagnosis of paralytic poliomyelitis as symptomless infections are so common. Virus isolation must be interpreted along with the clinical diagnosis.

(iv) Serology

The four fold rise of antibody titre can be demonstrated in paired sera by neutralisation test. Serodiagnosis is less often employed.

8. Prophylaxis

Two types of vaccines are available, killed polio vaccine (Salk) and live attenuated oral polio vaccine (Sabin). Killed vaccines induce only systemic antibody response but do not provide intestinal immunity. Live vaccine, on the other hand, induces both local secretory IgA antibodies in the intestine and also humoral antibodies (IgM and IgG).

(i) Salk's killed polio vaccine (Inactivated polio vaccine, IPV)

Killed polio vaccine was developed by *Salk* in 1953. It is a formalin inactivated preparation of the three types of the poliovirus grown in monkey kidney tissue culture. The vaccine is given by deep subcutaneous or intramuscular injection. Three doses are given 4-6 weeks apart followed by a booster to be given 6 months later. The first dose should be given after the age of six months so that antibody response is not impaired by the residual maternal antibodies. Immunity can be sustained by booster doses every 3–5 years thereafter.

This vaccine produces long-lasting immunity to all three poliovirus types. It induces serum antibodies (IgM, IgG). It does not induce secretory IgA in the intestine and therefore would not be able to prevent alimentary tract infections. The absence of live virus makes it safe to administer to immunocompromised individuals.

(ii) Live attenuated oral polio vaccine (OPV) (Sabin vaccine)

Oral polio vaccine was developed by *Sabin* in 1962. It contains live attenuated strains of polio virus types, 1, 2 and 3 grown either in monkey kidney cells or human diploid cell cultures and stabilised by magnesium chloride. The use of molar $MgCl_2$ stabilises the vaccine against heat inactivation. The shelf life of the vaccine is four months at 4–8°C but two years at –20°C. Improper storage conditions and cold chain failure may be partially responsible for failure to control poliomyelitis in the developing countries. Attenuated strains for live vaccine should have the following criteria:

1. They should not be neurovirulent.
2. They should be able to set up intestinal infection and should induce an immune response.
3. They should not acquire neurovirulence after serial enteric passage.
4. They should possess stable genetic markers by which they can be differentiated from the wild virulent strains.

The following markers are commonly used to differentiate wild strain from avirulent strain:

1. *d marker* : Wild strains will grow well in low levels of bicarbonate, but avirulent strains will not.
2. *rct 40*: Wild strains will grow well at 40°C, while avirulent strains grow poorly.
3. *MS*: Wild strains will grow well in stable cell line of monkey kidney but avirulent strains grow poorly.

Live attenuated oral polio vaccine contains Type 1 virus 10 lakh, Type 2 virus 2 lakh and Type 3 virus 3 lakh TCID50 per dose (0.5 ml). It is administered orally and parallels natural infection. It stimulates both local secretory IgA antibodies in the intestine as well as humoral antibodies (IgM and IgG). When the virus is excreted in the faeces, it may spread to close contacts and hence, it protects the individual and the community.

Vaccination schedule

It is administered orally. Three doses are given. In addition to three doses, zero dose is given at birth in institutional deliveries. First dose of OPV is given along with DPT at the age of 6 weeks. The second and third doses are administered at interval of 4–6 weeks. It has been recommended that in the tropics the number of doses of vaccine should be increased to five, in order to enhance the seroconversion rate. The booster dose is given at the age of 16–24 months. All the doses are generally given along with DPT.

Differences between killed polio vaccine and oral polio vaccine are shown in Table 57.3.

9. Epidemiology

Poliomyelitis is an exclusively human disease. Human beings are the only reservoirs, the patient or much more commonly the asymptomatic carrier. Faeces of patient or carrier is the important source of dissemination of polio virus in the community. Virus shed in the pharyngeal (throat) secretions in the early part of the disease is another equally important source of infection for the contacts of patients. Warm weather favours virus spread.

Table 57.3 Differentiating Features of Killed and Live Vaccines

	Killed polio vaccine	Oral polio vaccine
Virus	Killed virus	Live attenuated virus
Safety in immunodeficient persons	Safe	Not safe
Administration	Injectable (subcutaneous or intramuscular)	Orally
Economy	Costlier	Cheaper
Nature of immunity	Only systemic antibody response but no local (intestinal) immunity	Both local immunity and systemic antibody response
Duration of immunity	To be maintained by booster doses periodically	Life-long
Community protection	No	Yes
Storage	Does not require stringent storage conditions. Has a longer shelf life.	To be stored and transported in cold conditions
Usefulness in epidemics	Not useful	Useful

Poliovirus type 1 is responsible for most epidemics. Type 3 also causes epidemics to a lesser extent. Type 2 is quite common cause of paralytic poliomyelitis in India. In Western countries, type 2 usually causes inapparent infections. In India and other tropical countries, nearly 90 per cent of children have antibodies to all three types of poliovirus by the age of 5 years. In developing countries, 80 per cent of all paralytic cases occur before the age of three years. With improved sanitation and vaccination programme in advanced countries, the age distribution changed and paralytic cases in adults became more common. Vaccination of the whole population will have to be continued to eradicate the disease.

10. Eradication of Poliomyelitis and Pulse Polio Immunisation Programme

Poliomyelitis can be eradicated because man is the only host. A long-term carrier state is not known. Oral polio vaccine is suitable for poliomyelitis eradication because the live vaccine virus, by multiplying in the gut, can interrupt the transmission of the wild polioviruses. This vaccine virus is excreted in the vaccinated children, therefore, the live vaccine virus can also be transmitted to close contacts who have not been immunised. The simultaneous administration of vaccine to all children in a region on the same day (pulse immunisation) has been found to be useful to interrupt the transmission of wild poliovirus by displacing it from intestine, where the wild poliovirus multiply. This is the basis of Pulse Polio Immunisation Programme.

The World Health Organisation on 13th May, 1988 had passed a resolution committing the WHO to global eradication of poliomyelitis by the year 2000. Poor progress in immunisation in many countries has been a set back to this objective. Since the launch of Global Polio Eradication programme in 1988, polio cases have declined from 350,000 children paralysed or killed annually in 125 countries in 1988 to 620 cases reported in 16 countries in 2011. India launched the pulse polio immunisation (PPI) programme in 1995. Under this programme, all children under 5 years are given oral polio drops irrespective of their polio vaccination status. In 2006, India together with Nigeria, Pakistan and Afghanistan was one of the four polio-endemic countries (where wild poliovirus prospers and infects) left in the world. In 2009, India had 741 polio cases which were more than any other country in the world. There were 1604 polio cases reportedly globally during this year. However in 2010, India reported 42 cases which was the lowest ever since 66 cases reported in 2005. In 2011, India has had only one case of polio on January 13, 2011 in a two year-old-girl in Howrah district of West Bengal. On January 13, 2012, India achieved a major milestone by having no polio case for one year. Due to no polio case for one year, India's name was taken off the list of the World's four polio endemic countries by WHO. However there should be no case of polio infection over three consequative years for India to be declared as polio eradicated country. In 2014, India has been declared as polio eradicated country. WHO regions as a whole are certified as polio free and not single country. Thus with no polio case from India for last three years, WHO South East Asia Region (SEARO) has been certified as polio free. Now all countries in this region are polio free.

B. Coxsackieviruses

These viruses were first isolated in 1949 in Coxsackie village of New York state. They resemble polioviruses in properties and epidemiology. The morphology and the susceptibility of the virus to various agents is similar to those of other enteroviruses. The characteristic feature of the group is their ability to infect suckling mice. Based on the pathological changes produced in suckling mice, coxsackieviruses are classified into two groups, A and B (Table 57.4). Group A viruses produce a generalised myositis and flaccid paralysis leading to death of suckling mice within a week. Group B viruses produce a patchy focal myositis, spastic paralysis, necrosis of brown fat, pancreatitis, hepatitis and myocarditis. By neutralisation tests, group A viruses are classified into 21 types while group B into 6 types.

1. Clinical Features

Like other enteroviruses, coxsackieviruses inhabit the alimentary canal. Infection is transmitted by faeco-oral route. Incubation period varies from 2–9 days. They may cause following lesions:

Table 57.4 Differences between Coxsackie A and Coxsackie B

Feature	Coxsackie A	Coxsackie B
Pathological changes produced in suckling mice	Generalised myositis, flaccid paralysis, death within a week	Patchy focal myositis, spastic paralysis, necrosis of brown fat, pancreatitis, hepatitis and, myocarditis
Number of types	21 (1–24 except 15, 18 and 23)*	6 (1–6)

*Coxsackie A 23 is the same as ECHO 9

GROUP A VIRUSES

(*i*) *Aseptic meningitis* : It may be caused by most group A and all group B viruses. Occasionally, clinical picture may resemble paralytic poliomyelitis.

(*ii*) *Herpangina* (vesicular pharyngitis) : It is caused by types 2, 4, 5, 6, 8 and 10. It is characterised by abrupt onset of fever, pharyngitis, headache and pain in abdomen. It is usually seen in children.

(*iii*) *Hand-foot and mouth disease*: It is usually caused by type 5 and 16. The disease presents as a vesicular lesion involving mouth, hands and feet.

GROUP B VIRUSES

(*i*) *Epidemic myalgia (Bornholm disease)*: First described on Danish island of Bornholm, the disease is characterised by fever and stitch-like pain in the chest and abdomen. The disease may occur sporadically or as epidemics.

(*ii*) *Myocarditis and pericarditis*: Group B viruses cause severe and often fatal myocarditis in the newborn. The disease may also occur in children and adults.

(*iii*) *Aseptic meningitis* : Group B viruses may cause aseptic meningitis with paralysis.

(*iv*) *Juvenile diabetes*: Coxsackie B4 is reported to be associated with the disease.

(*v*) *Neonatal infections*: Transplacental passage may result in serious disseminated lesions such as hepatitis, myocarditis, meningoencephalitis and adrenocortical involvement.

(*vi*) *Postviral fatigue syndrome*: Coxsackie B viruses have been associated with this condition, but neither the condition nor the association has been clearly defined.

2. Laboratory Diagnosis

(i) Virus isolation from faeces or lesions

(*a*) *Inoculation into suckling mice*: Specimen is inoculated into suckling mice and the animals are then observed for illness. Identification is by studying the histopathology in infected mice. Typing can be done by neutralisation tests.

(*b*) *Tissue culture*: Monkey kidney cell line and human diploid embryonic lung fibroblasts support the growth of some coxsackie viruses. All coxsackie B viruses grow well in monkey kidney cell line, while in coxsackie A, only types 7 and 9 grow well. Cytopathic effects resemble those of poliovirus, but develop more slowly.

(ii) Serology

It is not practicable due to the existence of several antigenic types.

3. Prevention

Vaccination is not practicable because of several serotypes and immunity is type specific.

C. Echoviruses

Echoviruses (Enteric cytopathic human orphan viruses) infect only man. These were called *orphan viruses* because they were thought to be unrelated to any particular clinical disease. By neutralisation tests, they have been classified into 28 serotypes (1–33 except 8, 10, 22, 23 and 28). Type 10 has been reclassified as a reovirus and the type 28 as a rhinovirus. Echoviruses resemble other picornaviruses in their properties. Like other enteroviruses, they inhabit the alimentary tract and spread by the faeco-oral route.

1. Clinical Features

Most of the echoviruses produce asymptomatic infections. Only a few have been associated with clinical syndromes such as aseptic meningitis, paralysis, rash and fever, respiratory disease, infantile diarrhoea, pericarditis and myocarditis. Echoviruses perhaps constitute the most common cause of aseptic meningitis.

2. Laboratory Diagnosis

(i) Specimens

- Throat secretions
- Stool
- CSF

(ii) Isolation of virus

Specimen is inoculated on monkey kidney tissue cultures or human diploid embryonic lung fibroblasts. The cytopathic effect is similar to that of coxsackieviruses. Identification of the virus type is done by neutralisation tests.

(iii) Serology

It is difficult due to the existence of large number of serotypes.

D. Enteroviruses (Types 68–71)

Of the four enteroviruses in this group, three are associated with diseases in human beings (Table 57.5).

Table 57.5 Diseases Produced by Enteroviruses Types 68-71

Serotype	Disease
68	Pneumonia and bronchitis
69	—
70	Acute haemorrhagic conjunctivitis
71	Meningitis and encephalitis

Acute Haemorrhagic Conjunctivitis

In 1969, a pandemic of acute haemorrhagic conjunctivitis had spread widely involving several parts of Africa, India, South-East Asia, Japan, England and Europe. The incubation period is about 24 hours. The symptoms include sudden swelling, congestion, watering and pain in the eyes. Subconjunctival haemorrhage is a characteristic feature. Recovery is usually complete in 3-7 days.

The causative agent has been identified as enterovirus type 70. It can be grown on human embryonic kidney or HeLa cells on primary isolation, but can be adapted to grow on monkey kidney cells. Coxsackievirus type A 24 also produces the same disease.

IV. RHINOVIRUSES

Rhinoviruses are causative agents of common cold and are isolated commonly from nose and throat and rarely from faeces. They are named so because of their adaptation to grow in the nose (*Rhine*, nose). They can be differentiated from the enteroviruses on the basis of their acid lability (thus their inability to infect the intestinal tract) and their optimal temperature for growth (33°C) (Table 57.2). They are more heat stable.

1. Properties

Rhinoviruses resemble other picornaviruses in size and structure. They are small RNA viruses. They are destroyed at pH 3–5. They are more heat stable. They are fastidious growing only at 33°C (temperature of the nose). These properties differentiate rhinoviruses from other picornaviruses. On the basis of a type specific antigen in their capsid, rhinoviruses contain about 113 serotypes.

2. Host Range and Cultivation

Man is the only natural host. The viruses can be grown in human or monkey cell cultures with cytopathic changes, if good oxygenation (achieved by rolling), low pH (around 7) and low temperature (33°C) are provided. Based on growth in tissue culture, rhinoviruses are classified into three groups: M (Monkey) strains, H (Human) strains and O strains. M strains can grow in both human and monkey cells while H strains grow only in human cells. O strains could be grown only in nasal or tracheal ciliated epithelium.

3. Pathogenesis

Rhinoviruses cause common cold. The infection is transmitted by droplet infection via the upper respiratory tract. Incubation period is 2–4 days. Patient develops profuse watery discharge with nasal obstruction, sneezing, cough, headache, sore throat and malaise. Sometimes it is accompanied by fever. On an average, symptoms subside in about a week. A variety of other group of viruses which may also cause common cold include coronaviruses, respiratory syncytial virus, echoviruses, parainfluenza viruses and adenoviruses.

4. Laboratory Diagnosis

(i) Isolation of virus

Rhinoviruses may be isolated from nose and throat swabs or nasopharyngeal washings. Specimen is inoculated on human and monkey cell lines and incubated at 33°C.

(ii) Demonstration of antigen

Virus antigen may be demonstrated by ELISA.

(iii) Demonstration of RNA

Virus RNA may be demonstrated by PCR.

(iv) Serology

It is not practicable due to multiplicity of serotypes.

5. Prophylaxis

Rhinovirus 13, inactivated vaccine has been given trials with volunteers. It provided protection against some strains of virulent viruses. Preparation of effective vaccine is difficult due to multiplicity of antigenic types.

V. HEPATOVIRUS

Hepatitis A virus has been described with other hepatitis viruses in Chapter 62.

KEY POINTS

1. The picornavirus group comprises of a large number of very small viruses containing single stranded RNA. They are non-enveloped.
2. *Enterovirus*, *rhinovirus* and *hepatovirus* are three important genera of picornaviruses.
3. *Genus Enterovirus* contains *poliovirus*, *coxsackievirus*, *echovirus* and *enterovirus*.
4. Poliovirus is the causative agent of *poliomyelitis*. The poliovirus strains have been classified into three types : 1, 2 and 3.

5. Two types of vaccines are available for immunisation. These are *killed polio vaccine* (*Salk*) and *live attenuated oral polio vaccine* (*Sabin*). Killed vaccine induces only systemic antibody response but do not provide intestinal immunity. Live vaccine, on the other hand, induces both local secretory IgA antibodies in the intestine and also humoral antibody (IgM and IgG).
6. Live vaccine (Sabin) is administered orally. Three doses are given. First dose is given at the age of 6 weeks. The second and third doses are administered at interval of 4-6 weeks. The booster dose is given at the age of 16-24 months.
7. Oral polio vaccine is suitable for poliomyelitis eradication because the live vaccine virus, by multiplying in the gut, can interrupt the transmission of wild polioviruses. This vaccine virus is excreted in the vaccinated children, therefore, the live vaccine virus can also be transmitted to close contacts who have not been immunised.
8. The simultaneous administration of oral polio vaccine to all children in a region on the same day (*pulse immunisation*) has been found to be useful to interrupt the transmission of wild poliovirus by displacing it from intestine, where the wild poliovirus multiply
9. The characteristic feature of coxsackieviruses is their ability to infect suckling mice. Based on the pathological changes produced in suckling mice, they are classified into two group A and B. Group A viruses produce a generalised myositis and flaccid paralysis leading to death of suckling mice. Group B produces a patchy focal myositis, spastic paralysis, necrosis of brown fat, pancreatitis, hepatitis and myocarditis.
10. Coxsackieviruses may cause *aseptic meningitis*, *herpangina*, *hand-foot* and *mouth disease*, *epidemic myalgia*, *myocarditis*, *juvenile diabetes*, *postviral fatigue syndrome*.
11. *Echoviruses* (*Enteric cytopathic human orphan viruses*) infect only man. These were called *orphan viruses* because they were thought to be unrelated to any particular clinical disease.
12. Most of the echoviruses produce asymptomatic infections. Only a few have been associated with clinical syndromes such as aseptic meningitis, paralysis, rash and fever, respiratory disease, infantile diarrhoea, pericarditis and myocarditis.
13. Enterovirus type 70 causes *acute haemorrhagic conjunctivitis*.
14. Rhinoviruses are causative agents of *common cold*. They are named so because of their adaptation to grow in the nose (*Rhine*, nose). They can be differentiated from the enteroviruses on the basis of their acid lability (thus their inability to infect the intestinal tract) and their optimal temperature for growth (33°C). They are more heat stable.

YOU MUST KNOW

1. Vaccines for prevention of poliomyelitis.
2. Differentiating features of killed and live polio vaccines.
3. Differences between coxsackie A and coxsackie B viruses.
4. Diseases caused by coxsackie viruses.
5. Acute haemorrhagic conjunctivitis.
6. Rhinoviruses.

STUDY QUESTIONS

1. Classify picornaviruses. Discuss the pathogenesis and laboratory diagnosis of polioviruses.
2. Write short notes on:
   (a) Prophylaxis against poliomyelitis
   (b) Coxsackieviruses
   (c) Echoviruses
   (d) Enteroviruses
   (e) Acute haemorrhagic conjunctivitis
   (f) Rhinoviruses.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Which of the following genera belong to family *Picornaviridae*?
   (a) Enterovirus (b) Parechovirus
   (c) Hepatovirus (d) All of the above
2. How many types of poliovirus are there?
   (a) Two (b) Three
   (c) Four (d) Five
3. Which of the following cell cultures can be used to grow polioviruses?
   (a) Primary monkey kidney (b) Hep-2
   (c) HeLa (d) All of the above
4. How much percentage of poliovirus infections may develop into paralytic poliomyelitis?
   (a) 90–95% (b) 4–8%
   (c) 1–2% (d) 0.1–2%
5. Which of the following vaccines is killed vaccine?
   (a) Sabin polio vaccine (b) Salk polio vaccine
   (c) BCG (d) None of the above
6. Which of the following vaccines induce/s production of local secretory IgA antibodies?
   (a) Salk polio vaccine (b) Sabin polio vaccine
   (c) Both of the above (d) None of the above
7. Which vaccine is employed in pulse polio immunisation programme?
   (a) Salk vaccine (b) Sabin vaccine
   (c) BCG (d) None of the above
8. Which of the following coxsackieviruses can cause 'hand-foot and mouth disease'?
   (a) Group A (b) Group B
   (c) Both of the above (d) None of the above
9. Which of the following coxsackieviruses can cause 'Bornholm disease'?
   (a) Group A (b) Group B
   (c) Both of the above (d) None of the above
10. Which of the following viruses were called orphan viruses?
    (a) Polio virus (b) Coxsackievirus
    (c) Echovirus (d) Enterovirus
11. Which serotype of enteroviruses can cause acute haemorrhagic conjunctivitis?
    (a) Serotype 68 (b) Serotype 69
    (c) Serotype 70 (d) Serotype 71
12. Which of the following viruses are labile to acid?
    (a) Human enterovirus serotype 68 (b) Human enterovirus serotype 70
    (c) Rhinoviruses (d) Echoviruses

Answers (MCQs):

1. (d)	2. (b)	3. (d)	4. (d)	5. (b)
6. (b)	7. (b)	8. (a)	9. (b)	10. (c)
11. (c)	12. (c)			

Chapter 58

ORTHOMYXOVIRUSES

Influenza Viruses

A. Morphology
B. Antigenic Classification
C. Antigenic Structure
D. Antigenic Variation
E. Resistance
F. Cultivation
G. Pathogenesis
H. Laboratory Diagnosis
I. Immunity
J. Epidemiology
K. Prophylaxis
L. Treatment

Orthomyxoviruses are spherical or filamentous, enveloped viruses with single stranded and segmented RNA genome. They are so named because of their affinity for mucins (*ortho*: free, *myxa* : mucus). They have their ability to adsorb onto mucoprotein receptors on erythrocytes, causing haemagglutination. These viruses belong to family *orthomyxoviridae*. The viruses cause influenza, an acute respiratory illness. Besides humans, influenza viruses also infect pigs, horses and birds. The term myxovirus was originally proposed for those viruses which are able to attach to glycoprotein cell surface receptors with affinity for mucin. These viruses are now separated into two groups— *Orthomyxovirus* and *Paramyxovirus* due to differences in various properties (Table 58.1).

Table 58.1 Distinguishing Characteristics of Orthomyxovirus and Paramyxovirus

Property	Orthomyxovirus	Paramyxovirus
Size of virion	80–120nm	100–300 nm
Shape	Spherical; filamentous	Pleomorphic
Genome	Segmented; eight pieces* of single stranded RNA	Single piece of single stranded RNA
Nucleocapsid (diameter)	9 nm	18 nm
Site of ribonucleoprotein synthesis	Nucleus	Cytoplasm
Genetic recombination	Common	Absent
DNA-dependent RNA synthesis	Necessary for multiplication	Not required
Rate of antigenic change	High	Low
Haemolysin	Absent	Present

**Influenza virus* type C contains seven pieces of RNA

INFLUENZA VIRUSES

Influenza virus has three serotypes : type A, type B and type C. *Influenza virus* type A and type B are morphologically similar, but *Influenza virus* type C differs from them in certain respects, particularly in having only a single glycoprotein spike and RNA genome segmented into seven pieces.

A. Morphology

Morphology of influenza virus (Fig. 58.1) is as follows:

(i) Spherical Particle

It is typically spherical, 80–120 nm in diameter but pleomorphic and filamentous forms are frequent.

(ii) Helical Symmetry

The nucleocapsid has a helical symmetry.

(iii) RNA Genome

It contains a negative sense single stranded RNA genome which is segmented and exists as eight pieces. RNA genome is associated with an RNA dependent RNA polymerase (PA, PB1, PB2). However, *Influenza virus* type C genome exists as seven pieces in contrast to eight pieces in *Influenza virus* type A and that of *Influenza virus* type B.

(iv) Envelope

The nucleocapsid is enclosed by an inner protein layer called the matrix or 'M protein' and outer lipid layer.

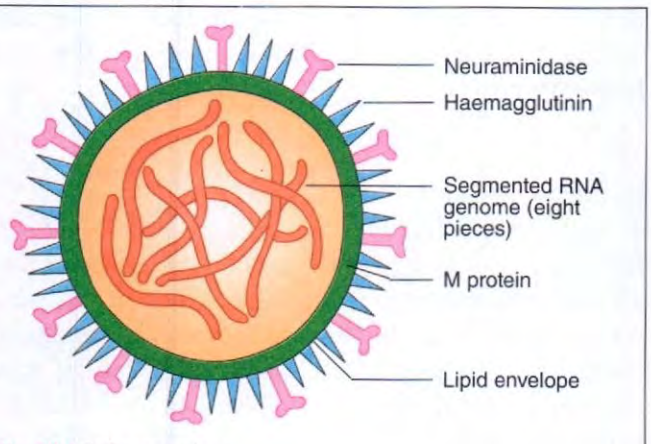


Fig. 58.1 Influenza virus.

The protein part of envelope is virus coded, but the lipid envelope is derived from the host cell membrane, during the process of replication by budding. This lipid envelope separates the peplomers (spikes) from the inner core structure.

(v) Peplomers

Attached to the lipid layer of the envelope are two types of glycoprotein peplomers or spikes, the haemagglutinin (HA) and the neuraminidase (NA). Haemagglutinin spikes are triangular in cross section while neuraminidase spikes are mushroom shaped which are less numerous. However, neuraminidase spikes are not present in *Influenza virus* type C.

B. Antigenic Classification

Influenza virus possesses four major structural antigens: the internal or nucleocapsid (RNP or ribonucleoprotein), matrix (M) protein of the viral envelope and two surface glycoproteins (HA, haemagglutinin and NA, neuraminidase) (Fig. 58.1). Three polymerase polypeptides, PA, PB1 and PB2 are also present.

Based on differences of antigenic structure of RNP and M proteins, influenza viruses are divided into three types: A, B and C. On the basis of antigenic variation of HA and NA antigens, these three types (A, B and C) can be divided into subtypes. Sixteen subtypes of haemagglutinins (H1–H16) and nine of neuraminidases (N1–N9) are known in birds, some of which have also been found in various combinations in humans. Human viruses show 3 HA (H1, H2, H3) and 2 NA (N1, N2) types (Table 58.2). All non-human influenza viruses belong to type A while types B and C viruses are exclusively human viruses except one report from China. Type A human virus contains two main subtypes A1 (H1N1) and A2 (H2N2). Generally non-human viruses do not infect man but they play an important role in emergence of pandemic influenza by genetic reassortment with human viruses.

Nomenclature system of influenza virus includes: type, host of origin, place of isolation, strain number, year of first isolation, followed in parenthesis by H and N subtypes e.g. A/swine/Iowa/3/70 (H1N1). If isolated from a human host, the origin is not indicated e.g. A/Shangai/24/90 (H3N2).

Table 58.2 Types and Subtypes of Orthomyxoviruses

Family	Types (Genus)	Subtypes (Species)
Orthomyxovirus	*Influenza virus A*	H1N1 (A1 human, Hsw N1), H2N2 (A2), H3N2 (A3, A Hong Kong)
	Influenza virus B	B (human)
	Influenza virus C	C (human)

C. Antigenic Structure

The influenza viruses have following antigens:

1. Ribonucleoprotein (RNP)

It is type (A, B or C) specific. All strains of a type possess the same antigen. Three types of influenza virus can be differentiated on the basis of this antigen. It is stable and does not exhibit any significant antigenic variation.

2. Matrix (M) Protein

It is also type specific. M protein is composed of two components, M1 and M2.

3. Haemagglutinin (HA)

It is strain specific and is capable of great antigenic variation. It is a glycoprotein composed of two polypeptides—HA1 and HA2. These are present as spikes on each influenza virus particle. When influenza virus is mixed with a suspension of fowl erythrocytes, the virus is adsorbed on to the mucoprotein receptors on the surface of erythrocyte by its haemagglutinin spikes. The virus links together adjacent erythrocytes resulting in haemagglutination. It is an important reaction in laboratory because it provides a simple and rapid method for detection of viruses in egg or tissue culture.

4. Neuraminidase (NA)

Like haemagglutinin, neuraminidase is also strain specific. The enzyme neuraminidase, a glycoprotein is present on the viral surface. It is present in the neuraminidase peplomers or spikes. The enzyme neuraminidase acts on specific red cell receptor, destroying it by splitting off N-acetylneuraminic acid from it. This specific receptor is the site for haemagglutinin on the surface of erythrocyte. Neuraminidases are also present in bacteria. Culture filtrates of *V. cholerae* are rich in neuraminidase activity. The red cells pretreated with these culture filtrates are resistant to haemagglutination by influenza viruses. The culture filtrate was, therefore, called the receptor destroying enzyme (RDE) of *V. cholerae*.

D. Antigenic Variation

A unique feature of influenza virus is its ability to undergo antigenic variation due to frequent changes in the antigenicity of HA and NA. The variation is highest in influenza virus type A and less in type B, while it has not been demonstrated in influenza viruses type C presumably due to lack of related viruses in animals. Depending on the degree of antigenic change of HA and NA, two distinct forms of antigenic variation are known.

1. Antigenic Drift

Antigenic drift refers to the minor antigenic changes in either the haemagglutinin or neuraminidase or both. This is a gradual sequential change occurring regularly at frequent intervals. Antigenic drift results from mutations in the HA and NA genes. The new antigens though different from the previous antigens, are yet related to them, so that antisera to the predecessor virus strains react with the mutant to a certain extent. Periodic epidemics of influenza are associated with antigenic drift.

2. Antigenic Shift

Major antigenic changes in HA (H2 to H3) or NA (N1 to N3) are called antigenic shift, resulting in emergence of a new subtype unrelated antigenically to predecessor strains. It is an abrupt, drastic, discontinuous variation in the antigenic structure. Such changes may involve haemagglutinin, neuraminidase or both. The changes involved in the antigenic shift are so extensive that they are unlikely to be due to mutation. The change probably results from gene reassortment (recombination) in doubly infected cells. The segmented genomes reassort between a human and animal strain. Antibodies to predecessor viruses cannot neutralise the new variants, therefore, they can spread widely in the population leading to major epidemics and pandemics. Antigenic shift of influenza virus A has occurred several times since 1900.

E. Resistance

Influenza virus is inactivated by heat (50°C for 30 minutes), ether, phenol, formaldehyde, salts of heavy metals and iodine. It remains viable at 0–4°C for about a week and can be preserved for years at –70°C or by freeze drying.

F. Cutivation

1. Egg Inoculation

Influenza virus is cultivated in amniotic cavity of 11 to 13 days old chick embryos. After a few egg passages, *Influenza virus* type A and type B grow well in allantoic cavity also, while *Influenza virus* type C can be grown only in amniotic cavity. Virus growth is detected by haemagglutination test performed with amniotic and allantoic fluids.

2. Tissue Culture

Influenza virus can also be isolated on primary monkey kidney, human embryo kidney cells, Madin-Darby canine kidney (MDCK) cells. MDCK cell line is more sensitive. Cytopathic effects are not prominent, therefore, virus

growth is detected by haemadsorption or demonstration of haemagglutinin in culture fluid.

3. Animal Inoculation

Influenza virus can cause experimental infection in ferrets and mice by intranasal inoculation.

G. Pathogenesis

Influenza virus enters the body by respiratory tract. The ciliated epithelial cells of respiratory tract are the main sites of infection. The viral neuraminidase facilitates infection by hydrolysing the mucus film lining the respiratory tract and exposing the specific mucoprotein receptors for virus adsorption. The death and sloughing of ciliated epithelial cells render the respiratory tract vulnerable to secondary bacterial infection.

Incubation period varies from 1–4 days. Subclinical infections are common. Influenza is an acute respiratory disease and clinical features vary from mild coryza to fulminating pneumonia. The illness is characterised by an abrupt onset of systemic symptoms such as chills, fever, sore throat, non-productive cough, myalgia, headache and malaise. The uncomplicated disease usually resolves within 7 days. Occasionally extrapulmonary lesions such as Guillain–Barre syndrome, Reye's syndrome and fatty liver may develop.

Reye's syndrome is a rare and serious complication in children, especially in those who have had influenza B. Aspirin, used to lower viral induced fever, contributes to the appearance of this syndrome. Therefore, acetaminophen is usually recommended for pyrexia of unknown origin (PUO) in children.

H. Laboratory Diagnosis

Laboratory diagnosis depends on demonstration of virus antigens, isolation of the virus and serology.

1. Demonstration of Virus Antigens

Viral antigens in clinical specimens can be detected by immunofluorescence. Smears of nasopharyngeal secretions and nasal swab or centrifuged deposit of throat garglings are prepared on slides and treated with fluorescent-tagged influenza antiserum and examined under the fluorescent microscope. The cells containing viral antigens will be found to fluoresce. It is a rapid method for diagnosis of influenza.

2. Isolation of the Virus

Influenza virus can be isolated during first 2-3 days of illness. Throat garglings are collected using suitable buffered salt solution. The specimen should be processed immediately. If short delay in processing is expected then specimen should be stored at 4°C, or if the delay is long, at –70°C. The specimen should be treated with antibiotics to destroy bacteria. The material is inoculated into:

(*i*) amniotic cavity of 11 to 13 days chick-embryo or
(*ii*) monolayers of monkey kidney cell cultures, human embryo kidney cells or MDCK cells.

After incubation of eggs at 35°C for three days, the amniotic and allantoic fluids are harvested separately. The fluids are tested for haemagglutination using guinea pig and fowl RBCs in parallel, at room temperature and at 4°C. Some strains of influenza virus type A agglutinate only guinea pig RBCs on initial isolation. The influenza virus type B agglutinates both cells, while type C agglutinates only fowl red cells at 4°C. The isolate is identified and typed with antisera to types A, B, C. Subtype identification is done by haemagglutination inhibition test. Some of type A strains can be isolated by direct allantoic inoculation of the clinical specimen into eggs but type B and C viruses will be missed if only allantoic inoculation is used. The isolate is identified and typed by complement fixation test with antisera to types A, B and C. Subtype identification is made by haemagglutination inhibition test.

Inoculated cell cultures are incubated at 33°C in roller drums and the virus growth can be identified by testing the culture fluid for haemagglutination or by haemadsorption. Rapid results can be obtained by detection of virus antigen in infected cell cultures by immunofluorescence.

3. Molecular method

Detection of the viral RNA by reverse transcriptase polymerase chain reaction (RT-PCR) is highly sensitive method. It can be used in clinical specimens.

4. Serology

The routinely employed serological tests for the diagnosis of influenza include:

(*i*) complement fixation test (CFT)
(*ii*) haemagglutination inhibition test (HAI test).

It is essential to demonstrate a four fold or higher rise in titre of antibodies between acute-phase and convalescent-phase sera.

Complement fixation tests with the RNP antigen of influenza virus are very useful as the antibodies are formed during infection, but not following immunisation with inactivated vaccines.

Haemagglutination inhibition (HAI) is a convenient and widely used test for the serological diagnosis of influenza. The major drawback is the frequent presence in the sera of non-specific inhibitors of haemagglutination.

In this technique, the sera are diluted serially and the influenza virus suspension containing 4 HA units is added. Fowl red cells are then added. The highest dilution of the serum which inhibits haemagglutination is its titre, named as HI titre.

I. Immunity

An attack of influenza confers protection effective for 1 to 2 years. Protection correlates with the concentration of serum antibodies and local antibodies in the respiratory tract. Local concentration of antihaemagglutinin (IgA) is more relevant in protection. The short duration of immunity is due to the antigenic variation that the virus undergoes frequently.

When an individual has repeated infections with different antigenic variants of influenza virus type A, he forms antibodies not only against each infecting strain, but also against the strain that he first came into contact with. The dominant antibody response will be shown against the strain that caused the earliest infection. This phenomenon has been called *the doctrine of original antigenic sin*.

J. Epidemiology

Influenza occurs sporadically, as epidemics or in pandemic form. Influenza A virus causes widespread epidemics and pandemics, B causes sporadic and sometimes epidemics but C does not cause epidemics. Influenza type C usually causes inapparent infections. The epidemics and pandemics of influenza are associated with antigenic shifts. It is now believed that pandemic strain originates from some animal or avian reservoir, either spreading to man directly by mutation, or as a result of recombination between human and non-human strains.

The most severe pandemic occurred in 1918-1919, during which over 20 million people perished. The next pandemic occurred in 1957 when the Asian strain originated in China and spread throughout the world. In 1968, the Hong Kong strain caused a pandemic, but it was much less severe. In 1977, epidemic influenza appeared in China and then in Russia (hence called the 'red flu'). The isolate was identified as H1N1 virus and has spread through most of the world, and with H3N2 virus, currently causes human influenza (Table 58.3).

A unique feature of influenza epidemiology was that once an antigenic variant emerged, it displaced completely the pre-existing strain. Thus, the A1 strains were displaced by Asian (H2N2) strains in 1957, and they, in turn, by the A2 Hong Kong (H3N2) strains in 1968. But this rule has not been observed in recent years. Even after the reappearance of Hsw1N1 strains in 1976 and the reemergence of H1N1 strain in 1977, the A3 Hong Kong (H3N2), strains continue to be prevalent. The reason for it is not known.

In April 2009, cases of influenza have appeared in Mexico and then in USA. These were first thought to be due to swine influenza A virus (H1N1). Later on, it had been identified as a new H1N1 virus which was different from swine influenza virus. It can be transmitted from human to human. It had spread to many countries including India across the world. WHO declared the situation as *pandemic*. On August 10, 2010, WHO

Table 58.3 Prevalence of Antigenic Sub-types of Influenza Virus Type A

Period of prevalence	Antigenic structure	Type of antigenic variation
1918–1919	H1N1	Antigenic shift
1918–1957		Progressive antigenic drift
1957	H2N2	Antigenic shift
1957–1968		Progressive antigenic drift
1968	H3N2	Antigenic shift
1968–1990		Progressive antigenic drift
1976	H1N1	Reappearance of Swine H1N1 virus.
1977	H1N1	Reappearance of H1N1 virus (resembling 1950 strain) first in China and Russia and spreading worldwide
1977–78		Progressive antigenic drift
1989	H1N1	Reappearance of Swine H1N1 virus
2009	H1N1	Appearance of a new subtype H1N1 in Mexico, USA and then spreading to many countries in America, Europe and other countries.

announced the end of HINI pandemic and also declared that it has moved into post pandemic period. However, localised outbreaks of various magnitudes are likely to occur.

As of 1st August 2010, more than 214 countries have reported laboratory confirmed cases including over 18449 deaths. This pandemic had occured 41 years after the last pandemic in 1968. Ostelamivir (Tamiflu) is the drug used for treatment. However, H1N1 strains resistant to ostelamivir have been reported from Denmark, Japan, Hongkong and China.

K. Prophylaxis

Vaccination is the primary means of prevention of influenza but the major difficulty in immunoprophylaxis is the frequent change in the antigenic structure of influenza virus. Vaccines cannot be made in bulk and stockpiled, as the appearance of a new variant strain will make the old vaccine useless. The most important indication for immunoprophylaxis is when a pandemic is threatened by a new virus. Vaccines are of two types:

1. Inactivated Vaccines

Influenza viruses are grown in allantoic cavity of chick embryos and inactivated by formalin or betapropiolactone. The vaccine can be further purified to contain only haemagglutinins and neuraminidases. This is called a *subunit vaccine*. The vaccine is administered parenterally in a single dose. Inactivated vaccine induces the formation of circulating antibodies. Local antibodies in the respiratory mucosa is only a fraction of the serum level.

Indications

It is administered to high risk individuals when influenza outbreak is imminent.

Protection

It gives protection for a short duration of one year.

Adverse reactions

Adverse reactions are few and mild. Occasionally cases of polyneuritis (Guillain-Barre syndrome) are reported.

2. Live Attenuated Vaccines

In order to get specific local immunisation, live vaccines have been employed. The earliest live vaccine was the virus attenuated by repeated egg passage. This vaccine sometimes gave rise to clinical disease. Another approach to live vaccine is the use of temperature sensitive mutants of influenza virus. They can grow at lower temperature (32-34°C) of the nasopharyngeal mucosa but not in the lungs (37°C). Such *ts* mutants are avirulent. They stimulate the production of local IgA antibodies. Recombinant live vaccines may be produced by hybridisation between *ts* mutants of established strains and a new antigenic variant strain. Live vaccine is administered intranasally or by aerosol spray.

3. Chemoprophylaxis

Rimantadine or amantadine may be given orally to unimmunised people during a major epidemic of *Influenza virus A*. These antiviral drugs block the viral M2 protein which functions as an ion channel. Influenza virus B lacks the M2 components thus these drugs do not act against type B.

L. Treatment

Amantadine and the rimantadine are useful in the treatment of influenza. They cause symptomatic improvement but virus shedding and antibody response are not affected.

Zanamivir and oseltamivir (Tamiflu), new drugs that block viral neuraminidase have been found effective in the treatment and prevention of influenza. Zanamivir is taken by inhalation. In contrast, oseltamivir is well absorbed when administered orally.

AVIAN INFLUENZA/ BIRD FLU

Avian influenza viruses do not normally infect other than birds and pigs. The virus may affect humans who have close contact with domestic birds such as chickens. H5N1 (an avian flu virus strain) was first isolated in 1997 from a human case in Hong Kong. Since then many reports of human disease have appeared. Bird to bird transmission does occur but it has not been shown to be passed from human to human. The symptoms of avian influenza in humans range from influenza like symptoms to pneumonia and acute respiratory distress.

Laboratory Diagnosis

1. Antigen detection

Antigen can be detected in nasal secretions by immunofluorescence test or ELISA

2. Isolation of the Virus

Virus isolation can be done by egg inoculation or by using cell culture.

3. Polymerase Chain Reaction (PCR)

KEY POINTS

1. Orthomyxoviruses are spherical or filamentous, enveloped viruses with single stranded and segmented RNA genome.
2. The viruses cause *influenza*, an acute respiratory illness. Besides humans, influenza viruses also infect pigs, horses and birds.
3. Influenza virus has three serotypes: type A, type B and type C. The virus possesses two surface glycoproteins, *haemagglutinin* (*HA*) and *neuraminidase* (*NA*). On the basis of antigenic variation of HA and NA antigens, these three types (A, B and C) can be divided into subtypes. Fifteen subtypes of haemagglutinins (H1-H15) and nine of neuraminidases (N1-N9) are known in birds, some of which have also been found in various combinations in humans. Human viruses show 3 HA (H1, H2, H3) and 2 NA (N1, N2) types.
4. *Antigenic drift* refers to the minor antigenic changes in either the haemagglutinin or neuraminidase or both. Antigenic drift results from *mutations* in the HA and NA genes. The new antigens though different from the previous antigens, are yet related to them, so that antisera to the predecessor virus strain react with the mutant to a certain extent.
5. Major antigenic changes in HA (H2 to H3) or NA (N1 to N3) are called *antigenic shift*, resulting in emergance of a new subtype unrelated antigenically to predecessor strains. The changes probably results from *gene reassortment* (*recombination*) in doubly infected cells. The segmented genomes reassort between a human and animal strain. Antibodies to predecessor viruses cannot neutralise the new variants, therefore, they can spread widely in the population leading to major epidemics and pandemics.
6. Laboratory diagnosis depends on *demonstration of virus antigens*, *isolation of the virus* and *serology*.
7. Viral antigens in clinical specimens can be detected by *immunofluorescence*.
8. Isolation of the virus can be done by *egg inoculation* (*amniotic cavity*) or in tissue culture (money kidney cell culture or human embryo kidney cells).
9. Serological tests employed for the diagnosis of influenza include *complement fixation test* (*CFT*) and *haemagglutination inhibition test* (*HAI test*).
10. Influenza A virus causes widespread epidemics and pandemics. Many pandemics have occurred since 1918.
11. Vaccination is the primary means of prevention of influenza but the major difficulty in the immunoprophylaxis is the frequent change in the antigenic structure of influenza virus. Inactivated vaccines and live attenuated vaccines are available. Vaccine is administered to high risk individuals when influenza outbreak is imminent.
12. *Amantadine* and *rimantadine* are useful in the treatment of influenza.

YOU MUST KNOW

1. Differences between orthomyxovirus and paramyxovirus.
2. Morphology of *influenza virus*.
3. Types and subtypes of orthomyxoviruses.
4. Haemagglutinin (HA) and neuraminidase (NA) of influenza virus.
5. Antigenic drift and antigenic shift.
6. Laboratory diagnosis of influenza.
7. Pandemics of influenza.
8. Vaccines used for prevention of influenza.

STUDY QUESTIONS

1. Tabulate the differences between orthomyxovirus and paramyxovirus. Discuss the morphology of *influenza virus*.
2. Write short notes on:
   (a) Antigenic shift
   (b) Antigenic drift
   (c) Laboratory diagnosis of influenza
   (d) Prophylaxis against influenza.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. How many serotypes of influenza are there?
   (a) Two (b) Three
   (c) Four (d) Five
2. In which of the following viruses, segmentation of genome is a characteristic feature?
   (a) Influenza virus (b) Parainfluenza virus
   (c) Mumps virus (d) Measles virus
3. Which of the following serotypes of influenza virus contain RNA genome segmented into seven pieces?
   (a) Influenza virus type A (b) Influenza virus type B
   (c) Influenza virus type C (d) None of the above
4. Which of the following structural antigen/s is/are present in influenza virus?
   (a) Ribonucleoprotein (b) Haemagglutinin
   (c) Matrix protein (d) All of the above
5. Which of the following serotypes of influenza virus does not contain neurominidase spikes in its structure?
   (a) Type A (b) Type B
   (c) Type C (d) Type D
6. In which of the following serotypes of influenza virus, antigenic variation has not been demonstrated?
   (a) Type A (b) Type B
   (c) Type C (d) Type D
7. Antigenic drift in influenza viruses results from mutations in:
   (a) Haemagglutnin genes (b) Neuraminidase genes
   (c) Both of the above (d) None of the above
8. Which of the following serotypes of influenza virus cannot be grown in allantoic cavity of chick embryos?
   (a) Type A (b) Type B
   (c) Type C (d) Type D
9. Which of the following can be used for isolation of influenza virus from clinical specimens?
   (a) Amniotic cavity of chick embryo (b) Monkey kidney cell cultures
   (c) Madin-Darby canine kidney cell lines (d) All of the above
10. Which avian influenza virus strain was first isolated from a human case of avian influenza in 1997 in Hongkong?
    (a) H2N2 (b) H3N2
    (c) H5N1 (d) H5N3
11. Which of the following drugs may be given for chemoprophylaxis during epidemic of influenza virus A?
    (a) Amantadine (b) Acyclovir
    (c) Ribavirin (d) Ganciclovir

Answers (MCQs):

1. (b)	2. (a)	3. (c)	4. (d)	5. (c)
6. (c)	7. (c)	8. (c)	9. (d)	10. (c)
11. (a)				

Chapter 59

PARAMYXOVIRUSES

Paramyxoviruses resemble *Orthomyxoviruses* in morphology but differ from the latter in structure and other properties (Table 58.1 in Chapter 58). They belong to family *Paramyxoviridae*. They possess single-stranded RNA genome as a single piece with helical nucleocapsid. They are enveloped. They are spherical in shape and size ranges from 100–300 nm in diameter with occasional giant forms up to 800 nm diameter. They are sensitive to lipid solvents. Haemagglutination and neuraminidase activity is exhibited by some members of the family.

I. CLASSIFICATION

The family *Paramyxoviridae* consists of six genera, *Respirovirus, Rubulavirus, Morbillivirus, Henipavirus, Pneumovirus* and *Metapneumovirus* (Table 59.1). They infect human beings as well as animal species. Parainfluenza virus, mumps virus, measles virus and respiratory syncytial virus are important for human infections. Newcastle disease virus is a natural pathogen of birds but man gets infection by handling these birds. It belongs to genus paramyxovirus. Canine distemper virus and Rinderpest virus infect animals.

II. DIFFERENTIATION OF GENERA

Parainfluenza virus and mumps virus have both haemagglutinin and neuraminidase, while measles virus has a haemagglutinin but no neuraminidase. Pneumovirus does not have any of the two. Measles virus has a haemolysin while respiratory syncytial virus (RSV) has a surface glycoprotein G, which has a function similar to that of haemagglutinin (Table 59.2). Unlike the orthomyxoviruses,

Table 59.1 Family Paramyxoviridae

Genus	Species	Origin
Respirovirus	Human parainfluenza viruses types 1 and 3	Human
	Newcastle disease virus	Animal
Rubulavirus	Human parainfluenza viruses types 2, 4a and 4b	Human
	Mumps virus	Human
Morbillivirus	Measles virus	Human
	Canine distemper virus	Animal
	Rinderpest virus	Animal
Henipavirus	Hendra virus, Nipah virus	Animal
Pneumovirus	Respiratory syncytial virus	Human
Metapneumovirus	Human metapneumovirus	Human

Table 59.2 Differentiation of various Paramyxoviruses

Virus	Haemagglutinin	Neuraminidase	Haemolysin	Surface glycoprotein
Parainfluenza viruses	+	+	–	–
Mumps virus	+	+	–	–
Measles virus	+	–	+	–
Respiratory syncytial virus	–	–	–	+

the paramyxoviruses with their unsegmented genome cannot exchange genetic information by recombination and variation depends on mutational change.

III. MORPHOLOGY OF PARAMYXOVIRUSES

The paramyxoviruses are spherical enveloped particles 100-300 nm diameter in size (Fig. 59.1). Occasionally there are filamentous forms and giant forms up to 800 nm in diameter. The envelope consists of lipoprotein membrane and covered by projections. These projections are of two types, HN (haemagglutinin, neuraminidase) and F (fusion protein) peplomers 12-14 nm long and 2-4 nm wide. The HN projections of paramyxovirus carry both haemagglutinin and neuraminidase functions, while in orthomyxoviruses, separate spikes carry each function. F glycoprotein causes fusion of cell membranes leading to the formation of syncytia. The inner surface of the envelope is lined by matrix (M) protein. Nucleocapsid is of helical symmetry and contains a single stranded negative sense RNA genome as a single piece and an RNA-dependent RNA polymerase. Measles virus envelope carries only haemagglutinin but not a neuraminidase.

IV. VIRUSES OF FAMILY PARAMYXOVIRIDAE

A. Parainfluenza Viruses

There are five serotypes of parainfluenza viruses, type, 1, 2, 3, 4a and 4b. They cause respiratory infections in children, and less often in adults. The parainfluenza viruses are spherical, 125-250 nm in diameter, enveloped RNA viruses. The outer envelope is lipoprotein in nature. Two types of spikes protrude from envelope, one of these has haemagglutinin and neuraminidase activities (the HN protein) while other protein mediates fusion and haemolytic properties of the virus (F protein). The RNA is single stranded.

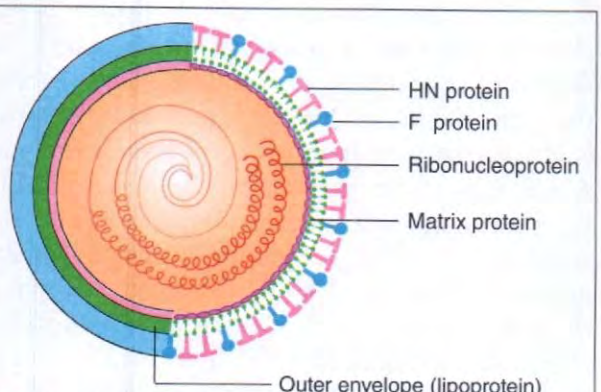


Fig. 59.1 Paramyxovirus.

1. Pathogenesis

Parainfluenza viruses are acquired by droplets and by contact with respiratory secretions. Incubation period varies from 2–6 days. These viruses are ubiquitous and produce upper respiratory tract infections. In infants and young children, they cause severe respiratory tract disease such as *laryngotracheobronchitis* (especially by types 1 and 2) or *croup*. The patient presents with fever, cough and respiratory obstruction as a result of swelling of larynx and related structures. Pneumonia or bronchiolitis may occur especially with type 3. Amongst all the four serotypes, type 4 produces only mild illness.

2. Laboratory Diagnosis

(i) Direct demonstration

(*a*) *Immunofluorescence*: Viral antigens can be demonstrated in exfoliated cells aspirated from respiratory tract by immunofluorescence using monoclonal antibodies. It is used for rapid diagnosis.

(*b*) *ELISA*: ELISA can also be used for detection of viral antigens using monoclonal antibodies.

(ii) Isolation

Mouth washings, samples collected from posterior pharynx or nasopharynx and throat swabs are inoculated in primary human or monkey kidney cells or in continuous cell lines such as H292 derived from human lung mucoepidermoid carcinoma. Visible cytopathic effect is minimal except with type 2 which induces syncytia formation. Virus in infected cell is identified by haemadsorption of guinea pig red cells or by use of specific immunofluorescent antibody. Typing is done by inhibition of haemadsorption by type-specific antisera.

(iii) Serology

Detection of the rising antibody titre by neutralisation, ELISA and complement fixation test are helpful in diagnosis.

B. Mumps Virus

Morphology of the mumps virus is same as described earlier under 'Morphology of paramyxoviruses' on page 473.

1. Pathogenesis

Mumps or epidemic parotitis is predominantly a disease of childhood and is acquired from direct contact with infected saliva or aerosols from infected patients. Respiratory tract is the portal of entry. Incubation period varies from 16-18 days. The virus multiplies in the upper respiratory tract and in local lymph nodes. It then enters the bloodstream and spreads to the salivary glands, testes, ovaries, pancreas, kidney and brain. The most characteristic feature is non-suppurative inflammation of the parotid glands in about 95% cases. Virus is shed in saliva from six days before to one week after onset of clinical parotitis.

Humans are the only natural hosts. No human carriers or animal reservoirs exist.

Complications such as meningitis, meningoencephalitis, orchitis, oophoritis, pancreatitis, and nephritis may occur. Orchitis is a common complication seen in postpubertal male patients.

2. Immunity

Immunity is life long after a single infection. There is only one serotype of mumps virus.

3. Laboratory Diagnosis

Most cases are diagnosed clinically. Mumps does not usually require to be established by laboratory study.

(i) Direct demonstration

Immunofluorescence may be used on secretions of throat and saliva for the demonstration of virus.

(ii) Isolation

Virus can be isolated from saliva, throat swab, CSF or urine by inoculating into primary monkey kidney, H292 and HEp-2 cell cultures. Virus produces a little cytopathic effect but can be detected by haemadsorption (of guinea pig red cells) which can be inhibited by specific antiserum. More rapid results can be obtained by immunofluorescence testing of infected cell cultures.

(iii) Serology

For rapid diagnosis, mumps specific IgM antibodies can be detected in serum by enzyme linked immunosorbent assay (ELISA).

4. Prophylaxis

A live attenuated vaccine, derived by passage in chick fibroblasts, is available. It can be administered subcutaneously in combination with attenuated measles and rubella vaccine (MMR vaccine). This vaccine is given to children aged 12-15 months. It provides effective protection for at least ten years. Contraindications are pregnancy, immunodeficiency and hypersensitivity to egg protein.

C. Newcastle Disease Virus

This is a natural pathogen of poultry. In India, it is known as Ranikhet virus. The virus may cause a self-limited conjunctivitis in poultry workers and other persons who are in contact with the infected birds. Control measures include vaccination, and slaughter of infected birds.

D. Measles Virus

Measles is highly infectious childhood disease spread by respiratory secretions. It is caused by measles virus. The virus resembles paramyxoviruses in morphology. It is spherical about 120–250 nm in diameter. It has tightly coiled helical nucleocapsid surrounded by lipoprotein envelope having haemagglutinin (H) spikes but neuraminidase spikes are absent. The envelope also has the F protein. Matrix M protein is located below the lipoprotein envelope.

1. Pathogenesis

Measles virus is acquired by inhalation. Incubation period varies from 10-12 days. The virus multiplies in lymphoid tissue of respiratory tract and invades bloodstream (*primary viraemia*). The virus spreads to the reticuloendothelial system through blood. After multiplication there, a *secondary viraemia* occurs. The virus is then transported to the epithelial surfaces including the skin, mouth, respiratory tract and conjunctiva. It is characterised by high fever, cough and conjunctivitis. *Koplik's spots* can be seen on the buccal mucosa and are pathognomic of measles. With the decline of acute symptoms in 1-2 days, widespread maculopapular rash appears first on the neck and then spreads to the rest of the body. The rash is due to delayed hypersensitivity (type IV) to viral antigens. The rash fades in about a week and the patient recovers uneventfully by 10-14 days.

2. Complications

(*i*) Since measles decreases the resistance of the respiratory epithelium, therefore, patient may develop secondary bacterial infections such as otitis media, bronchopneumonia and croup.

(*ii*) Giant cell pneumonia may occur in persons who have impaired cell mediated immunity.

(*iii*) Post-measles encephalitis and subacute sclerosing panencephalitis (SSPE) may occur in 1 : 1000 and 1 : 300,000 cases of measles respectively. SSPE may occur in children or adolescents who have had measles early in life. A defective form of measles virus persists within the infected cell. This defective form is not able to induce production of a functional M-protein and is not released as complete virus from the cells. Patient finally develops CNS symptoms. CSF contains antibodies to measles virus proteins.

3. Immunity

Infection confers life-long immunity. There is only one serotype of measles virus.

4. Laboratory Diagnosis

Most cases are diagnosed clinically. In atypical cases, and for differentiation from rubella, laboratory study may be necessary.

(i) Direct demonstration

Multinucleated giant cells can be demonstrated in Giemsa stained smears of nasal secretions. This is a simple test, which can be used even before the rash appears. Virus particles can be detected in exfoliated respiratory cells in nasal secretions by immunofluorescence.

(ii) Isolation

The measles virus can be isolated, with some difficulty, from throat washings, blood, nasopharyngeal swab and conjunctiva during the prodromal phase and upto about two days after appearance of the rash. Virus may be obtained from the urine for a few more days. These specimens are cultured in primary human embryo kidney, monkey kidney or human amnion cells. Cytopathic changes may take 7-10 days to develop. The appearance of multinucleated giant cells (cytopathic effect) containing both intranuclear and intracytoplasmic inclusion bodies suggests the presence of measles virus.

As cytopathic effects take about seven days to develop, earlier diagnosis of virus growth is possible by immunofluorescence staining with monoclonal antibody.

(iii) Serology

Measles specific IgM antibody in the patient serum can be detected by ELISA. Measles specific IgM antibody in a single serum specimen is confirmatory if the serum is drawn between one and two weeks after the onset of rash. Haemagglutination inhibition (HI), complement fixation test (CFT) and neutralisation tests can be carried out on acute and convalescent sera. A four fold rise in titre is diagnostic.

High titre measles antibody in the CSF is diagnostic of SSPE.

5. Epidemiology

Man is the only natural host of measles and monkeys acquire infection from man. Patients are infective from three days before to the onset of symptoms and remain infective till the rash desquamates. The virus enters the body through the respiratory tract and conjunctiva. The disease has maximum incidence in children 1-5 years of age. Measles is endemic throughout the world and epidemics usually occur in later winter and early spring.

6. Prophylaxis

(i) Active immunisation

Live attenuated vaccine is used in children at the age of nine months. Originally it was developed by passaging the *Edmonston* strain of measles virus through human kidney, amnion cell cultures and finally chick embryo culture. Due to its high risk of causing febrile rash (vaccination measles), further attenuation were done to develop strains named *Schwartz* and *Moraten*. These strains were safe but effective only in children older than 15 months. In the tropics, measles is a serious problem in children below 12 months. Hence, the *Edmonston-Zagreb* strain, attenuated by passage in human diploid cells, is preferred for vaccine because it is able to produce seroconversion even in infants of 4–6 months. The vaccine is administered in one dose by subcutaneous route. Measles vaccine is also being used in combination with mumps and rubella (MMR vaccine). This vaccine is administered at 12-15 months of age. MMR is also given in single dose by subcutaneous injection. It provides protection lasting for more than 20 years. These vaccines may not induce adequate antibody response in young babies who possess maternal antibodies.

Sabin and his colleagues have developed another live attenuated vaccine which can be used by intranasal aerosol and induces good antibody response irrespective of the presence of maternal antibodies.

(ii) Passive immunisation

Pooled sera containing antibody against measles virus confers passive immunity to children with immunodeficiency, pregnant women and others at special risk.

E. Respiratory Syncytial Virus

Respiratory syncytial virus (RSV) is pleomorphic and ranges in size from 150–300 nm. The viral envelope lacks both haemagglutinin and neuraminidase but contains a surface glycoprotein G by which the virus attaches to cell surfaces, and a F (fusion) protein that induces fusion of infected cells into large multinucleated syncytia, from which the virus gets its name. RSV differs from other paramyxoviruses in not possessing haemagglutinin activity. It also lacks neuraminidase or haemolytic properties (Table 59.2). Its nucleocapsid diameter (13 nm) is less than that of other paramyxoviruses (18 nm). It is the most important cause of bronchiolitis and pneumonitis in infants between one to six months of age. Infection in older children and adults results in rhinitis or common cold.

RSV is highly labile and is inactivated rapidly at room temperature. Lyophilisation can be used for its preservation. It is antigenically stable and only one antigenic type exists. However, studies with monoclonal antibodies revealed two subtypes, A and B. Subtype A is more virulent than B.

1. Pathogenesis

RSV is highly contagious. Infection is transmitted by contact with contaminated hands and surfaces. Nosocomial infections occur in nurseries and paediatric wards. Incubation period is 4–6 days. Virus multiplies in the mucous membranes of the nose and throat, from where they spread into lower respiratory tract causing bronchiolitis and pneumonitis. Virus is shed in respiratory secretions for several days or weeks.

2. Immunity

Secretory IgA in nasal secretions appears to be protective against reinfection but cell mediated immunity is more important than humoral antibodies in recovery from infection.

3. Laboratory Diagnosis

(i) Direct demonstration

Rapid identification of virus in nasopharyngeal aspirates can be done by immunofluorescence using monoclonal antibody. Viral antigens can also be detected in the specimen by ELISA.

(ii) Isolation

Virus can be isolated from nasopharyngeal secretions by inoculating the specimen in human (HeLa, HEp-2) or monkey cell cultures. Characteristic giant cells and syncytia formation occurs in 2-10 days. However, definitive identification can be done by immunofluorescence.

Samples should be inoculated immediately after collection. Freezing of clinical sample may destroy the virus.

(iii) Serology

It is not very helpful but CFT, neutralisation and ELISA techniques may detect rising antibody titre. It has proved useful in retrospective diagnosis of RSV infections.

4. Epidemiology and Prophylaxis

RSV is worldwide in distribution and causes winter epidemics in children. Both formalin killed whole cell vaccine and live vaccine (temperature sensitive) have been tried but none is found to be satisfactory. Attempts to prepare vaccine with purified F and G surface glycoproteins are in progress.

5. Treatment

Management is primarily of supportive care. Ribavirin by continuous aerosol has been found beneficial by decreasing the duration of illness and of virus shedding.

F. Human Metapneumovirus

Human metapneumovirus is a respiratory pathogen. It is an important cause of respiratory tract infection in children. It can also cause disease in adults. It causes a disease similar to that of human respiratory syncytial virus. Like other paramyxoviruses, it is a single stranded RNA virus. The virus is very difficult to grow. Polymerase chain reaction (PCR) can be used for diagnosis. Respiratory secretions are clinical specimens used for the test. No specific antiviral treatment or vaccine is available

KEY POINTS

1. Paramyxoviruses possess single-stranded RNA genome as a single piece with helical nucleocapsid. They are enveloped and spherical in shape. Haemagglutination and neuraminidase activity is exhibited by some members of the family.
2. *Parainfluenza virus*, *mumps virus*, *measles virus* and *respiratory syncytial* virus are important paramyxoviruses causing human infections.

3. Unlike the orthomyxoviruses, the paramyxoviruses with their unsegmented genome cannot exchange genetic information by recombination and variation depends on mutational change.
4. Parainfluenza viruses produce upper respiratory tract infections. Pneumonia or bronchitis may also occur. Mumps virus is the causative agent of mumps or epidemic parotitis. Measles is highly infectious childhood disease caused by measles virus. *Post-measles encephalitis* and *subacute sclerosing panencephalitis* (SSPE) may occur as serious complications in cases of measles.
5. Measles vaccine is a *live attenuated vaccine*. It is administered in one dose by *subcutaneous* route. It is given at the age of nine months. Measles vaccine is also being used in combination with mumps and rubella (*MMR vaccine*). This vaccine is administered at 12-15 months of age. MMR is also given in single dose by *subcutaneous* injection.
6. Respiratory syncytial virus (RSV) is highly contagious. Nosocomial infections occurs in nurseries and paediatric wards. It causes bronchiolitis and pneumonitis.

YOU MUST KNOW

1. Morphology of parainfluenza viruses.
2. Pathogenesis of mumps virus.
3. Pathogenesis and complications of measles.
4. Respiratory syncytial virus.
5. MMR vaccine.

STUDY QUESTIONS

1. Classify and discuss the morphology of paramyxoviruses.
2. Write short notes on:
   (a) Parainfluenza viruses
   (b) Mumps virus
   (c) Measles virus
   (d) Respiratory syncytial virus
   (e) MMR vaccine.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Which of the following viruses belong to family *Paramyxoviridae*?
   (a) Mumps virus
   (b) Measles virus
   (c) Respiratory syncytial virus
   (d) All of the above
2. Human respiratory syncytial virus belongs to which of the following genera of family *Paramyxoviridae*?
   (a) *Respirovirus*
   (b) *Rubulavirus*
   (c) *Pneumovirus*
   (d) *Metapneumovirus*
3. Envelope of which virus carries only haemagglutinin but not a neuraminidase?
   (a) Parainfluenza virus
   (b) Mumps virus
   (c) Measles virus
   (d) Pneumovirus
4. Which of the following conditions can occur by infection with mumps virus?
   (a) Inflammation of the parotid glands
   (b) Orchitis
   (c) Meningoencephalitis
   (d) All of the above
5. Which of the following viruses is known as Ranikhet virus in India?
   (a) Canine distemper virus
   (b) Rinderpest virus
   (c) Newcastle disease virus
   (d) Human metapneumovirus
6. Koplik's spots on the buccal mucosa is a characteristic feature of:
   (a) Mumps
   (b) Measles
   (c) Rubella
   (d) Respiratory syncytial virus

7. Subacute sclerosing panencephalitis may occur as complication in:
   (a) Mumps (b) Measles
   (c) Rubella (d) Respiratory syncytial virus
8. What type of vaccine is MMR?
   (a) Live attenuated vaccine (b) Inactivated vaccine
   (c) Subunit vaccine (d) DNA vaccine
9. What is the route of administration of MMR vaccine?
   (a) Intramuscular (b) Subcutaneous
   (c) Intracutaneous (d) Oral
10. Envelope of which virus lacks both haemagglutinin and neuraminidase but contains a fusion protein?
   (a) Measles virus (b) Mumps virus
   (c) Respiratory syncytial virus (d) Newcastle disease virus

Answers (MCQs):

1. (d)	2. (c)	3. (c)	4. (d)	5. (c)
6. (b)	7. (b)	8. (a)	9. (b)	10. (c)

Chapter 60

ARBOVIRUSES

Arboviruses (arthropod-borne viruses) are RNA viruses that are transmitted by blood-sucking arthropods from one vertebrate host to another. The viruses multiply in arthropods without producing disease. Inclusion in this group is based on ecological and epidemiological considerations and hence it contains viruses of diverse physical and chemical properties. Though taxonomically unacceptable, the name 'arbovirus' is a useful biological concept.

A similar ecological group is rodent-borne (robo) viruses which are maintained in nature by direct transmission between rodents, and sometimes infecting humans, by direct contact without the agency of arthropod vectors. Like arboviruses, roboviruses also belong to different taxonomical families, some of them in common with arboviruses.

I. CLASSIFICATION

Arboviruses were sometimes named according to the disease they are associated with such as yellow fever, Chikungunya (the local name for the disease) or after the name of the place of isolation of the virus (Kyasanur Forest disease). Some were named after the geographical area e.g. West Nile fever and St. Louis encephalitis. Taxonomically, arboviruses belong to six families : *Togaviridae, Flaviviridae, Bunyaviridae, Reoviridae, Rhabdoviridae* and *Orthomyxoviridae*. Most viruses of medical importance are togaviruses; some are bunyaviruses and a few are reoviruses or rhabdoviruses (Table 60.1). Togaviruses of medical importance include *Alphavirus* and *Rubivirus*. Rubivirus contains the rubella virus that possesses morphological and physicochemical features typical of togaviruses.

II. GENERAL PROPERTIES (Table 60.2)

The arboviruses share some common biological properties:

1. All members produce fatal encephalitis in suckling mice after intracerebral inoculation.
2. They possess haemagglutinin and agglutinate erythrocytes of goose or day-old chicks.

Table 60.1 Diseases Caused by Arboviruses

Family and Genus	Viruses causing		
	Encephalitis	Febrile illness	Haemorrhagic fever
I. *Togaviridae* *Alphavirus* (Mosquito-borne)	• Western equine encephalitis • Eastern equine encephalitis • Venezuelan equine encephalitis	• Chikungunya • O'nyong-nyong • Semliki Forest • Sindbis • Ross river virus	• Chikungunya
II. *Flaviviridae* *Flavivirus*			
(i) Mosquito-borne	• St. Louis encephalitis • Ilheus • West Nile • Murray Valley encephalitis • Japanese encephalitis	• Dengue, types 1-4	• Dengue • Yellow fever
(ii) Tick-borne	• Russian spring summer encephalitis • Powassan		• Kyasanur Forest disease • Omsk Haemorrhagic fever
III. *Bunyaviridae*			
a) *Orthobunya virus* (Mosquito-borne)	• California encephalitis • La Crossie	• Chittor virus	
b) *Phlebovirus* (Phlebotomus or mosquito-borne)		• Sandfly fever • Rift-valley fever	
c) *Nairovirus* (tick-borne)		• Nairobi sheep disease • Ganjam virus	
IV. *Reoviridae* *Orbivirus* (Tick-borne)		• Colorado tick fever virus	
V. *Rhabdoviridae* *Vesiculovirus* (Mosquito-borne, sandfly-borne)		• Vesicular stomatitis virus • Chandipura virus	
VI. *Orthomyxoviridae* (Tick-borne)	• Thogotovirus	• Thogotovirus	

Table 60.2 Properties of Arboviruses

Property	Alphavirus	Flavivirus	Bunyavirus	Rhabdovirus	Reovirus	Thogotovirus
Symmetry	Cubic	Cubic	Helical	Bullet-shaped	Cubic	Helical
Size (diameter in nm)	60-65	40-50	90-100	170 × 100	60-80	80-120
Nucleic Acid	Single stranded positive sense RNA	Single stranded positive sense RNA	Single stranded negative sense RNA	Single stranded negative sense RNA	Double stranded RNA	Single stranded negative sense RNA, segmented six pieces
Inactivation by diethyl ether/sodium deoxycholate	+	+	+	+	–	+

3. Mosquito-borne arboviruses multiply in aedes and culex mosquitoes while tick-borne arboviruses multiply in Ixodid ticks.
4. They can be grown in tissue cultures of primary cells like chick embryo fibroblasts or continuous cell lines like vero, and in cultures of appropriate insect tissues. They may also be isolated in the yolk sac or chorioallantoic membrane of chick embryo.
5. In general, arboviruses are readily inactivated at room temperature and by bile salts, ether and other lipid solvents.

Antigenic Structure

Three antigens are important in serological studies namely haemagglutinins, complement fixing and neutralising antigens. Considerable antigenic cross reactions occur among arboviruses.

Pathogenesis

The virus enters the body through the bite of the insect vector. It multiplies in the reticuloendothelial system and leads to viraemia. In some cases, the virus is transported to the target organs, such as the central nervous system (CNS) in encephalitis, the capillary endothelium in haemorrhagic fevers and the liver in yellow fever.

Arboviruses cause the following clinical syndromes such as febrile illness with or without rash and arthralgia, encephalitis, haemorrhagic fever; and the characteristic systemic disease, yellow fever (Table 60.1). All arbovirus infections occur with varying degree of severity, subclinical infections being common.

III. LABORATORY DIAGNOSIS OF ARBOVIRUSES

Diagnosis may be established by virus isolation or serology.

A. Specimens

Blood, CSF and brain may be used for isolation of virus. As all arboviruses are viraemic, blood is collected during the acute phase of the disease. CSF is useful in encephalitis cases but the best specimen is the brain.

B. Virus Isolation

1. Suckling Mice

Specimens are inoculated intracerebrally into suckling mice. The animal develops fatal encephalitis. This is the most sensitive method for isolation of arboviruses.

2. Tissue Culture

Some viruses may also be isolated in tissue cultures. Vero, BHK-21 and mosquito cell lines are inoculated with specimens. Growth of virus in cell cultures is identified by immunofluorescence, haemagglutination inhibition, complement fixation, ELISA or neutralisation tests.

3. Virus Isolation from Insect Vectors and Reservoir Animal

Such isolations are important to know the identification of arbovirus activity in the area.

C. Serology

Using ELISA, serotype specific IgM antibody may be detected in patient serum within 1-3 days after the onset of illness. The detection of a four fold rise or more in antibody titre by ELISA, complement fixation, haemagglutination inhibition or neutralisation test also provide a good evidence of infection.

IV. FAMILIES OF ARBOVIRUSES

A. Togaviridae

1. Morphology

Viruses of the family *Togaviridae* (from *Toga*, meaning mantle) are spherical, 60-70 nm in diameter with icosahedral capsid surrounded by lipoprotein envelope and contain single stranded positive sense RNA.

2. Classification

Togaviridae is the largest family of arboviruses. It contains two genera : *Alphavirus* and *Rubivirus*. The genus alphavirus has 32 members of which 13 have been shown to cause human diseases. They are transmitted principally by mosquitoes. Genus *Rubivirus*, which contains rubella virus, is not arthropod-borne.

3. Pathogenesis

The virus enters the body through skin by the bite of blood sucking mosquitoes. It multiplies in local lymph nodes and leads to *viraemia*. It may involve target organs leading to rash, arthritis, hepatitis, nephritis and encephalitis. Capillary endothelium is involved in haemorrhagic fevers, and liver in yellow fever.

4. Clinical Features

Incubation period varies from 4-21 days. Onset is sudden with headache, nausea, vomiting and sometimes arthralgia. Subclinical infections are common.

5. Viruses of Togaviridae

(i) Alphavirus (Mosquito-borne)

Encephalitis group

(*a*) Western equine encephalitis (WEE)
(*b*) Eastern equine encephalitis (EEE)
(*c*) Venezuelan equine encephalitis (VEE)

Febrile illness group

(*a*) Chikungunya virus
(*b*) O'nyong-nyong virus
(*c*) Semliki Forest
(*d*) Sindbis
(*e*) Ross river virus

(ii) Rubivirus

Rubella virus

ALPHAVIRUS

Encephalitis group

Three important viruses—Western, Eastern and Venezuelan equine encephalitis (WEE, EEE, VEE) viruses produce encephalitis in horses and humans. Wild birds are reservoirs and infection is transmitted by several species of *Culex* and *Anopheles mosquitoes*. These viruses are prevalent in USA and South America. Formalinised vaccines have been developed for WEE and EEE and a live attenuated vaccine for VEE.

Febrile illness group

(a) Chikungunya virus

The virus is transmitted by *Aedes aegypti*. The disease is characterised by fever, crippling joint pains, lymphadenopathy, conjunctivitis and rash. Haemorrhagic manifestations are seen in some patients. The fever is typically biphasic with a period of remission after 1–6 days. Chikungunya is the native word for the disease in which the patient lies 'doubled up' due to severe joint pains. The virus first appeared in India in 1963 when it caused extensive epidemics in Calcutta, Madras and other areas. Chikungunya outbreaks have occurred along the east coast of India and in Maharashtra till 1973. There is no animal reservoir for the virus. No vaccine is available.

(b) O'nyong-nyong virus

O'nyong-nyong virus was first isolated in Uganda. It is confined to Africa. It is closely related to the chikungunya virus antigenically and causes a similar disease. The disease is transmitted by the *Anopheles* species.

(c) Semliki forest virus

The virus has not been associated with clinical disease in man although neutralising antibodies to the virus have been demonstrated in Africans.

(d) Sindbis virus

It was originally isolated from *Culex mosquitoes* in 1952 in Sindbis district of Egypt. The virus has subsequently been recovered from other parts of Africa, India, Philippines and Australia. In India, antibodies have been detected but no association has been established with human disease.

(e) Ross River virus

It has been associated with epidemic polyarthritis in Australia.

RUBIVIRUS

Rubella virus has no known arthropod vector, therefore, it has been described in Chapter 64.

B. Flaviviridae

Originally these were named as group B arboviruses. Generic name of *Flavivirus* has now been assigned to members of the family *Flaviviridae*. Non-arthropod-borne viruses of this family belong to two genera : *Pestivirus* (contains a number of veterinary pathogens) and *hepatitis C* virus.

The genus flavivirus contains over 70 viruses of which 13 are capable of causing human disease. Some are mosquito-borne while others are tick-borne (Table 60.1). Hepatitis C virus is neither mosquito nor tick-borne.

Morphology

The viruses of the family *Flaviviridae* are spherical 40-50 nm in diameter. They contain a single stranded positive sense RNA. Inner viral core is surrounded by a lipid envelope which is covered with glycoprotein peplomers and matrix or membrane protein.

Mosquito-borne Flaviviruses

1. ENCEPHALITIS VIRUSES

Five members of this group cause encephalitis which are limited to certain geographical zone. These include:

(*i*) *St. Louis encephalitis virus* (North and Central America)
(*ii*) *Ilheus virus* (South and Central America)
(*iii*) *West Nile virus* (West Nile province of Uganda)
(*iv*) *Murray Valley encephalitis virus* (Australia and New Guinea)
(*v*) *Japanese B encephalitis virus* (Asia)

Culex mosquitoes are vectors in above group of encephalitis viruses.

(i) St. Louis encephalitis virus

This is the most important mosquito-borne disease in USA. Wild birds act as the reservoir and *Culex tarsalis* as the vector. No vaccine is available.

(ii) Ilheus virus

The virus is maintained in forests by a cycle involving birds and mosquitoes. Human infection is generally asymptomatic and leads rarely to encephalitis. The disease occurs in South and Central America.

(iii) West Nile virus

The virus is maintained in nature in wild birds by *Culex* mosquitoes. It is endemic in Africa, Central Asia and India. It causes a dengue-like illness in humans and very rarely encephalitis.

In India, the virus has been isolated from *Culex* mosquitoes. It has also been isolated from the brains of

three fatal cases of encephalitis in children in Karnataka during 1980-81.

(iv) Murray Valley encephalitis virus

Murray Valley encephalitis is believed to occur normally in an enzootic cycle involving wild birds and mosquitoes. *Culex annulirostris* is the vector. It is endemic in New Guinea and Australia. The virus was first isolated during an epidemic of encephalitis in the Murray River Valley in 1951.

(v) Japanese encephalitis virus

Japanese encephalitis virus was first isolated in Japan during an epidemic of encephalitis. The virus was named Japanese 'B' encephalitis to distinguish it from Japanese 'A' encephalitis virus (encephalitis lethargica, von Economo's disease) which was prevalent at that time. This virus is responsible for the most serious illness among five viruses of this group. In India, the Japanese B encephalitis was first recognised in 1955 during an outbreak of encephalitis in Tamil Nadu. Now a days it is named Japanese encephalitis virus.

(a) Pathogenesis

Natural infections of Japanese encephalitis occur in Adreid birds (herons and egrets) and the virus spread from bird to bird through *Culex tritaeniorhynchus*. Human infection occurs from these reservoir birds by several species of *Culicine mosquitoes*. Herons act as reservoir host and pigs as amplifier hosts. Other birds (ducks, pigeons and sparrows) may also be involved. Vertebrate hosts may include cattle and buffaloes, besides pigs. The high cattle-pig ratio in India has been suggested as a factor limiting human infection.

(b) Epidemiology

It is widely distributed in China, Japan, Korea, Philippines and Southeast Asia. Several outbreaks have occurred in Bankura and Burdwan in West Bengal, Dibrugarh in Assam, Gorakhpur in U.P., Goa, Kolar in Karnataka, South Arcot in Tamil Nadu, Pondicherry and various areas in Andhra Pradesh. Sporadic cases have been reported from different parts of the country. The disease has become a major public health problem of national importance.

(c) Clinical features

The disease has an abrupt onset and symptoms include fever, headache and vomiting. After 1-6 days, signs of encephalitis characterised by neck rigidity, convulsions, altered sensorium and coma appear. Mortality in some epidemics has been up to 50 per cent. Convalescence may take many weeks and residual neurological damage may persist in about 50 per cent of survivors. The large majority of infections are, however, asymptomatic. It has been estimated that 500-1000 asymptomatic infections occur for every case of clinical disease.

(d) Prophylaxis

Preventive measures include mosquito control and establishment of piggeries away from residential areas. A formalin inactivated mouse brain vaccine using the *Nakayama* strain has been employed for human immunisation in Japan and in India also. Two doses of vaccine are administered at two weeks interval, followed by a booster 6-12 months later. Immunity produced is short-lived. Vaccine is also useful for pig population to prevent epidemics. During major epidemics, slaughter of pigs has been employed as a containment measure. A live attenuated vaccine prepared in hamster kidney cell cultures is also available. The vaccine is administered in two doses, one year apart. It has been very effective in preventing clinical disease.

2. YELLOW FEVER

Yellow fever is a mosquito-borne acute febrile illness. It occurs in tropical Africa and Latin America. It does not exist in India. The name yellow fever does not indicate the association of the disease with jaundice. The name has been derived from the 'yellow quarantine flag' used by the ships during the 17th Century to warn the presence, on board, of this infection.

Virus infection occurs in two forms: *urban cycle* and *forest or sylvatic cycle*. In the urban cycle, man serves both as reservoir and as definitive case, the virus being transmitted by *Aedes aegypti* mosquito. In the forest or sylvatic cycle, wild monkeys act as the reservoirs and several species of forest mosquitoes (*Haemagogus spegazzinii* in South America and *Aedes africanus* in Africa) are vectors. Human cases occur only when humans trespass into the forest or when the monkeys raid villages.

(i) Clinical features

After an incubation period of 3-6 days, patient develops fever with chills, headache, myalgia and vomiting. Severe jaundice develops due to extensive destruction of liver. Death occurs in 20-50% cases of severe form of the disease.

(ii) Prophylaxis

The control of urban yellow fever can be achieved by eradicating the vector mosquito. Two vaccines have been developed for human use. The French neurotropic vaccine

(Dakar) produced from infected mouse brain carries a high risk of producing encephalitis in the vaccinees. A safe and equally effective vaccine is 17D vaccine. This was developed by *Theiler* in 1937 by passaging the *Asibi strain* serially in mouse embryo and whole chick embryo tissues and then in chick embryo tissue from which the central nervous tissue has been removed. The 17D vaccine is administered by subcutaneous inoculation. Vaccination which is mandatory for travel to or from endemic area is valid for ten years beginning ten days after administration of vaccine. In India, the 17D vaccine is manufactured at the Central Research Institute (CRI), Kasauli.

India has a receptive area for yellow fever, with a large population of *Aedes aegypti* and non-immune individuals, but still yellow fever does not exist in India. It may be due to strict vigilance on vaccination and quarantine for travellers from endemic area. This has checked the entry of the virus into India, but perhaps a more likely reason could be that the stray virus may not be able to get established in the vectors due to the prevalence in the *Aedes aegypti* of the Dengue fever virus.

3. DENGUE

Dengue is characterised by fever of sudden onset, headache, retrobulbar pain, conjunctival infection, pain in the back and joints (also called break-bone fever), lymphadenopathy and maculopapular rash. It is endemic and often epidemic in the tropics and subtropics, particularly in Asia, the Caribbean, the Pacific and some areas of West Africa. In India, dengue is common in the East coast.

Dengue virus has four serotypes : Dengue 1 (DEN 1), dengue 2 (DEN 2), dengue 3 (DEN 3) and dengue 4 (DEN 4). Like yellow fever, it is transmitted by *Aedes aegypti*. Although, the Dengue virus and yellow fever virus are antigenically related, yet there is no significant cross immunity.

(i) Clinical features

The disease may occur in two forms: classical dengue fever and dengue in more serious forms with haemorrhagic manifestations.

(*a*) Classical dengue fever: This usually affects older children and adults. It has relatively benign course with fever, headache and pain in muscles and bones. The fever is typically biphasic (saddle back). Incubation period is 5-8 days. A maculopapular rash generally appears on 3rd or 4th day. The febrile illness lasts for about 10 days after which recovery is generally complete. It is rarely fatal.

(*b*) Other manifestations: Dengue may also occur in more serious forms, with haemorrhagic manifestations or with shock. These are known as *dengue haemorrhagic fever* (DHF) and *dengue shock syndrome* (DSS). DHF/DSS remains mostly confined among children of 5-10 years age group in area where multiple dengue viruses cause disease. It appears to be hyperimmune response. It is seen in patients previously infected with dengue virus. On reinfection with a different serotype of dengue virus, antibody formed against the first virus reacts with the second serotype virus forming immune complexes (*virus-antibody complex*). Initial symptoms are like those of dengue fever but associated with haemorrhagic rash, thrombocytopenia and shock. The mortality rate is 5-10%. The disease is more often found in epidemic form in Thailand, S.E. Asia and India where dengue serotypes are regularly present. Dengue was initially confined to the east coast of India and has caused epidemics. In 1963, extensive outbreaks affected Calcutta and Madras. In the 1990s Surat and Delhi had major epidemics with deaths due to DHF and DSS. All four types of dengue virus are present in India.

(ii) Laboratory diagnosis

(a) Specimens

For antibody detection
- Serum

For antigen detection
- Serum

For isolation of virus and PCR
- Serum
- Plasma
- Whole blood (washed buffy coat)
- Autopsy tissues
- Mosquitoes collected in nature

(b) Haematological diagnosis

- Thrombocytopenia (100,000 cells or less per mm^3)
- Haemoconcentration (>20% rise in haematocrit)

(c) Microbiological diagnosis

Isolation of the virus is difficult but serology plays a major role in diagnosis.

- Detection of antibody

Demonstration of IgM antibody in serum provides early diagnosis. IgM antibody appears 5 days after onset of symptoms and persists for one to three months. ELISA is used for detection of IgM antibody. A strip immunochromatographic test for IgM is also available for rapid diagnosis.

IgG antibody appears later than IgM antibody. Detection of four fold rise in IgG titre in paired sera taken at an interval of ten days or more is

confirmatory. ELISA is used for detection of IgG antibody.

- Detection of NS1 antigen
  Immunochromatographic test is available for detection of NS1 antigen. It is a rapid test and detects antigen on the first day of fever before antibodies appear. It takes about 15 minutes.
- Isolation of Virus
  Virus isolation can be done by inoculating clinical specimen into mosquitoes, mosquitoes cell lines (C6/36 or AP-61 cells), or suckling mice. Further identification is done by using fluorescent antibody test. Live mosquito inoculation is the most sensitive technique for isolation of virus.
- Polymerase Chain Reaction (PCR)
  Viral RNA can be detected in clinical specimens by reverse transcriptase polymerase chain reaction (RTPCR). Viral genomic sequences can also be detected.

(iii) Prophylaxis

Control measures include elimination of mosquitoes. No effective vaccine is available. In order to avoid the DHF/DSS in immunised persons, a live attenuated vaccine containing all four dengue serotypes is under clinical trials.

Tick-borne Flaviviruses

These viruses produce two types of clinical syndrome—encephalitis and haemorrhagic fevers.

1. TICK-BORNE ENCEPHALITIS VIRUSES

(i) Russian spring-summer encephalitis

Russian spring-summer encephalitis (RSSE) is caused by RSSE virus in Central Europe, Eastern Europe and erstwhile Soviet Russia. Infection is transmitted by the bite of Ixodid ticks. The virus is transmitted transovarially in ticks so that they may also act as the reservoir hosts. Wild rodents and birds are other reservoirs. The virus is excreted in milk of infected goats. It may be transmitted to man by drinking the milk of infected goats. In Scotland, the disease is called 'louping ill' as it occurs primarily in sheep in which it causes a curious 'leaping' gait.

Control measures include avoidance of tick bites. A formalin-killed RSSE vaccine has been found useful.

(ii) Powassan virus

Powassan encephalitis is caused by another tick borne virus called the Powassan virus. It occurs in Canada and Northern U.S.A.

2. TICK BORNE HAEMORRHAGIC FEVERS

(i) Kyasanur Forest Disease (KFD)

This is an Indian haemorrhagic disease that appeared in Kyasanur Forest of Karnataka in 1957 as a fatal epizootic affecting monkeys, along with a severe prostrating illness in some of the villagers in the area. The causative virus, antigenically related to the RSSE complex, was isolated in National Institute of Virology, Pune, from the patients and dead monkeys. It was named the KFD virus.

Birds and small mammals are believed to be the reservoirs of the virus. It is transmitted by bite of tick (*Haemaphysalis spinigera*). Like RSSE, ticks may also act as the reservoir hosts as virus is transmitted transovarially in them. As infection in monkeys lead to fatal disease, therefore, they are unlikely to be reservoirs. They act only as amplifier hosts.

(a) Clinical features

Incubation period varies from 3-7 days. Patient develops fever of sudden onset with headache, vomiting, conjunctivitis, myalgia and severe prostration. Massive haemorrhages in alimentary canal, chest cavity and epistaxis may occur in some cases. Case fatality is about 5%.

(b) Epidemiology

For many years after its discovery, KFD remained confined to the areas contiguous to its original focus in Sagar, Sorab and Shikarpur taluks of Shimoga district in Karnataka. Between 1972 and 1975, a few other foci developed in the adjacent areas in North Kanara. In 1982, the epizootic and epidemic occurred in Belthangadi taluk in South Kanara. The outbreak, known locally as 'monkey fever', started with dead monkeys observed in October 1982. The first human case was seen in December 1982. During the next five months, 1142 human cases were reported with 104 deaths. The outbreak subsided in June with the onset of the monsoon but reappeared in the following December. The ecological disturbance caused by clear felling of the forest is believed to have activated a silent enzootic focus of the virus.

(c) Control

- Control of ticks.
- Vaccination—the population at risk should be vaccinated with killed KFD vaccine.
- Personnel protection—protection of individuals by adequate clothing and insect repellents.

(ii) Omsk haemorrhagic fever

It occurs in erstwhile Soviet Russia and Romania. It is clinically similar to KFD. It is caused by a virus related

to KFD. Water voles or muskrats are the reservoir hosts and *Dermacentor* ticks are the vectors.

3. HEPATITIS C VIRUS

It has been described in Chapter 62 on hepatitis viruses.

C. Bunyaviridae

Family *Bunyaviridae* contains more than 300 members. Bunyavirus was first isolated from a place Bunyamwera in Uganda, from which the name of virus is derived.

1. Morphology

They are spherical 90-100 nm in diameter. They contain a single stranded RNA genome and possess a lipid envelope with glycoprotein peplomers. They replicate in the cytoplasm.

2. Classification

Bunyaviridae contains four genera:

(*i*) *Orthobunyavirus*—Mosquito-borne
(*ii*) *Phlebovirus*—Phlebotomus or mosquito-borne
(*iii*) *Nairovirus*—Tick-borne
(*iv*) *Hantavirus*—Non-arthropod borne.

3. Viruses of Bunyaviridae

(i) Bunyavirus

This genus include *California encephalitis virus, La Crossie virus* and *Chittor virus*.

All the three viruses are closely related antigenically. First two were isolated in USA and *Chittor virus* was detected in Chittor, India. Clinical features are similar in all the three viruses except that *Chittor virus* causes only mild fever. Clinically, they present as fever, headache; central nervous system involvement and usually patient recovers completely. All are mosquito-borne infections.

(ii) Phlebovirus

(a) Sandfly fever (Phlebotomus fever)

It is transmitted by female sandfly (*Phlebotomus papatasii*). Incubation period varies from 3-5 days. Patient develops fever, malaise, headache and nausea which lasts for 3-4 days. The disease occurs in Mediterranean countries and Central Asia including India. Twenty antigenic types of the virus exist. Only five of twenty antigenic types cause human disease—Sicilian, Naples, Punta Toro, Chagres and Candiru. Other name for the disease is papattaci fever.

(b) Rift valley fever

This virus is primarily pathogenic for sheep and other domestic animals in Rift Valley (Kenya) and Middle East. Human beings are secondarily infected. It is mosquito-borne.

(iii) Nairovirus

Nairobi sheep disease affects sheep in large parts of equatorial Africa and causes considerable economic loss. The related *Ganjam* virus causes disease in sheep in India. *Haemaphysalis ticks* appear to be the vector. The virus occasionally infects man causing mild febrile illness.

Members of the Crimean-Congo haemorrhagic group are the major human pathogens in the genus *Nairovirus*. The Crimean Congo Haemorrhagic Fever (CCHF) virus is transmitted by Hyalomma ticks. The disease is endemic in Eastern Europe, Central Asia and many parts of Africa. Cattle, sheep, goats and other domestic animals act as natural reservoirs. The blood of the patients is highly infectious during the acute phase of the disease. Antibodies to this group of viruses have been detected in human and animal sera from India.

(iv) Hantavirus

Hantaviruses are transmitted to humans from rodent hosts (mice, rats) by direct contact with rodent excreta or contaminated fomites. The viruses included are Hantaan virus, Belgrade virus, Seoul virus, Puumala virus and Muerto Canyon virus. They cause haemorrhagic fever and nephropathy. The disease is named haemorrhagic fever with renal syndrome (HFRS). The clinical picture resembles leptospirosis and scrub typhus. Domestic rats appear to be the source of infection in urban cases of HFRS. The role of arthropods in the transmission of infection is not confirmed. Hence, it may be considered as a robovirus and not strictly an arbovirus infection.

A new syndrome, the *Hantavirus pulmonary syndrome* was identified in USA in 1993. It is caused by a newly identified hantavirus, *the Sin Nombre virus*, which is associated with the deer mouse and other rodents. Infection seems to be caused by inhalation of the virus aerosols in rodent faeces. Transmission of the virus has not been linked with any arthropod.

Laboratory diagnosis is done by demonstration of IgM antibody by ELISA. Rising titres of immune adherence haemagglutinating antibodies in paired sera can also be demonstrated.

D. Reoviridae

Family *Reoviridae* contains five genera—*Orthoreovirus, Coltivirus, Orbivirus, Rotavirus* and *Seadornavirus*. Only *Orbivirus* causes arthropod-borne infections. These viruses differ from other arboviruses in having double stranded RNA genomes. *Colorado tick fever virus* is the only recognised pathogen in this genus (orbivirus). It

causes Colorado tick fever and is spread by wood tick *Dermacentor andersoni*. Tick acts both as the vector and reservoir. Natural infection occurs in rodents. It is a self limiting disease. The disease in Western USA is limited to the habitat of the tick.

Palyam, Kasba and *Vellore* viruses belonging to the orbivirus, have been isolated from mosquitoes in India. Their pathogenic role is not known.

E. Rhabdoviridae

The Chandipura virus, belonging to the genus *Vesiculovirus* of family *Rhabdoviridae*, was isolated in Nagpur (India) in 1967 from the blood of patients during epidemic of dengue-chikungunya fever. The vectors are sandflies and *Aedes* mosquitoes, in which the virus multiplies. The pathogenic role of Chandipura virus is not yet fully established.

F. Orthomyxoviridae

Thogotovirus is structurally and genetically similar to influenza viruses. However, it is different in its ability to infect ticks as well as mammals. It is a single stranded RNA virus and segmented into six pieces.

G. Ungrouped Arboviruses

1. Bhanja Virus

This was isolated from goats in Ganjam, Orissa. Human infections with disease and death have been reported from Yugoslavia.

2. Wanowri Virus

This virus was isolated from Hyalomma ticks in India. It had also been isolated from the brain of a young girl who died after a two-day fever in Sri Lanka.

KEY POINTS

1. Arborivuses (arthropod-borne viruses) are RNA viruses that are transmitted by blood-sucking arthropods from one vertebrate host to another.
2. Taxonomically, arboviruses belong to six families: *Togaviridae*, *Flaviviridae*, *Bunyaviridae*, *Reoviridae*, *Rhabdoviridae* and *Orthomyxoviridae*.
3. Arboviruses may cause the *febrile illness with* or *without rash* and *arthralgia*, *encephalitis*, *haemorrhagic fever* and the characteristic systemic disease, *yellow fever*.
4. *Dengue* and *chikungunya* are two important viruses causing febrile illness. *Japanese encephalitis* is an important cause of encephalitis. *Dengue*, chikungunya and *Kyasanur Forest disease* (*KFD*) virus are responsible for haemorrhagic fever.
5. Dengue virus has four serotypes: dengue 1 (DEN 1), dengue 2 (DEN 2), dengue 3 (DEN 3) and dengue 4 (DEN 4). Dengue fever may occur as *classical* dengue fever or more serious forms known as *dengue haemorrhagic fever* (DHF) and *dengue shock syndrome* (*DSS*).
6. Both dengue virus and chikungunya virus are transmitted by *Aedes aegypti* mosquito. KFD is transmitted by bite of *tick*. Natural infections of Japanese encephalitis occur in Adreid birds which act as reservoirs. Human infection occurs from these reservoir birds by several species of *culicine mosquitoes*. Yellow fever virus is transmitted by *Aedes aegypti* mosquito. *The disease yellow fever does not occur in India.*
7. Laboratory diagnosis of arboviruses may be established by *virus isolation* or *serology*. Virus isolation may be done by intracerebral inoculation into *suckling mice* or by *tissue culture*. Using ELISA, serotype specific IgM antibody may be detected in patient serum.

YOU MUST KNOW

1. Different families of arboviruses.
2. List of mosquito-borne and tick-borne arboviruses.
3. Arboviruses causing encephalitis and those causing haemorrhagic fever.
4. Laboratory diagnosis of arbovirus infections.
5. Chikungunya virus.

STUDY QUESTIONS

1. Classify arboviruses. Discuss the methods used for laboratory diagnosis of arboviruses.
2. Name the arboviruses which cause encephalitis. Discribe briefly about Japanese encephalitis.
3. Name the arboviruses which cause haemorrhagic fever and discuss one such virus which is prevalent in India.
4. Write short notes on:
   (a) Yellow fever virus (b) Dengue virus (c) Kyasanur Forest disease
   (d) Bunyaviruses (e) Hantavirus.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Which of the following viruses can cause encephalitis?
   (a) West Nile virus (b) Japanese encephalitis virus
   (c) Powassan virus (d) All of the above
2. Which of the following viruses can cause haemorrhagic fever?
   (a) Chikungunya virus (b) Kyasanur Forest disease virus
   (c) Yellow fever virus (d) All of the above
3. Which of the following viruses belongs to family *Togaviridae*?
   (a) Dengue virus (b) Chikungunya virus (c) West Nile virus (d) All of the above
4. Which of the following viruses belongs to family *Flaviviridae*?
   (a) Dengue virus (b) Chikungunya virus (c) Semiliki Forest virus (d) All of the above
5. All of the following viruses belong to family *Bunyaviridae* except:
   (a) Sandfly fever virus (b) Chittor virus
   (c) California encephalitis virus (d) West Nile virus
6. All of the following viruses have single stranded RNA virus except:
   (a) Alphavirus (b) Flavivirus (c) Bunyavirus (d) Reovirus
7. Mosquitoes act as the vector in all of the following viruses except:
   (a) Dengue virus (b) Chikungunya virus
   (c) Semiliki Forest virus (d) Omsk haemorrhagic fever virus
8. Which of the following viruses is/are tick-borne?
   (a) Kyasanur Forest disease virus (b) Omsk haemorrhagic fever virus
   (c) Russian spring summer encephalitis virus (d) All of the above
9. Which of the following mosquitoes transmits chikungunya virus?
   (a) Anopheles (b) Aedes (c) Culex (d) None of the above
10. Which of the following mosquitoes transmits yellow fever virus?
    (a) Anopheles (b) Aedes (c) Culex (d) None of the above
11. Which of the following mosquitoes transmits Japanese encephalitis virus?
    (a) Anopheles (b) Aedes (c) Culex (d) None of the above
12. How many serotypes of dengue virus are there?
    (a) Three (b) Four (c) Five (d) Eight
13. Which of the following viruses is not transmitted by arthropods?
    (a) Alphavirus (b) Hantavirus (c) Flavivirus (d) Phlebovirus
14. Hantavirus pulmonary syndrome is caused by:
    (a) Chittor virus (b) Nairobi sheep disease virus
    (c) Sin Nombre virus (d) West Nile virus
15. Vaccine/s is/are available for which of the following diseases?
    (a) Japanese encephalitis (b) Yellow fever (c) Rubella (d) All of the above

Answers (MCQs):

1. (d) 2. (d) 3. (b) 4. (a) 5. (d) 6. (d) 7. (d) 8. (d) 9. (b) 10. (b)
11. (c) 12. (b) 13. (b) 14. (c) 15. (d)

Chapter 61

RHABDOVIRUSES

Rhabdoviruses are bullet or rod shaped (*rhabdos*, meaning rod) enveloped viruses with single stranded RNA genome. They are included in the family *Rhabdoviridae*.

I. CLASSIFICATION

Rhabdoviruses infecting mammals belong to two genera in *Rhabdoviridae* family.

1. *Vesiculovirus*: This genera contains *Vesicular stomatitis virus* and related viruses like Chandipura (arbovirus).
2. *Lyssavirus*: It contains rabies virus and related viruses, (*Lyssa*, meaning madness, synonym for rabies).

II. RABIES VIRUS

A. Morphology

1. Bullet shaped, 75 × 180 nm, with one end round and the other planar or concave (Fig. 61.1).
2. The outer lipoprotein envelope contains haemagglutinating peplomer spikes which do not cover the planar end of the virion.
3. Beneath the lipoprotein envelope is the matrix (M) protein layer which may be invaginated at the planar end.
4. Nucleocapsid shows helical symmetry containing a single stranded, negative sense RNA genome and a

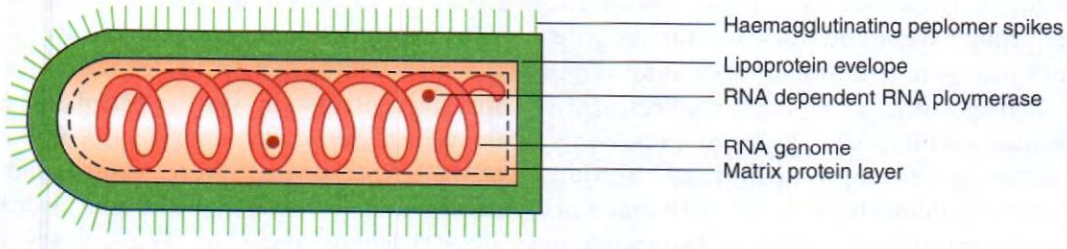


Fig. 61.1 Rabies virus.

RNA-dependent RNA polymerase. The genome is unsegmented.

B. Resistance

The virus is highly resistant against dryness, cold, decay etc. and remains infective for many weeks in the cadaver. Being enveloped virus, it is sensitive to lipid solvents such as ether, chloroform and acetone. The virus is sensitive to quarternary ammonium compounds, ethanol and iodine preparations, soaps and detergents. It is inactivated by phenol, formalin, betapropiolactone, sunlight, ultraviolet radiation and heat (50°C for one hour and 60° C for five minutes) but survives at 4°C for weeks. It can be preserved by lyophilisation or at –70°C. For storage in dry ice, the virus has to be sealed in containers or vials as it is inactivated on exposure to CO_2.

C. Antigenic Properties

Rabies virus of man and animals appears to be of a single antigenic type.

(*i*) The surface spikes are composed of glycoprotein G, which is strongly antigenic and antibody against it is protective. The purified glycoprotein may, therefore, provide an effective subunit vaccine.

(*ii*) The nucleoprotein induces antibodies which are not protective. This antigen is group specific, therefore, cross reactions occur with some rabies related viruses. Antiserum against nucleoprotein antigen is useful in diagnostic tests like immunofluorescence test.

(*iii*) Haemagglutinating activity is optimally seen with goose erythrocytes at 0–4°C and pH 6.2. Haemagglutination is a property of the glycoprotein spikes. The haemagglutinin antigen is species specific.

(*iv*) Other antigens include membrane proteins, glycolipid and RNA dependent RNA polymerase.

D. Animal Susceptibility and Culture

1. Animals

All warm blooded animals including man are susceptible to rabies infection, though some may be highly susceptible and other intermediate. Man and dogs are intermediate susceptible. Pups are more susceptible than adult dogs. Some species such as cattle, cats and foxes are highly susceptible, whereas certain species like skunks, opossums and fowls are relatively resistant. Experimental infection can be produced in any animal but mice are the animals of choice. They can be infected by any route. Intracerebral inoculation leads to encephalitis and the animal dies within 5-30 days.

Street virus

The rabies virus isolated from natural human or animal infection is called the *street virus*. Such viruses produce fatal encephalitis in laboratory animals after a long and variable incubation period of 1-12 weeks. Intracytoplasmic inclusion bodies (Negri bodies) can be demonstrated in brains of these animals.

Fixed virus

By several serial intracerebral passages in rabbits, the virus undergoes certain changes and is termed the *fixed virus*. The fixed virus is more neurotropic, it produces fatal encephalitis after intracerebral inoculation and the incubation period is shortened and fixed to 6-7 days. Negri bodies are usually not demonstrable in the brain of animals dying of fixed virus infection. The fixed virus is used in vaccine production.

2. Chick Embryos

The rabies virus grows in chick embryo and the usual mode of inoculation is into the yolk sac. Live attenuated vaccine strains, Flury and Kelev, have been developed by this method.

3. Tissue Culture

The rabies virus can grow in chick embryo fibroblast, hamster kidney cells, human diploid cells and vero cell cultures.

E. Pathogenesis

Rabies is a natural infection of dogs, foxes, cats, wolves and bats. Dogs, foxes and vampire bats are important maintenance hosts. Man is infected by the bite of rabid dog or other animals. Saliva containing viruses is deposited in the wound. Infection may also be acquired by the inhalation of massive virus aerosols generated in bat caves. The incubation period varies from 1-3 months, sometimes may be as short as 10 days particularly in children and with wounds on face and neck. The rabies virus multiplies in muscle or connective tissue at or near the site of introduction before it attaches to nerves. It spreads centripetally via the peripheral nerves towards the central nervous system (CNS). Following infection of the CNS, the virus spreads centrifugally to peripheral nerves, and involves skeletal and myocardial muscles, adrenal glands and skin. The salivary gland invasion is necessary to transmit the virus to another animal or human.

F. Laboratory Diagnosis

1. Diagnosis in Humans

Diagnosis can be made antemortem or postmortem. Corneal impression smears, facial skin biopsies or saliva are important specimens for antemortem diagnosis using immunofluorescence test. Antemortem diagnosis can also be made by virus isolation from saliva or CSF. Salivary glands, brain tissue, hippocampus or cerebellum are used for postmortem diagnosis by antigen detection, demonstration of negri bodies and isolation of virus. Details of tests used for antemortem and postmortem diagnosis are described below.

(i) Immunofluorescence test

Viral antigens can be detected in corneal impression smears and facial skin biopsies or saliva by direct immunofluorescence using antirabies serum tagged with fluorescein isothiocyanate. Diagnosis may be made postmortem using brain as a specimen. The use of monoclonal antibodies instead of crude antiserum makes the test very specific.

(ii) Demonstration of Negri bodies

This is demonstrated in brain but may be absent in some 20% of human cases. Negri bodies appear as intracytoplasmic, oval or round, purplish pink (3-27 mm) bodies with characteristic basophilic inner granules. *Seller's technique* (basic fuchsin and methylene blue in alcohol) is used to demonstrate these bodies. The advantage of this technique is that fixation and staining are done simultaneously.

(iii) Isolation of the virus

The virus can be isolated from specimens like CSF, saliva and urine, by intracerebral inoculation in mice. However, during postmortem, brain tissue is also used. The inoculated mice are examined for signs of illness, and their brains are examined at death or at 28 days after inoculation for Negri bodies. Brains can also be examined by immunofluorescence technique.

A more rapid and sensitive method is isolation of the virus by tissue culture. WI38, BHK-21 cell lines are used. Diagnosis is made by detection of rabies virus in inoculated tissue culture cells by immunofluorescence. A positive test can be obtained within 2-4 days.

(iv) Antibody detection

High titre antibodies in the CSF can be used for diagnosis.

(v) PCR

Reverse transcriptase PCR can be used for detection of rabies virus RNA. It is a sensitive method.

2. Diagnosis in Animals

The brain of rabid animal is removed carefully, part of which is preserved in 50% glycerol saline (for isolation of virus) and the other in Zenker's fixative (for demonstration of Negri bodies). Zenker's fluid contains potassium dichromate, mercuric chloride and distilled water. The portion of brain should include the hippocampus and cerebellum as Negri bodies are abundant there. The following tests are done :

(i) Immunofluorescence test

This is a rapid test done on brain tissue to demonstrate viral antigens. Examination of salivary glands is useful as it may indicate whether the animal was shedding virus in saliva. Technique is same as described earlier for diagnosis in humans.

(ii) Demonstration of Negri bodies

This is still the method most commonly used as facilities for immunofluorescence and virus isolation are not available in many laboratories. Impression smears of the brain are stained with Seller's technique and examined for Negri bodies. Failure to find Negri bodies does not exclude that animal is free of rabies.

(iii) Isolation of virus

The brain tissue suspension is injected into young mice by intracerebral route. They die within 3 weeks, showing Negri bodies in the brain. If several mice are injected per specimen, they can be sacrificed serially and viral antigen may be detected in brain tissue by immunofluorescence. This is useful as results are available quicker than Negri bodies.

3. Other Methods of Diagnosis

(i) Serum virus neutralisation test

It is very specific antigen-antibody reaction. This may be used for identification of the isolated virus or for antibodies detection in the serum or other body fluids.

(ii) Complement fixation test (CFT)

This can be performed for detection of rabies antigen and antibodies.

(iii) Counter Immunoelectrophoresis (CIE)

The test is used for assessment of antibodies in the serum.

(iv) Enzyme linked immunosorbent assay (ELISA)
It may be used for assessment of antibodies as well as detection of viral antigen.

(v) Immunoperoxidase test
It requires a peroxidase conjugated antibody for detection of antigen.

(vi) Haemagglutination inhibition test
It is used for assessment of antibodies. Erythrocytes of goose are used in the test.

(vii) Passive haemagglutination assay (PHA)
It is also used for detection of antibodies. Erythrocytes are coated with highly purified rabies antigen.

G. Prophylaxis

1. Pre-Exposure Prophylaxis

It is necessary for laboratory personnel and those who handle potentially infected animals.

2. Post-Exposure Prophylaxis

It includes : local treatment; hyperimmune serum; and vaccination. Post- exposure prophylaxis is given according to category of exposure (Table 61.1).

LOCAL TREATMENT

The wound should be thoroughly washed with soap and water for 15 minutes. It is very important as soap inactivates the virus by destroying its envelope. After washing, the wound is treated with quarternary ammonium compound (cetavlon) or tincture iodine or alcohol (40-70%). In severe wounds, antirabic hyperimmnue serum may be infiltrated around the wound. Damaged tissue is excised but wound is left unsutured. Antitetanus vaccination and antibiotics may be used to prevent sepsis.

The biting animal should be watched, if possible, for 10 days. If animal remains healthy after 10 days, rabies may be excluded.

HYPERIMMUNE SERUM

Hyperimmune serum (rabies immunoglobulin) is used alongwith vaccination in Category III exposure. However, in immunocompromised individual hyperinomune serum treatment alongwith vaccination is used in Category II and III exposure.

Human antirabies immunoglobulin (20 IU per kg body weight) is reserved for the high risk cases. It is the safest antirabies antiserum. Half dose of the antiserum is infiltrated locally in wound and other half is administered by intramuscular route. Antiserum should not be given to individuals who have had prior active immunisation. As antiserum may depress the active immune response to some extent, it is necessary to give booster doses of the vaccine after regular course is over.

Alternatively, hyperimmune serum prepared in horses is administered in a dose of 40 IU/kg body weight. Risk of hypersensitivity to horse serum is a disadvantage and should be administered with precautions.

VACCINATION

Vaccines are of two main categories—neural and non-neural (Table 61.2). Non-neural vaccines are being used increasingly because neural vaccines are associated with serious risk of neurological complications. Fixed virus is used for preparation of all vaccines. Manufacturing and use of neural vaccines have been discontinued in India.

(i) Neural vaccines
Pasteur (1885) first developed rabies vaccine by drying spinal cord of infected rabbit. The following are some of the infected brain vaccines.

(a) Semple vaccine: It was developed by *Semple* (1911) at the Central Research Institute (CRI), Kasauli, India. It was a 5% suspension of infected sheep brain and inactivated by 5% phenol at 37°C, leaving no residual live virus. It was widely used antirabic vaccine.

Table 61.1 Post-exposure prophylaxes according to categories of exposure

Category	Type and contact	Post-exposure prophylaxis (PEP)
I.	• Touching or feeding of animals • Licks on intact skin • Contact of intact skin with secretions/excretions of animal/human case	• PEP is not given if reliable case history is available
II.	• Nibbling of uncovered skin • Minor scratches or abrasions without bleeding	• Local wound treatment • Anti-rabies vaccination
III.	• Single or multiiple transdermal bites or scratches, licks on broken skin • Contamination of mucous membrane with saliva (i.e. licks)	• Local wound treatment • Anti serum (Rabies immunoglobulin) • Anti-rabies vaccine

Table 61.2 Antirabies Vaccines

Vaccine	Preparation	Type	Use
Neural			
1. Semple vaccine	Discontinued	—	Discontinued
2. Betapropiolactone (BPL) vaccine	Discontinued	—	Discontinued
3. Infant brain vaccine	Vaccine prepared in brain of suckling mice	Inactivated by UV radiation, BPL or phenol	Used widely in South America.
Non-neural			
1. Duck egg vaccine	Discontinued	—	Discontinued
2. Duck embryo vaccine	Purified duck embryo vaccine (PDEV)	Inactivated by BPL	Highly antigenic, free from side effects
3. Tissue culture vaccine	Fixed virus grown in human diploid cell strain vaccine (HDCV) e.g. WI-38 or MRC 5	Inactivated by BPL	Highly antigenic, free from side effects, but costly
4. Chick embryo vaccine			
(*i*) Low egg passage (LEP)	By 40-50 passages	Live attenuated	Used for dogs above 3 months old.
(*ii*) High egg passage (HEP)	By 180 passages	Live attenuated	Used for cattle and cats
Subunit vaccine			
	Surface glycoprotein cloned and recombinant vaccine	—	Experimental stage

(b) Beta propiolactone (BPL) vaccine: It was modified Semple vaccine with BPL as inactivating agent instead of phenol. It was believed to be more antigenic.

(c) Infant brain vaccine: Brain tissue vaccines are associated with neurological complications due to the presence of myelin. It is scanty or absent in non-myelinated neural tissue of infant brain and thus may reduce neurological complications. This type of vaccine is impractical in India due to very large quantities required. Occasional neurological reactions have also occurred with this vaccine.

(ii) Non-neural vaccines

(a) Egg vaccines

Duck egg vaccine: It is beta propiolactone inactivated vaccine. It has a poor immunogenicity and is therefore discontinued.

Duck embryo vaccine: Purified duck embryo vaccine (PDEV) is beta-propiolactone inactivated vaccine. It is as effective as cell culture vaccines and recommended by WHO.

Live attenuated chick embryo vaccine: It was used for vaccination of animals. Two types of vaccine were developed—the low egg passage (LEP) and high egg passage (HEP) at 40-50 egg passage level and at 180 passage level respectively. LEP was used for dogs of three months or more and HEP for cattle and cats. This vaccine is not recommended for humans as it contains live virus.

(b) Cell culture vaccines

The human diploid cell strain vaccine (HDCV) is prepared by growing fixed rabies virus on human diploid cells (WI 38 or MRC 5) and is inactivated with beta- propiolactone. This vaccine is highly antigenic and free of side effects. The only disadvantage is its high cost.

Other equally effective but more economical cell culture vaccines have been developed. The purified chick embryo cell culture vaccine (PCECV) is now widely used in the trade name of Rabipur. Purified vero cell rabies vaccine (PVRV) is the another economical cell culture vaccine.

(iii) Subunit vaccine

Surface glycoprotein, which is the protective antigen has been cloned and recombinant vaccine produced. It is still in the experimental stage.

Commonly used vaccines against rabies are shown in Table 61.3.

Table 61.3 Commonly used Non-neural Antirabies Vaccines

Vaccine	Substrate to grow fixed virus	Type
HDCV	Human diploid cell	Inactivated by BPL
PCECV	Chick embryo cells	Inactivated by BPL
PVRV	Vero cells	Inactivated by BPL
PDEV	Duck embryo	Inactivated by BPL

VACCINATION SCHEDULES

When the biting animal can be observed for ten days and the animal remains healthy, rabies may be excluded and vaccine, if already started, may be discontinued. This, of course, does not exclude the rare possibility of carrier state in dogs. The observation of animal for ten days is suggested because the virus may be present in the saliva 3-4 days before onset of symptoms and the animal usually dies within 5-6 days of developing the disease.

Neural vaccines

Use of neural vaccines have been discontinued. These are now of historical importance. The vaccine, used previously, was given subcutaneously on the anterior abdominal wall and the immunity lasted for six months only.

Complications of these vaccines included:

1. Mild local reactions
2. Neuroparalytic complications, the aetiology of these neurological complications is believed to be immune response to the injected brain tissue resulting in organ specific immunological damage.

Cell culture vaccines

All four cell culture vaccines available in India (HDCV, PCECV, PVRV and PDEV vaccines) have the same dose schedule. It is also same for both adults and children.

Preexposure vaccination

This is for persons at high risk like laboratory personnel handling rabies virus. Three doses of 1.0 ml on days 0, 7, and 21 or 28 are given by intramuscular route. For intradermal regimen, 0.1 ml is injected intradermally on days 0, 7 and 21 or 28. A booster dose is recommended when the titre of antibodies against rabies falls below 0.5 IU/ml. Serum sample should be collected for antibodies testing every six months for two years.

Postexposure vaccination

1.0 ml is given by intramuscular route in the deltoid region, on days 0, 3, 7, 14 and 28, with a booster dose (optional) on day 90. This course gives protection for at least five years, during which period any further exposure may require only one or two booster doses (on days 0, 3) depending on the degree of risk. After five years, full five injection course is advised if exposed to infection.

In children vaccination is given on the anterolateral aspect of the thigh. Gluteal injections are to be avoided as they are less immunogenic.

PCECV and PVRV vaccines are approved for intradermal use in India. These vaccines may be used for post-exposure prophylaxis. Regimen used is **Updated Thai Red Cross Schedule (2-2-2-0-2)**. 0.1 ml of vaccine is administered intradermally on two sites per visit (one on each deltoid area) on days 0,3,7 and 28.

VACCINE FOR ANIMALS

Cell culture vaccine containing BPL inactivated virus adsorbed on aluminium hydroxide is used by intramuscular route. Single dose of 1.0 ml, followed by booster at 1-3 years interval is given.

Neural vaccines are unsatisfactory as they are not adequately immunogenic and multiple doses are to be given.

H. Treatment

No specific antirabies agent is available but following may be beneficial.

1. Intensive supportive care
2. Management of complications
3. In non-immunised patients, hyperimmune serum may be administered.
4. Interferon has also been used.

I. Prevention and Control

1. Elimination of stray dogs
2. Vaccination of animals
3. Registration and licensing of all domestic animals
4. Control measures on wild life
5. Quarantine for about 6 months of imported dogs
6. Health education of people regarding care of dogs and prevention of rabies.

J. Epidemiology

Rabies virus is present in all parts of the world except Australia, Antarctica and some islands like United Kingdom. Most human cases follow dog bites but in endemic areas almost any animal can transmit rabies. In India, antirabic treatment is to be considered following the bite of any animal except rats. Direct man to man transmission, although rare, is possible. An unusual mode of transmission has occurred by corneal grafts.

In India, reservoir of infection in wild life are the jackal, fox, hyena and other wild life carriers. These animals transmit the infection to dogs and other domestic animals and also maintain a cycle among themselves. Incubation period of rabies is 3-8 weeks but may vary from 4 days to many years.

III. RABIES-RELATED VIRUSES

Rabies-related viruses include Lagos bat virus, Mokola virus, Duvenhage virus, European bat lyssavirus 1, European bat lyssavirus 2 and Australian bat lyssavirus (Table 61.4).

Some of these viruses cause human infections resembling rabies, which does not appear to be influenced by antirabies antibodies. However, a varying degree of cross reactions are observed between these viruses and rabies virus. They appear to be distinct from rabies virus. They are classified under Lyssavirus as different serotypes.

Table 61.4 Rabies and Rabies Related Viruses

Species		Serotype	Source	Distribution (first isolated from)
Rabies (prototype)		1	Carnivores, cattle, man, bats	World wide
Lagos bat virus	Rabies related viruses	2	Fruit eating bats, cat	West Africa (Lagos, Nigeria)
Mokola virus		3	Shrews, man, dogs, cats, rodents	Africa (Ibadan, Nigeria)
Duvenhage virus		4	Man, bat	South Africa, Europe
European bat lyssavirus 1		5	Bats, man	Europe
European bat lyssavirus 2		6	Bats, man	Europe
Australian bat lyssavirus		7	Bats, man	Australia

KEY POINTS

1. Rhabdoviruses are *bullet* or rod shaped, enveloped viruses with single stranded RNA genome.
2. Rabies virus is an important rhabdovirus. It causes *rabies*.
3. The rabies virus isolated from natural human or animal infection is called the *street virus*. By several serial intracerebral passages in rabbits, the virus undergoes certain changes and is termed the *fixed virus*.
4. Rabies is a natural infection of *dogs*, foxes, cats, wolves and bats. Man is infected by the bite of rabid dog or other animals.
5. Laboratory diagnosis of rabies in humans depends on *detection of viral antigens, demonstration of negri bodies in brain* and *isolation of the virus by tissue culture*.
6. Viral antigens can be detected in *corneal impression smears* and *facial skin biopsies* or *saliva* by *direct immunofluorescence*. It is a very useful for *antemortem* diagnosis. Same method may be used on brain for postmortem diagnosis.
7. Negri bodies can be demonstrated in brain by staining with *Seller's technique*.
8. Cell culture vaccines are commonly used antirabies vaccines. These include *human diploid cell strain vaccine* (*HDCV*), *purified chick embryo cell culture vaccine* (*PCECV*) and *purified vero cell rabies vaccine* (*PVRV*). These are *non-neural* vaccines.
9. All three cell culture vaccines available in India (HDCV, PCECV and PVRV vaccines) have the same dose schedule. It is also same for both adults and children. For *postexposure vaccination*, 1.0 ml is given by intramuscular route in the deltoid region, on days *0*, *3*, *7*, *14* and *28*, with a booster dose (optional) on day 90. This course gives protection for at least five years.

YOU MUST KNOW

1. Morphology of rabies virus.
2. Street virus and fixed virus.
3. Laboratory diagnosis of rabies.
4. Negri bodies.
5. Non-neural vaccines and their schedule for pre-exposure and post-exposure vaccination.

STUDY QUESTIONS

1. Draw a labelled diagram of rabies virus. Discuss the laboratory diagnosis of rabies.
2. Describe the prophylaxis of rabies.
3. Write short notes on:
   (a) Negri bodies (b) Fixed virus (c) Cell culture vaccines (d) Rabies related viruses.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Which of the following viruses is bullet shaped?
   (a) Herpes virus (b) Variola virus
   (c) Rabies virus (d) Parvovirus
2. Which of the following viruses is enveloped?
   (a) Human adenovirus (b) Rabies virus
   (c) Human reovirus (d) Colarado tick fever virus
3. Which animals are susceptible to rabies infection?
   (a) Cattle (b) Cats
   (c) Foxes (d) All of the above
4. Negri bodies can be demonstrated in brain of animals who are infected with:
   (a) Street virus (b) Fixed virus
   (c) Both of the above (d) None of the above
5. Which of the following rabies virus is used for vaccine production?
   (a) Street virus (b) Fixed virus
   (c) Both of the above (d) None of the above
6. Which of the following clinical specimens can be used for antemortem diagnosis of rabies in humans by direct immunofluorescence test?
   (a) Corneal impression smear (b) Facial skin biopsy
   (c) Saliva (d) All of the above
7. Which of the following tests can be used in diagnosis of rabies?
   (a) Direct immunofluorescence test (b) Demonstration of negri bodies
   (c) Isolation of virus (d) All of the above
8. Which of the following is non-neural antirabies vaccine?
   (a) Semple vaccine (b) Purified vero cell vaccine
   (c) Betapropiolactone vaccine (d) Infant brain vaccine
9. All of the following antirabies vaccine are inactivated vaccines except:
   (a) Human diploid cell strain vaccine (b) Purified chick embryo cell culture vaccine
   (c) Purified vero cell vaccine (d) Chick embryo vaccine
10. Which of the following measures can be required for prevention of rabies in humans exposed to bite of rabid animal?
    (a) Local treatment of wound (b) Hyperimmune serum administration
    (c) Vaccination (d) All of the above

Answers (MCQs):

1. (c)	2. (b)	3. (d)	4. (a)	5. (b)
6. (d)	7. (d)	8. (b)	9. (d)	10. (d)

Chapter 62

HEPATITIS VIRUSES

Viral hepatitis is a systemic disease with primary inflammation in the liver. Till now, there are six hepatitis viruses i.e. hepatitis A, B, C, D, E, and G (Type F is proved to be a mutant of type B virus and not a separate entity, Type F was therefore deleted as a separate hepatitis virus). The infection caused by hepatitis B is most severe and at times fatal. *Hepatitis* B and C viruses are also responsible for many cases of primary hepatocellular carcinoma. Hepatitis B is a DNA virus while others (A, C, D, E, G) contain RNA genome (Table 62.1).

Table 62.1 Characteristic Features of Major Hepatitis Viruses

Feature	HAV	HBV	HCV	HDV	HEV	HGV
Genome	RNA	DNA	RNA	RNA	RNA	RNA
Nomenclature	*Picornaviridae*	*Hepadnaviridae*	*Flaviviridae*	*Deltavirus*	*Hepeviridae*	*Flaviviridae*
Mode of transmission	Enteric	Parenteral, sexual, perinatal	Parenteral, sexual	Parenteral	Enteric	Parenteral, sexual, perinatal
Antigen in blood	HAV	HBsAg, HBeAg	HCV	HDAg	HEV	?
Antibodies in blood	Anti-HAV	Anti-HBs, Anti-HBe, Anti-HBc	Anti-HCV	Anti-HDV	Anti-HEV	Anti-HGV envelope
Chronic carrier state	No	Yes	Yes	Yes	No	?
Hepatic carcinoma	No	Yes	Yes	No	No	No
?, not known						

I. HEPATITIS A VIRUS (HAV) (INFECTIOUS HEPATITIS)

Infectious hepatitis is a subacute disease occurring mainly in children and young adults. The hepatitis A virus enters the body by oral route, multiplies in the intestinal epithelium and reaches the liver by haematogenous spread. The large majority of infections are asymptomatic. The incubation period of the disease is 2–6 weeks. The clinical disease has two stages: the prodromal or preicteric and the icteric stages. Symptoms include fever, malaise, anorexia, nausea, vomiting and liver tenderness. These usually subside with the onset of jaundice. Recovery occurs over a period of 4–6 weeks. The virus is shed in faeces during the late incubation period and prodromal stage of the disease. Once jaundice develops, it is rarely detectable in faeces.

Natural infection with HAV is seen only in humans. There is no evidence of extra human source of the virus in nature. Virus persistence in nature depends on continuing inapparent infections. Chronic carriers are not seen.

A. Morphology

HAV is 27 nm non-enveloped single stranded RNA virus with an icosahedral symmetry (Fig. 62.1). It belongs to the picornavirus family. It was originally designated as 'enterovirus 72'. It is now recognised as the prototype of a genus *Hepatovirus*. Only one serotype of the virus exists.

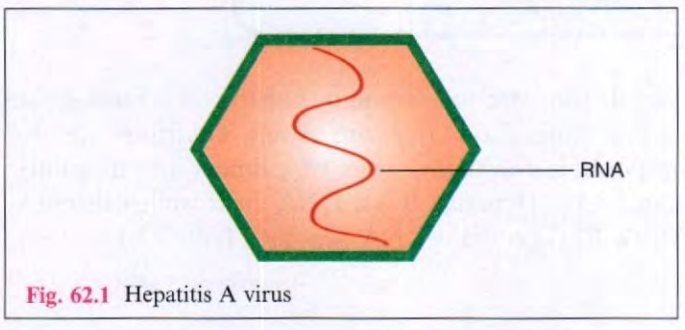


Fig. 62.1 Hepatitis A virus

B. Resistance

The virus is resistant to inactivation by heat at 60°C for one hour, ether and to pH 3, but is destroyed by autoclaving, boiling for five minutes, formaldehyde (1: 4000) and by chlorine at 10-15 ppm for 30 minutes. It survives at 4°C or below.

C. Laboratory Diagnosis

Laboratory diagnosis can be made by

1. Demonstration of virus
2. Detection of antibody
3. Biochemical tests

1. Demonstration of Virus

The virus may be demonstrated by following methods:

(i) Immunoelectron microscopy (IEM)

The virus can be visualised by IEM in faeces during the late incubation period and preicteric phase but seldom later.

(ii) Enzyme-linked immunosorbent assay (ELISA)

HAV antigen can be detected in faeces by ELISA using monoclonal antibodies.

(iii) Isolation

The virus has been grown in human and simian cell cultures but it is not possible to grow them routinely from faeces of patients.

2. Detection of Antibody

Because of difficulties of isolation and detection of virus, diagnosis depends on demonstration of specific IgM

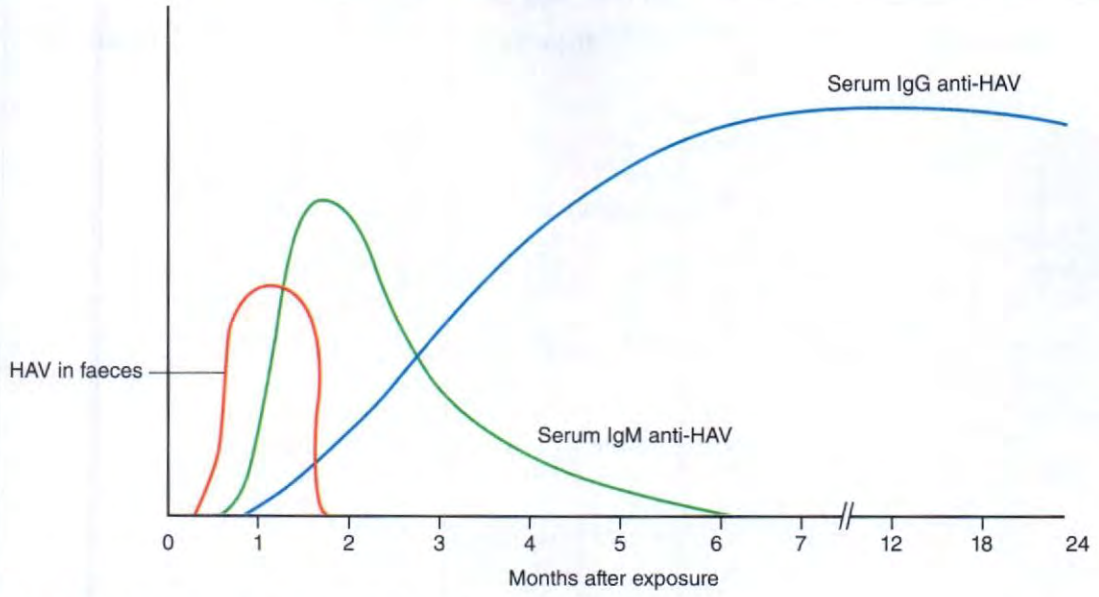


Fig. 62.2 Typical course of hepatitis A virus (HAV) infection.

antibody to HAV in the blood. Antibody appears early in the clinical illness and certainly by the time jaundice appears. IgM antibody is diagnostic of a recent infection and is detectable in serum for 2-6 months after onset of symptoms. IgG antibody to HAV appears at about the same time, usually persists for many years and is a useful indicator of past infection (Fig. 62.2). ELISA kits for detecing IgM and IgG antibodies are available.

3. Biochemical Tests

Liver function tests such as ALT and bilirubin supplement the diagnosis.

(i) Alanine aminotransferase (ALT)

Alanine aminotransferase (ALT, previously designated as SGPT) occurs in much higher concentration in hepatic cells than elsewhere and hence a rise in serum ALT activity reflects liver damage more specifically.

(ii) Bilirubin and protein

Serum bilirubin level usually rises with the appearance of jaundice. Serum globulin is increased with fall in albumin level.

D. Epidemiology

Infection spreads from case to case and through contaminated food, water or milk. In India, type A hepatitis is the most common cause of acute hepatitis in children.

E. Prophylaxis

1. As the disease is transmitted by the faeco-oral route, general prophylaxis includes improved sanitation and prevention of faecal contamination of food and water.
2. Passive prophylaxis with normal pooled human immunoglobulin before exposure to the virus or in the early incubation period may prevent or attenuate a clinical illness, while not necessarily prevent virus excretion.
3. A formalin inactivated, alum conjugated vaccine containing HAV grown in human diploid cell culture is available. It is safe and effective. A full course consists of two intramuscular injections of the vaccine. Second dose is administered 6 months after first dose. Protection lasts for 10–20 years.
4. One attack of the disease gives life-long protection against HAV.

F. Treatment

Treatment is symptomatic. No specific antiviral therapy is available.

II. HEPATITIS B VIRUS (HBV) (SERUM HEPATITIS)

Hepatitis B virus (HBV) belongs to the family *Hepadnaviridae* which also includes certain viruses causing infection to animals. It differs from hepatitis A in various aspects (Table 62.2).

A. Morphology

HBV is a complex 42 nm double shelled particle. The outer surface or envelope of virus contains hepatitis B surface antigen (HBs Ag). It encloses an inner icosahedral 27 nm nucleocapsid (core), which contains hepatitis B

Table 62.2 Differentiating Features of Hepatitis A and B Viruses

Feature	Hepatitis A	Hepatitis B
1. Virus		
Diameter	27 nm	42 nm
Genome	RNA	DNA
Symmetry	Icosahedral	Icosahedral
Envelope	Non-enveloped	Enveloped
Stability		
60°C for 60 mins	Survives	Survives
100°C for 5mins	Inactivated	Inactivated
2. Usual mode of infection	Faeco-oral	Parenteral
3. Clinical		
Incubation period	2-6 weeks	2-6 months
Onset	Usually acute	Insidious
Fever less than 38°C	Usual	Rare
Chronicity	Rare	Common
Age incidence	Children and young adults	All ages: commoner in adult
Seasonal distribution	Post monsoon in India Autumn-winter	All the year round
4. Laboratory diagnosis		
HBsAg	Absent	Present
Raised serum IgM	Common	Rare
Raised serum transaminase	For few days	For many weeks
Virus in faeces	Present early	Absent
5. Mortality	0.1%	1-10%
6. Carrier state		
Blood	Up to 8 months	Up to 5 years
Faeces	Up to 1-3 month	Not known (or negative)

core antigen (HBc Ag). Inside the core is the genome, a circular double stranded DNA and a DNA polymerase (Fig. 62.3).

Blumberg and coworkers (1965) described a protein antigen in serum of an Australian aborigine, which gave a positive precipitation reaction with sera from two haemophiliacs who had received multiple transfusions. This antigen was named *Australia antigen*. It was subsequently established to be the surface component of hepatitis B virus (HBsAg). Electron microscopy of sera of hepatitis B patients shows three types of particles (Fig. 62.3). The most abundant form is a spherical particle (22 nm in diameter) and the second type is tubular (22 nm in diameter) particle of varying length. These two types are antigenically identical and are the surface subunits of hepatitis B virus (HBsAg). The third type is a double shelled spherical structure (42 nm in diameter). This particle is the complete hepatitis B virus or *Dane particle*. It was first described by Dane and coworkers in 1970 and was therefore known as Dane particle.

B. Antigenic structure

1. HBsAg

Surface antigens (envelope protein)

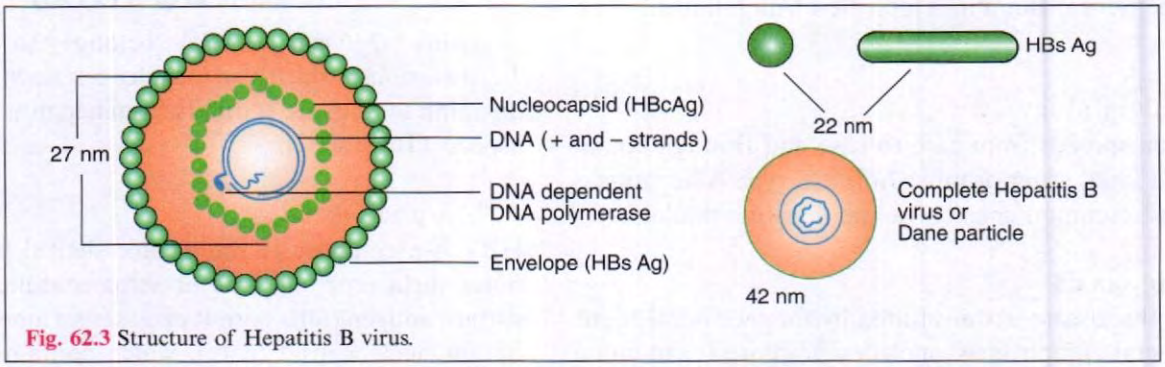


Fig. 62.3 Structure of Hepatitis B virus.

2. *HBcAg*

It is the core (nucleocapsid) antigen of the virus. It contains group specific protein and is not detectable in patient's blood.

3. *HBeAg*

It appears in serum along with HBsAg but disappears within a few weeks. It is the hidden antigenic component of core. HBcAg and HBeAg, though immunologically distinct are coded by the same gene.

HBsAg carries a group-specific antigen 'a' and two types of specific antigens, d or y and w or r. Thus, there are four antigenic types of HBsAg—*adw*, *adr*, *ayw* and *ayr*. These are useful markers of epidemiology. Type adw is predominant in Europe and USA and type adr in Asia. Type ayw is predominant in Africa, Russia and India (Table 62.3).

Table 62.3 Antigenic Types of HBs Ag

Antigenic types	Distribution
adw	Worldwide
adr	Asia
ayw	Africa, India, Russia
ayr	Africa, India, Russia

Additional surface antigens of HBV (q, x, f, t, j, n, g) are described, but they have not been characterised.

4. *Viral Genes and Antigens*

The viral genome consists of two linear strands of DNA held in a circular configuration. One of the strands (the plus strand) is incomplete while other is complete. This gives the appearance of partially double stranded and partially single stranded DNA. Associated with the *plus* strand is a viral *DNA polymerase* (Fig. 62.3). This polymerase can repair the gap in the incomplete (the plus strand) strand and render the genome fully double stranded.

The genome has four genes (with different regions) coding for different antigens (Table 62.4). HBxAg and its antibody are found to be present in patients with severe chronic hepatitis and hepatocellular carcinoma.

Antigenic variation occurs in HBV due to genetic heterogeneity. On this basis, HBV has been classified into eight genotypes, A to H.

C. Cultivation and Stability

HBV has not been cultivated in the laboratory. However, limited production of the virus and its proteins can be obtained from cell lines transfected with HBV DNA. HBV proteins have been cloned in yeast and bacteria. The virus survives heating of 60°C for 60 minutes but gets inactivated at 100°C for 5 minutes. It is inactivated by formaldehyde (1:4000) and 2% glutaraldehyde.

D. Modes of Transmission

There are three important modes of transmission of HBV infection:

1. Parenteral
2. Perinatal
3. Sexual.

1. *Parenteral Transmission*

Transmission of infection may result from accidental inoculation of minute amounts of blood, blood products or fluid containing HBV during medical, surgical or dental procedures.

2. *Perinatal Transmission*

Transmission probably occurs when carrier mother's blood contaminates the mucous membranes of the newborn during birth.

3. *Sexual Transmission*

HBV is present in body fluids such as semen and vaginal secretions, hence, it can be transmitted by sexual contact. Male homosexuals are at higher risk of acquiring infection.

E. Clinical Features

Onset is slow, usually insidious but more severe. The incubation period varies from 6 weeks to 6 months. Fever

Table 62.4 Genes Coding for Antigens of HBV

Gene	Regions	Antigen
S (Having three regions S, Pre-S1 and Pre-S2)	S S + Pre-S2 S + Pre-S1 & S2	Major protein (S) Middle protein (M) } Surface antigen (HBsAg) Large protein (L)—Present only in virion
C (Having two regions C and Pre-C)	C C + Pre-C	Core antigen (HBcAg) HBe Ag
P		DNA polymerase
X		HBx Ag (Non-particulate antigen which leads to enhanced replication of HBV)

is less common and of low grade, jaundice is rarely seen in children but is present more often in adults. The course of acute HBV infection can be divided into three phases: preicteric, icteric and convalescent.

1. Preicteric Phase
Patient develops malaise, anorexia, weakness, myalgia, nausea and vomiting. A minority of patients develop arthralgia, serum sickness, polyarteritis nodosa and glomerulonephritis. These features may be related to circulating immune complexes.

2. Icteric Phase
Patient develops jaundice, pale stools and dark urine (bilirubinuria).

3. Convalescent Phase
This phase is long and drawn out with malaise and fatigue. The duration of uncomplicated hepatitis is usually 8-10 weeks, but mild symptoms may persists for more than one year.

F. Hepatitis B Carriers
There are two types of hepatitis B carriers : super carriers and simple carriers.

1. *Super carriers*: They have HBe Ag in blood and are highly infectious. Their blood contains high titre of HBsAg and DNA polymerase. HBV may also be demonstrable in blood. Very minute amount of serum or blood can transmit the infection. These are called super carriers.
2. *Simple carriers*: They are more common type of carriers who have no HBeAg and a low level of HBsAg in blood. HBV and DNA polymerase are absent. They transmit the infection only when large volumes of blood or serum are transferred, as in blood transfusion. These are named simple carriers.

G. Immune Response
Both humoral and cellular responses occur during the infection.

1. Humoral Response (Antibody to HBV) (Fig. 62.4)
(i) Antibody to HBs Ag
It is associated with resistance to infection. It is a useful indicator of past infection or recent immunity.

(ii) Antibody to HBc Ag
It rises rapidly following infection. It is not protective. It appears to be related to the amount and duration of replication of the virus. The highest titre of anti-HBc are found in persistent HBs Ag carriers.

(iii) Antibody to HBe Ag
It is seen in sera of patients with low infectivity.

2. Cell Mediated Immunity
In self-limited course of hepatitis B infection, T-cell function remains within normal limit. However, defective function of T-cell may favour the development of chronic liver damage.

H. Laboratory Diagnosis
Laboratory diagnosis of HBV infections can be carried out by detection of hepatitis B antigens and antibodies. These can be detected by sensitive and specific tests like ELISA and RIA. Sequence of appearance of viral markers (hepatitis B antigens and antibodies) in blood is important in diagnosis (Fig. 62.4). The presence of HBsAg in a patient's serum indicates that the patient may have an active infection, carrier or is in incubation period. IgM anti-HBc indicates an acute infection and appears early in the course of the disease. Anti-HBs in the serum indicates a past infection or immunity following vaccination. The presence of HBsAg after 6 months of acute infection indicates that the patient is a chronic carrier. The appearance of HBcAg alongwith HBeAg positivity is a indication of chronic infection and high infectivity (Table 62.5).

1. Detection of Viral Markers
(i) HBsAg
HBsAg is recognised as a specific marker for HBV infection. It is the first marker to appear in blood after infection. It is detectable in blood even before elevation of transaminases and onset of clinical illness. Peak

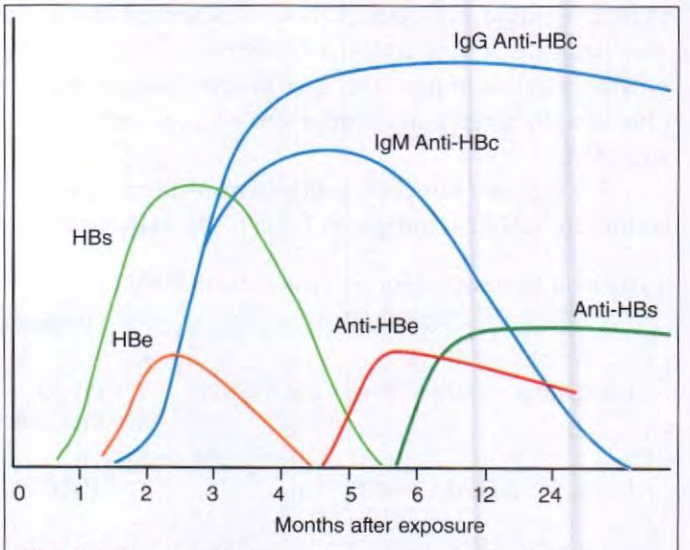


Fig. 62.4 Hepatitis B virus antigens and antibodies in acute HBV infection.

Table 62.5 Serological Tests in Hepatitis B Infection

Clinical condition	Serological tests					
	HBs Ag	HBe Ag	Anti-HBs	Anti-HBe	Anti-HBc	
					IgM	IgG
Late incubation period or early hepatitis	+	+	–	–	–	–
Acute hepatitis	+	+	–	–	+	–
Late/chronic HBV infection	+	±*	–	–	–	+
Simple carrier	+	–	–	–	–	+
Super carrier	+	+	–	–	–	+
Past infection	–	–	+	+	–	+
Immunity following vaccination	–	–	+	–	–	–

*When +, it indicates high infectivity while – indicates low infectivity.

levels of HBs Ag are seen in the pre-icteric phase of the disease. It remains in circulation throughout the icteric or symptomatic course of the disease. HBsAg disappears with recovery from clinical disease in most patients, however , it persists for years in carriers. Antibody to HBsAg appears within weeks after the disappearance of HBsAg and persists for very long periods. Anti-HBs is the protective antibody.

(ii) HBeAg

HBeAg appears in the serum at the same time as HBsAg, but in most cases it disappears within a few weeks. Sera containing HBeAg are believed to be highly infectious and those with anti-HBe of little infectivity. HBeAg is an indicator of active intrahepatic viral replication, and the presence in blood of HBV DNA, virions and DNA polymerase. The presence of HBeAg is thought to be an adverse prognostic sign. The disappearance of *HBeAg* is followed by appearance of anti-*HBe*.

(iii) HBcAg

It is not detectable in the serum but can be demonstrated in liver cells by immunofluorescence. Anti-HBc antibody usually appears in serum a week or two after the appearance of HBsAg. It is the earliest antibody to appear in the blood. It remains lifelong and thus serves as a useful indicator of prior infection with HBV, even after all the other markers become undetectable. Initially, anti-HBc is predominantly IgM type, but later on it is mainly IgG type. Hence, recent or remote infection can be differentiated by selective tests for IgM (recent infection) or IgG (remote infection) anti-HBc antibody.

2. Viral DNA Polymerase

It appears transiently in serum during pre-icteric phase.

3. Polymerase Chain Reaction (PCR)

HBV DNA level can be detected in serum by PCR. It is a highly sensitive test. HBV DNA is also an indicator of viral replication in the liver and so helps to assess the progress of patients under antiviral chemotherapy.

4. Biochemical Tests

In acute viral hepatitis caused by hepatitis A, B, C, D, or E viruses, transaminase values range between 500 to 2000 units (SGPT is always higher than SGOT). Serum bilirubin level indicates the degree of jaundice and may rise up to 25-fold.

I. Prophylaxis

Hepatitis B infection occurs mainly following blood transfusion, injection of blood products, in drug addicts, in male homosexuals, in medical laboratory personnel handling infected patients and blood and in infants of carrier mothers. Prophylaxis includes:

1. General preventive measures
2. Immunisation.

1. General Preventive Measures

These include health education, improvement of personal hygiene and strict attention to sterility. An important preventive measure is the screening for HBsAg and HBeAg in blood donors. Use of unsterile needles, syringes and other material must be avoided to prevent hepatitis B infection.

2. Immunisation

(i) Passive immunisation

Passive immunisation may be employed following any accidental exposure to hepatitis B infection. Hepatitis B immunoglobulin (HBIG) is prepared from donors with high titres of anti-HBs. It can be given in doses of 300-

500 IU intramuscularly. HBIG should be administered as early as possible after exposure, preferably within 48 hours. A second dose is usually given at interval of 4 weeks after the first dose. It may not prevent infection but protects against illness and the development of carrier state.

(ii) Active immunisation

Following vaccines are available.

(a) *Plasma derived vaccine*: Vaccine is prepared by purifying 22 nm particle of HBs Ag from the plasma of healthy carriers. The particles are separated by ultracentrifugation and inactivated with formaldehyde. The vaccine is immunogenic and safe. It is still being produced and used, particularly in developing countries.

(b) *Recombinant yeast hepatitis B vaccine*: It is produced by a recombinant DNA in yeasts in which a plasmid containing the gene of HBsAg has been incorporated. HBsAg particles produced are extracted and purified for use as vaccine. The vaccine is as immunogenic as plasma-derived vaccine. It is safe and free from side effects.

Both vaccines are adsorbed with aluminium hydroxide as adjuvant, stored in cold but not frozen. Three doses at 0, 1 and 6 months are administered intramuscularly into deltoid muscle. Local swelling and reddening may occur in some 20% cases accompanied by slight fever.

(c) *Recombinant Chinese hamster ovary (CHO) cell hepatitis B vaccine:* CHO cells have been used for preparation of vaccine. It is first vaccine using mammalian cell expression system. It is commercially available.

(d) *Synthetic peptide vaccine*: These are chemically synthesised polypeptide vaccines. They are still under experimental stage.

J. Treatment

No specific antiviral treatment is available. Interferon-α alone or in combination with other antiviral agents (e.g. lamivudine, telbivudine and entecavir) has been beneficial in some chronic hepatitis cases.

III. HEPATITIS C VIRUS (HCV)

Hepatitis C virus (HCV) belongs to the family *Flaviviridae*. It is a 50–60 nm virus with a linear single stranded RNA of positive polarity, enclosed within a core and surrounded by an envelope, carrying glycoprotein spikes. It has been classified in a separate genus called *Hepacivirus* under family *Flaviviridae*.

The virus shows considerable genetic and antigenic diversity. Six differnt genotypes and many subtypes have been identified, indicating high mutability. Due to this diversity there is little heterologous or even homologous postinfection immunity in hepatitis C infection.

Hepatitis C virus has not been grown in culture, but has been cloned in *Escherichia coli*.

A. Modes of Infection

It is transmitted by needlestick injuries, use of contaminated needles and syringes, transfusion of infected blood and blood products, and sexual intercourse. Maternal-neonatal transmission has also been reported.

HCV infection is seen only in humans and occurs throughout the world. The source of the infection is the large number of carriers.

B. Clinical Features

The incubation period varies from 15–160 days, with an average of 50 days. About 75% infections are subclinical. Clinical infection with hepatitis C is generally less severe, with milder symptoms, absent or less marked jaundice. About 50 to 80% of patients develop chronic hepatitis. The affected individuals have persistence of the virus in their blood. They are at risk, of developing cirrhosis and hepatocellular carcinoma.

C. Laboratory Diagnosis

It can be established by detection of anti-HCV (antibody) by ELISA which is available as screening test. Antibody detection becomes positive only months after the infection and shows non-specific reactions. Confirmation by immunoblot assay is therefore recommended. A recombinant immunoblot assay (RIBA) can be performed to confirm the positive results obtained with a screening test such as ELISA. Viral genome (HCV RNA) can be detected by polymerase chain reaction (PCR) and by immunofluorescence. It can be detected in-situ on biopsy and autopsy specimens. Viral genome can also be detected in blood within a few days of exposure to HCV. It is more sensitive and specific test.

D. Prophylaxis

Only general prophylaxis, such as blood or blood products screening, is possible. Avoidance of use of unsterile needles, syringes and other material is another important general prophylactic measure.

E. Treatment

Treatment with interferon-α, either alone or in combination with antiviral agents such as ribavirin has been found to be useful in some cases.

IV. HEPATITIS D VIRUS (HDV) (DELTA ANTIGEN)

The HDV is a defective virus as it is dependent on the helper function of HBV for its replication and expression. It belongs to genus *Deltavirus*. It is spherical, 36-38 nm diameter, RNA particle surrounded by HBsAg envelope (Fig. 62.5). The genome is a single stranded small circular molecule of RNA. It encodes its own nucleoprotein, the *delta antigen* or HDAg, but the outer envelope (HBs Ag) of HDV is encoded by the genome of HBV coinfecting the same cell. HBV is necessary for the production of HDV virions.

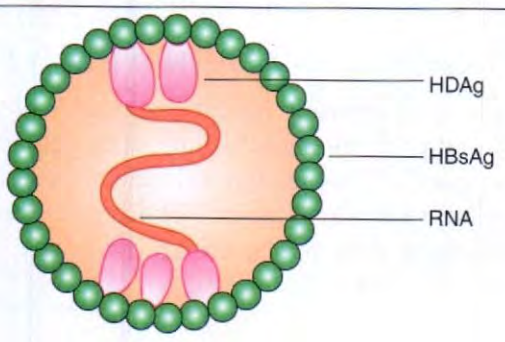


Fig. 62.5 Hepatitis D virus

A. Clinical Features

HDV infection can occur in presence of HBV under two situations:

(*i*) Simultaneous infection with both HDV and HBV (*coinfection*)

(*ii*) Superinfection of an HBsAg carrier by HDV.

Transmission occurs parenterally. Coinfection with HBV and HDV results in hepatitis of increased severity than the disease caused by HBV alone. Once infection due to HDV is established, it interferes with HBV multiplication and utilises HBV for its own replication. No association has been noted between HDV and hepatocellular carcinoma.

B. Laboratory Diagnosis

Diagnosis can be made by detecting the IgM anti-delta antibody in serum. ELISA and RIA kit are commercially available for detection of antibodies to HDV. The *IgM* antibody appears 2-3 weeks after infection and is soon replaced by the *IgG* antibody in acute infections. *HDAg* (Delta antigen) is primarily expressed in liver cell nuclei, where it can be detected by immunofluorescence. It is occasionally present in serum. HDVRNA can be detected by hybridisation using a radiolabelled probe.

C. Prophylaxis

No specific prophylaxis exists, but immunisation with the hepatitis B vaccine is effective because delta antigen cannot infect persons immune to HBV. Screening of blood donors for HBsAg will also limit blood borne HDV infection.

V. HEPATITIS E VIRUS (HEV)

Hepatitis E virus belongs to family *Hepeviridae* and genus *Hepevirus*. They are spherical, nonenveloped and 27-38 nm in diameter. They possess single stranded RNA genome, which is surrounded by icosahedral capsid with characteristic surface depressions.

A. Pathogenesis

Hepatitis E has been shown to occur in epidemic, endemic and sporadic forms almost exclusively in developing countries. It is primarily associated with ingestion of faecally contaminated drinking water. Incubation period ranges from 2-8 weeks, with an average of 6 weeks. It occurs predominantly in young to middle-aged adults. Clinically the disease resembles that of hepatitis A. The disease is generally mild and self limiting. It has a low case fatality of about one per cent. Like hepatitis A, hepatitis E does not lead to chronic hepatitis, cirrhosis, cancer or carrier state. In hepatitis E infection, secondary attack rate among household contacts is very low (2–3%) as against 10–20% in HAV infection.

The epidemic of viral hepatitis which occurred in Delhi in 1955 involving 30,000 cases of icteric hepatitis is now believed to have been of this type. It occurred following widespread faecal contamination of drinking water. A similar epidemic occurred in 1975-76 in Ahmedabad city.

B. Laboratory Diagnosis

1. Exclusion of hepatitis A and hepatitis B

Hepatitis A can be excluded by IgM serology and hepatitis B by absence of HBsAg and anti HBc-IgM.

2. Immunoelectron microscopy

Faeces is examined by electron microscopy for aggregated hepevirus particles, using monoclonal antibodies.

3. ELISA test and Western blot assay

These are used for detection of IgM and IgG antibodies.

4. Polymerase chain reaction (PCR)

HEV RNA can be detected in faeces or acute phase sera of patients by RT-PCR. It is a gold standard test for diagnosis of acute HEV infection.

C. Prophylaxis

Hepatitis E can be prevented by

1. improved standards of sanitation
2. chlorinated water

No vaccine is available.

VI. HEPATITIS G VIRUS (HGV)

In 1996, this virus was first isolated from a patient with chronic hepatitis. This has been called hepatitis G virus. It has been placed in family *Flaviviridae* and genus *Hepacivirus*. HGV is distinct from HCV, GBV-A and GBV-B agents, while GBV-C represents an isolate of HGV. It has not been grown in cell lines, but its RNA has been cloned. HGV RNA has been found in patients with acute, chronic and fulminant hepatitis, haemophiliacs, patients with multiple transfusions, blood donors and intravenous drug addicts. Its role in hepatitis is not clear.

The genome of HGV consists of single stranded RNA. HGV replicates in peripheral blood cells. The virus is transmitted parenterally, sexually and from mother to child. A significant proportion of HIV-infected individuals are also HGV-coinfected. There is no evidence of a relationship between HGV infection and hepatic carcinoma. HGV infection subsides after several years and anti-hepatitis G envelope antibody develops.

A. Laboratory Diagnosis

HGV infection can be detected by reverse transcriptase polymerase chain reaction (RT-PCR). Recently an immunoassay has been developed to detect antibody against hepatitis G envelope. Serum HGV RNA indicates viraemia while antibody is associated with recovery.

B. Prophylaxis

HGV infection can be prevented by employing the general prophylactic measures used for HBV and HCV.

VII. NON-A, NON-B (NANB) HEPATITIS

It refers to viral hepatitis resembling type A or type B clinically and epidemiologically but not caused by either of these viruses. Now four types (hepatitis C, D, E, and G) of NANB viruses are known. The diagnosis is possible with serological tests but a few are diagnosed by the process of exclusion (Table 62.6).

Table 62.6 Serological Tests in Viral Hepatitis

HBs Ag	IgM anti-HBc	IgM anti-HAV	Anti-HDV	Diagnosis
–	–	+	–	Acute hepatitis A
+	+	–	–	Acute hepatitis B
–	+	–	–	Acute hepatitis B (HBs Ag below detectable level)
±	+	–	+	Acute hepatitis B and delta co-infection
+	–	–	+	Acute delta infection in hepatitis B carrier
–	–	–	–	Non-A, Non-B hepatitis

KEY POINTS

1. There are six hepatitis viruses, i.e., hepatitis A, B, C, D, E and G.
2. The infection caused by hepatitis B is most severe and at times fatal. Hepatitis B and C viruses are also responsible for many cases of *primary hepatocellular carcinoma*.
3. Hepatitis B is a *DNA virus* while others (A, C, D, E, G) contain *RNA* genome.
4. Mode of transmission is *enteric* in hepatitis A and hepatitis E viruses while it is *parenteral* and *sexual* in both hepatitis B and hepatitis C viruses.
5. Hepatitis A virus (HAV) can be demonstrated in faeces by *immunoelectron microscopy* (*IEM*) and ELISA. Specific IgM antibody to HAV can also be detected in blood for diagnosis of HAV infected.
6. A formalin inactivated vaccine is available for prophylaxis against HAV. Two intramuscular injections of the vaccine are given.
7. Hepatitis B virus (HBV) is a complex 42 nm double shelled particle. The outer surface or envelope of virus contains hepatitis B surface antigen (HBsAg). It encloses an inner icosahedral 27 nm nucleocapsid (core), which contains hepatitis B core antigen (HBcAg). Inside the core is the genome, a circular double stranded DNA and a DNA polymerase.

8. HBsAg is also named as *Australia antigen.*
9. There are three types of antigen namely *HBsAg* (surface antigen), *HBcAg* (core antigen) and *HBeAg.*
10. *Parenteral, perinatal* and *sexual* are three important *modes of transmission* of HBV infection.
11. Laboratory diagnosis of HBV infections can be carried out by detection of hepatitis B antigens and antibodies. These can be detected by ELISA and RIA. Sequence of appearance of viral markers (hepatitis B antigens and antibodies) in blood is important in diagnosis.
12. Viral markers include HBsAg, HBeAg, Anti-HBs, Anti-HBe and Anti-HBc.
13. HBV DNA can be detected in serum by PCR. It is a highly sensitive test. HBV DNA is also an indicator of viral replication in the liver.
14. HBV infection can be prevented by *general measures* or *immunisation.* General measures include the *screening of HBsAg in blood donors, avoidance of use of unsterile needles, syringes* and other material.
15. Vaccine is prepared by purifying *HBsAg particles.* Three doses of vaccine at *0, 1* and *6* months are administered intramuscularly into deltoid muscle.
16. Passive immunisation may be employed following any accidental exposure to hepatitis B infection. Hepatitis B immunoglobulin (HBIG) should be administered as early as possible after exposure, preferably within 48 hours.
17. Hepatitis C virus (HCV) can also be transmitted by *needle stick injuries*, use of *contaminated needles* and *syringes*, transfusion of *infected blood* and *blood products* and *sexual intercourse.*
18. Laboratory diagnosis of HCV infection can be established by detection of *anti-HCV* (*antibody*) by *ELISA.* Viral genome (HCV RNA) can be detected by *polymerase chain reaction* (*PCR*) and by Immunofluorescence.
19. Hepatitis D virus (HDV) is a defective virus as it is dependent on the helper function of HBV for its replication and expression. It is spherical, RNA particle surrounded by HB_SAg envelop. Transmission of HDV occurs parenterally.
20. Hepatitis E has been shown to occur in epidemic, endemic and sporadic forms almost exclusively in developing countries. It is primarily associated with ingestion of faecally *contaminated drinking water.*
21. Hepatitis E infection can be prevented by *improved standards of sanitation* and *chlorinated water.* No vaccine is available.

YOU MUST KNOW

1. Characteristic features of major hepatitis viruses.
2. Differentiating features of hepatitis A and B viruses.
3. Morphology of hepatitis B virus.
4. Antigenic structure of hepatitis B virus.
5. Modes of transmission of hepatitis B virus.
6. Hepatitis B carriers.
7. Laboratory diagnosis of infections caused by hepatitis B virus.
8. Vaccines for prevention of hepatitis B infections.
9. Hepatitis C Virus (HCV).
10. Delta antigen.
11. Hepatitis E virus (HEV).

STUDY QUESTIONS

1. Discuss the morphology and antigenic structure of hepatitis B virus.
2. Classify hepatitis viruses. Describe the laboratory diagnosis of infections caused by hepatitis B virus.
3. Write short notes on:
   (a) Hepatitis A virus (HAV)
   (b) HBs Ag
   (c) Hepatitis B carriers
   (d) Hepatitis B vaccines
   (e) Hepatitis B markers
   (f) Hepatitis C virus (HCV)
   (g) Deltavirus
   (h) Hepatitis E virus (HEV)
   (i) Non-A, Non-B hepatitis

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Which of the following hepatitis viruses is DNA virus?
   (a) Hepatitis A virus (b) Hepatitis B virus
   (c) Hepatitis C virus (d) Hepatitis E virus
2. Which of the following hepatitis viruses can be transmitted by sexual route?
   (a) Hepatitis A virus (b) Hepatitis C virus
   (c) Hepatitis E virus (d) All of the above
3. Which of the following hepatitis viruses may cause hepatic carcinoma?
   (a) Hepatitis A virus (b) Hepatitis C virus
   (c) Hepatitis E virus (d) Hepatitis G virus
4. Which of the following hepatitis viruses is non-enveloped?
   (a) Hepatitis A virus (b) Hepatitis B virus
   (c) Hepatitis C virus (d) Hepatitis D virus
5. Which of the following methods is preferred for diagnosis of hepatitis A virus (HAV) infection?
   (a) Alanine aminotransferase levels (b) Detection of IgM antibody to HAV by ELISA
   (c) Detection of IgG antibody to HAV by ELISA (d) Cell cultures for growing HAV
6. Which of the following antigens is present in the envelope of hepatitis B virus?
   (a) HBsAg (b) HBcAg
   (c) HBeAg (d) None of the above
7. Which of the following viral markers is first to appear in blood after infection with hepatitis B virus?
   (a) HBsAg (b) HBcAg
   (c) HBeAg (d) Antibody to HBsAg
8. Which of the following viral markers is/are positive in simple carriers of hepatitis B?
   (a) HBsAg (b) HBeAg
   (c) Both of the above (d) None of the above
9. Which of the following viral markers is/are positive in super carriers of hepatitis B?
   (a) HBsAg (b) HBeAg
   (c) Both of the above (d) None of the above
10. Which of the following markers when positive indicate high infectivity of hepatitis B virus?
    (a) HBsAg (b) HBeAg
    (c) HBcAg (d) None of the above
11. Which of the following hepatitis B genes are used for preparation of recombinant hepatitis B vaccine?
    (a) HBsAg gene (b) HBeAg gene
    (c) HBcAg gene (d) All of the above
12. What is the mode of transmission of hepatitis E virus?
    (a) Faecally contaminated drinking water (b) Parenteral route
    (c) Sexual route (d) None of the above
13. Clinically, hepatitis E virus infection resembles to:
    (a) Hepatitis A infection (b) Hepatitis B infection
    (c) Hepatitis C infection (d) None of the above
14. What is the mode of transmission of hepatitis G virus?
    (a) Parenteral route (b) Sexual route
    (c) Mother to child (d) All of the above

Answers (MCQs):

1. (b)	2. (b)	3. (b)	4. (a)	5. (b)
6. (a)	7. (a)	8. (a)	9. (c)	10. (b)
11. (a)	12. (a)	13. (a)	14. (d)	

Chapter 63

RETROVIRUSES: HIV

I. RETROVIRUSES

These are RNA viruses that belong to family *Retroviridae* (*retro* L. backward). Members of this family possess reverse transcriptase (RNA directed DNA polymerase) enzyme which prepares a DNA copy of the RNA genome in host cell. The presence of enzyme reverse transcriptase is a characteristic feature.

II. CLASSIFICATION

The family *Retroviridae* has been divided into two sub families: *Orthoretrovirinae*, *Spumaretrovirinae*. Of the seven genera included in this family, two contain human retroviruses (Table 63.1).

III. HUMAN IMMUNODEFICIENCY VIRUS (HIV)

Acquired immunodeficiency syndrome (AIDS) was first recognised in USA in 1981 amongst homosexuals and drug addicts in whom the incidence of Kaposi's sarcoma and *Pneumocystis jiroveci* pneumonia were alarmingly

Table 63.1 Retroviruses Infecting Humans

Subfamily	Genus	Virus	Disease
Orthoretrovirinae	*Retrovirus*	Human T-cell Lymphotropic Virus type 1 (HTLV-1)–oncogenic virus	Adult T-cell leukaemia/lymphoma
		HTLV-2	Not associated with any disease but prevalent in intravenous drug users
	Lentivirus	Human Immunodeficiency Virus type 1 (HIV-1)	AIDS
		HIV-2	AIDS
Spumaretrovirinae	*Spumavirus*	Foamy virus	Found in primary cell culture but not associated with disease

high. The causative agent of AIDS was first reported by *Luc Montagnier* and colleagues of the Pasteur Institute, Paris in 1983. They called it *lymphadenopathy associated virus* (LAV). *Robert Gallo* and colleagues from National Institute of Health, USA, reported isolation of a retrovirus and called it *human T-cell lymphotropic virus-III* (HLTV-III). In 1986, the International Committee on Virus Nomenclature gave a name human immunodeficiency virus (HIV). HIV occurs in two main types, HIV-1 isolated from USA, Europe and Central Africa and HIV-2 from West Africa. In 2009, *Luc Montagnier* was awarded Nobel prize for the discovery of HIV.

A. Morphology

HIV is a spherical enveloped virus, about 90-120 nm in diameter (Fig. 63.1). It contains two identical copies of single stranded, positive sense RNA genome. In association with viral RNA is the reverse transcriptase enzyme. The virus core is surrounded by a nucleocapsid composed of protein. The virus contains a lipoprotein envelope, which consists of lipid derived from the host cell membrane and glycoproteins which are virus coded. The major virus coded envelope glycoproteins are the projecting spikes on the surface and the anchoring transmembrane pedicles. These spikes bind to the CD4 receptors on susceptible host cells. Transmembrane pedicles cause cell fusion.

B. Viral Genes and Antigens

HIV genome contains the three structural genes (*gag*, *pol* and *env*) characteristic of all retroviruses. Five non-structural genes (*tat, rev, nef, vif* and *vpr*) are present in both HIV-1 and HIV-2. Other than these, HIV-1 contains *vpu* and HIV-2 has *vpx*. The products of these genes, both structural and non-structural, act as antigens. Infected person's serum contains antibodies to these antigens. Detection of these antigens and antibodies is of great importance in the diagnosis and prognosis of HIV infections.

1. Genes Coding for Structural Proteins (Table 63.2)

(i) The gag gene

(a) The *gag* gene encodes for core and shell proteins of the virus.
(b) It is expressed as a precursor protein, p 55.
(c) This precursor protein is cleaved into three proteins p 15, p 18 and p 24.
(d) The p 24 antigen (the major core antigen) can be detected in serum during the early stages of infection till the appearance of antibodies.
(e) The decline of anti-p24 antibody from circulation indicates progression of illness and is an indication for antiviral treatment.

Table 63.2 Gene Products of HIV (HIV Antigens)

Gene	Coding of HIV antigens	Antigens
gag	(a) Core antigen	p 24 (principal core antigen), p 15, p 55
	(b) Shell antigen	p 18 (nucleocapsid protein)
env	Envelope antigen	gp 120 (principal envelope spike antigen) gp 41 (transmembrane antigen)
pol	Reverse transcriptase antigen	p 31, p 51, p 64

(ii) The env gene

(a) The *env* gene encodes for envelope glycoprotein 160 (gp 160).
(b) The glycoprotein 160 (gp 160) is cleaved into the two envelope components, gp 120 which forms the

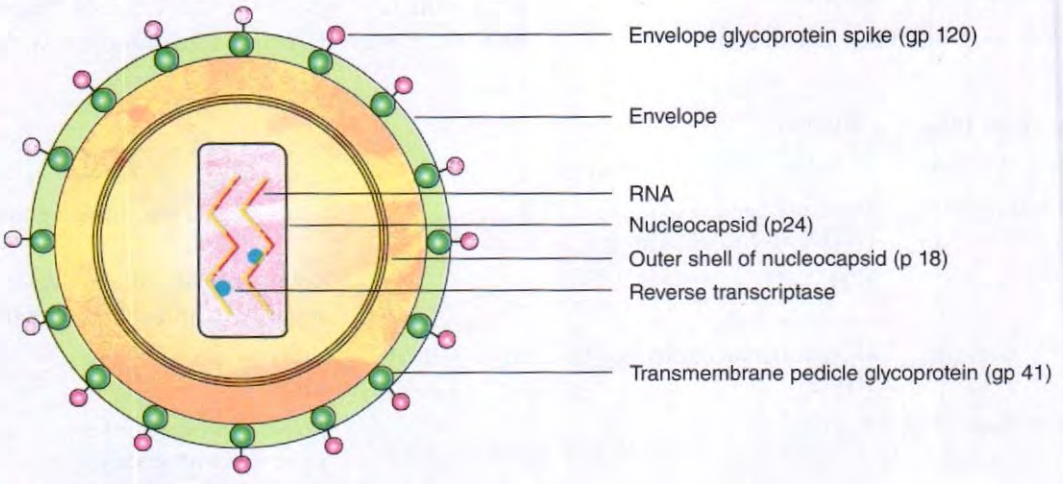


Fig. 63.1 Structure of Human immunodeficiency virus

surface spikes and gp 41 which is the transmembrane pedicle protein.
(c) The spike glycoprotein gp 120 is the major envelope antigen.
(d) The antibodies to gp 120 are the first to appear after HIV infection and are present in circulation till the terminal stage of the infection.

(iii) The pol gene

(a) The *pol* gene encodes for the reverse transcriptase and other viral enzymes.
(b) It is expressed as precursor protein p 100.
(c) This precursor protein is cleaved into three proteins p 31, p 51 and p 64.

2. Non-Structural and Regulatory Genes

Structural genes are present in all retroviruses while non-structural and regulatory genes are specific for HIV only.

(*i*) The *tat* (transactivation) gene: It specifies a transactivating factor that enchances viral protein synthesis.
(*ii*) The *rev* (regulatory of virus) gene: It is required for expression of structural genes.
(*iii*) The *nef* (negative factor) gene: This gene may be responsible for the regulation of latent state of virus.
(*iv*) The *vif* (viral infectivity factor) gene: The gene product confers the infectivity to the virus.
(*v*) The *vpr* gene stimulates promoter region of the virus.
(*vi*) The *vpu* (only in HIV-1) and the *vpx* (only in HIV-2) genes code for small viral proteins of unknown function. They also enhance maturation and release of progeny virus from cells.
(*vii*) LTR (long terminal repeat) sequences, one at either end, give promoter, enhancer and integration signals.

C. Antigenic Variation

HIV undergoes frequent antigenic variation of core and envelope antigens. Two distinct antigenic types of HIV have been identified—HIV-1 and HIV-2. Antigenic variation occurs within both HIV-1 and HIV-2. HIV-1 represents the original isolate from America, Europe and other Western countries whereas HIV-2 represents isolates from West Africa, which weakly reacts with HIV-1 antisera. The envelope antigens of the two types are different. Their core polypeptides show some cross reactivity. HIV-2 is more closely related to simian immunodeficiency virus than to HIV-1.

HIV-1 strains have been classified into ten subtypes, A to J, based on sequence analysis of their *gag* and *env* genes. Subtype A is found worldwide, while B is the most common in the Americas and Europe. Subtypes A, C and D are common in Africa, while in Asia the common subtypes are E, C and B. In Thailand, subtype E is the most common. Subtype C is the most prevalent in India and China. Subtypes A to J constitute Group M (for 'major') but some HIV-1 strains from West Africa do not fall within this group. These strains from West Africa have been designated as Group O (for 'Outlier') and Group N (for 'new').

Antigenic differences between HIV strains may be important in serodiagnosis. Infections by HIV-1 or HIV-2 may not be differentiated unless the corresponding type is represented in the test antigen. It is also important to use antigens containing the prevalent subtypes in different countries.

The subtypes seem to vary in frequency of transmission by different routes. Subtypes common in Asia and Africa (C and E) are more readily transmitted by heterosexual contact while the American strains (subtype B) are preferentially blood-borne—by injection and homosexual contact (in homosexuals, the virus is likely to enter directly into the blood through minor tears).

D. Cell Tropism

1. HIV infects all cells expressing at their surface the CD4 antigen, which is the receptor for the virus.
2. The spikes (gp120) of the viral envelope selectively binds to the CD4 antigen and antibodies to CD4 protein block the virus binding site.
3. The T4 (helper/inducer) lymphocytes are principally involved in HIV infection but other human cells expressing CD4 are also susceptible. These cells are 5-10 per cent of B lymphocytes, 10-20% of monocytes and macrophages. Glial cells and microglia in the central nervous system (CNS) are also found infected.
4. After binding to CD4 of host cell, the gp 41 terminus is exposed and the host cell membrane fuses with the viral membrane. Thus the viral core directly enters the cytoplasm of host cell. Cell fusion and virus entry also requires a coreceptor molecule, identified as CXCR4 for T cell-tropic HIV strains and CCR5 for macrophage-tropic strains.
5. The infected CD4 cells express a high level of gp 120 on their surface. The gp 120 on the surface of infected cells leads to fusion of these cells with CD4 protein of uninfected neighbouring cells, with formation of *multinucleated syncytial cells*. The lysis of fused cells finally occur resulting in depletion of large number of uninfected cells from the circulation.

E. Resistance

1. *Temperature*: HIV is thermolabile, being inactivated at 56°C in 30 minutes and in seconds at 100°C. At room temperature, it may survive up to seven days.
2. *Disinfectants*: It is inactivated in 10 minutes by treatment with 35% isopropyl alcohol, 70% ethanol, 0.5% lysol, 2% freshly prepared glutaraldehyde, 0.5% sodium hypochlorite and 3% hydrogen peroxide. For treatment of contaminated medical instruments, a 2% glutaraldehyde solution is useful.
3. *Detergents*: Because of presence of lipid membrane envelope, it is highly susceptible to detergents, therefore, washing with detergents is adequate for decontaminating clothes and household utensils.
4. *Lyophilisation*: The virus withstands lyophilisation.

F. Modes of Transmission

There are three modes of transmission: sexual contact, parenteral and perinatal.

1. *Sexual contact*: This is the most important mode of transmission. Sexual transmission occurs among both homosexual as well as heterosexual individuals. HIV has been isolated from semen, vaginal and cervical secretions and breast milk which are important vehicles of transmission. The risk of acquiring HIV infection enhances if genital ulcers are present, as in syphilis or chancroid. Risk is also greatly enhanced if the sexual partners are more than one.
2. *Parenteral transmission*: It may occur through blood after receiving infected blood transfusions, blood products, sharing contaminated syringes and needles as in intravenous drug abusers or accidental inoculation.
3. *Perinatal transmission*: Infection may be transmitted from an infected mother to her child either transplacentally or perinatally. During birth, infection may also develop from the genital secretions and from mother's milk after birth.

Types of exposure and their relative risk is shown in Table 63.3

Table 63.3 Types of Exposure and their Relative Risk

Types of exposure	Relative risk% per exposure
1. Sexual intercourse: anal, vaginal, oral	0.1-1.0
2. Transfusion of blood and blood products	> 90
3. Tissue and organ donations	50-90
4. Injections and injuries: injections with unsterile syringes and needles, sharing contaminated syringes and needles by drug abusers, needle-stick and other accidental injuries in health personnel	0.5-1.0
5. Mother to baby: transplacentally or perinatally	30

G. Pathogenesis

After entry into blood stream, HIV comes into contact with suitable host cells—principally the CD4 lymphocytes. Once in the cell, RNA is transcribed by reverse transcriptase into DNA (*provirus*). The provirus is integrated into the genome of the infected cell causing a *latent infection*. The long and variable incubation period of HIV infection is because of the latency. From time to time, lytic infection is initiated and releases progeny virions to infect other cells. In an infected person, HIV can be isolated from blood, lymphocytes, cell-free plasma, cervical secretions, semen, saliva, urine, tears and breast milk.

The infection causes damage to the T4 lymphocyte. T4 cells are depleted in numbers and the T4:T8 (helper : suppressor) ratio is reversed. Viral infection can suppress the function of infected cells without causing any structural damage. This leads to a marked damping effect on cell mediated immune response. Function of other cells (monocyte, macrophage) is also affected apparently due to the lack of secretion of activating factors by T4 lymphocytes.

Clinical manifestations in HIV infections are mainly due to failure of immune responses. This renders the patient susceptible to life threatening opportunistic infections and malignancies. Dementia and other degenerative neurological lesions may also be seen in AIDS. These may be due to the direct effect of HIV on the central nervous system.

H. Clinical Features

The clinical course of HIV infection can present as follows:

1. *Acute HIV infection*: The illness is characterised by acute onset of fever, malaise, sore throat, myalgia, arthralgia, skin rash and lymphadenopathy. Peripheral blood shows lymphocytosis. Virus, viral nucleic acid or viral p 24 antigen may be detected during acute infection. HIV antibodies are usually negative at the onset of illness but become positive during its course.
2. *Asymptomatic infection*: This includes all infected persons who are usually well. They show positive HIV antibody tests, and are infectious.
3. *Persistent generalised lymphadenopathy (PGL)*: This group is characterised by enlarged nodes (more than 1 cm) at two or more extragenital sites for at least

three months. PGL must be differentiated from other causes of lymphadenopathy such as the lymphomas.

4. *Symptomatic HIV infection*: When CD4+ T lymphocyte count falls below 400 per mm^3, the patient may develop symptoms like fever, diarrhoea, weight loss, night sweats and opportunistic infections. During this phase, some patients develop an illness which is known as AIDS related complex or condition (ARC).

When CD4+ cells fall below 200 per mm^3, the titre of virus increases markedly and there is irreversible breakdown of immune defence mechanisms, it is defined as AIDS. Most of the patients with HIV disease die of infections other than HIV e.g. opportunistic infections and malignancies (Table 63.4). AIDS is the end stage of HIV infection.

In addition to the opportunistic infections, patient may also develop primary CNS lymphoma and progressive multifocal leukoencephalopathy. Dementia, severe encephalopathy, myelopathy, peripheral neuropathy, diminished concentration and motor disturbances may develop in patients with HIV infection.

Clinical Types

Incubation period of AIDS varies from 1-14 years, with an average of 6 years. Clinical responses to HIV in adults are shown in Fig. 63.2.

1. AIDS in adults

Risk factors for AIDS include:

(*i*) Anal and genital sex
(*ii*) Intravenous drug abusers
(*iii*) Haemophiliacs treated with blood or blood products
(*iv*) Blood transfusion

2. Paediatric AIDS

Infection is transmitted from

(*i*) infected mothers
(*ii*) transfused blood or blood products

Kaposi's sarcoma, toxoplasmosis and cryptococcosis are frequently seen in adults but less common in children.

Table 63.4 Opportunistic Infections and Malignancies Commonly Associated with HIV Infection.

I. Bacterial
1. Mycobacterial infections—Tuberculosis and non-tuberculous infections
2. *M. avium* complex
3. Salmonellosis

II. Viral
1. CMV
2. Herpes simplex
3. Varicella-zoster
4. Epstein-Barr (EB) virus
5. Human herpes virus 6 (HHV6)
6. Human herpes virus 8 (HHV8)

III. Mycotic
1 *Pneumocystis jiroveci* pneumonia
2. Candidiasis
3. Cryptococcosis
4. Aspergillosis
5. Histoplasmosis
6. Coccidioidomycosis

IV. Parasitic
1. Toxoplasmosis
2. Cryptosporidiosis
3. Isosporiasis
4. Generalised strongyloidiasis

V. Malignancies
1. Kaposi's sarcoma
2. B-cell lymphoma or non-Hodgkin's lymphoma

Four clinical stages (clinical stage 1 to 4) of HIV/AIDS for adults and adolescents with confirmed HIV infection have been defined by WHO (Table 63.5). Initiation of antiretroviral therapy is also based on these clinical stages.

I. Laboratory Diagnosis

Laboratory diagnosis of HIV infection includes specific tests for HIV and tests for immunodeficiency.

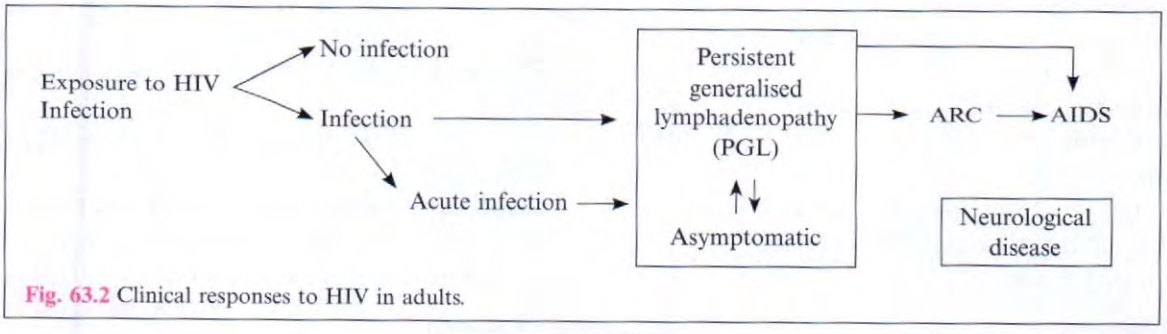


Fig. 63.2 Clinical responses to HIV in adults.

Table 63.5 WHO Clinical Staging of HIV/AIDS in Adults and Adolescents with confirmed HIV infection.

Clinical Stage 1
- Asymptomatic
- Persistent generalised lymphadenopathy

Clinical Stage 2
- Moderate unexplained weight loss (<10% of presumed or measured body weight)
- Recurrent respiratory tract infections (e.g., sinusitis, tonsillitis, otitis media, pharyngitis)
- Herpes zoster
- Angular cheilitis
- Recurrent oral ulceration
- Papular pruritic eruptions
- Seborrheic dermatitis
- Fungal nail infections

Clinical Stage 3
- Unexplained* severe weight loss (>10% of presumed or measured body weight)
- Unexplained chronic diarrhoea for longer than 1 month
- Unexplained persistent fever (above 37.6°C, intermittent or constant, for longer than 1 month)
- Persistent oral candidiasis
- Oral hairy leukoplakia
- Pulmonary tuberculosis (current)
- Severe bacterial infections (e.g., pneumonia, empyema, pyomyositis, bone or joint infection, meningitis or bacteraemia)
- Acute necrotizing ulcerative stomatitis, gingivitis, or periodontitis
- Unexplained anaemia (<8 g/dL), neutropenia (<0.5 × 10^9/L), or chronic thrombocytopenia (<50 × 10^9/L)

Clinical Stage 4
- HIV wasting syndrome
- *Pneumocystis jirovecii*
- Recurrent severe bacterial pneumonia
- Chronic herpes simplex infection (orolabial, genital or anorectal, longer than 1 month's duration, or visceral at any site)
- Oesophageal candidiasis (or candidiasis of trachea, bronchi, or lungs)
- Extrapulmonary tuberculosis
- Kaposi's sarcoma
- Cytomegalovirus infection (retinitis or infection of other organs)
- Central nervous system toxoplasmosis
- HIV encephalopathy
- Extrapulmonary cryptococcosis, including meningitis
- Disseminated nontuberculous mycobacterial infection
- Progressive multifocal leukoencephalopathy
- Chronic cryptosporidiosis (with diarrhoea)
- Chronic isosporiasis
- Disseminated mycosis (coccidiomycosis or histoplasmosis)
- Recurrent nontyphoidal *Salmonella bacteraemia*
- Lymphoma (cerebral or B-cell non-Hodgkin's) or other solid HIV-associated tumors
- Invasive cervical carcinoma
- Atypical disseminated leishmaniasis
- Symptomatic HIV-associated nephropathy or symptomatic HIV-associated cardiomyopathy

* Unexplained refers to where the condition is not explained by other causes.

1. Specific tests for HIV infections
   (*i*) Antigen detection: p24 antigen
   (*ii*) Virus isolation
   (*iii*) Detection of viral nucleic acid
   (*iv*) Antibody detection.
2. Non-specific tests
   (*i*) Total and differential leucocyte count
   (*ii*) T-lymphocyte subset assays
   (*iii*) Platelet count
   (*iv*) IgG and IgA levels
   (*v*) Skin tests for CMI.
3. Tests for opportunistic infections and tumour

1. Specific Tests for HIV Infections (Table 63.6)

(i) Antigen detection

Following a single massive infection, the virus antigen (p24) and reverse transcriptase (RT) may be detected in blood after about two weeks. The p24 antigen is the earliest

Table 63.6 Specific Tests for Laboratory Diagnosis of HIV Infection

Test	Window period	Acute infection	Asymptomatic infection	ARC and AIDS
Antigen: p24, RT	+*	+	–	+
virus isolation	++	±	–	+
Antibody				
ELISA test	–	+	+	+
Western blot test	–	+ (Partial p24 and/or gp120)	+ (Full pattern)	+ (Absence of p24 antibody)

* Positive in less than 50 per cent cases.

virus marker to appear in the blood. With seroconversion, antibody becomes detectable and antigen p24 disappears from circulation and remains absent during the long asymptomatic phase. The p24 antigenemia reappears with the onset of clinical disease which corresponds to loss of anti p24 antibody. The p24 antigen capture assay (ELISA) using anti-p24 antibody as the solid phase can be used for detection of this antigen.

(ii) Virus isolation

For diagnosis, virus is not routinely isolated. Once infected with HIV, a person remains infected for life. The virus is present in blood and body fluids, mostly within CD4 lymphocytes. It can be isolated from CD4 lymphocytes of peripheral blood, bone-marrow and serum.

Patient's lymphocytes are co-cultivated with uninfected human lymphocytes in the presence of interleukin-2. Viral replication can be detected by demonstration of reverse transcriptase activity and presence of viral antigen p24 in the culture fluid.

Virus titres are high early in infection before antibodies appear. Antibodies do not neutralise the virus and the two can coexist in the body. During asymptomatic infection, virus titre is low and may not be detectable, but when clinical disease sets in, the titre rises once again.

(iii) Detection of viral nucleic acid

Viral nucleic acid can be detected by polymerase chain reaction (PCR). It is also useful for diagnosis in window period. Two types of PCR have been used, DNA PCR and RNA PCR. In the DNA PCR, peripheral lymphocytes are lysed and the proviral DNA is amplified. The test is highly sensitive and specific. A related test, RNA PCR can be used for diagnosis and also for monitoring the level of viraemia. The PCR tests are costly and are indicated only when other methods give inconclusive result.

(iv) Antibody detection

Demonstration of antibodies is the simplest and most commonly employed technique for diagnosis. It may take several weeks to months for antibodies to appear after infection. IgM antibodies appear first usually in about 3-4 weeks after infection, to be followed by IgG antibodies. IgM antibodies disappear in 8-10 weeks while IgG antibodies remain throughout life. With severe immunodeficiency in AIDS, some components of anti-HIV antibody (e.g. anti-p24) may disappear.

HIV infected persons remain negative for antibodies during *window period*, when initial viral replication takes place for about 2-3 weeks.

The diagnosis of HIV infection is made by detecting serum antibodies to viral proteins, both core (p24) or envelope (gp120, gp41). There are two types of serological tests—screening and supplemental (Table 63.7).

Table 63.7 Laboratory Tests for Detection of Specific Antibodies in HIV Infection

1. Screening (E/R/S) tests
   (a) ELISA
   (b) Rapid tests
      — Dot blot assay
      — Lateral flow assay (Immunochromatography)
      — Particle agglutination (latex, gelatin)
      — HIV spot and comb tests
   (c) Simple tests
      — These are based on ELISA principle
2. Supplemental tests
   — Western blot test
   — Indirect immunofluorescence test
   — Radio Immuno Precipitation assay

Screening tests

(a) *ELISA test*: Direct solid phase ELISA is the method most commonly used. The antigen is prepared from HIV grown in continuous T-lymphocyte cell line or by recombinant technique. The viral antigen is coated on surface of microtitre wells. The test serum is added, and if antibody is present it binds to the viral antigen. The unbound serum is washed away, anti-human goat immunoglobulin linked to a suitable enzyme is added, followed by a colour forming substrate. If the test is

positive, photometrically detectable colour is formed which can be read by ELISA reader. It is highly sensitive and specific test.

The early diagnostic tests (*first generation*) used purified lysate as antigens. The *second generation* tests used recombinant viral proteins. The *third generation* tests are double-antigen sandwich assay. In this, viral antigen is attached to a solid phase to which binds antibody to HIV from patient's serum. Then labelled HIV antigen is added which attaches to the patient's antibody and measured. *Fourth generation* tests detect both antibody and antigen.

ELISA test is an extremely good screening test and most laboratories use a commercial ELISA kit that contains both HIV-1 and HIV-2.

Saliva is an acceptable alternative to serum for antibody testing by ELISA. It is specially useful for injectable drug users who may have collapsed blood vessels.

(*b*) *Rapid tests*: These tests take less than 30 minutes and do not require expensive equipment. The rapid tests include dot-blot assay, lateral flow assays, particle agglutination, HIV spot and comb tests.

(*c*) *Simple tests*: They take 1–2 hours and do not require expensive equipment.

Supplemental tests

(*a*) *Western blot test*: In this test, HIV proteins are separated by polyacrylamide gel electrophoresis. The separated proteins are blotted on to strips of nitrocellulose paper. These strips are reacted with test sera. Antibodies to HIV proteins, if present in test serum, combine with different fragments of HIV. The strips are washed and reacted with enzyme-conjugated anti-human globulin. A suitable substrate is then added which produces colour bands. The position of the colour band on the strip indicates the fragment of antigen with which antibodies have reacted (Fig. 63.3). In a positive serum, bands will be seen with multiple proteins. Antibodies to p24 (*gag* gene, core protein), p31 (*pol* gene, reverse transcriptase) and gp41, gp120 or gp160 (*env* gene, envelope protein) are commonly detected. A positive reaction with proteins representing the three genes (*gag, pol, env*) is conclusive evidence of HIV infection. The test is also considered positive if it shows bands against at least two of following proteins : p24, p31, gp41, gp120/160.

Interpretation of Western blot test is sometimes difficult when bands appear only at one site as with p24 or gp120. This may happen in early infection but may also be nonspecific.

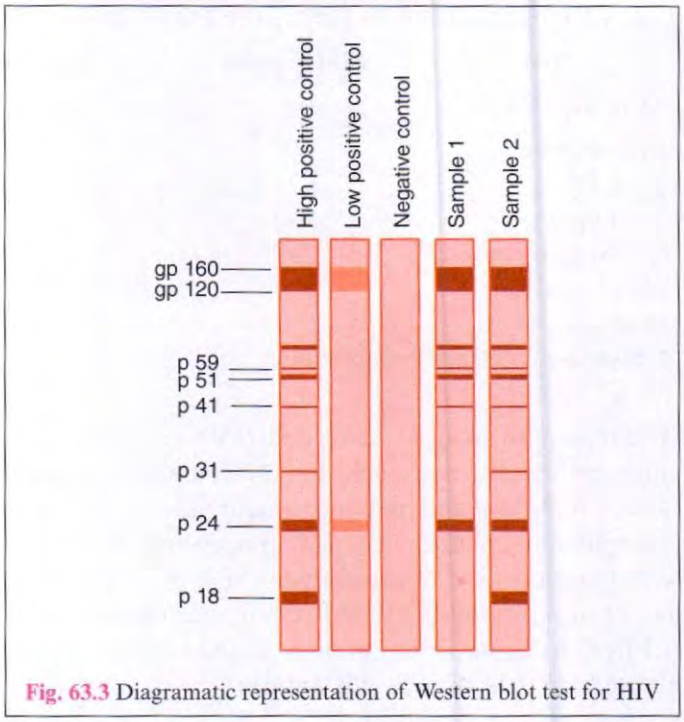


Fig. 63.3 Diagramatic representation of Western blot test for HIV

A positive result in any one screening test may not be accepted without confirmation. It was the practice to use the Western blot test for confirmation. As the test is cumbersome, costly and not readily available, different strategies are followed for confirmation. The practice now is to perform either two different types of ELISA or an ELISA with any of the rapid tests. A serum positive in both tests is considered positive. In case of doubt, sample is retested after 1 or 2 months.

(*b*) *Indirect immunofluorescence test*: HIV infected cells are fixed onto glass slides and then reacted with serum followed by fluorescein conjugated antihuman gamma globulin. In a positive test, apple-green fluorescence appears when examined under fluorescent microscope.

STRATEGIES FOR HIV TESTING IN INDIA

Strategy I: Serum is subjected once to E/R/S test and if positive, the sample is taken as HIV infected and if negative, the serum is considered to be free of HIV. This strategy is used for ensuring donation safety (Blood, organ, tissues etc.). For this purpose, a highly sensitive and very reliable test kit must be used.

Strategy IIA: A serum sample is considered negative if the first E/R/S test reports it so, but if positive, it is retested with a second E/R/S test based on a different antigen preparation and/or different test principle. If found positive on second E/R/S test also, it is reported as positive, otherwise as negative. This strategy is used for HIV surveillance.

Strategy IIB: The serum sample is processed as in strategy IIA, but a sample positive with first E/R/S test and negative with the second test is subjected to the third E/R/S test. If the third test is positive, sample is considered equivocal. Such persons should be retested after 2-4 weeks. If the third test is negative, it is reported as negative. Two to three different E/R/S tests based on a different antigen preparation and/or different test principle are used. If the first two tests are positive, a positive report can be given to the patient after post-test counselling. The strategy IIB is used for diagnosis of an individual with symptoms suggestive of AIDS clinically.

Strategy III: It is similar to strategy IIA, with the added confirmation by a third E/R/S test. The third test should again be based on different antigen preparation or test principle. A serum testing positive on all three E/R/S tests is reported positive. A serum specimen negative in third E/R/S is considered equivocal. Such persons should be retested after three weeks. If this specimen also provides an equivocal result, the individual is considered to be negative for HIV antibody. This strategy is used for diagnosis of HIV infection in asymptomatic individuals.

The first test selected for any of these strategies should be of highest sensitivity and second and third test should be of highest specificity to eliminate any chances of false positive results.

2. Non-specific Tests

(i) Total and differential leucocyte count

In AIDS, there is leucopenia with a lymphocyte count less than 400 per mm^3.

(ii) T-lymphocyte subset assays

The normal CD4:CD8 T-cell ratio of 2:1, is reversed to 0.5:1 in cases of AIDS. The count of CD4 lymphocytes falls below 200 per mm^3.

(iii) Platelet count

There is thrombocytopenia in patients of AIDS.

(iv) IgG and IgA levels

Both IgG and IgA levels are raised in blood.

(v) Skin tests for CMI

Cell mediated immunity is diminished as evident from tuberculin test or other skin tests for CMI.

3. Tests for Opportunistic Infections and Tumour

(i) Opportunistic infections

Most of these infections are diagnosed by direct microscopy and only in some cases culture is necessary. Serological diagnosis may not be reliable due to diminished immune response.

(ii) Tumour

Malignant tumours are more frequently associated in homosexuals who are having AIDS. Most important of these are Kaposi's sarcoma, B-cell lymphoma or non-Hodgkin's lymphoma. Kaposi's sarcoma is a malignant vascular tumour that arises from endothelial cells of blood vessels. It causes bluish purple, painless spots usually on the skin and mucous membranes but also on internal organs.

Applications of Serological Tests

Serological tests for HIV infection are applied in the following situations.

1. Screening
2. Seroepidemiology
3. Diagnosis
4. Prognosis

1. *Screening*: Screening is done for all donors of blood, blood products, semen, cells, tissues and organs. As antibody tests are negative during the early stage of infection when the person is infectious, screening may not rule out all dangerous donors, but can still eliminate the large majority of them. An individual found positive for HIV-antibody should never donate blood or other biological materials. HIV infection can be transmitted from mother to baby before, during or after birth, therefore, antenatal screening may be considered.
2. *Seroepidemiology*: Serological tests have been useful in identifying the geographical extent of HIV infection.
3. *Diagnosis*: Antibody testing may help to check whether infection has taken place following an exposure. It may however be negative in acute illness and sometimes in the very late cases when the immune system is non-reactive. HIV-2 infections are likely to be missed if antibody testing is done with HIV-1 antigen only. Serology after two months and, if negative, after six months would be sufficient. If antibody testing is negative six months after exposure, infection is unlikely to have occurred.
4. *Prognosis*: In a case of HIV infection, loss of detectable anti-p24 antibody indicates clinical deterioration. This is associated with HIV antigenemia and increased virus titre in blood.

Laboratory Monitoring of HIV Infection

The tests used for monitoring the course of HIV infection include:

(*i*) CD4+ T cell count
(*ii*) Measurement of HIV RNA

The most important among these is CD4+ T cell count which reflects the immunological competence of the patient. The count below 350 per mm^3 is an indication of disease progression and the need for antiretroviral therapy. However antiretroviral therapy is started irrespective of CD4 + T cell count in certain conditions. These conditions include tuberculosis and hepatitis B co-infections and also advanced clinical stages (WHO clinical stages 3 and 4). When the count falls below 200 per mm^3, it denotes risk of serious infections.

Measurement of HIV RNA is done by the reverse transcriptase PCR (RT-PCR) assay. During the course of treatment, it becomes necessary to measure HIV RNA.

J. Epidemiology

HIV is transmitted through blood, semen, vaginal fluid and from an infected mother to her baby. It is predominantly a sexually transmitted disease (STD). It can occur in homosexuals as well as heterosexuals. Different modes of transmissions have been described earlier in this chapter (page 512).

The danger of needle-stick injury remains in medical and paramedical personnel, though the risk of infection has been estimated to be about one percent. Medical and paramedical staff are to be educated on caring for patients with HIV infection.

Two serotypes of HIV are recognised, HIV-1 and HIV-2, HIV-1 is worldwide in distribution, while HIV-2 is principally found in West Africa. AIDS cases resulting from HIV-1 or HIV-2 infection are clinically indistinguishable. About 60% adults infected with HIV will develop AIDS within 5 to 10 years and vast majority of infected individuals will develop AIDS eventually. Virtually all persons diagnosed as having AIDS die of the disease.

In Africa, the major manifestation of AIDS is pronounced wasting so that it has been named the *slim disease*.

In India, the first case of HIV infection was found in female sex workers in Madras (Chennai) in 1986 and the first AIDS patient the same year from Bombay (Mumbai). Since then HIV infection has spread throughout the country.

K. Prevention

The following preventive measures are recommended.

1. *Sexual contact*: The use of condoms can prevent transmission of the virus.
2. *Sharing needles*: Contaminated syringes or needles should not be shared.
3. *Blood*: All blood and blood products are to be screened for HIV. This also applies to donation of cornea, semen, marrow, kidney and other organs.
4. Isolation of AIDS patient and initiation of treatment.
5. *Control of infection*: Screening of individuals within risk groups helps to identify the HIV infected persons.

L. Prophylaxis

No effective vaccine has yet been found out. High rate of mutation of the virus has made difficulty in developing the vaccine. Several strategies have been explored for vaccine preparation. These include immunisation with (*i*) modified whole virus (*ii*) subunits, based on envelope glycoproteins (*iii*) target cell protection by anti-CD4 antibody. A number of these candidate vaccines are under clinical trials in humans.

M. Antiretroviral Therapy (ART)

Specific treatment with antiretroviral drugs is the mainstay in the management of HIV infection. *Highly active antiretroviral therapy (HAART)* is effective in inhibition of HIV replication in most of the HIV-infected individuals but major drawback with this therapy is the selection of resistant mutants. Antiretroviral drugs include both nucleoside and non-nucleoside inhibitors of enzyme reverse transcriptase, viral protease inhibitors, fusion inhibitor, integrase inhibitor and entry inhibitor. (Table 63.8). These drugs have been used as monotherapy or in various combinations. HARRT includes combinations such as two nucleoside reverse transcriptase inhibitors combined with a protease inhibitor. Adverse reactions and high cost restrict their wide use in developing countries.

Apart from specific antiretroviral therapy, other measures in the treatment of AIDS include (*i*) treatment and prophylaxis of opportunistic infections and tumours (*ii*) general management and (*iii*) immuno-restorative measures.

N. Postexposure Prophylaxis (PEP)

Exposure to blood, body fluid, other potentially infected material or an instrument contaminated with one of these materials may lead to risk of acquiring HIV infection. The risk of infection varies with the type of exposure and other factors. Most exposures do not result in infection. Health workers are normally at very low risk of acquiring infection during management of infected patients. Following exposure, postexposure prophylaxis (PEP) may be required depending upon the category of exposure and HIV status of exposure source (Table 63.9). Basic PEP

Table 63.8 Antiretroviral Drugs

Nucleoside reverse transcriptase inhibitors (NRTIs)	Non-nucleoside reverse transcriptase inhibitors (NNRTIs)	Protease inhibitors	Fusion inhibitor	Integrase Inhibitor	Entry inhibitor (CCR5-Co-receptor antagonist
Zidovudine (AZT, azidothymidine) *Didanosine (ddI)* *Zalcitabine (ddc)* *Stavudine (d4T)* *Lamivudine (3TC)* *Abacavir (ABC)* *Tenofovir* *Emitricitabine* (FTC)	*Nevirapine (NVP)* *Delaviridine (DLV)* *Efavirnez (EFV)* *Etravirine (ETR)*	*Saquinavir (SQV)* *Ritonavir (RTV)* *Indinavir (IDV)* *Nelfinavir (NFV)* *Lopinavir (LPV)* *Amprenavir* *Tipranavir (TPV)* *Atazanavir (ATV)* *Fosamprenavir (FPV)* *Darunavir (DRV)*	*Enfuvirtide (T20)*	*Raltegravir (RAL)*	Maraviroc *(MVC)*

Table 63.9 PEP regimen according to exposure and status of source

Category of exposure	Status of Source		
	HIV positive and asymptomatic	HIV positive and clinically symptomatic	HIV status not known
i. Mild exposure (Mucous membrane/non-intact skin with small volumes)	Consider two drug PEP regimen	Start two drug PEP regimen	Usually no PEP or consider two drug PEP regimen
ii. Moderate exposure (Mucous membrane/non-intact skin with large volume or percutaneous superficial exposure with solid needle)	Start two drug PEP regimen	Start three drug PEP regimen	
iii. Severe exposure (Percutaneous with large volume)	Start three drug PEP regimen		

regimen consists of two drug combination while expanded PEP regimen is a combination of three drugs. Zidovudine 300 mg BD and Lamivudine 150 mg BD are used in basic two drug regimen. In expanded three drug PEP regimen, a protease inhibitor is added to this combination of drugs. Among protease inhibitors, lopinavir 400 mg BD or 800 mg OD or ritonavir 100 mg BD or 200 mg OD are preferred as third drug. To be effective these drugs must be started within the first 72 hours and ideally within 2 hours. The PEP should be continued for a period of four weeks. Both risk of infection and possible side-effects of antiretroviral drugs should be carefully considered when deciding to start PEP. Besides PEP, injured site on the wound should be thoroughly washed with soap and water. Antiseptics may also be used.

Exposed persons should have post PEP HIV testing, at three months and at 6 months. If the test at six months is negative, no further testing is required.

KEY POINTS

1. Retroviruses possess *reverse transcriptase* (*RNA directed DNA polymerase*) *enzyme* which prepares a DNA copy of the RNA genome in host cell. The presence of enzyme reverse transcriptase is a characteristic feature.
2. Human immunodeficiency virus (HIV), the causative agent of AIDS, belongs to retroviruses.
3. HIV is a spherical enveloped virus containing two identical copies of single stranded positive sense RNA genome. In association with viral RNA is the reverse transcriptase enzyme. The virus core is surrounded by a nucleocapsid composed of protein. The virus contains a lipoprotein envelope.
4. HIV genome contains the three structural genes (*gag*, *pol* and *env*). Five non-structural genes (*tat, rev, nef, vif* and *vpr*) are present in both HIV-1 and HIV-2. Other than these, HIV-1 contains *vpu* and HIV-2 has *vpx*. The products of these genes, both structural and non-structural, act as antigens. Infected person's serum contains antibodies to these antigens. Detection of these antigens and antibodies is of great importance in the diagnosis and prognosis of HIV infections.

5. There are three modes of transmission of HIV infection. These are *sexual contact*, *parenteral* and *perinatal*.
6. HIV infects principally the *CD4 lymphocytes*. The infection causes damage to T helper (T4) lymphocytes. *T4 cells* are *depleted* in numbers and the *T4:T8* (helper : suppressor) *ratio* is *reversed*.
7. When CD4+ cells fall below 200 per mm^3, the titre of virus increases markedly and there is irreversible breakdown of immune defence mechanisms. Most of the patients with HIV disease die of infections other than HIV e.g. opportunistic infections and malignancies. AIDS is the end stage of HIV infection.
8. Laboratory diagnosis of HIV infection includes specific tests for HIV and tests for immunodeficiency. Specific tests include *antigen* (*p24*) *detection*, *virus isolation*, detection of *viral nucleic acid* and *antibody detection*.
9. The p24 antigen is the earliest virus marker to appear in the blood. Viral isolation, detection of viral nucleic acid by *polymerase chain reaction* (PCR) and p24 antigen detection are useful for diagnosis *in window period*. HIV infected persons remain negative for antibodies during window period.
10. Demonstration of antibodies is the simplest and most commonly employed technique for diagnosis. It may take several weeks to months for antibodies to appear after infection. The diagnosis of HIV infection is made by detecting serum antibodies to viral proteins, both core (*p24*) or envelope (*gp120, gp41*). There are two types of serological tests available for antibody detection—*screening tests* and *supplemental tests*.
11. Screening (E/R/S) tests include *ELISA*, *rapid* tests and *simple tests*. *Western blot* test and *indirect Immunofluorescence* test are supplemental tests used for HIV antibody detection.
12. *ELISA* is the method *most commonly used*.
13. There are three strategies (*strategy I to III*) for HIV testing in India.
14. Serological tests for HIV infection are applied for *screening*, *seroepidemiology*, *diagnosis* and *prognosis*.
15. Specific treatment with *antiretroviral drugs* is the mainstay in the management of HIV infection.
16. *Postexposure prophylaxis* (PEP) may be required when there is exposure to blood, body fluids, other potentially infected material or an instrument contaminated with HIV.

YOU MUST KNOW

1. Morphology of human immunodeficiency virus (HIV).
2. Genes coding for structural proteins.
3. Modes of transmission of HIV infection.
4. Types of exposure and their relative risk.
5. Opportunistic infections associated with HIV infection.
6. Laboratory diagnosis of HIV infection.
7. Laboratory tests for detection of specific antibodies in HIV infection.
8. Strategies for HIV testing in India.
9. Postexposture prophylaxis (PEP) for HIV.
10. Antiretroviral drugs for HIV.

STUDY QUESTIONS

1. Describe the morphology of HIV and laboratory diagnosis of HIV infection.
2. Discuss the modes of transmission and the pathogenesis of HIV infection.
3. Write short notes on:
   (a) Genes coding for HIV antigens
   (b) Opportunistic infections associated with HIV infection
   (c) Strategies of HIV testing in India
   (d) Control of HIV
   (e) Antiretroviral therapy for HIV
   (f) Postexposure prophylaxis for HIV.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. What is the characteristic feature of viruses belonging to family *Retroviridae*?
   (a) Presence of enzyme reverse transcriptase (b) Presence of envelope
   (c) Presence of nucleic acid (d) Presence of lipid
2. Human immunodeficiency virus belongs to which of the following subfamilies of family *Retroviridae*?
   (a) Oncovirinae (b) Lentivirinae
   (c) Spumavirinae (d) All of the above
3. Which structural genes are present in the genome of human immunodeficiency virus-1 and not in human immunodeficiency virus-2?
   (a) *gag* gene (b) *pol* gene
   (c) *env* gene (d) All of the above
4. Which of the following non-structural genes is present only in human immunodeficiency virus-1 and not in human immunodeficiency virus-2?
   (a) *tat* gene (b) *rev* gene
   (c) *vpu* gene (d) *vpx* gene
5. Which of the following non-structural genes is present only in human immunodeficiency virus-2 and not in human immunodeficiency virus-1?
   (a) *tat* gene (b) *rev* gene
   (c) *vpu* gene (d) *vpx* gene
6. Which is the principal envelope spike antigen of HIV-1?
   (a) gp 120 (b) gp 140
   (c) gp 41 (d) gp 38
7. Which is the transmembrane pedicle antigen of HIV-1?
   (a) gp 120 (b) gp 140
   (c) gp 41 (d) gp 38
8. What is the mode of transmission of human immunodeficiency virus?
   (a) Sexual contact (b) Parenteral transmission
   (c) Perinatal transmission (d) All of the above
9. What is the relative risk of transmission of HIV by the sexual contact?
   (a) 0.1-1.0% per exposure (b) 2-20% per exposure
   (c) 30-50% per exposure (d) 50-90% per exposure
10. Which type of cells are most often infected by HIV?
    (a) CD4+T lymphocytes (b) CD8+T lymphocytes
    (c) B lymphocytes (d) None of the above
11. Which of the following opportunistic infections is/are associated with HIV infection?
    (a) Tuberculosis (b) *Pneumocystis jiroveci* pneumonia
    (c) Toxoplasmosis (d) All of the above
12. Which of the following opportunistic parasitic infections is/are associated with HIV infection?
    (a) Toxoplasmosis (b) Cryptosporidiosis
    (c) Isosporiasis (d) All of the above
13. Which of the following opportunistic fungal infections is/are associated with HIV infection?
    (a) *Pneumocystis jiroveci* pneumonia (b) Candidiasis
    (c) Cryptococcosis (d) All of the above
14. Which of the following opportunistic viral infections is/are associated with HIV infection?
    (a) Cytomegalovirus infections (b) Herpes simplex infections
    (c) Varicella-zoster infections (d) All of the above
15. Which of the following tests may be used for diagnosis of HIV infection?
    (a) P24 antigen detection (b) Virus nucleic acid detection
    (c) Antibody detection (d) All of the above

16. Which of the following tests is the most commonly employed for diagnosis of HIV infection?
(a) Virus isolation
(b) Antibody detection
(c) Viral nucleic acid detection
(d) None of the above

17. Which of the following tests is/are screening test/s used for diagnosis of HIV infection?
(a) ELISA
(b) HIV spot test
(c) Comb test
(d) All of the above

18. Which of the following tests is/are rapid tests for diagnosis of HIV infection?
(a) Dot blot assay
(b) Latex agglutination test
(c) Comb test
(d) All of the above

19. Which of the following tests may be useful for diagnosis of HIV infection in window period?
(a) P24 antigen detection
(b) DNA PCR
(c) RNA PCR
(d) All of the above

20. How many strategies for HIV testing are there in India?
(a) One
(b) Two
(c) Three
(d) Five

21. Which HIV testing strategy is used for ensuring blood transfusion safety?
(a) Strategy I
(b) Strategy II
(c) Strategy III
(d) None of the above

22. Which HIV testing strategy is used for HIV surveillance?
(a) Strategy I
(b) Strategy IIA
(c) Strategy IIB
(d) Strategy III

23. Which HIV testing strategy is used for diagnosis of an individual with symptoms suggestive of AIDS clinically?
(a) Strategy I
(b) Strategy IIA
(c) Strategy IIB
(d) Strategy III

24. Which HIV testing strategy is used for diagnosis of HIV infection in asymptomatic individuals?
(a) Strategy I
(b) Strategy IIA
(c) Strategy IIB
(d) Strategy III

25. In which year, the first case of HIV infection was found in India?
(a) 1984
(b) 1985
(c) 1986
(d) 1987

26. Which of the following antiretroviral drugs may be used for management of HIV infection?
(a) Nucleoside reverse transcriptase inhibitors
(b) Non-nucleoside reverse transcriptase inhibitors
(c) Protease inhibitors
(d) All of the above

27. Which of the following antiretroviral drugs has been most widely used for management of HIV infection?
(a) Azidothymidine
(b) Nevirapine
(c) Enfuvirtide
(d) Indinavir

28. Which of the following antiretroviral drugs may be used for postexposure prophylaxis in individuals exposed to potentially HIV infected blood?
(a) Azidothymidine
(b) Lamivudine
(c) Lopinavir
(d) All of the above

29. After exposure to blood potentially infected with HIV, post exposure prophylaxis with antiretroviral drugs should ideally be started within:
(a) Two hours
(b) Four hours
(c) Six hours
(d) Eight hours

Answers (MCQs):

1. (a)	2. (b)	3. (d)	4. (c)	5. (d)	6. (a)	7. (c)	8. (d)	9. (a)	10. (a)
11. (d)	12. (d)	13. (d)	14. (d)	15. (d)	16. (b)	17. (d)	18. (d)	19. (d)	20. (c)
21. (a)	22. (b)	23. (c)	24. (d)	25. (c)	26. (d)	27. (a)	28. (d)	29. (a)	

Chapter 64

MISCELLANEOUS VIRUSES

I. PAPOVAVIRUSES

Papova (from *pa*, papilloma; *po*, polyoma; *va*, vacuolating agent) viruses contain papillomaviruses and polyomaviruses. These viruses contain a small icosahedron composed of 72 capsomers and double stranded DNA genome. Papillomaviruses and Polyomaviruses belong to family *Papillomaviridae* and family *Polyomaviridae* respectively. They induce both lytic infections and tumours.

A. Papillomaviruses

Papillomaviruses are icosahedral, 55 nm in diameter, non-enveloped viruses which contain double stranded circular DNA. There are over 100 types of human papilloma viruses (HPV). They infect the squamous epithelia and mucous membranes.

1. Pathogenesis

They may cause cutaneous warts, genital warts, recurrent respiratory papillomatosis, oral papillomatosis and cancer. These infections are transmitted by indirect or direct contact including sexual contact. Incubation period varies from 2 to 8 months. Regression of most lesions is spontaneous.

(i) Cutaneous warts

Cutaneous warts are commonly found in children. They disappear spontaneously within 2 years of onset. Recurrence is believed to be due to persistence of virus in the skin surrounding the original wart. The virus leads to hypertrophy of all the layers of dermis with hyperkeratosis of horny layer. Cutaneous warts are generally caused by HPV types 2, 4 and 7.

(ii) Genital warts

These lesions are also known as condyloma acuminata. These are common in sexually active adults. In females, lesions appear in vulva, within vagina and on the cervix. In men, the lesions are found on shaft of penis, perianal skin and anal canal. Genital warts are generally caused by HPV types 6 and 11.

(iii) Recurrent respiratory papillomatosis

Benign squamous papillomas appear on the mucosa of respiratory tract, particularly on the larynx. Children below 5 years of age acquire the lesion by passage through an infected birth canal. Adults are infected by orogenital contact with an infected sexual partner. HPV types 6 and 11 are involved in recurrent respiratory papillomatosis.

(iv) Oral papillomatosis

Infection is usually acquired by orogenital contact with infected sexual partner. Multiple papillomatous lesions may develop on the buccal mucosa. The condition is also known as oral florid papillomatosis. It is caused by HPV types 6, 7, 11, 13, 16 and 32. Oral papillomas sometimes progress to malignancy. HPV DNA has been demonstrated in a minority of oral carcinomas. HPV has also been thought to cause hairy leukoplakia on the tongue in the patients infected with human immunodeficiency virus.

(v) Cancer

An aetiological relationship has been established between cervical cancers and human papilloma viruses. DNA of HPV has been detected in premalignant lesions of the female and male genital tract (HPV types 6, 11). HPV (types 16,18 and 31) has been detected in 60 to 100% of cervical cancers. In 2008, Herald Zur Hausen was awarded the Nobel Prize for the research in human papilloma viruses causing cervical cancers.

2. Laboratory Diagnosis

Laboratory diagnosis can be made by

(i) *Histology and cytopathology*

(ii) *Electron microscopy*

(iii) *Immunocytochemistry*

The HPV capsid antigen in sections of tissues or in cell smears can be detected by immunoperoxidase test using a commercially available antiserum. It detects all the genital HPV types.

(iv) *Detection of viral nucleic acid*

Viral DNA in tissues and exfoliated cells can be detected by DNA hybridisation and polymerase chain reaction.

3. Prophylaxis

Quadrivalent HPV vaccine containing HPV types 6, 11, 16 and 18 has been recommended for administration to girls and young women of 9 to 26 years of age. It can prevent infection and hence reduce the incidence of anogenital warts and cervical cancers. HPV types 6 and 11 are responsible for genital warts in 90% of cases while HPV types 16 and 18 are responsible for more than 70% of cervical cancers. Three doses of 0.5 ml each are given intramuscularly. First dose is administered at age 11-12 years (may be started at 9 year of age). The second dose is given 2 months after the first dose and the third dose 6 months after first dose. Booster doses are not recommended.

Another bivalent HPV vaccine is also available containing HPV types 16 and 18. Three doses at 0, 1 and 6 months are given. 0.5 ml is given intramuscularly

B. Polyomaviruses

Polyomaviruses produce a variety of malignant tumours in newborn mice, hamsters and guinea pigs, hence the name polyoma (*poly*, many; *oma*, tumour). They are non-enveloped, 42-45 nm in diameter with a 72 capsomer icosahedral capsid. Viral genome, like that of papilloma-viruses, is a double-stranded DNA molecule. Polyomaviruses include:

(i) *Mouse polyomavirus*: It causes harmless infections in mice by natural routes. However, when injected into infant rodents such as hamsters it induces different types of malignant tumours.

(ii) *Simian virus 40* (SV 40): It was isolated from uninoculated rhesus and cynomolgus monkey kidney tissue cultures. It causes subclinical infections in monkeys. SV 40 is oncogenic in newborn hamsters. Its only medical importance is that, because of its oncogenic potential, live viral vaccines should be prepared only in monkey kidney tissue cultures found free from SV40 infection.

(*iii*) *JC polyomavirus*: It was first isolated from the brain of a male patient with Hodgkin's disease and progressive multifocal leucoencephalopathy (PML). The name is derived from the initials of the person from whom it was isolated. Progressive multifocal leucoencephalopathy (PML) is a rare, subacute demyelinating disease of nervous system that results from JC virus infection. It occurs mainly as a complication of advanced disseminated malignant conditions such as Hodgkin's disease or chronic lymphocytic leukaemia.

(*iv*) *BK polyomavirus*: It was isolated from the urine of a patient with kidney transplant and was named after his initials. The virus persists for life in kidneys. Reactivation may occur if the immune system is impaired in later life following disease or transplantation. Both JC and BK viruses may remain as latent infections. On reactivation these may lead to PML (JC virus) and renal disease (BK virus).

(*v*) *Merkel cell polyomavirus*: It is a new polyomavirus discovered in 2008. It has been established as cause of Merkel cell carcinoma, a rare skin cancer of humans.

Laboratory Diagnosis

(i) Electron microscopy

Human polyomaviruses can be detected by electron microscopy from brain tissue in a case of PML (JC virus) and from the urine of a renal transplant case (BK virus).

(ii) Virus isolation

JC polyomavirus can be isolated from the urine or the brain and BK polyomavirus from the urine. Human foetal glial cell culture and human diploid fibroblasts are used for the isolation of JC polyomavirus and BK virus respectively. These two viruses can be differentiated by haemagglutination inhibition.

(iii) Viral antigen detection

Following brain biopsy, JC polyomavirus antigen can be detected by immunofluorescence. In urine, viral antigen can be detected by ELISA.

(iv) Viral nucleic acid detection

Viral nucleic acid can be detected by nucleic acid hybridisation and polymerase chain reaction (PCR).

(v) Cytopathology

Exfoliated urinary epithelial cells show the presence of enlarged deeply stained basophilic nuclei, with a single inclusion.

II. PARVOVIRUS

Parvoviruses belong to the family *Parvoviridae* and are very small (about 20 nm), non-enveloped, icosahedral viruses containing a single stranded DNA. There are three genera in the family: *Bocavirus, Erythrovirus* and *Dependovirus*. Members of genus *Dependovirus* contain viruses which are defective and require another virus (helper) for their own replication. They are usually found in association with an adenovirus (as helper virus) and are known as *adeno-associated viruses* (AAV). Parvovirus B19 has now been classified in a separate genus *Erythrovirus*.

Parvovirus B19

It was first isolated from the blood of asymptomatic donors in 1983. Parvovirus B19 has an affinity for immature red blood cell precursors. It has been shown to be associated with aplastic crisis in sickle cell disease and other haemolytic anaemias. In the immunodeficient, it may cause persistent anaemia. It also causes *erythema infectiosum* characterised by an erythematous skin eruption of the cheeks (*slapped cheek appearance*), spreading to the trunk and limbs, followed by lymphadenopathy and arthralgia. It is a common childhood febrile illness with rash and has been called the *fifth disease*, as it was the fifth in the old list of six exanthematous fevers of children.

If a pregnant woman develops primary parvovirus B19 infection, fatal erythroblastosis foetalis with hydrops may result.

Transmission appears to be respiratory, though it may also be through blood. Parvovirus B19 is highly contagious.

Laboratory Diagnosis

(i) Virus isolation

Parvovirus B19 may be cultured in cells from human bone marrow or foetal liver. By electron microscopy, it can also be detected in patient's blood.

(ii) Detection of nucleic acid

Nucleic acid can be detected by hybridisation technique or by polymerase chain reaction.

(iii) Antigen detection

Antigen can be detected by ELISA, RIA or indirect immunofluorescence.

(iv) Antibody detection

IgM antibodies or a significant rise in IgG antibodies can be detected by ELISA or RIA. It is the most successful diagnostic technique.

III. CORONAVIRUSES

A group of spherical or pleomorphic medium sized (100-150 nm), enveloped RNA viruses, containing petal or club shaped peplomers on the surface (Fig. 64.1), has been classified as coronaviruses (*corona*, meaning crown). They belong to the family *Coronaviridae*. These viruses infect mammals and birds causing diseases of the respiratory tract, gastrointestinal tract, liver, kidneys and nervous system. Only two species of the genus *Coronavirus*, human coronavirus 229E and human coronavirus OC43, are responsible for human respiratory disease.

Coronaviruses are sensitive to heat, lipid solvents, formaldehyde, non-ionic detergents and oxidising agents.

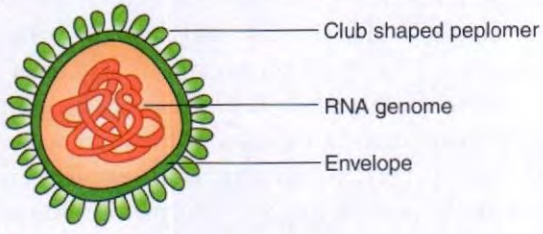


Fig. 64.1 Coronavirus.

Human Coronavirus

In man, they infect the upper respiratory tract causing common cold. Human coronaviruses are responsible for 30% cases of common cold. They cause nasal discharge more than that in rhinovirus infection. The incubation period varies from 2-5 days and symptoms persist for about a week. The resulting immunity is poor and reinfections can occur even with the same serotype.

By electron microscopy, virus particles resembling coronoviruses have been observed in faeces of patients with gastroenteritis. However, their role in the causation of gastroenteritis in humans is not known.

Laboratory Diagnosis

(i) Isolation of virus

Nasopharyngeal washings are used for the isolation of virus. Human coronaviruses can be cultured in human foetal tracheal organ culture. Some strains may grow on monolayers of diploid human embryonic fibroblasts, with minimal cytopathic effects.

(ii) Demonstration of antigen

Coronavirus antigens can be detected in respiratory secretions by ELISA test.

(iii)) Antibody detection

Antibodies in serum may be detected by ELISA.

Severe Acute Respiratory Syndrome (SARS)

In November 2002, South China had an outbreak of an unusual respiratory infection, with many deaths. In February 2003, a physician from China visited Hong Kong, fell ill and died after infecting twelve persons. These twelve persons went to their countries to fall ill and also initiated outbreaks there. In February in Hanoi, Vietnam, an unusual case of pneumonia was reported by a hospital which sought the help of a WHO office in this connection. Dr. Carlo Urbani, the WHO infectious disease specialist advised for quarantine but by then outbreaks had involved many countries including USA, Canada, Ireland, Hong Kong, China, Taiwan, some European countries and many countries in South East Asia. This new disease was named 'Severe Acute Respiratory Syndrome' (SARS). It affected over 30 countries, with many thousand cases and about 800 deaths. A few cases have also been reported from India. Dr. Carlo Urbani, who identified this new epidemic participated in its control and died of the disease.

A coronavirus was found in the respiratory secretions of these patients by electron microscopy and confirmed by growth in Vero cell culture, cloning, sequencing and animal inoculation. This coronavirus is named severe acute respiratory syndrome-related *coronavirus* (SARS-CoV). It belongs to family *Coronaviridae* and the genus is *Betacoronovirus*. It is the causative agent of SARS. It may be a recombinant of some animal and human coronoviruses.

Mode of Infection

SARS spreads by inhalation of the virus present in respiratory secretions of patients. Facial aerosols also may be infectious. Incubation period is 10 days or less. The disease starts with fever and cough or other respiratory symptoms. The chest X-ray shows pneumonia like changes. The death occurs due to respiratory failure.

Laboratory Diagnosis

Nasopharyngeal swab or aspirate, throat swab or stool specimens may be collected for laboratory diagnosis. Serum is used for antibody detection.

(i) Polymerase Chain Reaction (PCR)

Reverse transcriptase PCR (RT-PCR) has been used for early diagnosis.

(ii) Virus culture

Virus in clinical specimens can be cultured on Vero cell lines.

(iii) Antibody detection

The rise in titre of antibodies in paired serum samples can be demonstrated by ELISA or indirect immunofluorescent test. However, for early diagnosis PCR is preferred.

Treatment and Prophylaxis

No specific treatment or prophylaxis is available. The virus is highly mutable, hence vaccine may not be easy to prepare. Strict isolation and quarantine are the only ways to control the disease. However, ribavirin and steroids have been shown to be useful in treatment of critical patients.

Middle East Respiratory Syndrome (MERS)

Middle East Respiratory Syndrome (MERS) is viral respiratory disease first reported in Saudi Arabia in 2012. It is caused by novel coronavirus called MERS-CoV. Most patients develop severe acute respiratory illness. Symptoms include fever, cough, and breathlessness. All the cases reported have been linked to countries in and near the Arabian Peninsula. This virus has spread from patients to others through close contact.

In 2014, a number of cases have been reported from Saudi Arabia and other countries. It has been reported from 22 countries. As of January 6, 2015, the WHO has reported 945 human cases including 348 deaths.

IV. ARENAVIRUSES

Several rodent-borne heamorrhagic fever viruses are classified as *Arenaviruses* (*Arena* (Latin), meaning sand) because of sand sprinkled appearance of viruses under electron microscope. This appearance is due to cellular ribosomes picked up by the virus presumably during maturation by budding from host cells. The virions are spherical or pleomorphic, 50-300 nm in diameter and contain single stranded RNA genome. They possess envelope into which are embedded glycoprotein peplomers.

Arenaviruses are maintained in nature from rodent to rodent without any help from arthropod vectors. Man gets infection by contact with excreta (urine or faeces) of infected rodents.

A. Arenaviruses Causing Human Disease

1. Lymphocytic Choriomeningitis (LCM)

It is a natural parasite of mice. Natural infection occurs in dogs, monkeys and guinea pigs. Human infection with LCM virus is often inapparent, but some may develop influenza like illness, aseptic meningitis and rarely severe encephalomyelitis. Incubation period varies from 1-2 weeks. Man acquires the infection probably from the excreta of rodents.

2. Lassa Fever

It was first noticed in 1969 in an American Mission station in Lassa, Nigeria. It causes haemorrhagic fever in humans and mortality rate is very high. The incubation period varies from 3 to 16 days. Rodent excreta probably act as the source of infection. The virus is present in the throat, urine and blood of patients. Person to person transmission may also occur by droplet infection. Ribavirin has proved useful in treatment.

Lassa virus is endemic widely in West Africa, causing asymptomatic infection in the native population . Many outbreaks of Lassa fever have occurred in West Africa.

3. South American Haemorrhagic Fevers

Three related viruses, the *Junin*, *Machupo* and *Guanarito* viruses cause Argentinian, Bolivian and Venezuelan haemorrhagic fevers respectively. The incubation period is 1-2 weeks. Mortality rate varies from 5-30%. Death is due to hypovolaemic shock.

B. Laboratory Diagnosis

1. Virus Isolation

Arenaviruses can be isolated from specimens, by inoculation of suckling mice, hamsters and guinea pigs. Blood, CSF, throat washings, pleural fluid and urine are the specimens used for diagnosis. Virus can also be isolated by inoculation of Vero E6 and BHK-21 cells.

2. Antigen Detection

Viral antigen can be detected in the blood by indirect immunofluorescence.

3. Serology

Indirect immunofluorescence test may be used for detection of IgM antibody and/or a rising titre of antibody in paired sera.

V. FILOVIRUS

The Marburg virus was first recognised simultaneously in laboratory workers in Marburg (Germany) and Belgrade (Yugoslavia) in 1967. The infection arose from tissues of African green monkeys to which these workers had been exposed. In 1976, a morphologically similar but antigenically distinct virus was isolated from several cases of haemorrhagic fever in Sudan and Zaire. It was named *Ebola virus*. In 1979 Ebola reemerged in Sudan, with serial man to man spread. These viruses belong to the family *Filoviridae*.

A. Morphology

Marburg virus and Ebola virus are enveloped negative

sense single stranded RNA viruses with long tubular or filamentous forms (800-1500 nm × 80 nm). They possess 7 nm surface spikes.

B. Pathogenesis

These viruses cause haemorrhagic fever. The natural reservoir of Marburg and Ebola viruses is not known, but is presumably some African rodents. These viruses are transmitted to man by aerosol route and by direct contact with blood, organs or tissue cultures. However, secondary spread between humans occur by contact with body fluids from an acute case, sexual intercourse and the use of contaminated injection. The liver is the major site of infection but the virus can infect most tissues in the body. The incubation period is 3-15 days.

C. Laboratory Diagnosis

1. Electron Microscopy

Filamentous viruses can be seen in the blood and in the cytoplasm of the affected cells by electron microscopy.

2. Isolation of Virus

Virus can be cultured in Vero cells from the blood during the febrile phase. Virus isolate is identified by electron microscopy and direct immunofluorescence. Virus culture must only be attempted in laboratories with required biosafety level.

3. Serology

Antibodies in the serum can be detected by indirect immunofluorescence.

VI. RUBIVIRUS

Rubella Virus

Rubella or German measles is primarily a mild childhood disease. It may be acquired congenitally or postnatally. Although rubella virus is a member of togaviridae family, it is not transmitted by arthropods. Clinical features of the disease are similar to those of paramyxoviruses.

Morphology

Rubella virus is a pleomorphic, roughly spherical, 50-70 nm in diameter, enveloped RNA virus. It has single stranded RNA genome and envelope carries haemagglutinin peplomers.

Properties

The virus agglutinates erythrocytes of goose, pigeon, day-old chick and human at 4°C.

Resistance

The virus is destroyed by heating at 56°C, but survives for several years at –60°C. It is inactivated by chemicals such as ether, chloroform, formaldehyde and beta propiolactone.

Pathogenesis

Postnatal rubella

Infection is acquired by inhalation. Virus multiplies locally and in the cervical lymph nodes. After an incubation period of 2 to 3 weeks, viraemia occurs. The virus is disseminated throughout the body by blood- stream. Patient develops fever, and fine, pink, macular rash, which first appears on the face and then spreads to the trunk and legs. The illness is of short duration and recovery is usually complete. About 50% of the infections in children are asymptomatic but asymptomatic rubella is less common in adults. Complications like thrombocytopenic purpura, post infection encephalomyelitis though rare are sometimes observed.

Congenital rubella

Rubella virus can cross the placental barrier and can replicate in differentiating cells of the embryo. The foetal cells are not destroyed by the virus but their rate of growth is reduced. It may result in congenital abnormalities or even death of the foetus. The classical congenital rubella syndrome (CRS) includes cataract, deafness and patent ductus arteriosus (PDA).

Laboratory Diagnosis

Routine diagnosis of rubella is not called for but the laboratory diagnosis becomes important in pregnant women suspected to have rubella. Diagnosis can be established by virus isolation or serology.

Virus isolation

Rubella virus can be isolated from adult throat swab. In infants with congenital rubella, the throat swab, urine or CSF can be used for isolation. Virus can be grown in cell lines of rabbit kidney (RK13), rabbit cornea (SIRC) and Vero cells, but cytopathic effects (CPE) develop only in a few cell lines such as RK 13. In others, rubella virus is detected by interference, using a challenge virus (ECHO 11) or by immunofluorescence for detection of antigen in such cells. The virus grows better if cultures are incubated at lower temperature i.e. 33–35°C. The virus isolation is not commonly employed for rubella diagnosis because of the difficulties and the delay involved.

Serology

Serological diagnosis is the method of choice in rubella. Antibodies in blood are detected by ELISA. Demonstration of rubella IgM antibody in a single specimen of blood

has a diagnostic value. In case of rubella IgG antibody, four-fold rise in titre must be demonstrated between two serum samples collected at 10 days interval. In a newborn baby, rubella specific IgM antibody is diagnostic of congenital rubella as IgM antibodies do not cross the placenta.

Other serological tests for detection of antibodies include latex agglutination and immunofluorescence assay.

Immunity

One attack of rubella confers life-long immunity.

Prophylaxis

Live attenuated vaccine, RA 27/3 strain grown in human diploid cells is widely used. It is administered by subcutaneous injection. A combined measles-mumps-rubella (MMR) vaccine is recommended for all infants at the age of 15 months, followed by a booster at the age of 4-6 years. The vaccine should not be given to immunodeficient individuals. Pregnancy is an absolute contraindication. In women, pregnancy should be avoided for three months after vaccination.

VII. REOVIRIDAE

The name Reoviridae is derived from the 'Respiratory Enteric Orphan Virus' because of their frequent occurrence in respiratory and enteric tracts, and it was considered an 'orphan' as its pathogenicity to humans was not proven.

A. Classification

The family *Reoviridae* has three genera which contain human pathogens. These are *Rotavirus, Coltivirus* and *Orthoreovirus*. *Coltivirus* causes Colorado tick fever in North America and *Orthoreovirus* may cause upper respiratory infections. Members of genus *Rotavirus* are recognised as important cause of gastroenteritis throughout the world.

B. Morphology

Members of the family *Reoviridae* are non-enveloped, double shelled icosahedral viruses, 55-75 nm in diameter. They contain double stranded RNA in 10-12 pieces, a feature unique among animal viruses. These segments have been found to perform diverse functions.

C. Rotaviruses

Rotaviruses are the commonest cause of gastroenteritis in children. Rotavirus (*Rota*, meaning wheel) is a double shelled icosahedral capsid resembling a wheel with short spokes radiating from a wide hub to a clearly defined outer rim (Fig. 64.2). The genome consists of double stranded segmented RNA. The complete rotavirus particle is 65-70 nm in diameter and has a smooth surface. The incomplete virus particle is smaller, about 60 nm, with a rough surface and is believed to be rotavirus that has lost the outer shell. 'Empty' particles without the RNA core are also seen. Based on group specific antigens of the major viral structural protein VP6, rotaviruses are classified into seven serogroups (A to G). Most human rotaviruses fall into serogroup A. Serogroup A has been classified into subgroups (I and II) by ELISA, CFT or immune adherence agglutination and into serotypes (1, 2, 3 etc.) by neutralisation tests.The virus multiplies in epithelial cells of small intestine and are excreted in the stool during the first few days of diarrhoea.

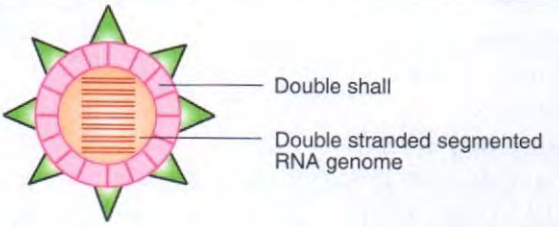


Fig. 64.2 Rotavirus

Human rotavirus does not grow readily in cell cultures. However, some strains have been adapted for serial growth in tissue cultures. As calf and simian rotaviruses grow readily in cell cultures, they have been used as antigens for serological tests.

1. Epidemiology

Rotavirus diarrhoea is usually seen in children below the age of five years, but is most frequent between six months and two years of age. Infection is acquired by the faeco-oral route. Incubation period is 2-3 days. Vomiting and diarrhoea occur with little or no fever. There is no blood or mucus present in the stools. The disease is self limiting and recovery occurs within 5-10 days. Mortality is low. The disease occurs throughout the year but predominates in winter months.

Outbreaks of rotavirus diarrhoea in adults and older children have been reported from China. This virus is named—adult diarrhoea rotavirus (ADRV). These strains belong to serogroup B of rotaviruses.

2. Laboratory Diagnosis

(i) Demonstration of virus

During acute stage of the disease, 10^{11} virus particles may be present in the faeces. These can be demonstrated by:

(a) ELISA
(b) Latex agglutination test

(c) CIEP
(d) Electron microscopy
(e) Immunoelectron microscopy.

(ii) Antibody detection

IgM and IgG antibodies can be detected in the blood by ELISA and complement fixation test.

(iii) Polymerase Chain Reaction (PCR)

3. Treatment

Rehydration is the only treatment needed.

4. Prophylaxis

Apart from improvement in hygiene, an oral rotavirus vaccine has been used for prophylaxis. Three doses at 2, 4 and 6 months of age are given. This vaccine has been approved in USA and the European Union.

VIII. VIRUSES CAUSING GASTROENTERITIS

In addition to rotaviruses, other viruses that produce diarrhoea in children and adults include the Norovirus, Sapovirus, adenovirus, astrovirus, coronavirus Torovirus, Aichivirus, Picobirnavirus and Bocavirus (Table 64.1). *Norovirus* and *Sapovirus* genera cause mainly gastroenteritis in humans. They belong to family *Calciviridae*. They were also named "Norwalk-like viruses" and "Sapporo-like viruses" after the protype viruses, the Norwalk and Sapporo viruses respectively. Norwalk virus has been described below.

Table 64.1 Viruses causing gastroenteritis

Virus	Age group involved	Genome
Rotavirus	Children < 5 years	Double stranded RNA
Norovirus	All ages	Single stranded RNA
Sapovirus	Children	Single stranded RNA
Adenovirus	Children < 5 years	Double stranded DNA
Astrovirus	Children	Single stranded RNA
Coronavirus	All ages	Single stranded RNA
Torovirus	All ages	Single stranded RNA
Aichivirus	Both children and adults	Single stranded RNA
Picobirnavirus	Both children and adults	Double stranded RNA
Bocavirus	Mainly children but also adults	Single stranded RNA

A. Norwalk virus

It is a 27 nm virus and was first isolated from an epidemic of gastroenteritis in Norwalk, Ohio, in 1968. Epidemics of Norwalk virus diarrhoea due to consumption of raw oysters have been reported from Australia and America. Serological studies have shown that infection with this virus is widespread in many countries. It has been included in the family *Caliciviridae*. It is a small round RNA virus. It appears to be an important cause of diarrhoea in adults and children. Infection is acquired via faecal contamination of water and food.

Laboratory Diagnosis

(i) Electron microscopy

The virus can be detected in faeces by electron microscopy.

(ii) Antigen and antibody detection

ELISA test has been developed for detection of viral antigens and antibodies.

B. Other caliciviruses

As described earlier, *Norovirus* and *Sapovirus* are two important calciviruses causing gastroenteritis. These are heterogenous group of 'small round viruses' (30-38 nm) causing localised outbreaks of diarrhoea. Norovirus is highly contagious. They cause acute gastroenteritis in children but all ages can be infected. Sapoviruses cause disease mainly in children. Laboratory diagnosis is same as that of Norwalk virus. Human caliciviruses have not been grown on cultured cells.

C. Adenovirus

Adenovirus associated diarrhoea has been often seen in summer. These adenoviruses can be grown with difficulty in tissue culture. They have been designated types 40 and 41.

D. Astrovirus

Astroviruses are small (28-30 nm in diameter) spherical, RNA viruses with a star shaped outline. They belong to the family *Astroviridae*. They are transmitted by the faeco-oral route and cause diarrhoea principally in young children. The incubation period is 1–4 days.

Laboratory diagnosis

Demonstration of virus: The virus in the faeces can be demonstrated by immunoelectron microscopy and ELISA test.

E. Other Viruses

Other viruses which are implicated in acute gastroenteritis of humans include coronaviruses and toroviruses which

belong to family *Coronaviridae*; Aichivirus of family *Picornaviridae*; *Picobirnavirus* in the family *Picobirnaviridae* and *Bocavirus* of family *Parvoviridae*.

IX. VIRAL HAEMORRHAGIC FEVERS

Haemorrhagic manifestations may occur in patients suffering from several virus infections. These include:

1. Exanthematous fevers
- (*i*) Smallpox
- (*ii*) Chickenpox
- (*iii*) Measles.

2. Mosquito borne diseases
- (*i*) Yellow fever
- (*ii*) Dengue
- (*iii*) Chikungunya.

3. Tick borne fevers
- (*i*) Kyasanur forest disease (KFD)
- (*ii*) Omsk haemorrhagic fever
- (*iii*) Crimean-Congo haemorrhagic fever.

4. Arenaviruses
- (*i*) Lassa fever
- (*ii*) South American haemorrhagic fever.

5. Filovirus
- (*i*) Marburg virus
- (*ii*) Ebola virus.

6. Hantavirus
- (*i*) Hantaan virus
- (*ii*) Belgrade virus
- (*iii*) Seoul virus.

For details of these viruses, refer to respective chapters.

X. SLOW VIRUS DISEASES

A number of viral agents cause slow infections in animals and man, characterised by a very long incubation period, slow course, predilection for involvement of central nervous system and terminating fatally. The term *slow infection* was first proposed by *Sigurdsson* (1954), a veterinary pathologist for slowly progressive infections of sheep, such as scrapie, visna and maedi.

A. Characteristics of Slow Virus Infections

1. Long incubation period ranging from months to years
2. Course of illness lasting for months or years
3. Predilection for involvement of the central nervous system
4. Absence of immune response
5. Genetic predisposition
6. Fatal termination

Slow virus diseases may infect animals or human beings (Table 64.2).

Table 64.2 Diseases Caused by Slow Viruses

Disease	Host	Virus
Animal infections		
1. Visna	Sheep	Retrovirus
2. Maedi	Sheep	Retrovirus
3. Scrapie	Sheep	Prion
4. Mink encephalopathy	Mink	Prion
Human infections		
1. Creutzfeldt-Jakob disease (CJ disease)		
(i) Sporadic CJ Disease	Man	Prion
(ii) Inherited CJ Disease		
(a) Gerstmann-Straussler-Scheinker syndrome (GSS)	Man	Prion
(b) Fatal familial insomnia (FFI)	Man	Prion
(iii) Acquired CJ Disease		
(a) Variable CJ disease	Man	Prion
(b) Iatrogenic CJ disease	Man	Prion
2. Kuru	Man	Prion
3. SSPE	Man	Measles, Rubella virus
4. PML	Man	Papova virus (BK, JC, SV40)

B. Prions

Now it is clear that all human infections and some animal infections are due to prions. Prions are unique in being protein in nature, devoid of DNA and RNA, and unusually resistant to physical and chemical agents. *Stanley B. Prusiner* was awarded the Nobel prize in 1997 for discovery of prions.

The pathogenic mechanism seems to be proliferation of an abnormal prion protein (PrP^{sc}) which is derived from the normal prion protein (PrP^{c}). The accumulation of PrP^{sc} in the central nervous system disrupts the architecture and function of the brain, causing disease. In humans, these diseases are named as "Transmissible spongiform encephalopathies".

C. Animal Infections

1. Visna

It is a demyelinating disease of sheep with an incubation period of about two years. It involves mainly brain, lungs and reticuloendothelial system. The disease has an insidious onset with pareses, progressing to total paralysis and death. The virus can be isolated from CSF,

saliva or blood of affected animals, by growing in sheep choroid plexus tissue cultures. CNS lesions appear to be an antigen-antibody reaction on the surface of infected glial cells.

2. Maedi

It is a slowly progressive fatal haemorrhagic pneumonia of sheep. The incubation period is about 2-3 years. Viruses of visna and maedi are closely related and belong to the subfamily lentivirinae. Human immunodeficiency virus (HIV), the causative agent of AIDS, also shows many features of a slow virus disease.

3. Scrapie

It is a slow virus infection of sheep. The incubation period is two years. It remains confined to nervous system. The diseased animals are irritable and later die of paralysis. Different breeds of sheep show marked genetic differences in susceptibility to infection. The causative agent has been maintained in brain tissue explant cultures through serial passages. The agent is reported to be a prion. Scrapie can be transmitted to sheep and other experimental animals by injection of suspensions of brain and spinal cord from affected animals.

4. Mink Encephalopathy

It resembles scrapie of sheep in all respects except that it occurs in minks. The causative agent may be a strain of scrapie virus, transferred to mink by feeding on scrapie infected sheep meat.

5. Bovine Spongiform Encephalopathy (BSE, 'mad cow disease')

It had been enzootic in U.K. from 1986 and by 1995 there had been about 1,50,000 cases. This disease is similar to scrapie. The outbreak in U.K. was due to infection of cattle with scrapie agent via feed which contained meat infected with scrapie agent.

D. Human Infections

1. Creutzfeldt-Jakob Disease (CJ disease)

CJ disease is caused by a prion. It is a subacute presenile encephalopathy, characterised by progressive dementia due to spongiform degeneration of the brain.

CJ disease has been divided into *sporadic CJ disease*, *inherited CJ disease* and *acquired CJ disease*.

(i) Sporadic CJ disease

It presents as a rapidly progressive dementia, usually leads to death within 6 months. Age at onset of disease is 60-70 years.

(ii) Inherited CJ disease

It has been divided into three types: genetic CJ disease, Gerstmann-Straussler-Scheinker Syndrome (GSS) and fatal familal insomnia (FFI). The mode of inheritance in all of these diseases, is autosomal dominant and it occurs by mutations in PrP^C gene. Age at onset of disease is 50-60 years. Disease duration is 6 months in genetic CJ disease, 5-6 years in GSS and 13-15 months in FFI.

(iii) Acquired CJ disease

It is of two types: variant CJ disease and iatrogenic CJ disease.

(a) Variant CJ disease

It is relatively new member of human prion diseases, first reported in 1996 in Britain. It differs from classical CJ disease in affecting young persons (26 years) as compared to 60 years in classical CJ disease. Disease duration is 14 months. Studies have indicated that variant CJ disease occurs by transmission of bovine spongiform encephalopathy (BSE) prions to humans. In 1996, many thousands of British cattle suffering from BSE had to be slaughtered in Britain.

(b) Iatrogenic CJ disease

Iatrogenic CJ disease is caused by accidental transmission of prion during medical or neurological procedures such as implantation of human duramater or treatment with human cadaveric pituatory extrcts. It is a rare disease. It affects mainly younger persons of less than 39 years. Disease duration is similar to sporadic CJ disease.

2. Kuru

Kuru is caused by a prion. It is a disease which occurs only in Fore tribe of the eastern highlands of New Guinea. The incubation period is 5-10 years. It is transmitted by the tribal practice of eating infected brains of relatives after ritual 'non-sterilising' cooking. Symptoms include progressive cerebellar ataxia and tremors. It is not accompanied by any biochemical or cellular change in the blood or CSF nor by any inflammatory or immune response. There is degeneration of grey matter of central nervous system, most marked in cerebellum. The disease ends fatally in 3-6 months. The disease is predominantly seen in women and children because only they partook the brain of carcasses. The disease has disappeared following the prohibition of ritual cannibalism. The disease can be produced experimentally in chimpanzees by inoculation of brain material from Kuru victims, after incubation period of 1-3 years. *Carlton Gajdusek* was awarded the Nobel prize in 1976 for his contributions on Kuru.

3. Subacute Sclerosing Pan-encephalitis (SSPE)

It is a very rare delayed sequel to infection with measles virus, many years after the initial infection. The disease is characterised by slowly progressive demyelination in the central nervous system with gradual deterioration of mental and motor functions. Death occurs 1-3 years after onset of symptoms.

Brain cells from SSPE patients show electron microscopic and serological evidence of measles virus infection, but the virus cannot be isolated by routine cultures. A defective virus resembling measles virus has been isolated from brain material by co-cultivation with HeLa cells. Patients show very high titres of measles virus antibody in serum. Antibody is regularly found in CSF and is pathognomonic. Cell mediated immunity to measles virus is absent in SSPE patients.

SSPE may also develop as a very rare late complication of live measles virus vaccination. Similar illness may rarely occur as a complication of rubella infection.

4. Progressive Multifocal Leucoencephalopathy (PML)

It is a rare subacute demyelinating disease seen in old patients whose immune system is impaired as a result of malignancy or immunosuppression. The disease results from infection of oligodendrocytes by *Papovavirus*. It is characterised by progressive loss of motor functions, vision and speech. Death occurs in 3-4 months. A papovavirus has been demonstrated by electron microscopy and cultured from brain material of patients.

KEY POINTS

1. Papovaviruses (from *pa*, papilloma, *po*, polyoma, *va*, vacuolating agent) are double stranded DNA viruses. There are two genera, *Papillomavirus* and *Polyomavirus*. They induce both lytic infections and tumours.
2. Papillomaviruses cause *cutaneous warts*, *genital warts*, *recurrent respiratory papillomatosis*, *oral papillomatosis* and *cervical cancers*.
3. *Polyomaviruses* include *mouse polyomavirus*, *simian virus 40* (SV40), *JC polyomavirus* and *BK polyomavirus*.
4. Parvoviruses are very small (about 20 nm), non-enveloped, icosahedral viruses containing a single stranded DNA.
5. Parvovirus B19 has been shown to be associated with aplastic crisis in sickle cell disease and other haemolytic anaemias. It also causes *fifth disease*.
6. Coronaviruses are spherical or pleomorphic, enveloped RNA viruses, containing petal or club shaped peplomers on the surface. Only two species of the genus *Coronavirus*, human coronavirus 229E and human coronavirus OC43, are responsible for human respiratory disease. '*Severe acute respiratory syndrome*' (*SARS*) is a disease caused by a new coronavirus different from other coronoviruses.
7. Rubella virus is a pleomorphic, roughly spherical, enveloped RNA virus. It has single stranded RNA genome and envelope carries haemagglutinin peplomers. It causes *rubella* or *German measles*. It may be acquired congenitally or postnatally.
8. *Measles-mumps-rubella* (*MMR*) vaccine is recommended for prophylaxis. It is given at age of 15 months, followed by a booster at the age of 4-6 years.
9. The name *Reoviridae* is derived from the '*Respiratory Enteric Orphan Virus*' because of their frequent occurrence in respiratory and enteric tracts, and it was considered an 'orphan' as its pathogenicity to humans was not proven. *Rotavirus* is an important member of this group of viruses. It is the commonest cause of *gastroenteritis in children*.
10. Rotavirus (*Rota*, meaning wheel) is a double shelled icosahedral capsid resembling a wheel with short spokes radiating from a wide hub to a clearly defined outer rim. The genome consists of double stranded segmented RNA.
11. Laboratory diagnosis of rotavirus diarrhoea can be done by *demonstration of virus* in *faeces* by *ELISA*, *latex agglutination test*, *CIEP* and *electron microscopy*.
12. In addition to rotaviruses, other viruses that produce diarrhoea in children and adults include the *Norwalk virus*, *adenovirus*, *astrovirus* and *caliciviruses*.
13. A number of viral agents cause slow infections in animals and man, characterised by a *very long incubation period*, slow course, predilection for *involvement of central nervous system* and terminating fatally.
14. Human slow virus infections include *Creutzfeldt-Jakob disease* (*CJ disease*), *subacute sclerosing panencephalitis* (*SSPE*) and *progressive multifocal leucoencephalopathy* (*PML*).
15. CJ disease is caused by *prion*. SSPE is a very rare delayed sequel to infection with measles virus, many years after the initial infection. *Papova virus* is responsible for PML.

YOU MUST KNOW

1. Human papilloma viruses and their association with cervical cancers.
2. SV 40.
3. JC polyomavirus.
4. BK polyomavirus.
5. Parvovirus.
6. Severe acute respiratory syndrome (SARS).
7. Lassa fever.
8. Rubella virus.
9. Structure of rotavirus.
10. Laboratory diagnosis of rotavirus infections.
11. List of viruses causing gastroenteritis.
12. List of slow viruses causing human infections.

STUDY QUESTIONS

1. Describe the pathogenesis and laboratory diagnosis of infections caused by Papillomaviruses.
2. Write short notes on:
   (a) SV 40
   (b) JC polyomavirus
   (c) BK polyomavirus
   (d) Parvovirus
   (e) Coronavirus
   (f) Ebola virus
   (g) Rubella virus
   (h) Rotaviruses
   (i) Viruses causing gastroenteritis
   (j) Slow virus diseases
   (k) CJ disease
   (l) SSPE.
   (m)Lymphocytic choriomeningitis virus (LCM)
   (n) Severe acute respiratory syndrome (SARS)

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Which of the following viruses can cause cervical cancers?
   (a) Human papilloma virus
   (b) Herpes simplex virus
   (c) Epstein-Barr virus
   (d) Norwalk virus
2. Which of the following methods can be used for diagnosis of human papilloma virus infections?
   (a) Electron microscopy
   (b) Immunocytochemistry
   (c) Detection of viral nucleic acid
   (d) All of the above
3. Which of the following viruses is associated with progressive multifocal leucoencephalopathy?
   (a) Simian virus 40
   (b) BK polyomavirus
   (c) JC polyomavirus
   (d) Mouse polyomavirus
4. Which of the following viruses is defective virus?
   (a) Adeno-associated virus
   (b) Coronaviruses
   (c) Herpes simplex virus
   (d) Rotaviruses
5. Which of the following viruses can cause erythema infectiosum?
   (a) Coronaviruses
   (b) Parvovirus B 19
   (c) BK polyomavirus
   (d) JK polyomavirus
6. Which of the following tests can be used for diagnosis of severe acute respiratory syndrome (SARS)?
   (a) Electron microscopy
   (b) Growth in Vero cell culture
   (c) Cloning
   (d) All of the above

7. Lassa fever is caused by:
   (a) Arenaviruses (b) Coronaviruses
   (c) Parvoviruses (d) Rubivirus
8. All of the following viruses can cause haemorrhagic fevers except:
   (a) Junin virus (b) Machupo virus
   (c) Ebola virus (d) Norwalk virus
9. German measles is caused by:
   (a) Measles virus (b) Rubella virus
   (c) Ebola virus (d) Parvovirus
10. Which of the following can cause congenital infections?
   (a) *Toxoplasma gondii* (b) Rubella virus
   (c) Cytomegalovirus (d) All of the above
11. Which of the following viruses can cause diarohoea?
   (a) Rotaviruses (b) Norwalk virus
   (c) Adenoviruses (d) All of the above
12. What type of nucleic acid is there in rotavirus?
   (a) Single stranded RNA (b) Double stranded RNA
   (c) Single stranded DNA (d) Double stranded DNA
13. Which of the following methods can be used for diagnosis of rotavirus infection?
   (a) ELISA (b) Latex agglutination test
   (c) Electron microscopy (d) All of the above
14. Which of the following slow virus diseases occur/s in humans?
   (a) Creutzfeldt Jakob disease (b) Kuru
   (c) Gerstmann-Straussler-Scheinker syndrome (d) All of the above
15. Which of the following slow virus diseases does not occur in humans?
   (a) Visna (b) Kuru
   (c) Creutzfeldt-Jakob disease (d) Fatal familial insomnia
16. Who discovered prions?
   (a) Carlton Gajdusek (b) Stanley B. Prusiner
   (c) Sigurdsson (d) Karry mullis
17. Subacute sclerosing panencephalitis is a late complication of:
   (a) Hepatitis C infection (b) Measles
   (c) Mumps (d) Rubella

Answers (MCQs):

1. (a)	2. (d)	3. (c)	4. (a)	5. (b)
6. (d)	7. (a)	8. (d)	9. (b)	10. (d)
11. (d)	12. (b)	13. (d)	14. (d)	15. (a)
16. (b)	17. (b)			

◆◆◆

Chapter 65

ONCOGENIC VIRUSES

Viral aetiology of fowl leukaemia was first discovered in 1908 by *Ellerman and Bang*. In 1911, *Rous* showed that a virus caused fowl sarcoma, a discovery for which he was awarded the Nobel prize in 1966. It has now been estimated that 20% of human tumours have a viral risk factor.

Viruses that produce tumours in their natural hosts or in experimental animals, or induce malignant transformation of cells in culture, are known as *oncogenic viruses* (Table 65.1). Many genera of DNA viruses are found to produce tumours whereas only one family of RNA viruses *(Retroviridae)* can do so. These oncogenic RNA viruses were formerly named as *oncornaviruses*.

I. INTERACTION OF ONCOGENIC VIRUSES WITH HOST CELLS

Oncogenic viruses cause mutation-like changes in the host cells by transformation. Transformation represents the various changes that accompany the conversion of a normal cell into the malignant cell. Transformed culture cells exhibit some special properties.

Properties of cells transformed by viruses:

1. Alteration in shape
2. Increased growth rate
3. Loss of contact inhibition so that, instead of growing as monolayer, they grow piled up, one over another, forming 'microtumours'.
4. Appearance of new virus specified antigens (T antigen–TSTA)
5. Capacity to induce tumours in susceptible animals

II. MECHANISMS OF VIRAL ONCOGENESIS

In case of oncogenic DNA viruses, the viral DNA (or portion of it) is integrated with the host cell genome as prophage. As the integrated viral DNA is incomplete or 'defective', no infectious virus is produced. However, under its influence, the host cell undergoes neoplastic transformation.

Table 65.1 Oncogenic Viruses

I. DNA viruses
A. Papovavirus
1. Human papilloma virus (HPV)
2. Polyoma virus
3. Simian virus 40 (SV40)
4. BK and JC viruses
B. Herpesvirus
1. Herpes simplex virus (HSV) types 1 and 2
2. Cytomegalovirus (CMV)
3. Epstein-Barr (EB) virus
4. Marek's disease virus
5. Herpesvirus saimiri
C. Hepatitis B virus
D. Poxvirus
1. Molluscum contagiosum
2. Shope fibroma
3. Yaba virus
E. Adenovirus
II. RNA viruses
A. Retroviruses
1. The Avian sarcoma leukosis complex
2. Murine leukosis viruses
3. Mammary tumour virus of mice
4. Leukosis-sarcoma viruses of various animals
5. Human T cell leukaemia viruses
B. Hepatitis C virus

In the case of oncogenic retroviruses, the viral RNA is converted into double stranded DNA form by the action of an enzyme reverse transcriptase (RNA directed DNA polymerase) which is characteristic of all retroviruses. This double stranded DNA form of the retrovirus genome is called *provirus* which becomes integrated with the cell genome. The integrated provirus may remain latent for variable periods. Only when activated, it acts as a template for viral RNA synthesis and also induces cell transformation. The standard oncogenic retroviruses, such as chronic leukaemia viruses, are generally slow transforming viruses i.e. they cause malignancy after a long latent period. In contrast, *the acute transforming viruses*, are highly oncogenic and induce malignant change after a short latent period. However, most acute transforming viruses are *replicative defective* because they carry on their genome an additional gene (*V–onc gene*, refer 'oncogenes' *vide infra*) which replaces some of the genes essential for viral replication. Such V-onc viruses replicate only if co-infected with a standard helper retrovirus. However, Rous sarcoma virus is an exception among acute transforming viruses. It can replicate without a helper retrovirus because it possesses all the genes essential for viral replication even when it carries the oncogenic *src* gene.

Oncogenes

Genes that are responsible for the induction of tumours are called *oncogenes*. Many of them not only play an important role in malignant transformation but also in normal cell growth. The oncogenes present in viruses are called *viral oncogenes (V–onc)*. Genes with sequence similar to viral oncogenes exist in host cell also, not only in cancer cells but also in normal cells of man and animals. These oncogenes isolated from cancer cells are called *cellular oncogenes (C–onc)* and are not of viral origin (Table 65.2). Similar genes found in normal cells are called *proto-oncogenes*. Cellular oncogenes are believed to have some important controlling function on cell growth and regulation.

It appears that viral oncogenes are of host cell origin. It is believed that viruses may have picked up the proto-oncogenes from host cells during infection at some remote time during evolution. It has been suggested that cancer results when cellular genes overact following infection with oncogenic viruses.

Table 65.2 Oncogenes

Viral oncogene	Origin	Tumour in natural host	Human gene	Chromosomal location in human beings
V-*src*	Chicken	Sarcoma	C-*src*	20
V-*ras*	Rat	Sarcoma	C-*ras*	11
V-fes	Cat	Sarcoma	C-*fes*	15
V-*myc*	Chicken	Myelomatosis	C-*myc*	8
V-*sis*	Monkey	Sarcoma	C-*sis*	22
V-*mos*	Mouse	Sarcoma	C-*mos*	8

V–viral, C–cellular, *src*-sarcoma of chicken, *ras*–rat sarcoma, *myc*-myelomatosis of chicken, *sis*–simian sarcoma, *fes*–feline sarcoma, *mos*-mouse sarcoma

Proto-oncogenes serve some essential functions in normal cells. They have been found to code for proteins involved in regulating cell growth and differentiation. For example, the oncogene *src* is related to tyrosine-specific protein kinases and *myc* to DNA binding proteins.

Oncogenes can be studied by a method named *transfection*. Certain mouse fibroblast cell lines (e.g. NIH 3T3) can take up foreign DNA, incorporate them into their genome and express them. This is called *transfection*.

A new class of human cancer genes called *tumour suppressor genes, growth suppressor genes* or *anti-oncogenes* have been identified. This gene has to be inactivated for tumour formation in contrast to activation of cellular oncogenes. Retinoblastoma (Rb) gene is a prototype of the suppressor class of genes. Loss of this gene leads to development of retinoblastoma.

III. ONCOGENIC DNA VIRUSES

A. Papovaviruses

Papilloma viruses produce benign warts and papillomas in their natural hosts. Human papilloma virus (HPV) has been incriminated in premalignant lesions of the female and male genital tract. Cancer of cervix uteri is one of the important cancers associated with HPV types 16 and 18.

The polyoma virus causes natural latent infection in laboratory and domestic mice. However, it induces tumours when injected into infant mice or other rodents. In 2008, Chang and Moore alongwith other colleagues discovered the association of Merkel cell polyomavirus with Merkel cell carcinoma in humans. It is a rare skin cancer. This is the first polyomavirus to be well established as cause of human cancer.

Simian virus 40 (SV 40) causes an inapparent infection in rhesus and cynomolgus monkeys but does not cause cytopathic effects in cell cultures derived from these monkeys. However, when fluid from such cultures is inoculated into cell cultures from African green monkeys, cytopathogenic changes with cytoplasmic vacuolation results. Injection into newborn hamster produces tumours. SV40 was discovered in monkey kidney cultures used for production of polio vaccine. However, there is no evidence that injection of polio vaccine containing SV40 virus has induced cancer in man.

BK and JC viruses cause asymptomatic human infections but can induce tumours in immunodeficient subjects.

B. Herpes Virus

Herpes simplex virus and cytomegalovirus have been found to transform cultured cells. In human beings, *herpes simplex type 2* has been implicated in cancer of uterine cervix. It is believed that the virus causes insertional mutagenesis. It has been suggested that herpes simplex type 1 may be associated with cancer of the lip. Herpesvirus type 8 has been implicated in Kaposi's sarcoma. *Cytomegalovirus* infection has been suggested to be associated with Kaposi's sarcoma and carcinoma of prostate. Epstein-Barr (EB) virus is associated with Burkitt's lymphoma and nasopharyngeal carcinoma in the Chinese male population in south-east Asia and East Africa. EB virus is believed to transform normal lymphocytes into lymphoblasts in children whose immune system is immunocompromised. Cells that grow indefinitely carrying EB virus express an antigen EBNA (Epstein-Barr nucleic antigen) which is virus specific.

Other herpesviruses have also been associated with natural cancer in animals. Marek's disease is a neurolymphomatosis of chickens. The disease can be induced in young chickens by the injection of the virus. It can be prevented by live avirulent vaccine. This is the first instance when a malignant disease can be controlled by a viral vaccine. *Herpesvirus saimiri* causes fatal lymphoma or reticulum cell sarcoma when injected into owl monkeys or rabbits.

C. Hepatitis B Virus

Hepatitis B virus is strongly implicated in the development of hepatocellular carcinoma. Tumour cells contain *Hepatitis B virus* DNA which probably acts indirectly by insertional mutagenesis.

D. Poxvirus

Yaba virus causes naturally occurring benign histiocytomas in monkeys and *Shope fibroma viruses* produce fibromas in rabbits. In humans, benign growth is produced by *Molluscum contagiosum* virus.

E. Adenovirus

Though some types (12, 18, 31) of human adenoviruses may produce sarcomas in hamsters after experimental inoculation, they do not appear to have any association with human cancer.

IV. ONCOGENIC RNA VIRUSES

All oncogenic RNA viruses except Hepatitis C virus belong to the family *Retroviridae* but all retroviruses are not oncogenic. The oncogenic retroviruses are mainly responsible for leukaemias and sarcomas in their hosts. Hepatitis C virus has been associated with primary hepatocellular carcinoma. Oncogenic RNA viruses include

oncogenic retroviruses (subheadings A to E below) and hepatitis C virus (subheading F) as follows.

A. The Avian Sarcoma Leukosis Complex

These are antigenically related viruses which induce avian leukosis (lymphomatosis, myeloblastosis and erythroblastosis viruses) or sarcoma in fowls (Rous sarcoma virus).

B. Murine Leukosis Viruses

This group includes several strains of murine leukaemia and sarcoma viruses.

C. Mammary Tumour Virus of Mice

This virus occurs in certain strains of mice with higher natural incidence of breast cancer. It used to be known as the 'Bittner virus' or 'milk factor'. Mammary cancer develops only in susceptible strains of mice, after a latent period of 6-12 months.

D. Leukosis-Sarcoma Viruses of Other Animals

A number of viruses have been isolated from leukosis and sarcomas in various species of animals like cat, rat, guinea pig, hamster and monkey.

E. Human T Cell Leukaemia Viruses (HTLV)

These viruses produce T cell leukaemia in human beings. HTLV-I causes adult T-cell leukaemia and HTLV-II induces hairy cell leukaemia of T-cell type.

F. Hepatitis C Virus

Hepatitis C virus infection has also been reported to lead to hepatocellular carcinoma.

V. VIRUSES ASSOCIATED WITH HUMAN CANCER

Viruses which are strongly associated with human cancers are listed in Table 65.3.

Table 65.3 Viruses Associated with Human Cancer

Virus	Associated cancers
DNA Viruses	
Human papilloma viruses (HPV)	Genital (cervical, vulvar, penile) warts, may progress to carcinoma
Epstein-Barr virus (EBV)	Nasopharyngeal carcinoma, Burkitt's lymphoma, B-cell lymphoma
Human hepesvirus 8 (HHV-8)	Kaposi's sarcoma
Hepatitis B virus (HBV)	Primary hepatocellular carcinoma
Merkel cell polyomavirus	Merkel cell carcinoma
RNA Viruses	
Human T cell leukaemia virus (HTLV-I)	T-cell leukaemia / lymphoma
Hepatitis C virus (HCV)	Primary hepatocellular carcinoma

KEY POINTS

1. Viruses that produce tumours in their natural hosts or in experimental animals, or induce malignant transformation of cells in culture, are known as *oncogenic viruses*.
2. Oncogenic viruses cause mutation – like changes in the host cells by transformation. Transformation represents the various changes that accompany the conversion of a normal cell into the malignant cell.
3. In case of oncogenic DNA viruses, the viral DNA (or portion of it) is integrated with the host cell genome as prophage. As the integrated viral DNA is incomplete or 'defective', no infectious virus is produced. However, under its influence, the host cell undergoes neoplastic transformation.
4. In the case of oncogenic retroviruses, the viral RNA is converted into double stranded DNA form by the action of an enzyme reverse transcriptase. This double stranded DNA form of retrovirus genome is called *provirus* which becomes integrated with the cell genome. The integrated provirus may remain latent for variable periods. Only when activated, it acts as a template for viral RNA synthesis and also induces cell transformation.
5. Genes that are responsible for the induction of tumours are called *oncogenes*. The oncogenes present in viruses are called *viral oncogenes* (*V-onc*). The oncogenes isolated from cancer cells are called *cellular oncogenes* (*C-onc*) and are not of viral origin.

6. *Papilloma virus*, *Epstein-Barr virus* (*EBV*), *hepatitis B virus* (*HBV*), *hepatitis C virus* (*HCV*), *Merkel cell polyoma virus* and *human T-cell leukaemia virus* (*HTLV-1*) are some viruses which are strongly associated with human cancers.

YOU MUST KNOW

1. List of oncogenic viruses.
2. Oncogenes and mechanisms of viral oncogenesis.
3. List of viruses associated with human cancers.

STUDY QUESTIONS

1. Name different oncogenic viruses and discuss the mechanisms of viral oncogenesis.
2. Write short notes on:
   (a) Oncogenes (b) Viruses associated with human cancer.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Which of the following viruses can be oncogenic?
   (a) Polyomavirus (b) Simian virus 40
   (c) Marek's disease virus (d) All of the above
2. Which of the following viruses are associated with human cancer?
   (a) Human papilloma viruses (b) Human herpesvirus 8
   (c) Hepatitis C virus (d) All of the above

Answers (MCQs):

1. (d) 2. (d)

UNIT V

MYCOLOGY

Chapter 66

MEDICAL MYCOLOGY

I. INTRODUCTION

1. Study of fungi is called *Mycology*.
2. Name is derived from *Mykos* meaning mushroom.
3. All fungi are eukaryotic.
4. Water, soil and decaying organic debris are natural habitat.
5. Fungi are obligate or facultative aerobe.
6. They are chemotrophic organisms i.e. obtaining their nutrients from chemicals in nature.
7. Their cell walls contain chitin, mannan and other polysaccharides.
8. Cytoplasmic membrane contains sterols.
9. They divide asexually, sexually or by both processes.
10. Some fungi are useful to man such as edible mushrooms. Certain yeasts are used in fermentation of fruit juices and some fungi in elaborating antibiotics *(Penicillium)*.

II. DIFFERENCES OF FUNGI FROM BACTERIA

1. They possess rigid cell walls containing chitin, mannan and other polysaccharides.
2. The cytoplasmic membrane contains sterols.
3. The cytoplasm contains true nuclei with nuclear membrane, mitochondria and endoplasmic reticulum.

4. Fungi may be unicellular or multi-cellular.
5. They divide asexually, sexually or by both processes.

III. CLASSIFICATION OF FUNGI

A. Taxonomical Classification

Fungi are placed in the phylum *Thallophyta*. There are four classes of fungi. Their characteristics are described in a 'flow chart' shown below.

B. Morphological Classification

Based on the morphology, there are four main groups of fungi (Fig. 66.1).

1. Yeasts
2. Yeasts like fungi
3. Moulds
4. Dimorphic fungi

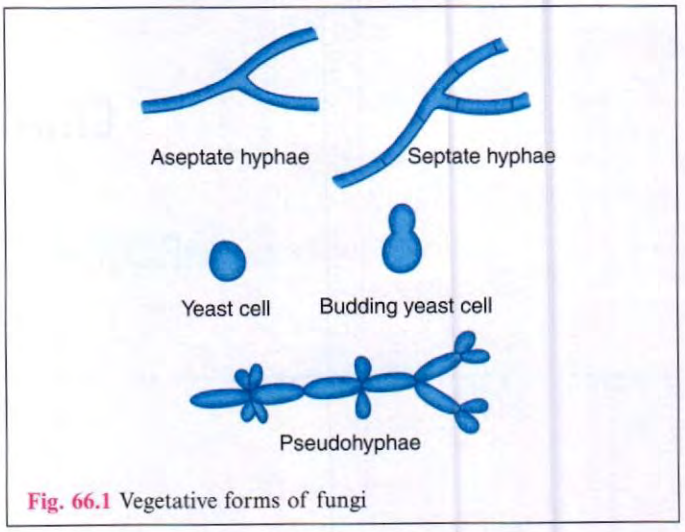


Fig. 66.1 Vegetative forms of fungi

1. Yeasts

(*i*) Round to oval unicellular fungi.
(*ii*) Reproduce by budding.
(*iii*) Form creamy mucoid colonies on culture media.
(*iv*) The important pathogenic yeast is *Cryptococcus neoformans*.

2. Yeasts Like Fungi

These yeasts grow partly as yeasts and partly as chains of elongated budding cells joined end to end forming *pseudohyphae*. Example is *Candida albicans*.

3. Moulds

(*i*) They grow as branching filaments called hyphae usually 2-10 μm in width. Hyphae may be septate or non-septate.
(*ii*) The hyphae continue to grow and branch to form tangled mass of growth known as *mycelium*.
(*iii*) *Aerial mycelium*—In the culture medium, the part of the mycelium which projects above the surface is called aerial mycelium.
(*iv*) *Vegetative mycelium*—The part of mycelium growing in the culture medium is called vegetative mycelium.
(*v*) They reproduce by formation of different types of sexual and asexual spores that develop from the mycelium.
(*vi*) Dermatophytes, *Aspergillus, Penicillium* and *Rhizopus* are few examples of moulds.

4. Dimorphic Fungi

(*i*) Dimorphic fungi exist as yeasts in the host tissue and in the cultures at 37°C and as hyphal (mycelial) forms in the soil and in the cultures at 22-25°C.

FLOW CHART

Thallophyta
(Irregular plant masses lacking definite root, stem and leaf structures)

- Fungi (No chlorophyll)
  - 4 Classes
    - **Zygomycetes**
      - Lower fungi having non-septate hyphae.
      - Forms asexual spores called sporangiospores.
      - Sexual spores are known as zygospores and oospores.
    - **Ascomycetes**
      - Septate hyphae
      - Sexual spores (ascospores) are present within a sac or ascus.
    - **Basidiomycetes**
      - Septate hypha
      - Sexual spores are basidiospores on a basidium.
    - **Deuteromycetes or fungi imperfecti**
      - Septate hyphae
      - Lack a known sexual state. Most fungi of medical importance belong to this class.
- Algae (Chlorophyll)

(*ii*) *Blastomyces dermatitidis, Paracoccidioides brasiliensis, Coccidioides immitis, Histoplasma capsulatum* and *Sporothrix schenckii* are examples of dimorphic fungi.

(*iii*) Most of the systemic infections are due to dimorphic fungi.

IV. REPRODUCTION AND SPORULATION

1. Fungal spores are of two types, sexual and asexual spores.
2. Sexual spores are of four types—oospore, ascospore, zygospore and basidiospore (Fig. 66.2).
3. Sexual spore is formed by fusion of cells and meiosis as in all forms of higher life.
4. Asexual spores are produced by mitosis. These may be *vegetative spores* or *aerial spores.*

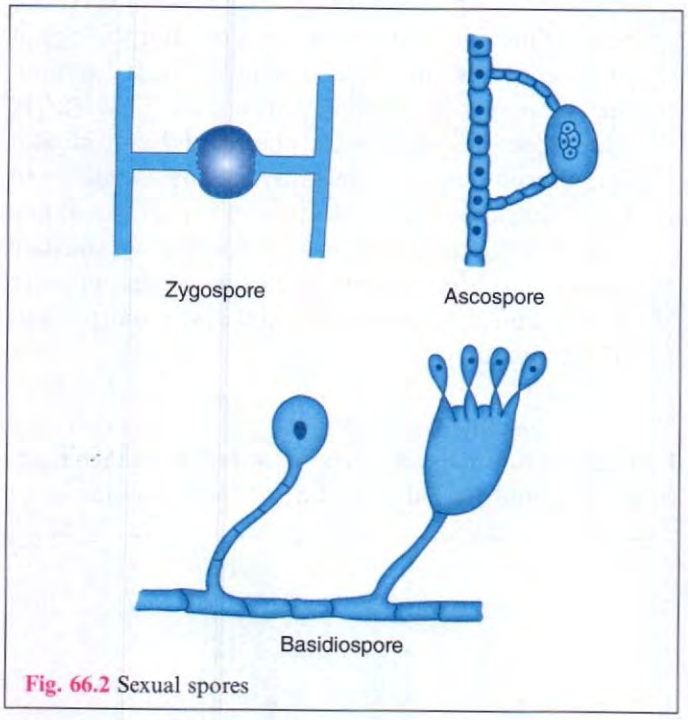


Fig. 66.2 Sexual spores

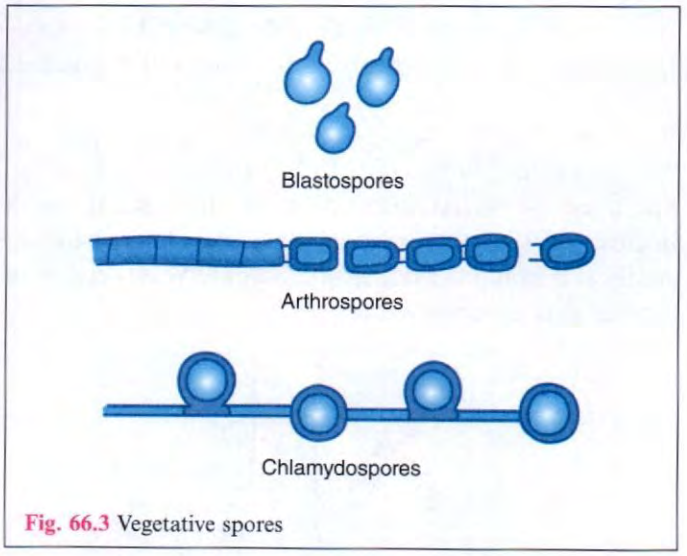


Fig. 66.3 Vegetative spores

5. Vegetative spores (Fig. 66.3)

Blastospores: These are formed by budding from parent cell, as in yeasts.

Arthrospores: These are formed by the production of cross-septa into hyphae resulting in rectangular thick-walled spores.

Chlamydospores: These are thick-walled resting spores developed by rounding up and thickening of hyphal segments.

6. Aerial spores (Fig. 66.4)

Conidiospores: Spores borne externally on sides or tips of hyphae are called *conidiospores* or simply conidia.

Microconidia: When conidia are small and single, these are called *microconidia.*

Macroconidia: These are large and septate conidia and are often multicellular.

Sporangiospores: These are spores formed within the sporangium. They develop on the ends of hyphae called sporangiophores. Examples are *Mucor* and *Rhizopus.*

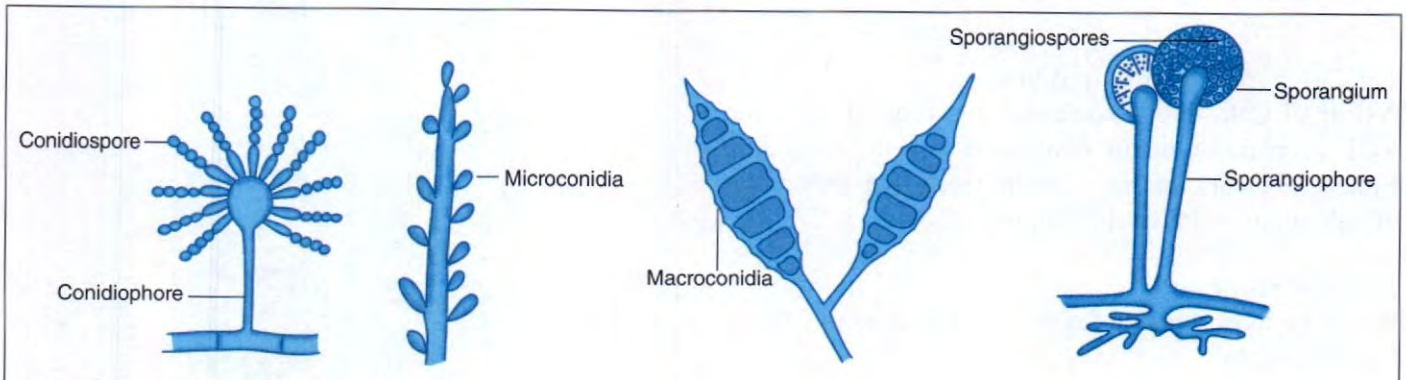


Fig. 66.4 Aerial spores

V. LABORATORY DIAGNOSIS

Laboratory diagnosis of mycoses consists of following:

A. Direct Microscopy

1. Potassium Hydroxide (KOH) Preparation

Specimen is placed in a drop of 10% KOH on a microscopic slide and covered with a coverslip. It is heated gently and examined under microscope. Yeasts cells and hyphae may be observed (Fig. 66.5).

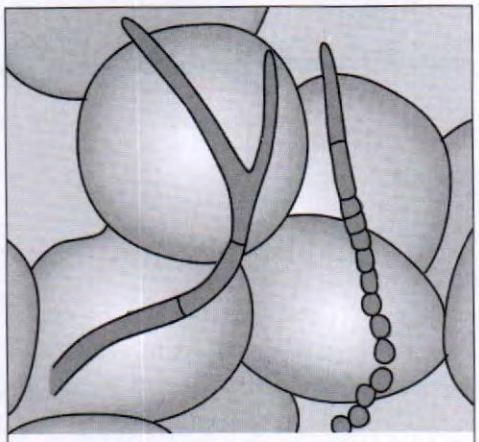


Fig. 66.5 KOH preparation showing hyphae

Hyphal diameter, presence or absence of septa and of special structures help in diagnosis. Special hyphal structures frequently found are spring like helical coils (*spiral hyphae*), resembling tennis racquets (*racquet hyphae*) and numerous short branches appearing at the ends of hyphae (*favic chandelier*) (Fig. 66.6).

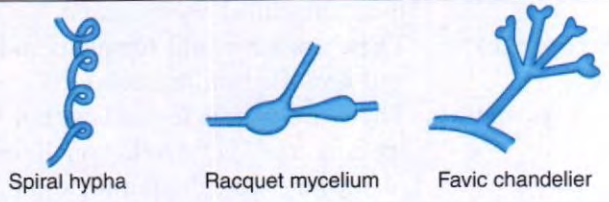


Fig. 66.6 Different forms of hypha

2. KOH with Calcofluor White

A drop of Calcofluor white solution can be added to the KOH preparation before covering it with the cover slip. Fungal elements fluoresce bright green due to binding of calcofluor white to the fungus.

3. Gram Staining

It is done to observe Gram positive yeasts as in case of Candida species.

4. India Ink Preparation

India ink preparation may be used for detection of capsulated yeast such as *Cryptococcus neoformans* in cerebrospinal fluid (CSF).

B. Culture

1. Sabouraud's dextrose agar (SDA) and SDA medium with antibiotics are inoculated and incubated at 25°C and 37°C for three weeks (Fig. 66.7). Chloramphenicol is added in the culture medium to suppress the growth of contaminating bacteria while cycloheximide (actidione) is incorporated to suppress the contaminating fungi.
   Brain heart infusion (BHI) agar with blood and antibiotics is another medium used for primary isolation of fungi.
2. Growth on the medium is identified by rapidity of growth, colour and morphology of the colony on the obverse and pigmentation on the reverse.
3. Microscopy is performed from fungal colony (in teased mounts or slide cultures) to study the morphology of hyphae, spores and other structures. Teased mounts are prepared in lactophenol cotton blue (LCB) which contains lactic acid, phenol and cotton blue. The morphology of asexual spores or conidia is of diagnostic importance. The type of spore formation is distinctive for different fungi. Morphology of different spores has been described earlier in this chapter.
4. Slide culture is done for studying the exact morphology of the fungus.

C. Tissue Sections

Fungal elements in tissue can be identified by methenamine silver stain and Periodic Acid Schiff (PAS) stain.

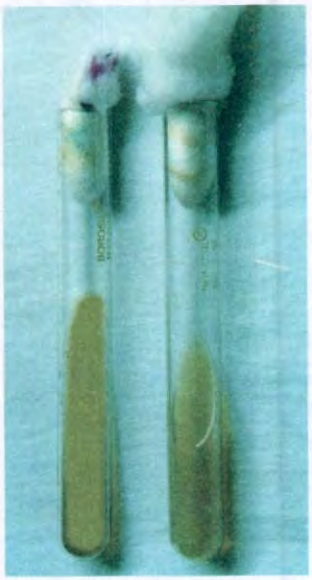


Fig. 66.7 Sabouraud's dextrose agar (SDA) slope with and without antibiotics

VI. CLASSIFICATION OF FUNGAL DISEASES

Infection caused by fungus is known as mycoses. Fungal infections are of three principal clinical types: superficial mycoses, subcutaneous mycoses and systemic mycoses.

Superficial Mycoses

1. These are strictly surface infections involving skin, hair, nail and mucosa.
2. These include infections by dermatophytes, *Pityrosporum orbiculare* (Pityriasis versicolor), *Exophiala werneckii* (tinea nigra), *Piedria hortae* (black piedra) and *Trichosporon beigelii*.

Subcutaneous Mycoses

1. Saprophytic fungi of soil or decaying vegetation are usually introduced into subcutaneous tissue and produce a progressive local disease with tissue destruction and sinus formation.
2. Subcutaneous mycoses include mycetoma, chromoblastomycosis, sporotrichosis and rhinosporidiosis.

Systemic Mycoses

1. These are caused by fungi of soil and is acquired by inhalation.
2. Fungus may disseminate to CNS, bones and other internal organs.
3. Systemic mycoses include blastomycosis, paracoccidioidomycosis, coccidioidomycosis, histoplasmosis and cryptococcosis.
4. Systemic mycoses and subcutaneous mycoses collectively are also named as *Deep mycoses*.

A. Superficial Mycoses

Superficial mycoses

Surface infections	Cutaneous infections
e.g. Tinea versicolor, Tinea nigra, Piedra	e.g. Dermatophytes

In surface infections, the fungi live exclusively on the dead layers of the skin and its appendages. As there is no contact of fungi with the living tissue, no inflammatory response is elicited. On the other hand, cutaneous infections are generally confined to the cornified layer of the skin and its appendages. Inflammatory and allergic responses are induced due to the presence of the fungi and their metabolic products.

1. Tinea Versicolor (Pityriasis Versicolor)

Fungus: *Pityrosporum orbiculare* (*Malassezia furfur*), lipophilic yeast-like fungus

(*i*) It is characterised by mild chronic infection of the stratum corneum.
(*ii*) There is patchy discolouration of the skin. The commonest sites involved include chest, back, abdomen, neck and upper arms.
(*iii*) *Pityrosporum orbiculare* forms the normal skin flora and most infections are thought to be endogenous.
(*iv*) The disease is worldwide in distribution.

LABORATORY DIAGNOSIS

Direct microscopy

(*i*) Skin scales are examined microscopically by KOH preparation.
(*ii*) Clusters of round yeast cells along with short, curved, non-branched hyphae are characteristic (Fig. 66.8).

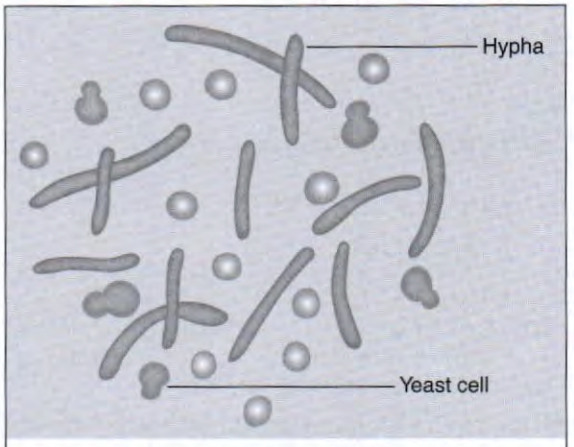


Fig. 66.8 Morphology of *Pityrosporum orbiculare*

Culture

(*i*) It is rarely necessary for diagnosis.
(*ii*) SDA overlaid with a layer of olive oil (as it is lipophilic fungus) is used.
(*iii*) Creamy colonies develop within 5-7 days at 37°C.
(*iv*) Lactophenol cotton blue wet mount of the colonies show budding yeast cells along with a number of bottle shaped cells. Hyphae are occasionally seen in culture.

2. Tinea Nigra

Fungus: *Hortaea wernickii* (*Cladosporium wernickii*)
It is characterised by black brown macular patches affecting the thickly keratinised sites such as palms and soles.

LABORATORY DIAGNOSIS

Microscopy

Skin scrapings in 10% KOH mount show brown, septate, branching hyphae and budding cells (Fig. 66.9).

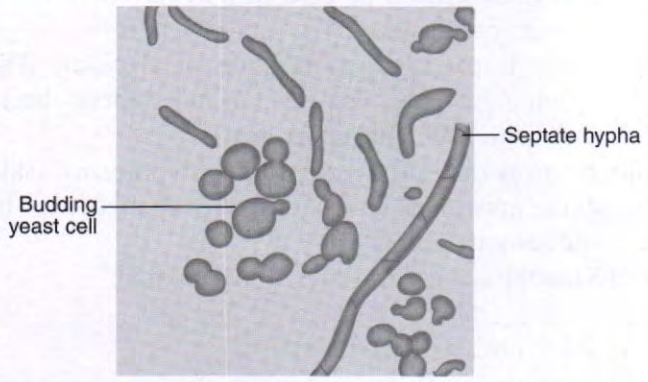


Fig. 66.9 *Hortaea wernickii*

Culture
On SDA, moist, yeast like white to dark coloured colonies appear.

3. Piedra
(*i*) Piedra is a fungal infection of the hair.

Piedra

Black piedra	White piedra
Fungus: *Piedraia hortae* (Dematoceous fungus)	Fungus: *Trichosporon beigelii*

(*ii*) Black piedra is characterised by black hard nodules on hair shaft of beard and scalp.

(*iii*) White piedra is characterised by white nodules on hair shaft of axilla, moustache, beard and sometimes pubic hair.

LABORATORY DIAGNOSIS
Direct microscopy
Hair mounted in 10% KOH reveals nodules containing asci having ascospores.

In white piedra
In addition to above findings, arthroconidia (arthrospores) can be seen.

4. Dermatophytes
(*i*) Dermatophytes are a group of fungi that infect only superficial keratinised tissue (skin, hair and nails) without involving the living tissue.

(*ii*) They break down and utilise keratin.

(*iii*) They are incapable of penetrating subcutaneous tissue.

(*iv*) They cause *dermatophytoses*, also known as tinea (Latin for worm or ringworm) or ringworm.

(*v*) Hypersensitivity to fungus antigens is probably responsible for the sterile vesicular lesions sometimes seen in sites distant from the ringworm. These lesions are named *dermatophytids* (or the 'id' reaction).

Classification
Dermatophytes are classified into three genera as follows:

Genus		Infection of
1. *Trichophyton*	—	Hair, skin, nail
2. *Microsporum*	—	Hair, skin
3. *Epidermophyton*	—	Skin, nail

On the basis of their natural habitat and host preferences, these can be classified into three groups:

- Anthropophilic — Man
- Zoophilic — Animals
- Geophilic — Soil

T. rubrum, T. mentagrophytes, E. floccosum and *M. audouinii* are examples of anthropophilic dermatophytes. Example of zoophilic dermatophytes is *M. canis* in dogs and cats. *M. gypseum* is a geophilic species, which occurs naturally in soil.

Important species
Trichophyton:
- *T. rubrum*
- *T. mentagrophytes*
- *T. tonsurans*
- *T. schoenleinii*
- *T. violaceum*
- *T. verrucosum*

Microsporum:
- *M. gypseum*
- *M. canis*
- *M. audouinii*

Epidermophyton:
- *E. floccosum*

Clinical types
Clinically, ringworm can be classified depending on the site involved. These include Tinea capitis (scalp), Tinea corporis (non-hairy skin of the body), Tinea cruris (groin), Tinea pedis (foot) or athlete's foot and Tinea barbae or barber's itch (bearded areas of the face and neck). Favus is a chronic type of ringworm involving the hair follicles. It leads to alopecia and scarring. Some of the clinical types of dermatophytosis and their common causative agents are listed below.

Clinical type		Causative agents
Tinea capitis	:	Microsporum any spp. Trichophyton most spp.
Favus	:	*T. schoenleinii* *T. violaceum* *M. gypseum*
Tinea corporis	:	*T. rubrum* and other dermatophytes
Ectothrix	:	Microsporum spp. *T. rubrum* *T. mentagrophytes*
Endothrix	:	*T. schoenleinii* *T. tonsurans* *T. violaceum*

- In favus, there is sparse hyphal growth and formation of air spaces within the hair shaft.
- Two types of hair infection may be present, ectothrix and endothrix. In ectothrix, a sheath of arthospores is present on the surface of hair shaft, while the arthospore formation occurs entirely within the hair shaft in endothrix (Fig. 66.10).

Laboratory diagnosis

Specimens

Skin
Hair
Nail

Direct microscopy

- Direct 10% KOH mount may show fungal hyphae.

Culture

- SDA and SDA with antibiotics are used.
- Culture media are incubated at 25–30°C for three weeks.
- Identification of dermatophytes is based on
  — colony morphology
  — pigment production
  — microconidia and macroconidia
- Colony characters
  — White to creamy, cottony growth
  — Reverse of media is red in *T. rubrum*.
- Microscopy
  — Lactophenol cotton blue preparation from colony reveals microconidia, macroconidia or both. The following are the characterstics of three genera:

Genus *Trichophyton*—More microconidia, very few macroconidia

Genus *Microsporum*—Predominant macroconidia

Genus *Epidermophyton*—Macroconidia

Differentiation at species level

T. rubrum and *T. mentagrophytes* are the two most commonly isolated Trichophyton species.

T. rubrum

- Tear shaped microconidia along the hyphae (Fig. 66.11)
- Red pigment on the reverse side of the medium
- Urease negative
- Club shaped, thin walled macroconidia—scanty

T. mentagrophytes

- Grape like clusters of microconidia (Fig. 66.11)
- No red pigment
- Urease positive
- Hair perforation test is positive.
- Cigar shaped macroconidia
- Bromocresol purple (BCP)–milk solid–glucose (BCPMSG) medium may be used to differentiate *T. mentagrophytes* from *T. rubrum*. On this medium, *T. mentagrophytes* shows profuse growth and alkaline reaction. A colour change from pale blue to violet purple (due to indicator, BCP) indicates alkaline reaction. *T. rubrum* shows restricted growth and no alkaline reaction (no change in colour).

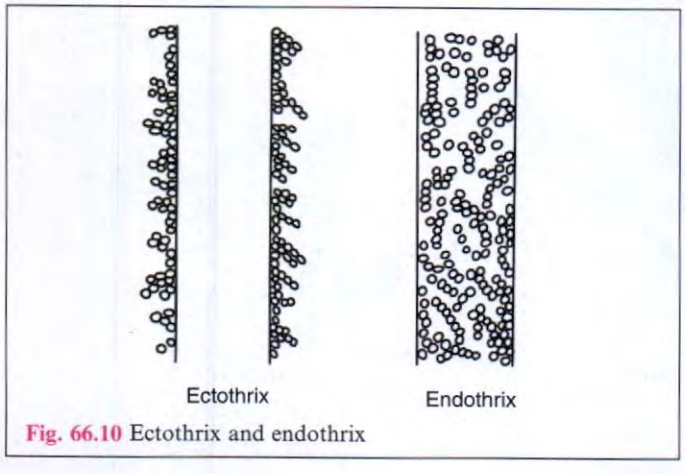


Fig. 66.10 Ectothrix and endothrix

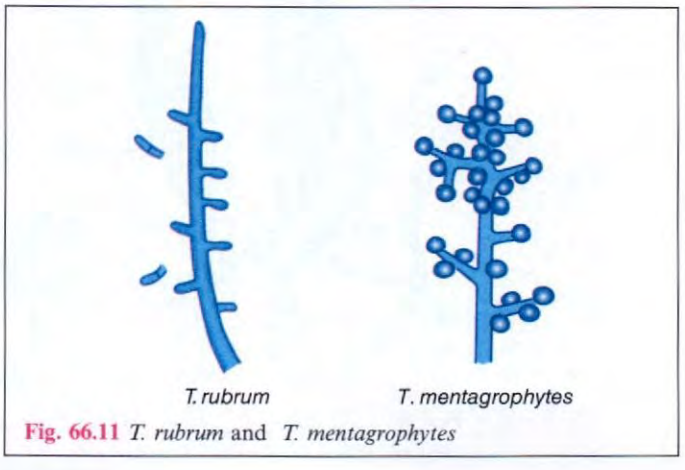


Fig. 66.11 *T. rubrum* and *T. mentagrophytes*

T. violaceum, T. schoenleinii, T. verrucosum

- These are slow growing.
- Conidia are usually not present.
- Only hyphae are seen.

Apart from above mentioned common features, these three species have also some specific characteristics. *T. violaceum* has violet coloured colonies.

Antler hyphae (favic chandelier) are present in *T. schoenleinii.* Colony of *T. verrucosum* is partially submerged in media and it contains chain of chlamydospore.

Microsporum audouinii

- Anthropophilic
- Hair fluoresce yellow-green when examined under wood's lamp.
- Velvety, brown coloured colony
- Macroconidia—distorted shape or absent
- Terminal chlamydospore present

M. canis

- Zoophilic (Dogs and cats)
- Hair fluoresce yellow-green.
- Spindle shaped, rough walled, multisegmented, curved end, warty projections macroconidia (Fig. 66.12) are abundant.
- Microconidia are very few.

M. gypseum

- Geophilic
- Hair do not fluoresce.
- Spindle shaped, round end macroconidia (Fig. 66.12) are abundant. There is no curve at the end of macroconidia.

Epidermophyton floccosum

- Macroconidia are numerous. These are smooth thin walled, club shaped, multiseptate and round at tip (Fig. 66.12).
- Microconidia are absent.
- Chlamydospores are numerous.

TREATMENT OF DERMATOPHYTOSES

Topical antifungal agents are generally used for treatment. *T. rubrum* infections may be resistant. Oral griseofulvin is the drug of choice.

B. Subcutaneous Mycoses

1. Mycetoma

(*i*) Mycetoma is a chronic granulomatous infection of the subcutaneous tissue, usually affects the foot and rarely the other parts of body.

(*ii*) The disease was first described by *Gill* (1842) from Madurai, South India and *Carter* (1860) established the fungal origin of the disease. It is therefore commonly referred to as *Madura foot* or *Maduramycosis*.

(*iii*) It is seen mainly in the tropical countries.

(*iv*) The disease is quite common in Tamil Nadu.

Aetiology

- Mycetoma is caused by a number of actinomycetes and filamentous fungi (Table 66.1).
- Botryomycosis, a condition caused by *Staphylococcus aureus*, clinically resembles mycetoma and for that reason aetiological diagnosis is necessary for proper treatment.

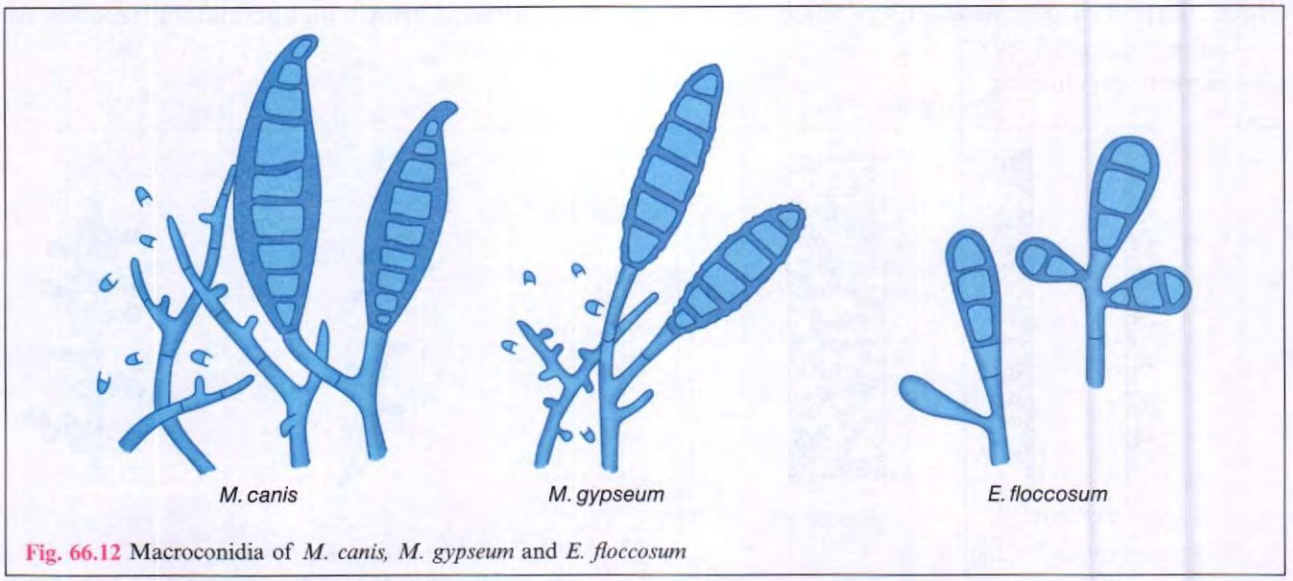


Fig. 66.12 Macroconidia of *M. canis, M. gypseum* and *E. floccosum*

Pathogenesis

- The causative organism is believed to enter the body through minor trauma.
- The disease begins as a subcutaneous swelling usually of foot, which enlarges and burrows into the deeper tissues producing characteristic abscess. The abscess bursts with the formation of chronic multiple sinuses discharging viscid, seropurulent fluid containing granules.
- The colour and consistency of the granules vary with the different causative agents (Table 66.1).

Table 66.1 Important Causative Agents of Mycetoma

Causative agent	Colour of the grains
A. Eumycetoma	
Phaeacremonium sp.	White-yellow
Leptosphaeria sp.	Black
Madurella mycetomatis	Black
M. grisea	Black
Pseudoallescheria boydii	White or Black
Exophiala jeanselmei	Black
B. Actinomycetoma	
Actinomadura madurae	White-yellow
A. pelletieri	Red
Nocardia brasiliensis	White
Streptomyces somaliensis	Yellow

Laboratory diagnosis

- Diagnosis is made from examination of granules.
- The granules are composed of very thin (less than 1 μm in diameter) filaments in actinomycotic mycetoma while they are broader and often show septae and chlamydospores in mycotic mycetoma.

Treatment

Actinomycotic mycetoma usually respond well to sulphonamides and antibiotics, but mycotic mycetoma may require amputation.

2. Chromomycosis

(*i*) It is a chronic, localised infection of skin and subcutaneous tissue.

(*ii*) The fungus enters the body through a wound.

Aetiological agents

- It is caused by several darkly pigmented fungi of the family *Dematiaceae*. These include:
  Fonsecaea pedrosoi
  F. compactum
  Cladosporium carrionii
  Phialophora verrucosa
- The disease is more common in tropical and subtropical countries.
- The term chromomycosis includes chromoblastomycosis and other infections caused by dematiaceous fungi (Phaeohyphomycosis). Chromoblastomycosis is usually confined to the subcutaneous tissue of the feet and lower legs. The lesions appear as warty cutaneous nodules which resemble the florets of cauliflower. Phaeohyphomycosis may affect cutaneous, subcutaneous, deeper tissues or organs like brain or lung.

Laboratory diagnosis

Chromoblastomycosis

- Histologically, in H and E staining they appear as yeast like bodies with septae, called *sclerotic bodies* (Fig. 66.13).
- These sclerotic bodies can be demonstrated in KOH mounts and by culture on SDA.

Phaeohyphomycosis

- The fungi appear in lesions as distorted hyphal strands. Sclerotic bodies are absent.

Treatment

- Amphotericin B and 5-fluorocytosine have been found useful.

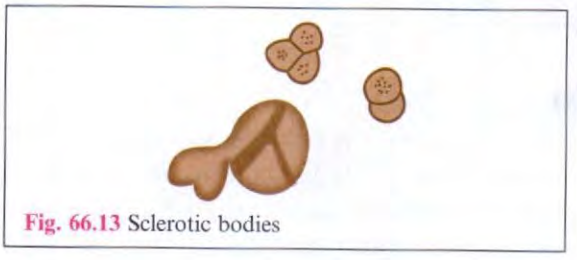


Fig. 66.13 Sclerotic bodies

3. Sporotrichosis

(*i*) Sporotrichosis is a nodular, ulcerating disease of skin and subcutaneous tissue.

(*ii*) It usually affects the hand or the forearm.

(*iii*) The fungus gains access through thorn pricks or some injuries. It is more common in gardeners and farmers.

(*iv*) The fungus spreads through lymphatics up to regional lymph nodes and rarely beyond that.

(*v*) The disease is worldwide, though more common in USA.

Causative agent

- *Sporothrix schenckii*—a dimorphic fungus

Laboratory diagnosis

- Diagnosis is made by culture as the fungus may not be demonstrable in pus or tissues.
- *S. schenckii* occurs in the yeast phase in the tissues and in cultures at 37°C, and in the mycelial phase in cultures at 22°C–25°C (*dimorphic fungus*).
- Yeast phase appears as cigar-shaped cells and mould form contains hyphae carrying flower like clusters of small conidia borne on delicate sterigmata (Fig. 66.14).
- The fungus produces progressive disease in rats on intraperitoneal or intratesticular inoculation.

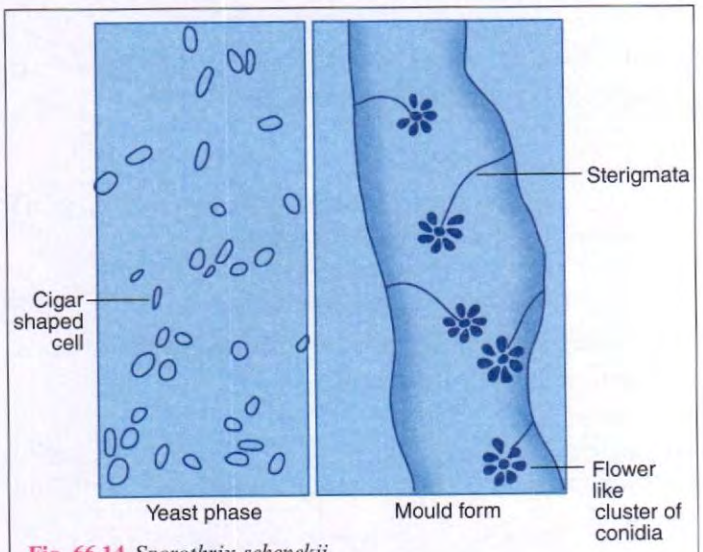


Fig. 66.14 *Sporothrix schenckii*

4. Rhinosporidiosis

(*i*) Rhinosporidiosis is a chronic granulomatous disease characterised by formation of friable polyps, usually confined to the nose, mouth or eye and rarely seen on other mucous membranes.

(*ii*) Causative agent is *Rhinosporidium seeberi*.

(*iii*) More than 80% cases are reported in India and Sri Lanka.

(*iv*) The mode of infection is not known but most infections occur in males who have frequent contact with stagnant water or aquatic life.

Laboratory diagnosis

- The fungus has not been cultivated.
- Diagnosis depends on the demonstration of sporangia.
- Tissue sections stained with H&E stain show large number of endospores within the sporangia embedded in a stroma of connective tissue and capillaries. The sporangium (10–200 μm) contains thousands of endospores (6-7 μm in diameter) (Fig. 66.15). These spores when released develop into new sporangia.

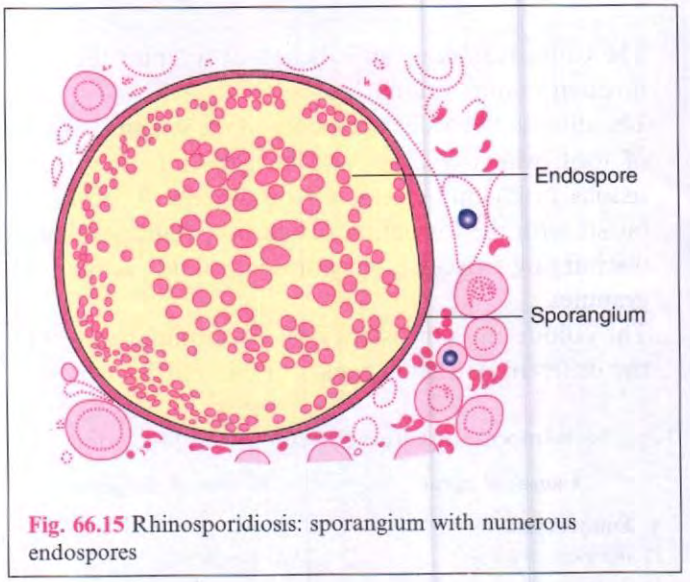


Fig. 66.15 Rhinosporidiosis: sporangium with numerous endospores

C. Systemic Mycoses

1. Histoplasmosis

(*i*) Causative fungus: *Histoplasma capsulatum*, a dimorphic fungus

(*ii*) It is primarily a disease of reticuloendothelial system.

(*iii*) *H. capsulatum* is an intracellular parasite.

(*iv*) The disease is worldwide in distribution but is most common in America.

Source of infection

- The fungus is present in the soil enriched with excreta of birds or bats.
- Human infection results from inhalation of spores.

Clinical features

- The large majority of infections are asymptomatic.
- Some individuals develop pulmonary disease which resembles tuberculosis.
- Disseminated histoplasmosis develops only in a minority of infected individuals.
- Involvement of reticuloendothelial system results in lymphadenopathy, hepatosplenomegaly, fever, anaemia and a high rate of fatality.
- Granulomatous and ulcerative lesions may develop on the skin or mucosa.

Laboratory diagnosis

Specimens

- Sputum
- Bone-marrow aspirate
- Peripheral blood
- Scrapings from dermal or mucosal ulcers
- Biopsies of lymph nodes and other organs

Direct examination

- Smears of sputum or pus are stained with Giemsa or Wright stains. On microscopy, *H. capsulatum* appears as small oval yeast cell, (2-4 μm in diameter) packed within the cytoplasm of macrophages or monocytes (Fig. 66.16).

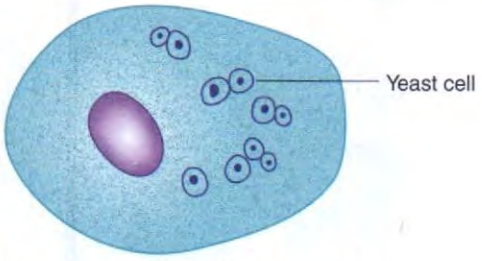


Fig. 66.16 H. capsulatum: yeast cells in macrophage

Culture

- SDA or brain heart infusion (BHI) agar with cycloheximide and chloramphenicol are inoculated.
- The yeast phase is formed in cultures at 37°C. White cottony mycelial growth containing large (8-20 μm) thick walled, spherical spores with tubercles or finger like projections (Fig. 66.17) appears at 25°C.

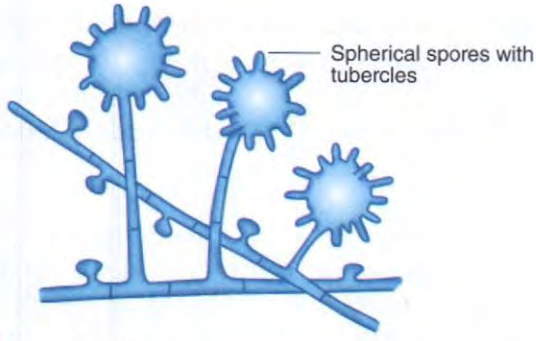


Fig. 66.17 H. capsulatum : mycelial form

Serological tests

- Latex agglutination, precipitation and complement fixation tests become positive two weeks after infection.
- Increase in titre of antibody indicates a progressive disease.

Histoplasmin skin test

- Delayed hypersensitivity test
- The test is similar to tuberculin test but antigen used is histoplasmin.
- Histoplasmin is a culture filtrate antigen of mycelial phase of *H. capsulatum*.
- A positive 'histoplasmin skin test' indicates past or present infection, but does not differentiate active and past infections.

Treatment

- Amphotericin B has been found useful in treatment of histoplasmosis.

African Histoplasmosis

(*i*) Causative fungus: *Histoplasma duboisii*
(*ii*) It is mainly confined within the continent of Africa.
(*iii*) It primarily involves skin and subcutaneous tissues.
(*iv*) The lungs are not commonly affected.
(*v*) It is morphologically identical to *H. capsulatum* in its mycelial phase but differs in the yeast phase by forming much larger yeast like cells (7-15 μm).

2. Blastomycosis

(*i*) Causative fungus : *Blastomyces dermatitidis*, a dimorphic fungus
(*ii*) It is a chronic infection of the lungs which may spread to other tissues, particularly skin, bone and genitourinary tract.
(*iii*) As the infection is confined to the North American Continent, it is also known as *North American blastomycosis*.

Source of infection

- Inhalation of conidia of fungus growing as saprophytes in the soil.

Clinical features

- Asymptomatic
- Mild primary pulmonary disease
- Disseminated disease—It disseminates to most organs including bones.
- Disseminated lesions have been found in immuno-compromised patients including AIDS.
- Cutaneous blastomycosis—It is usually observed on the skin of the face or other exposed parts. The initial lesion is a papule. Around this papule, secondary nodules develop and coalesce, leading to large elevated ulcerative lesions.

Morphology

B. dermatitidis is a dimorphic fungus. In tissues and in cultures at 37°C, the fungus appears as spherical or oval budding yeast cells (7–20 μm) with thick, double contoured walls. Each cell has only a single broad based bud. At 25°C, the culture is filamentous with septate hyphae and many round or oval conidia (Fig. 66.18).

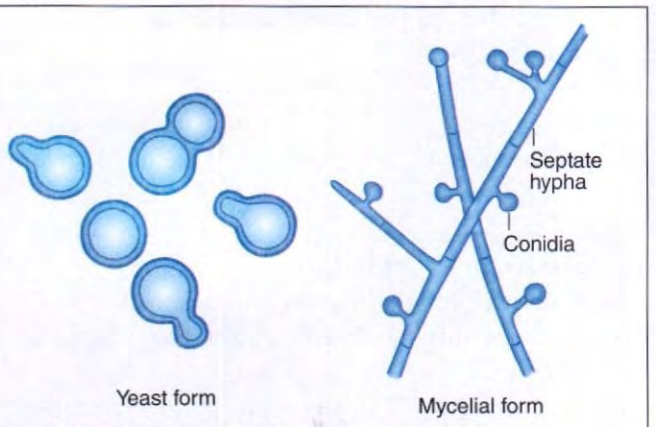


Fig. 66.18 *B.dermatitidis* : yeast and mycelial forms

Laboratory diagnosis

Specimens

- Sputum
- Pus
- Scrapings from skin lesions

Direct microscopy

- 10% KOH mount shows thick walled yeast cells with a single broad based bud.
- H&E and PAS stains also show yeast cells in sections.

Culture

- SDA or blood agar
- Mycelial phase occurs slowly on incubation at 25°C. Yeast phase is seen in cultures incubated at 37°C
- Cultures should be incubated for at least six weeks before discarding them as negative.

Treatment

Amphotericin B is the drug of choice.

3. Paracoccidioidomycosis

(*i*) Causative fungus: *Paracoccidioides brasiliensis*, a dimorphic fungus.

(*ii*) It is a chronic granulomatous disease involving lungs, mucosa, skin and lymphatic system.

(*iii*) As the disease is confined to South America, it was formerly called *South American blastomycosis*.

Source of infection

- By inhalation of spores from environmental sources.

Clinical features

- Primary pulmonary infection that spreads by haematogenous route to mucous membranes of mouth, nose, lymph node and adjacent skin, producing chronic granulomatous reaction.
- Ulcerative granulomas of the buccal and nasal mucosa are a prominent feature.

Morphology

- As a dimorphic fungus, it grows as yeast cells with multiple buds in tissues and in cultures at 37°C and in the mycelial phase at 25°C (Fig. 66.19).

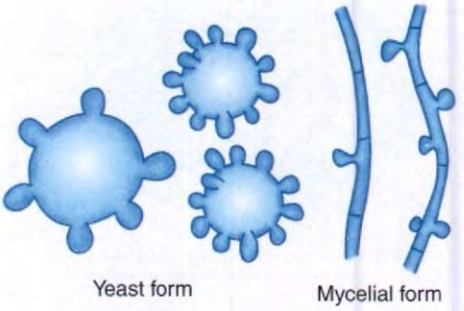


Fig. 66.19 *P. brasiliensis* : yeast and mycelial forms

Laboratory diagnosis

Specimens

- Pus
- Sputum
- Biopsies from granulomatous lesions

Direct microscopy

- Microscopical examination in KOH mount of the specimen shows numerous yeast cells with multiple buds.
- Tissue sections should be stained with H & E and PAS stains.

Culture

- Mycelial phase of the fungus develops on SDA incubated at 25°C.
- Yeast phase may be obtained by inoculating specimen on enriched media such as BHI agar and incubating it at 37°C.

Treatment

- Amphotericin B is the drug of choice.

4. Coccidioidomycosis

(*i*) Causative fungus: *Coccidioides immitis*, a dimorphic fungus.

(*ii*) It occurs as primary infection of the lung in endemic areas of South-West USA and northern Mexico.

Source of infection

- By inhalation of dust containing arthrospores of the fungus.

Clinical features

- Asymptomatic
- Primary pulmonary disease—mild influenza-like fever (known as *valley fever*) to severe pneumonia
- Disseminated disease—It may disseminate to involve virtually every tissue of the body including central nervous system, skin and bones. Disseminated disease is uncommon (about 1%) but is highly fatal.

Morphology

- Being a dimorphic fungus, it occurs in the tissue as a yeast and in culture (both at 37°C and at 25°C) as the mycelial form.
- The yeast form is a spherule (15-75 µm in diameter) with a thick doubly refractile wall and filled with endospores (Fig. 66.20). Each endospore gives rise to a new spherule.
- The mycelial phase contains pseudohyphae which fragment into arthrospores (Fig. 66.20) which are highly infectious.

Laboratory diagnosis

Specimens

- Sputum
- Pus
- Biopsy material

Direct microscopy

- Microscopic examination of the specimen detects large number of mature spherules.

Culture

- Specimen is inoculated on SDA medium in the test tube.
- Since arthrospores are highly infectious and readily airborne, therefore, petri dish should not be used for the isolation of *C. immitis*.
- Inoculated media are incubated at 25°C for 3 weeks.
- Septate hyphae which fragment into thick walled arthrospores in chains are found.

Skin test

- It is an intradermal skin test using 'coccidioidin', an antigen from the fungus.
- The test becomes positive (5 mm diameter of induration at 48 hours) between 3-21 days of symptoms.
- About 90% of inhabitants in endemic areas exhibit positive skin test.

5. *Cryptococcosis*

(*i*) Causative fungus: *Cryptococcus neoformans*, a capsulated yeast

(*ii*) It is a soil saprophyte and is particularly abundant in the faeces of pigeons.

(*iii*) *C. neoformans* does not appear to infect birds, probably because of their high body temperature.

(*iv*) Cryptococcal infection occurs throughout the world.

(*v*) Several cases of cryptococcosis have been identified in India.

(*vi*) This is the only deep mycosis common in India.

Morphology

- Spherical budding cell (5-15 µm diameter) having a prominent polysaccharide capsule (Fig. 66.21).
- It is a true yeast and is Gram positive.
- Capsule may be demonstrated by India-ink or nigrosin staining.

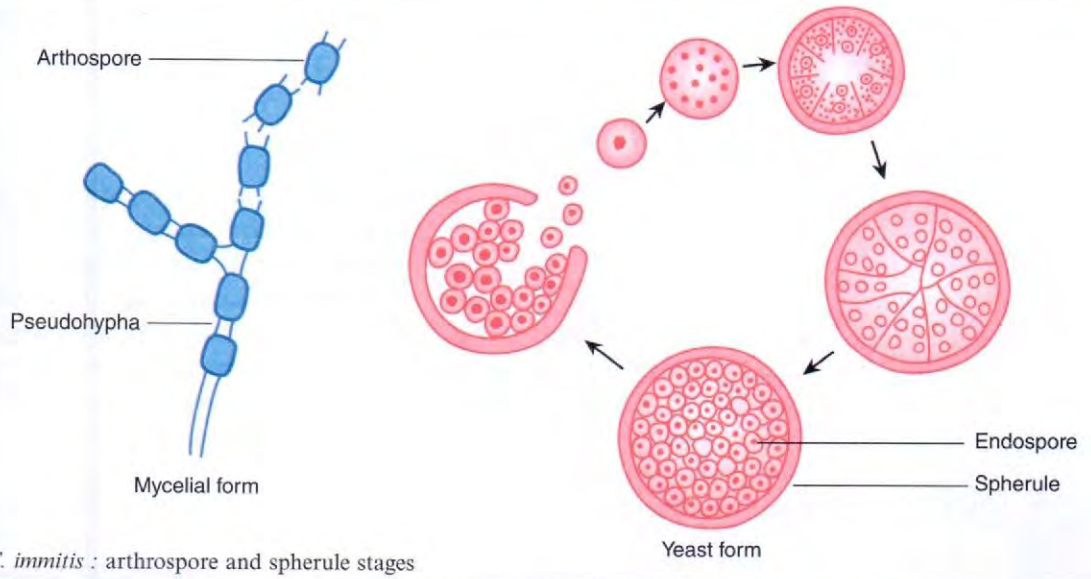


Fig. 66.20 *C. immitis* : arthrospore and spherule stages

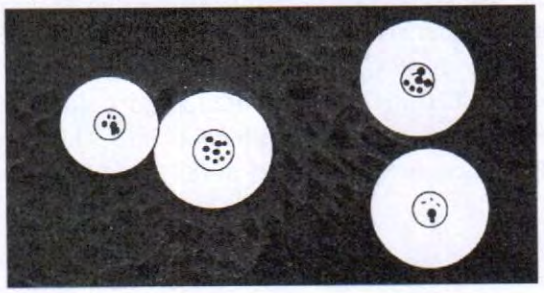


Fig. 66.21 *C. neoformans*

Antigenicity

- On the basis of cryptococcal polysaccharide antigen, *C. neoformans* has four serotypes (A, B, C and D).

Source of infection

- Infection is usually acquired by inhalation of dust containing yeast cells.

Pathogenesis

- *C. neoformans* is pathogenic in humans and animals.
- The disease is usually seen in immunocompromised host.
- Most infections are asymptomatic.
- Pulmonary cryptococcosis may lead to a mild pneumonitis.
- Cryptococcal meningitis occurs by haematogenous spread. It is often seen in AIDS patients.
- Skin, lymph nodes, bones and other organs may be involved when dissemination of infection occurs. Cutaneous cryptococcosis varies from small ulcers to large granulomas.
- Visceral forms of cryptococcal infection simulate tuberculosis and cancer clinically.

Laboratory diagnosis

Specimens

- CSF
- Sputum
- Pus
- Brain tissue

Direct microscopy

- Specimen mixed with a drop of India ink or nigrosin shows round budding yeast cells. In India ink, capsule appears as a clear halo around the yeast cells.
- Gram staining shows Gram positive yeast cells.
- The histopathological examination of tissue can be done by staining with H& E, PAS and mucicarmine stains.

Culture

- Sediment from a centrifuged CSF is inoculated on SDA and incubated at 37°C. Other specimens may also be inoculated on SDA.
- *C. neoformans* grows to form smooth, mucoid, cream coloured colonies.
- Lactophenol cotton blue mount shows budding yeast cells.
- Niger seed (bird seed) agar is a differential medium for presumptive identification of *C. neoformans*. It produces brown colonies on this medium within one week when incubated at 30°C. *C.neoformans* produces phenoloxidase, which oxidises the caffeic acid in the niger seed into melanin.

Latex agglutination test

- Cryptococcal capsular polysaccharide antigen can be detected in CSF, serum or urine by latex agglutination test.

Animal inoculation test

- Intracerebral or intraperitoneal inoculation into mice leads to a fatal infection in case of *C. neoformans*. Capsulated budding yeast cells can be demonstrated in the brain of the infected mice.

Other tests

- *C. neoformans* hydrolyses urea.

Differentiation of C.neoformans (pathogenic) from other non-pathogenic Cryptococci

C. neoformans has ability to

- grow at 37°C.
- hydrolyse urea.
- produce brown colonies on niger seed agar.
- produce disease in mice (animal inoculation test positive).

VII. OPPORTUNISTIC MYCOSES

- Some saprophytic fungi usually do not produce disease but may cause infection under special conditions such as immunocompromised individuals and in terminal stages of chronic disease. The incidence of these fungal infections has increased with widespread use of antibiotics, corticosteroids and immunosuppressive drugs. These are called *opportunistic fungi*.
- Some of these are common laboratory contaminants in culture media and grow on virtually anything. *Aspergillus* and *Penicillium* grow on damp bread and other organic matter.

- These fungi can produce serious and even fatal infections in persons who are otherwise debilitated.

A. Candidiasis

1. Causative fungus: *Candida albicans* (80-90% of cases)
2. Candidiasis is an infection of skin, mucosa and internal organs, caused by yeast like fungus *Candida albicans*, and occasionally by other candida species.
3. *Candida albicans* is the normal inhabitant of skin, gastrointestinal tract, oral and vaginal cavities.
4. Candidiasis is an opportunistic endogenous infection.

Morphology

(*i*) *C. albicans* is an ovoid or spherical budding yeast cell, 3–5 μm in diameter (Fig. 66.22).

(*ii*) It produces pseudohyphae (elongated filamentous cells joined end to end, resembling hyphae) both in culture and in tissues (Fig. 66.22).

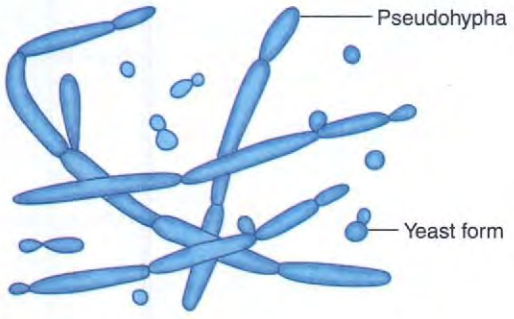


Fig. 66.22 *Candida albicans:* yeast form and pseudohyphae

Species of Candida

Important species of *Candida* found in man are :

(*i*) *C. albicans*
(*ii*) *C. stellatoidea*
(*iii*) *C. tropicalis*
(*iv*) *C. krusei*
(*v*) *C. guilliermondii*
(*vi*) *C. parapsilosis*
(*vii*) *C. glabrata*
(*viii*) *C. viswanathii*

Pathogenesis

(*i*) Candidiasis is an opportunistic endogenous infection.

(*ii*) Predisposing factors for candidiasis are diabetes, immunodeficiency, malignancy, prolonged administration of antibiotics, patients on immunosuppressive drugs and intravenous catheters.

(*iii*) Lesions caused by candida are as follows:

(a) Mucocutaneous lesions

- Oral thrush
- Vulvovaginitis
- Balanitis
- Conjunctivitis
- Keratitis

(b) Skin and nail infections

- Skin—Infections of axillae, groin, perineum and submammary folds where the skin is macerated by perspiration.
- Nails—Infections of finger webs, nail folds and nails may occur. Paronychia and onychia are seen in those persons who frequently immerse their hands in water.
- In infants it may lead to napkin dermatitis.

(c) Chronic mucocutaneous candidiasis

This is serious manifestation seen in immunodeficiencies.

(d) Systemic candidiasis

- Urinary tract infection
- Intestinal candidiasis: It is a frequent sequel to oral antibiotic therapy and present as diarrhoea.
- Pulmonary candidiasis
- Endocarditis
- Meningitis
- Septicaemia

Laboratory Diagnosis

(i) Direct microscopy

(*a*) Gram stained smears and KOH mounts from lesions of skin, nail or mucous membranes show budding Gram positive yeast cells.

(*b*) Since candida is normally present on skin and mucosa, only their abundant presence is of significance.

(*c*) Demonstration of pseudohyphae indicates colonisation and tissue invasion.

(ii) Culture

(*a*) Candida species grow well on SDA and ordinary bacteriological culture media e.g. blood agar.

(b) They grow at 25-37°C within 24 hours.

(c) Cream coloured, smooth, pasty colonies appear (Fig. 66.23).

(d) Gram stained smear from colonies shows Gram positive budding yeast cells (Fig. 66.24).

(iii) Identification

To differentiate *C. albicans* from other species, the following tests are done.

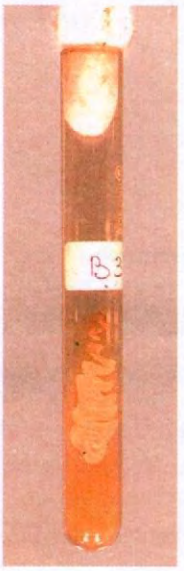


Fig. 66.23 Growth of *Candida albicans* on SDA slope

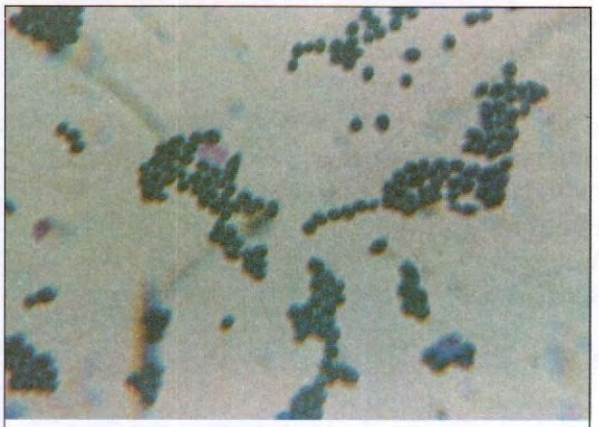


Fig. 66.24 Gram stained smear of *Candida albicans*

(*a*) Germ tube test : *C. albicans* has ability to form germ tubes within two hours when incubated in human serum at 37°C (*Reynolds-Braude phenomenon*) (Figs. 66.25 and 66.26).

(*b*) Chlamydospores : Chlamydospores develop in a nutritionally deficient medium such as *cornmeal agar* at 20°C. They can be seen at the end of pseudohyphae (Fig. 66.27).

(*c*) Carbohydrate fermentation and carbohydrate assimilation tests : These are used in identification of *C. albicans* and other species of candida. Depending on fermentation and assimilation of various carbohydrates, species of candida can be identified.

(iv) Serology

Precipitation test with a carbohydrate extract of candida can be used. Tests for precipitins in serum are available for *C. albicans* e.g. CIEP.

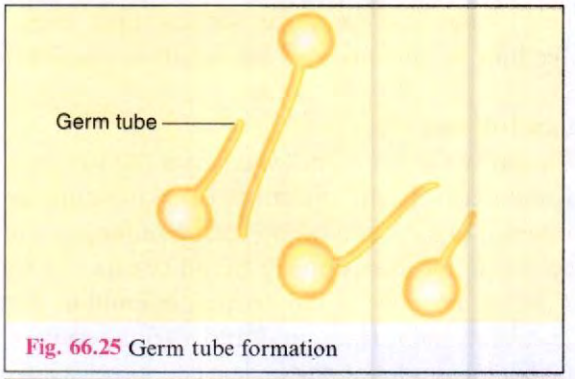


Fig. 66.25 Germ tube formation

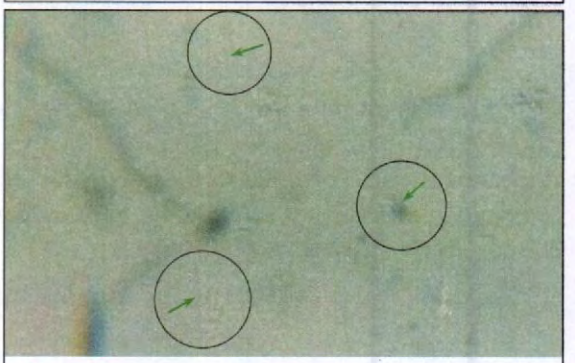


Fig. 66.26 *Candida albicans* showing Germ tubes

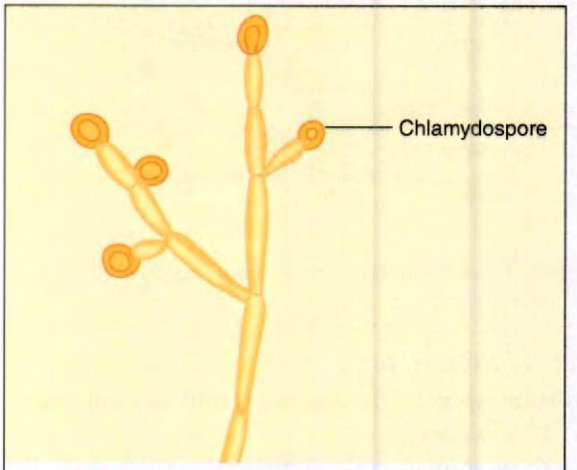


Fig. 66.27 Chlamydospore formation

(v) Antigen detection

ELISA is available for detection of candidal antigens such as cell wall mannan or cytoplasmic constituents.

(vi) Skin test

It shows universal positivity and is useful as an indicator of intensity of cell mediated immunity (CMI).

Treatment

(*i*) Predisposing factors are to be removed in all cases.

(*ii*) Topical application of polyene (nystatin) or imidazole (miconazole, clotrimazole) is effective in superficial infections.

(*iii*) Amphotericin B is administered along-with 5-fluorocytosine in systemic infections.

B. Aspergillosis

1. Aspergilli are ubiquitous in nature.
2. *Aspergillus fumigatus* is the main opportunistic pathogen.
3. Other important species are *A. niger, A. flavus, A. terreus* and *A. nidulans*

Pathogenesis

(*i*) Aspergillosis is caused by inhalation of *Aspergillus* conidia or mycelial fragments which are present on the decaying matter, soil or air.

(*ii*) When the host defence is compromised, aspergillosis may develop.

(*iii*) There are three clinical forms of systemic aspergillosis as follows:

(a) Respiratory disease

- Aspergillus asthma—Hypersensitivity to aspergilli may occur in atopic individuals following inhalation of spores of aspergilli.
- Bronchopulmonary aspergillosis—The fungus grows within the lumen of the bronchioles, which may be occluded by fungus plugs. Some patients may expectorate mucus plugs containing fungus.
- Aspergilloma—The fungus colonises in the pre-existing pulmonary cavities such as in tuberculosis or cystic disease. It is often called *fungus ball.*

(b) Invasive aspergillosis

Invasive or disseminated aspergillosis occurs in severely immunocompromised individuals. The fungus first establishes in lung tissue and then disseminate to involve other organs particularly brain, kidney and heart.

(c) Superficial infections

- Sinusitis—*A. flavus* and *A. fumigatus*
- Mycotic keratitis—*A. flavus* and *A. fumigatus*
- Otomycosis—Species of Aspergillus, particularly *A. niger*

Laboratory Diagnosis

(i) Specimens

- Sputum
- Bronchoalveolar lavage
- Biopsy

(ii) Direct microscopy

- KOH preparation of the specimen reveals non-pigmented septate hyphae (3-5 μm in diameter) with characteristic dichotomous branching (at an angle of approximately 45°).
- Biopsy sections can be stained with H & E and PAS staining and examined for the characteristic hyphae.

(iii) Culture

- The clinical specimen is inoculated on SDA without cycloheximide and incubated at 25°C.
- Colonies appear within 1–2 days and show a velvety to powdery surface. Colonies are coloured.
- *A. fumigatus*—green coloured colonies (Fig. 66.28), *A. niger*—black colonies (Fig. 66.29) and *A. flavus*—golden-yellow coloured colonies.
- Identification of aspergillus is based on growth characteristics and morphology.
- Lactophenol cotton blue preparation from colonies shows branching and septate hyphae. Asexual conidia are arranged in chains, carried on sterigmata, borne on the expanded ends (vesicles) of conidiophores (Fig. 66.30).
- Since *Aspergillus* species are common laboratory contaminants, their isolation has to be interpreted

Fig. 66.28 Growth of *Aspergillus fumigatus* on SDA slope

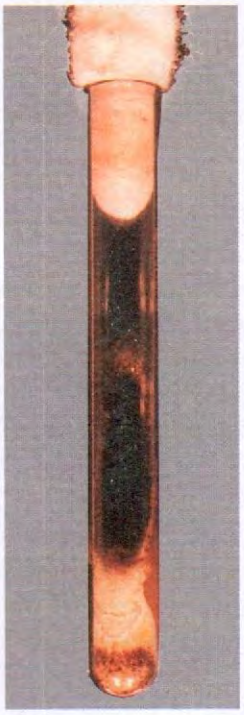


Fig. 66.29 Growth of *Aspergillus niger* on SDA slope

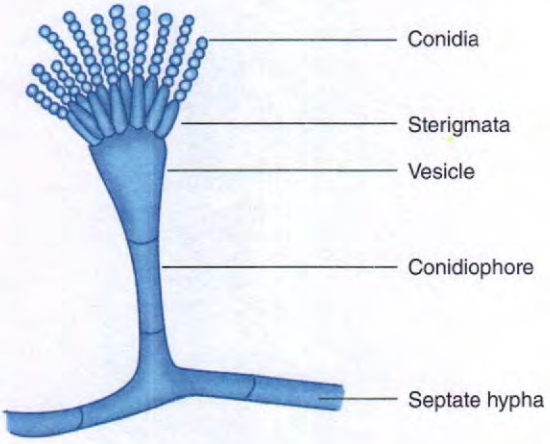


Fig. 66.30 Aspergillus

with care. Repeated isolation may be of help to find out its pathogenic role.

C. Mucormycosis (Zygomycosis)

1. Three genera *Mucor, Rhizopus* and *Lichtheimia* (formerly *Absidia*) are associated with mucormycosis.
2. These fungi are saprophytes of soil, manure and decaying vegetables.
3. These are normally avirulent and are able to cause disease only when general resistance is extremely low.
4. The primary infection is usually in upper respiratory tract or nose.
5. Rhinocerebral form of disease is characterised by spreading lesion from nasal mucosa to turbinate bone, paranasal sinuses, orbit and brain.
6. *Mucor* and *Lichtheimia* are most commonly isolated from mucormycosis.
7. Rhizopus causes mucormycosis and otomycosis.
8. Lichtheimia may also cause keratitis.
9. Pulmonary mucormycosis is a progressive severe pneumonia. The fungi may spread haematogenously to other areas of the lung and to other organs such as brain.
10. Gastrointestinal mucormycosis may occur in malnutrition, uraemia and diarrhoeal diseases.

Laboratory Diagnosis

(i) Specimens

- Scrapings from the lesions
- Pus
- Sputum
- Nasal discharge

(ii) Direct microscopy

- KOH wet mounts of specimens show non-septate hyphae.
- Histological sections stained with H&E stain reveal the presence of hyphae.

(iii) Culture

- Fungi can be readily grown on SDA without cycloheximide at 37°C.
- Lactophenol cotton blue preparation of colonies shows branched sporangiophores arising randomly along aerial mycelium. In the case of mucor rhizoids are absent. Rhizopus has rhizoids, and sporangiospore arise in groups directly above the rhizoids. Lichtheimia has also rhizoids but sporangiophores arise from the aerial mycelium in between the rhizoids (Fig. 66.31).

D. Penicillosis

1. Penicillium species, rarely cause opportunistic human infections.
2. It causes penicillosis, keratitis, otomycosis and rarely deep infections.
3. This saprophytic fungus contains septate hyphae and bears flask-shaped phialides which in turn supports the chains of round conidia. Members of this genus are identified by the brush or broom like arrangement of conidia (Fig. 66.32).
4. The fungus grows rapidly on SDA.
5. The colonies are initially velvety and white but later on become powdery and blue green.

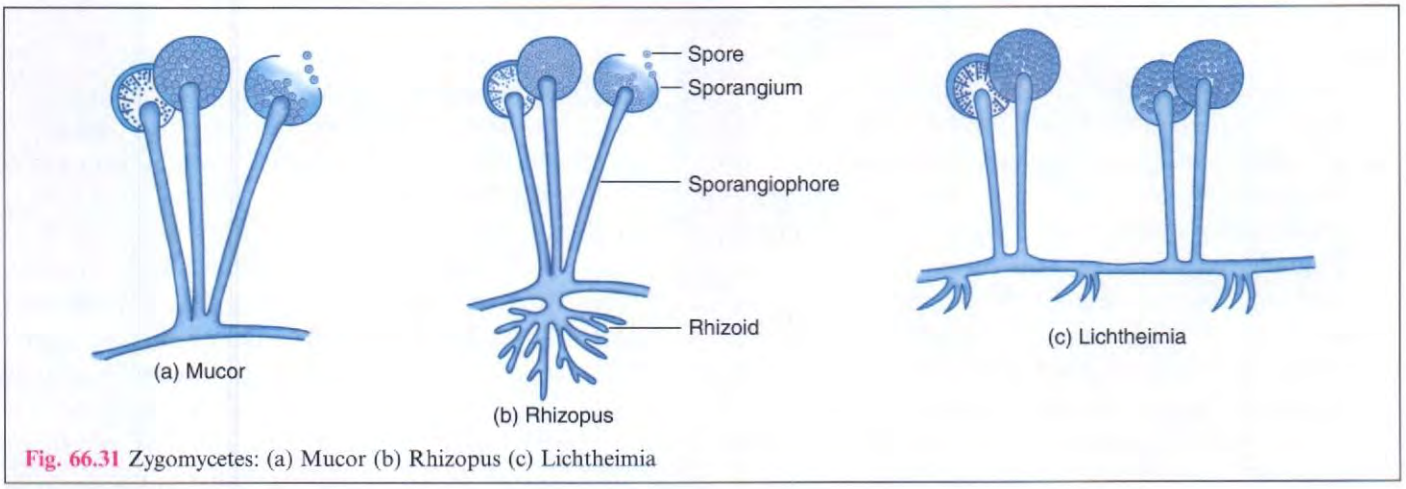


Fig. 66.31 Zygomycetes: (a) Mucor (b) Rhizopus (c) Lichtheimia

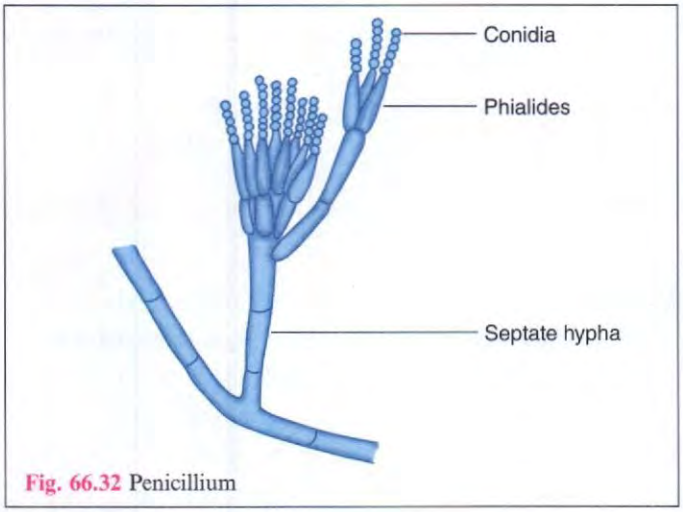


Fig. 66.32 Penicillium

6. *P. marneffei* has been reported as an important opportunist pathogen in the HIV infected individuals. Infections are usually disseminated with multiple organ involvement. It can be isolated from cutaneous lesions. The fungus is unique among the *Penicillium* being dimorphic, and is the only true pathogen in the genus. The yeast form of *P. marneffei* can be detected in Wright-stained smears from skin lesions or biopsy specimens. The yeast forms resemble those of *H. capsulatum*. The mould form is having sparse green aerial and reddish-brown vegetative hyphae and produces a red diffusible pigment. Polymerase chain reaction (PCR) tests have been described for identification and confirmation.

PNEUMOCYSTIS JIROVECI

1. *Pneumocystis jiroveci* causes pneumonia in immunocompromised patients. Until recently, it was thought to be a protozoan, but now it has been included in fungi. Molecular studies proved it as a fungus, an ascomycete. Prior to the introduction of chemoprophylaxis, it was a major cause of death among AIDS patients. *P. jiroveci* is a human species and *P. carinii* is found only in rats.
2. *P. jiroveci* has three stages: thin walled trophozoite (1-5 μm), the precyst (5-8 μm) and thick walled spherical cyst (about 8 μm). Cyst contains up to 8 intracystic bodies.
3. Transmission of the organism occurs through the respiratory route, with the cyst being infective stage.
4. Bronchoalveolar lavage, lung biopsy and induced sputum (by administration of a saline mist to induce production of sputum) are the specimens used for diagnosis.
5. Direct demonstration of trophozoites and cysts can be done by Giemsa, toludine blue, methenamine silver and calcofluor white stains. Cyst wall stains black with methenamine silver staining. Cysts often have a punched out "ping-pong ball" appearance. Fluorescent monoclonal antibody staining shows 'honeycomb' appearance of the cyst.
6. Serological tests can be used for diagnosis in suspected cases. Complement fixation titres of 1:4 or more is indicative of active disease. Latex agglutination test is also used.
7. Polymerase chain reaction (PCR) for amplification of *P. jiroveci* DNA is a rapid method for detection of early infection.
8. Trimethoprim-sulphamethoxazole or pentamidine are used in treatment of acute cases of pneumonia.
9. Trimethoprim-sulphamethoxazole or aerosolized pentamidine are used for chemoprophylaxis.

VIII. SOME CLINICAL PRESENTATIONS OF FUNGAL INFECTION

A. Otomycosis

1. Otomycosis is a fungal infection of the external auditory canal.

2. It is a common disease and is usually caused by species of aspergilli (*A. niger, A. fumigatus*), *Penicillium, Candida albicans, C. tropicalis* and *C. krusei.*
3. The symptoms include itching, pain and deafness.
4. Secondary bacterial infections, usually due to *Pseudomonas* and *Proteus* may occur.
5. Laboratory diagnosis is done by demonstration of fungi in the scrapings and by culture. Potassium hydroxide (KOH) preparation is used for demonstration of fungi in the scrapings. Culture is done on Sabouraud's dextrose agar (SDA) medium. Growth on the medium is identified by rapidity of growth, colour and morphology of the colony. Microscopy is performed from fungal colony (in teased mounts) to study the morphology of hyphae, spores and other structures. Teased mounts are prepared in lactophenol cotton blue (LCB).

B. Keratomycosis (Mycotic keratitis or fungal keratitis)

1. It is an invasive fungal infection of the cornea.
2. It usually follows corneal trauma.
3. Fungal spores colonise the injured tissue and initiate an inflammatory reaction which leads to hypopyon ulcer and endophthalmitis.
4. The incidence of keratomycosis has increased because of widespread use of corticosteroids.
5. Many saprophytic fungi can cause keratomycosis. However, Aspergillus species (*A. fumigatus, A. flavus* and *A. niger*), *Fusarium* and *Candida albicans* are most often responsible.
6. Laboratory diagnosis is done by microscopic examination and culture of scrapings taken from the base of edge of the ulcer.
7. Local application of amphotericin B and nystatin may be useful in treatment.

C. Mycotic Poisoning

1. Many fungi produce poisonous substances.
2. Mycotic poisoning:
   (*i*) *Mycetism*—fungus itself causes toxic effects.
   (*ii*) *Mycotoxicosis*—fungal toxins contaminate some article or food.
3. Toxin produced by fungus is known as *mycotoxin.*
4. Mycetism may cause gastrointestinal disease, dermatitis or death. Several varieties of poisonous mushrooms are inedible. The hallucinogenic agents (d-lysergic acid, *psilocybin*) are produced by *Psilocybe* species.
5. The best known mycotoxin is *Aflatoxin* produced by *A. flavus.* It is frequently present in groundnuts, corns and peas. It can cause hepatomas in animals, and its possible carcinogenic effect in human beings is of great concern.
6. *Ergotoxicosis* (ergotism) is due to the toxic ergot alkaloids produced by the fungus *Claviceps purpurea.*
7. There are also other fungi responsible for mycotoxicosis (Table 66.2).

Table 66.2 Fungi Responsible for Mycotoxicosis

Fungus	Mycotoxin produced
Aspergillus flavus	Aflatoxin
Fusarium graminarum	Mycotoxin
Mushrooms (toad stools)	Mycotoxin
Claviceps species	Ergot alkaloids
A. fumigatus	Fumigatin
A. ochraceus	Ochratoxin
Penicillium rubrum	Rubratoxin B
Penicillium puberulum	Penicillic acid
Fusarium nivale	Sciepenols
Amanita muscaria	Muscarine

KEY POINTS

1. Study of fungi is called *Mycology.*
2. Fungi possess rigid cell walls containing chitin, mannan and other polysaccharides. The cytoplasmic membrane contains sterols. The cytoplasm contains true nuclei with nuclear membrane, mitochondria and endoplasmic reticulum.
3. Based on the morphology, there are four main groups of fungi namely *yeasts, yeasts like fungi, moulds* and *dimorphic fungi.*
4. Infections caused by fungus is known as *mycoses.* Fungal infections are of three principal clinical types: *superficial mycoses, subcutaneous mycoses* and *systemic mycoses.*
5. Dermatophytes are a group of fungi that infect only superficial keratinised tissue (*skin, hair* and *nails*) without involving the living tissue. Three genera of dermatophytes include *Trichophyton, Microsporum* and *Epidermophyton.*

6. *Mycetoma* is a chronic granulomatous infection of the subcutaneous tissue, usually affects the foot and rarely the other parts of body. The disease was first described from Madurai, South India. It is therefore commonly referred to as *Madura foot* or *Maduramycosis*.
7. *Sporotrichosis* is a nodular, ulcerating disease of skin and *subcutaneous* tissue. It is caused by *Sporothrix schenckii*, a *dimorphic* fungus.
8. *Rhinosporidiosis* is a chronic granulomatous disease characterised by formation of friable polyps, usually confined to the *nose,* mouth or eye. Causative agent is *Rhinosporidium seeberi*. This fungus has *not* been *cultivated.*
9. *Dimorphic fungi* exist as *yeasts* in the host tissue and in the cultures at *37°C* and as *hyphal* (*mycelial*) forms in the soil and in the cultures at *22-25°C*. *Sporothrix schenckii, Histoplasma capsulatum, Blastomyces dermatitidis, Paracoccidioides brasiliensis*, and *Coccidioides immitis* are examples of dimorphic fungi.
10. *Histoplasmosis* is primarily a disease of *reticuloendothelial system*. It is caused by *Histoplasma capsulatum*. Mycelial growth containing *thick walled, spherical spores* with *tubercles* or *finger like projections* is a characteristic feature.
11. *Blastomycosis* is a chronic infection of the lungs which may spread to other tissues. It is also known as *North American blastomycosis*. Causative fungus is *Blastomyces dermatitidis*. *Spherical* or *oval* budding yeast cell with thick, *double contoured wall* is a characteristic feature. Each cell has only a *single broad based bud.*
12. *Paracoccidioidomycosis* is a chronic granulomatous disease involving lungs, mucosa, skin and lymphatic system. It was formerly called *South American blastomycosis*. Causative fungus is *Paracoccidioides brasiliensis*. Yeast cell with *multiple buds* is a typical feature.
13. *Coccidioidomycosis* is a primary infection of the lung. It occurs by inhalation of dust containing *arthrospores* of the fungus. The causative fungus is *Coccidioides immitis*. The yeast form is a *spherule* with a *thick doubly refractile wall* and filled with *endospores*. The mycelial phase contains *pseudohyphae* which fragment into *arthrospores* which are highly infectious.
14. *Cryptococcosis* is caused by *Cryptococcus neoformans*, a *capsulated yeast*. The fungus is a soil saprophyte and is particularly abundant in the *faeces* of *pigeons*. Cryptococcal meningitis is an important manifestation caused by *C. neoformans*. The disease is usually seen in immunocompromised host. *C. neoformans* has ability to *grow at 37°C, hydrolyse urea*, produce *brown colonies* on *niger seed agar* and produce disease in *mice* (*animal inoculation test positive*).
15. Some saprophytic fungi usually do not produce disease but may cause infection under special conditions such as immunocompromised individuals and in terminal stages of chronic disease. These are called *opportunistic fungi. Candida albicans, Aspergillus fumigatus, Aspergillus niger, Penicillium sp., Rhizopus* and *Mucor* are some examples of opportunistic fungi.
16. *Candida albicans* is an ovoid or spherical budding *yeast* cell. It causes *oral thrush, vulvovaginitis, keratitis, paronychia, onychia, napkin dermatitis in infants, chronic mucocutaneous candidiasis, urinary tract infection, intestinal candidiasis, pulmonary candidiasis, meningitis* and *septicaemia*. *C. albicans* has ability to form *germ tube*.
17. *Aspergillus fumigatus* may cause *aspergillus asthma, bronchopulmonary aspergillosis, aspergilloma and invasive aspergillosis*. *A. niger* causes *otomycosis*.
18. *Mucor, Rhizopus* and *Lichtheimia* are associated with mucormycosis.
19. *Penicillium marneffei* has been reported as an important opportunist pathogen in the *HIV infected* individuals.
20. *Pneumocystis jiroveci* causes *pneumonia* in *immunocompromised* patients.
21. Laboratory diagnosis of fungal infections consists of *direct microscopy*, *culture* on *Sabouraud's dextrose agar* (*SDA*) or *brain heart infusion* (*BHI*) *agar,* and to identify fungal elements in *tissue sections*.

YOU MUST KNOW

1. Differences of fungi from bacteria.
2. Classification of fungi.
3. Laboratory diagnosis of fungal infections.
4. Causative agents of ectothrix and endothrix.
5. List of dermatophytes.

6. Causative agents of mycetoma.
7. List of dimorphic fungi.
8. List of fungi causing systemic infections.
9. Histoplasmosis.
10. Rhinosporidiosis.
11. Cryptococcosis.
12. Diseases caused by *Candida albicans*.
13. Laboratory diagnosis of candidiasis.
14. Germ tube test.
15. List of opportunistic fungi.
16. Different species of Aspergillus.
17. Causative fungi of zygomycosis.
18. Fungi responsible for otomycosis.
19. Fungi responsible for mycotic keratitis.
20. Fungi responsible for mycotoxicosis.

STUDY QUESTIONS

1. Name various genera of dermatophytes. Discuss the laboratory diagnosis of infections caused by dermatophytes.
2. Write short notes on:
   (a) Classification of fungi
   (b) Superficial mycoses
   (c) Dermatophytes
   (d) Subcutaneous mycoses
   (e) Rhinosporidiosis
   (f) Mycetoma
   (g) Chromomycosis
   (h) Sporotrichosis
   (i) Histoplasmosis
   (j) Blastomycosis
   (k) Paracoccidioidomycosis
   (l) Coccidioidomycosis
   (m) Dimorphic fungi
   (n) Cryptococcosis
   (o) Opportunistic mycoses
   (p) Candidiasis
   (q) Aspergillosis
   (r) Zygomycosis
   (s) *Pneumocystis jiroveci*
   (t) Keratomycosis
   (u) Mycotic poisoning

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Which of the following fungi is not a mould?
   (a) *Aspergillus fumigatus*
   (b) *Rhizopus*
   (c) *Cryptococcus neoformans*
   (d) *Absidia*
2. Which of the following fungi is/are dimorphic?
   (a) *Paracoccidioides brasiliensis*
   (b) *Coccidioides immitis*
   (c) *Sporothrix schenckii*
   (d) All of the above
3. Which of the following methods can be used for laboratory diagnosis of fungal infections?
   (a) Direct microscopy of specimens
   (b) Culture
   (c) Demonstration of fungal elements in tissue
   (d) All of the above
4. Which of the following stains can be used for diagnosis of fungal infections?
   (a) Gram stain
   (b) India ink
   (c) Lactophenol cotton blue
   (d) All of the above
5. Which of the following culture media can be used for growing fungi?
   (a) Sabouraud's dextrose agar
   (b) Brain heart infusion agar
   (c) Both of the above
   (d) None of the above
6. Which of the following dermatophytes can infect hair, skin and nail?
   (a) Trichophyton
   (b) Microsporum
   (c) Epidermophyton
   (d) All of the above

7. Which of the following is/are anthropophilic dermatophytes?
   (a) *Trichophyton rubrum* (b) *Epidermophyton floccosum*
   (c) *Microsporum audouinii* (d) All of the above
8. Which of the following is zoophilic dermatophyte?
   (a) *Microsporum audouinii* (b) *M. canis*
   (c) *M. gypseum* (d) None of the above
9. Which of the following is geophilic dermatophyte?
   (a) *Microsporum gypseum* (b) *M. audouinii*
   (c) *M. canis* (d) None of the above
10. Microconidia are absent in which of the following dermatophytes:
   (a) *Trichophyton rubrum* (b) *Microsporum canis*
   (c) *Epidermophyton floccosum* (d) None of the above
11. Which of the following fungi can cause mycetoma?
   (a) *Madurella mycetomi* (b) *Pseudoallescheria boydii*
   (c) *Acremonium falciforme* (d) All of the above
12. Which of the following are pigmented fungi?
   (a) *Fonsecaea* (b) *Cladosporium*
   (c) *Phialophora* (d) All of the above
13. Sclerotic bodies are present in:
   (a) Mycetoma (b) Sporotrichosis
   (c) Chromoblastomycosis (d) Rhinosporidiosis
14. Which of the following fungi has not been cultured?
   (a) *Sporothrix* (b) *Rhinosporidium*
   (c) *Acremonium* (d) *Blastomyces*
15. Which of the following fungi infects reticuloendothelial system?
   (a) *Aspergillus fumigatus* (b) *Histoplasma capsulatum*
   (c) *Trichophyton rubrum* (d) All of the above
16. Which fungus contains highly infectious arthrospores?
   (a) *Histoplasma capsulatum* (b) *Blastomyces dermatitidis*
   (c) *Paracoccidioides brasiliensis* (d) *Coccidioides immitis*
17. Which of the following fungi is a capsulated?
   (a) *Candida albicans* (b) *Cryptococcus neoformans*
   (c) *Aspergillus fumigatus* (d) None of the above
18. Which of the following fungi is present abundantly in the faeces of pigeons?
   (a) *Cryptococcus neoformans* (b) *Candida albicans*
   (c) *Candida tropicalis* (d) *Candida rugosa*
19. Which animal is used for pathogenicity test in *Cryptococcus neoformans*
   (a) Mice (b) Rabbit
   (c) Guinea pig (d) None of the above
20. *Cryptococcus neoformans* can be differentiated from non-pathogenic cryptococci by:
   (a) Growth at 37°C (b) Urea hydrolysis
   (c) Production of brown colonies on niger seed agar (d) All of the above
21. Which of the following species of *Candida* is responsible for most of the cases in human infections?
   (a) *Candida albicans* (b) *C. krusei*
   (c) *C. glabrata* (d) *C. stellatoidea*
22. Which of the following infections may occur by *candida albicans*?
   (a) Oral thrush (b) Vulvovaginitis
   (c) Poronychia (d) All of the above
23. *Candida albicans* can be differentiated from other species of candida by:
   (a) Germ tube test (b) Chlamydospore formation
   (c) Carbohydrate fermentation and assimilation tests (d) All of the above

24. Which of the following species of *Aspergillus* is the main opportunistic pathogen in human disease?
(a) *Aspergillus fumigatus* (b) *A. niger*
(c) *A. flavus* (d) None of the above

25. Which of the following diseases can be caused by *Aspergillus* species?
(a) Fungal ball (b) Keratitis
(c) Sinusitis (d) All of the above

26. Which of the following *Aspergillus* species produce green coloured colonies on Sabouraud's dextrose agar
(a) *Aspergillus fumigatus* (b) *A. niger*
(c) *A. flavus* (d) None of the above

27. Which of the following *Aspergillus* species produce black coloured colonies on Sabouraud's dextrose agar?
(a) *Aspergillus niger* (b) *A. fumigatus*
(c) *A. flavus* (d) None of the above

28. Which of the following fungi is/are associated with zygomycosis?
(a) Mucor (b) Rhizopus
(c) Absidia (d) All of the above

29. Absence of rhizoids is a characteristic feature of:
(a) Mucor (b) Rhizopus
(c) Absidia (d) All of the above

30. Which of the following fungi are important opportunistic pathogens in immunocompromised patients?
(a) *Pneumocystis jiroveci* (b) *Penicillium marneffei*
(c) *Candida albicans* (d) All of the above

31. Otomycosis is caused by
(a) *Aspergillus niger* (b) *A. fumigatus*
(c) *Penicillium species* (d) All of the above

32. Which of the following fungi can cause keratomycosis?
(a) *Aspergillus niger* (b) *A. fumigatus*
(c) *Fusarium species* (d) All of the above

33. Which mycotoxin is produced by *Aspergillus flavus*?
(a) Aflatoxin (b) Ergot alkaloids
(c) Penicillic acid (d) Muscarine

34. Ergot alkaloids are toxins produced by:
(a) *Aspergillus flavus* (b) *Fusarium nivale*
(c) *Claviceps purpurea* (d) *Penicillium rubrum*

Answers (MCQs):

1. (c)	2. (d)	3. (d)	4. (d)	5. (c)
6. (a)	7. (d)	8. (b)	9. (a)	10. (c)
11. (d)	12. (d)	13. (c)	14. (b)	15. (b)
16. (d)	17. (b)	18. (a)	19. (a)	20. (d)
21. (a)	22. (d)	23. (d)	24. (a)	25. (d)
26. (a)	27. (a)	28. (d)	29. (a)	30. (d)
31. (d)	32. (d)	33. (a)	34. (c)	

UNIT VI

CLINICAL MICROBIOLOGY

Chapter 67

NORMAL MICROBIAL FLORA OF THE HUMAN BODY

Normal flora refers to the population of microorganisms that inhabit skin and mucous membranes of normal human body. A healthy foetus in-utero is free from microorganisms. During birth the infant is exposed to vaginal flora. Within a few hours of birth, oral and nasopharyngeal flora develops and in a day or two resident flora of the lower intestine appears. The normal microbial flora is more or less constant for each species of animal and can be divided into two groups, *resident flora* and *transient flora*.

Resident Flora

It consists of organisms which are regularly present in a particular area and when disturbed it re-establishes itself. For example, *Escherichia coli* is a normal inhabitant of the intestine.

Role of Resident Flora

Beneficial role

1. They prevent or suppress the colonisation/invasion of the body by pathogens.
2. The bacterial flora of the intestinal tract synthesise vitamins, especially vitamin K and several B vitamins.
3. Antibodies produced in response to commensals cross-react with pathogens having related or shared antigens and thus raise the overall immune status of the host against pathogens.
4. Colicins, produced by some organisms of normal flora, have a harmful effect on pathogens.
5. The endotoxins liberated by them may help the defence mechanism of the body by triggering the alternative complement pathway.

Disease production

1. They become pathogenic when the host resistance is lowered.
2. They may act as pathogens in tissue outside their normal habitat e.g. normal flora of the intestine may cause urinary tract infection.
3. Streptococcus mutans may cause dental caries.
4. Penicillinase producing organisms can aggravate infection by interfering with therapy.
5. Use of broad spectrum antibiotics, affects the normal flora by inhibiting sensitive bacteria and thereby allowing overgrowth of resistant bacteria.
6. Normal flora may cause confusion in diagnosis due

to their ubiquitous presence in the body and their resemblance to some of the pathogens.

Transient Flora

It consists of both non-pathogenic and potentially pathogenic bacteria that inhabit the body surface or mucous membrane for a limited period. They can be eliminated from the body surface by mechanical means. Pathogens such as the pneumococcus and the meningococcus may be found in nasopharynx of humans from time to time.

I. NORMAL FLORA OF THE SKIN

The skin contains 10^2 to 10^4 organisms per cm^2. Bathing has little effect on the resident flora of the skin. Low pH, fatty acids in sebaceous secretions and presence of lysozyme are important factors for eliminating non-resident microorganisms from the skin. *Staph. epidermidis* and *diphtheroids* are numerous and most constant in the skin. Other microorganisms include Peptococcus, *Str. viridans,* Enterococcus, Micrococcus, *Esch. coli,* Proteus, *Candida albicans, Pityrosporum orbiculare and Propionibacterium acne*. Penicillin resistant staphylococci are seen in individuals working in hospitals.

II. NORMAL FLORA OF THE CONJUNCTIVA

The conjunctiva is relatively free from bacteria due to the flushing action of tears and due to the presence of lysozyme in it. The predominant organisms are *Corynebacterium xerosis, Staph. epidermidis,* Moraxella species and non-haemolytic streptococci.

III. NORMAL FLORA OF THE NOSE AND NASOPHARYNX

The flora of nose harbours diphtheroids, staphylococci, streptococci and *Haemophilus* species.

The nasopharynx of the infant is sterile at birth but, within 2-3 days after birth, acquires the flora carried by the mother and the attendents. The nasopharynx is a natural habitat of the common pathogens which cause infections of the nose, throat, bronchi and lungs. *Pseudomonas aeruginosa, Esch. coli* and Proteus are also occasionally found in normal persons.

IV. NORMAL FLORA OF THE MOUTH

The mouth contains micrococci, Gram positive aerobic spore bearing bacilli, coliforms, Proteus and lactobacilli. The gum pockets between the teeth have a wide spectrum of anaerobic bacilli, anaerobic micrococci, microaerophilic and anaerobic streptococci, vibrios, fusiform bacilli, Corynebacterium species, actinomyces, mycoplasma and bacteroides are all found in varying extent. Candida and geotrichum have also been reported.

The mouth of infant is not sterile at birth. It generally contains the same organisms as those present in mother's vagina i.e. a mixture of micrococci, streptococci, coliform bacilli and Doderlein's bacilli. These organisms diminish in number during the first 2-5 days after birth and are replaced by the bacteria present in the mouth of the mother.

V. NORMAL FLORA OF THE UPPER RESPIRATORY TRACT

Within 12 hours after birth alpha haemolytic streptococci are present in the upper respiratory tract. They become the dominant organism of the oropharynx and remain so for life. In the pharynx and trachea, flora is similar to that of the mouth. Few bacteria are present in normal bronchi, but smaller bronchi and alveoli are normally sterile.

VI. NORMAL FLORA OF THE GASTROINTESTINAL TRACT

The gastrointestinal tract of the foetus in-utero is sterile. It becomes contaminated with organisms shortly after birth. In breast fed infants, the intestine contains lactobacilli, enterococci, colon bacilli and staphylococci. In bottle fed infants, the intestine contains *Leptotrichia*, anaerobic lactobacilli, colon bacilli and aerobic and anaerobic spore-bearing organisms. With the change of food, the flora change. Diet has a marked influence on the composition of the intestinal and faecal flora.

In normal adult, the surface of oesophageal wall contains microorganisms which are swallowed with saliva and food. Due to low pH of the stomach, it is virtually sterile except soon after eating. In gastric carcinoma and pyloric obstruction, there is proliferation of Gram positive cocci and bacilli due to stagnation of food-stuff.

As the acidic pH of the stomach becomes alkaline in the intestine, the number of bacteria increases progressively beyond the duodenum to the colon. The bacterial count is low in small intestine compared to that in large intestine.

The bacterial count in adult duodenum is 10^3-10^6 per gram, in the jejunum and proximal ileum 10^5-10^8 per gram and in the lower ileum and caecum 10^8-10^{10} per gram of contents. Lactobacilli and enterococci

predominate in the duodenum and upper ileum, but in the lower ileum and caecum the flora resemble the faecal flora. Colon and rectum contains about 10^{11} bacteria per gram of contents, constituting 10-20 per cent of the faecal mass. The normal flora of adult colon contains mostly (96-99 per cent) anaerobes and only 1-4 per cent aerobes. The anaerobic flora includes Bifidobacteria, anaerobic lactobacilli, Bacteroides species, anaerobic streptococci, and clostridia. The anaerobic condition of colon is maintained by aerobes, which utilise free oxygen. Enterococci, coliforms and small numbers of Proteus, Pseudomonas, mycoplasma, lactobacilli and candida are predominant aerobes present in normal colon.

VII. NORMAL FLORA OF THE GENITOURINARY TRACT

Mycobacterium smegmatis, a harmless commensal, is found in the secretions (smegma) of the genitalia of both males and females. This may, by its presence in the urine specimens, cause confusion with tubercle bacilli. Strains of Mycoplasma and Ureaplasma are frequently present as part of normal flora of genitalia of both sexes. *Gardnerella vaginalis,* Bacteroides species and alpha haemolytic streptococci have been found in penile urethra. The female urethra is either sterile or contains *Staph. epidermidis.*

The vagina of newly born child is sterile. Within 24 hours it is colonised with micrococci, enterococci and diphtheroids. In 2-3 days, the maternal oestrogen induces glycogen deposition in the vaginal epithelium. Glycogen facilitates the growth of a lactobacillus (Doderlein's bacillus) which ferments glycogen to produce acid. The vaginal secretion now becomes acidic and the flora for a few weeks is similar to that of the adult. After the passively transferred oestrogen has been eliminated in the urine, the glycogen disappears, along with Doderlein's bacillus and the pH of the vagina becomes alkaline. Thereafter, the vaginal flora changes to micrococci, alpha and non-haemolytic streptococci, coliforms and diphtheroids.

At puberty, there is again deposition of glycogen and the pH changes to acid due to the metabolic activity of Doderlein's bacilli. The pubertal flora persists throughout the reproductive period till menopause. After menopause, the flora resembles that found before puberty. The normal vaginal flora includes clostridia, anaerobic streptococci, *Bacteroides* species, *Gardnerella vaginalis*, diphtheroids, listeria and *Candida albicans*.

VIII. EXTERNAL AUDITORY MEATUS

Being an extension of skin, it is profusely colonised by *Staph. epidermidis* and diphtheroids.

KEY POINTS

1. *Normal flora* refers to the population of microorganisms that inhabit skin and mucous membranes of normal human body.
2. *Resident flora* consists of organisms which are regularly present in a particular area and when disturbed it re-establishes itself. They prevent or suppress the colonisation/invasion of the body by pathogens.
3. *Transient flora* consists of both non-pathogenic and potentially pathogenic bacteria that inhabit the body surface or mucous membrane for a limited period.
4. Different bacteria are present as normal flora on different sites e.g. skin, nose, mouth, upper respiratory tract, gastrointestinal tract, genitourinary tract etc.

YOU MUST KNOW

1. Role of normal flora in human body
2. List of organisms present as normal flora in upper respiratory tract and gastrointestinal tract.

STUDY QUESTIONS

1. Define normal flora. What is the role of this flora in human body.
2. Write short notes on :
   (a) Normal flora of the skin
   (b) Normal flora of the mouth and upper respiratory tract
   (c) Normal flora of the gastrointestinal tract
   (d) Normal flora of the genitourinary tract

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Which of the following bacteria can be present normally in the conjunctiva?
   (a) *Corynebacterium xerosis*
   (b) *Staphylococcus epidermidis*
   (c) *Moraxella species*
   (d) All of the above
2. Which bacteria is responsible for producing acidic pH in adult vagina?
   (a) *Lactobacillus*
   (b) *Bacteroides* species
   (c) *Diphtheroids*
   (d) *Gardnerella vaginalis*

Answers (MCQs):

1. (d) 2. (a)

Chapter 68

SORE THROAT AND PNEUMONIA

Sore Throat

I. Causative Agents

II. Laboratory Diagnosis

A. Specimen
B. Collection
C. Direct Microscopy
D. Culture
E. Identification
F. Antibiotic Sensitivity

Pneumonia

I. Types

II. Laboratory Diagnosis

A. Specimens
B. Direct Microscopy
C. Culture
D. Detection of Bacterial Antigens
E. Serology

SORE THROAT

Sore throat is essentially an acute tonsillitis and/or pharyngitis. It is characterised by redness and oedema of mucosa, exudation of tonsils, pseudomembrane formation, oedema of uvula and enlargement of cervical lymph nodes. Viruses are more common cause of sore throat. About two-third cases are of viral origin.

I. CAUSATIVE AGENTS

Bacteria

Streptococcus pyogenes—This is the most common bacterial cause.
Streptococcus groups C and G
Corynebacterium diphtheriae
Haemophilus influenzae
Bordetella pertussis
Treponema vincentii
Leptotrichia buccalis

Fungus

Candida albicans

Viruses

Epstein-Barr virus
Adenoviruses
Coxsackievirus A

Of these *C. diphtheriae, Candida albicans, Streptococcus pyogenes, Treponema vincentii* and *Leptotrichia buccalis* may lead to pseudomembrane formation.

II. LABORATORY DIAGNOSIS

The signs and symptoms of sore throat caused by streptococci and viruses are similar. These include pain on swallowing, congested tonsils and pharynx, enlarged lymph nodes and pyrexia. If untreated, streptococcal tonsillitis may give rise to complications such as

peritonsillar abscess, sinusitis or immune complex diseases (rheumatic fever, glomerulonephritis).

A. Specimen

Throat swab from fauces

B. Collection

Two sterile swabs should be used, one for direct microscopy and other for culture. Swabs should be rubbed over the tonsillar fossa, pseudomembrane if present, and finally at the post pharyngeal wall. These swabs should include exudate present in the throat. They should be quickly sent to the laboratory. If delay is inevitable, swabs should be refrigerated.

C. Direct Microscopy

Gram staining and Albert staining may be used for staining the smears.

1. Gram Staining

It is helpful in Vincent's organisms or *Candida albicans.* Vincent's infection shows Gram negative spirochaetes (*Borrelia vincentii*) and Gram negative fusiform bacilli (*Fusobacterium* spp.). When *Candida albicans* is suspected, it appears as Gram positive oval budding yeast cells. Gram staining is not helpful in the diagnosis of other causative organisms.

2. Albert Staining

Albert staining is helpful for presumptive diagnosis of *C. diphtheriae*. It shows green coloured, V or L shaped (Chinese letter pattern) bacilli with bluish-black metachromatic granules.

D. Culture

Culture media are selected according to the organism suspected to be the causative agent of sore throat. Following media may be used for culture:

Blood agar—All the organisms will grow on this medium.

Crystal violet blood agar—It is a selective medium for *Str. pyogenes.*

Loeffler's serum slope—If *C. diphtheriae* is suspected, it should be included.

Potassium tellurite blood agar—It is a selective medium for growing *C. diphtheriae.*

Sabouraud's dextrose agar (SDA)—When suspecting *Candida albicans,* SDA should be included.

These culture media are incubated at 37°C for overnight. In case of potassium tellurite blood agar, it should be incubated for 48 hours. After 6-8 hours, a subculture should be made from Loeffler's serum slope onto potassium tellurite blood agar, which is then incubated at 37°C for 48 hours.

E. Identification

1. Colony Morphology

(*i*) *Str. pyogenes*—Pin-point, round or convex colonies with entire margins, having β-haemolysis are observed.

(*ii*) *C. diphtheriae*—On potassium tellurite blood agar, black coloured round colonies are seen.

(*iii*) *Candida albicans*—White or cream coloured colonies may be seen.

2. Gram Staining or Albert Staining

(i) Gram staining

— Small Gram positive cocci which may be present in chains is characteristic of *Str. pyogenes*

— *Candida albicans* reveals Gram positive budding yeast cells.

— *C. diphtheriae* is seen as Gram positive bacilli.

(ii) Albert staining

C. diphtheriae shows green coloured V or L shaped bacilli with bluish-black metachromatic granules.

3. Other tests for confirmation

(i) For β-haemolytic streptococci

(a) Bacitracin sensitivity test—for *Str. pyogenes*

(b) Lancefield grouping—for all β-haemolytic streptococci

(ii) For C. diphtheriae

(a) Biochemical tests

(*b*) Toxigenicity tests

- Elek's gel precipitation test
- Animal inoculation test

(iii) For Candida albicans

(a) Germ tube test

(b) Carbohydrate fermentation and assimilation tests.

(iv) For other causative agents

Special culture media and different biochemical reactions or serological tests may be required as described in respective chapters.

For details of tests mentioned above, refer to the corresponding chapters.

F. Antibiotic Sensitivity

All β-haemolytic group A streptococci are sensitive to penicillin G, and most are sensitive to erythromycin. *C. diphtheriae* is sensitive to penicillin.

PNEUMONIA

Pneumonia may be defined as inflammation and consolidation of the lung substance. Bacterial causes for pneumonia are listed in Table 68.1.

Table 68.1 Bacterial Causes of Pneumonia

Lobar Pneumonia	
	Streptococcus pneumoniae
Bronchopneumonia	
	Streptococcus pneumoniae
	Haemophilus influenzae
	(*rarely Kleb. pneumoniae, Staph. aureus*)
Atypical pneumonia	
	Mycoplasma pneumoniae
	Chlamydophila psittaci
	Chlamydophila pneumoniae
	Coxiella burnetii
Legionnaire's disease	
	Legionella pneumophila

I. TYPES

Different types of pneumonia include:

1. Lobar Pneumonia

It is an acute inflammation characterised by homogenous consolidation of one or more lobes.

2. Bronchopneumonia

It is almost always a secondary infection and generally follows viral infections of the respiratory tract. It is an acute inflammation of bronchi and the consolidation is scattered

3. Atypical Pneumonia

There is patchy consolidation of lungs. It is caused by *Mycoplasma pneumoniae*.

4. Pneumonia in Immunocompromised Patients

The common causative agents include *Pneumocystis jiroveci, Staph. aureus, Ps. aeruginosa*, viral infections (CMV and herpes) and *M. tuberculosis*. Respiratory syncytial virus causes bronchiolitis and bronchopneumonia in young children. Other viruses include influenza, parainfluenza viruses, adenovirus and measles virus.

II. LABORATORY DIAGNOSIS

A. Specimens

- Sputum
- Pleural fluid
- Blood
- Blood for serological tests

B. Direct Microscopy

1. Gram Staining

Adequate number of pus cells alongwith presence of predominant organisms gives a clue to the probable pathogen.

2. Ziehl-Neelsen Staining

Presence of acid-fast bacilli (AFB) gives a presumptive diagnosis of tuberculosis.

3. Giemsa Staining

Giemsa stain of sputum is useful to detect cysts and trophozoites of *Pneumocystis jiroveci*.

C. Culture

Sputum, blood and pleural fluid can be cultured on blood agar and chocolate agar. Purulent portion of sputum is best for culture. If the sputum is too viscid, it may be homogenised.

1. Blood agar—It is incubated aerobically at 37°C under 5-10 per cent CO_2.
2. Chocolate agar—It is also incubated at 37°C with 5-10 per cent CO_2.
3. Lowenstein-Jensen (LJ) medium—Three specimens of sputum are collected on three successive days for culture on LJ media which are then incubated at 37°C for 6-8 weeks.
4. Selective media are required for culture of *L. pneumophila* (see Chapter 38).
5. Isolation of chlamydophila can be done on cell-lines (see Chapter 50).

D. Detection of Bacterial Antigens

Direct Immunofluorescence Test

Direct immunofluorescence examination of sputum is done to detect antigens in *L. pneumophila*.

E. Serology

Diagnosis of some causative organisms is made by demonstration of antibody in patient's serum. Most often these are diagnosed by high titres in a single sample, but demonstration of a four fold rise in titre of antibody is better.

1. Mycoplasma pneumoniae

- Complement fixation test (CFT)
- Cold agglutinin test

2. Chlamydophila pneumoniae

- Microimmunofluorescence with a TWAR antigen
- CFT

3. Legionella pneumophila

- Immunofluorescent antibody test

4. Coxiella burnetii

- CFT

KEY POINTS

1. *Sore throat* is essentially an acute tonsilitis and/or pharyngitis. It is characterised by redness and oedema of mucosa, exudation of tonsils, pseudomembrane formation, oedema of uvula and enlargement of cervical lymph nodes.
2. *Viruses* are more common cause of sore throat. About two-third cases are of viral origin.
3. *Streptococcus pyogenes* is the *most common bacterial* cause.
4. Laboratory diagnosis of sore throat caused by bacteria depends on *direct microscopy* and *culture*.
5. *Pneumonia* may be defined as inflammation and consolidation of the lung substance.
6. *Streptococcus pneumoniae* is the most important bacterial cause of pneumonia. Other bacterial causes include *Haemophilus influenzae, Mycoplasma pneumoniae, Chlamydophila pneumoniae* and *Legionella pneumophila*.
7. Laboratory diagnosis of pneumonia depends on *direct microscopy* and *culture*.

YOU MUST KNOW

1. Causative agents of sore throat.
3. Laboratory diagnosis of sore throat.
4. Bacterial causes of pneumonia.

STUDY QUESTIONS

1. Define normal flora. What is the role of this flora in human body.
2. Write short notes on :
   (a) Normal flora of the skin
   (b) Normal flora of the mouth and upper respiratory tract
   (c) Normal flora of the gastro-intestinal tract
   (d) Normal flora of the genitourinary tract

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Which is the most common bacterial cause of sore throat?
   (a) *Streptococcus pyogenes*
   (b) Streptococcus group C
   (c) Streptococcus group G
   (d) Streptococcus group D
2. Which of the following may cause pseudomembrane formation in throat?
   (a) *Corynebacterium diphtheriae*
   (b) *Streptococcus pyogenes*
   (c) *Candida albicans*
   (d) All of the above
3. Which of the following bacteria can cause pneumonia?
   (a) *Streptococcus pneumoniae*
   (b) *Haemophilus influenzae*
   (c) Both of the above
   (d) None of the above
4. Which of the following agents may cause pneumonia in immunocompromised patients?
   (a) *Pneumocystis jiroveci*
   (b) *Pseudomonas aeruginosa*
   (c) *Staphylococcus aureus*
   (d) None of the above

Answers (MCQs):

1. (a) 2. (d) 3. (c) 4. (d)

Chapter 69

URINARY TRACT INFECTIONS

Urinary tract infection is the second most common infection after respiratory tract infection. Urinary tract infection (UTI) is defined as a disease caused by microbial invasion of the genitourinary tract that extends from the renal cortex of the kidney to the urethral meatus. The presence of detectable bacteria in the urine is named as bacteriuria. Presence of pus cells in urine denotes *pyuria* which most often accompanies UTI.

Normally, kidneys, ureters, urinary bladder and proximal urethra are sterile, but bacteria may be present in distal urethra as transient flora, most of which are derived from the faecal flora.

I. TYPES OF UTI

It is divided into two broad categories:

1. Lower UTI

(i) Urethritis
(ii) Cystitis
(iii) Prostatitis

Lower UTI is due to ascending infection caused by faecal coliforms.

2. Upper UTI

(i) Acute pyelitis—infection of pelvis of kidney
(*ii*) Acute pyelonephritis—infection of parenchyma of kidney.

Pyelonephritis is probably due to haematogenous infection.

II. PREDISPOSING FACTORS

1. Gender
   Females are more frequently affected by UTI due to the following reasons:
   (a) short length of urethra and its proximity to anus
   (b) sexual intercourse leads to introduction of bacteria into the bladder.
2. Pregnancy
   It predisposes to upper UTI.
3. Obstruction to flow of urine (stone, stricture, prostatic hypertrophy and tumour)
4. Neurogenic bladder dysfunction (spinal cord injury, multiple sclerosis)
5. Bacterial virulence

6. Reflux of urine from bladder up into ureters and sometimes into the renal pelvis
7. Genetic factors (genetically determined receptors on uroepithelial cells)

III. CLINICAL FEATURES

1. Asymptomatic

Symptomless urinary tract infection is not uncommon and can be detected only by urine culture.

2. Symptomatic

Symptoms include frequency, dysuria, suprapubic pain alongwith loin pain. There may be fever with rigors. UTI is more frequent in women than men. In males over 60 years, in whom, UTI due to enlarged prostate is relatively common.

IV. CAUSATIVE ORGANISMS

A. Gram Negative Bacilli

Gram negative bacilli are by far the most common infecting agents.

1. *Escherichia coli* is the commonest cause of UTI. It is responsible for about 70–80% of acute infections in general population and 50% of hospital-acquired infections. Certain strains of *Esch. Coli* (serogroups O2, O4, O6, O7, O8, O75) appear well-adapted to invade the urinary tract. These strains are normally found in the faeces.
   Virulence of *Esch. coli* may be associated with:
   (a) K antigens which inhibit phagocytosis and bactericidal effect of complement.
   (b) bacterial adhesion to receptors on uroepithelium by specialised fimbriae.
2. *Klebsiella* spp.
3. *Proteus* spp., especially *P. mirabilis*
4. *Enterobacter*
5. *Pseudomonas aeruginosa*
6. *Serratia*

B. Gram Positive Cocci

1. *Enterococci* e.g. *E. faecalis*
2. *Staph. saprophyticus*
3. *Staph. aureus*
4. *Staph. epidermidis*—It is found in urine as a contaminant but may occur as pathogen in immunocompromised individuals.

C. Miscellaneous

1. *M. tuberculosis*
2. Citrobacter
3. Salmonellae
4. *Str. pyogenes*
5. *Str. agalactiae*
6. *Gardnerella vaginalis*

D. Fungus

Candida albicans may cause UTI in diabetes and immunocompromised patients.

V. LABORATORY DIAGNOSIS

Culture of the urine is necessary for the identification of the organism and its antimicrobial susceptibility test.

A. Specimen Collection

1. Midstream Urine Specimen (MSU)

It is collected preferably prior to administering antibiotics. Specimen is collected in a sterile container. Before collecting a sample, genitalia should be cleaned with soap and water and men are instructed to retract the foreskin of glans penis whereas women should keep the labia apart. The first portion of urine is allowed to pass then without interrupting the urine flow, mid-portion of the stream is collected. The first portion of urine adequately flushes out the normal urethral flora.

2. Catheter Specimen

Urine should be collected directly from the catheter and not from the collection bag. The catheter should not touch the container. Although a catheter specimen yields excellent results but catheterisation to obtain urine is not justified because of risk of introducing infection.

3. Urine Specimens from Infants

A clean catch specimen after cleansing of genitalia is preferred. Another procedure of collecting specimen in infants is suprapubic aspiration. This procedure may also be used in adult women when uncontaminated specimen cannot be obtained by other methods.

B. Transport

As urine is a good culture medium, specimens after collection should reach the laboratory with minimum delay, if this is not possible, the specimen is to be refrigerated at 4°C.

C. Laboratory Methods

Part of the specimen is used for bacteriological culture and the rest is examined immediately under the microscope.

1. Microscopy

Urine is centrifuged and deposit is examined under microscope for detecting pus cells, epithelial cells, erythrocytes and bacteria. *Pyuria* is associated with most clinical infections but may be absent in symptomless

bacteriuria. Pyuria without bacteriuria may be an indicator of renal tuberculosis.

Presence of urinary casts, red cells, tubular epithelial cells or atypical cells will indicate non-infective lesions such as glomerulonephritis or tumour.

2. Culture

Uncentrifuged urine is inoculated on blood agar and MacConkey's agar. Culture plates are incubated at 37°C for 24 hours. Bacteria isolated on culture are identified. Most laboratories use a semiquantitative method (standard loop technique) for culture of urine specimens.

Standard Loop Technique

A standard calibrated loop is used to culture a fixed volume of uncentrifuged urine. Blood agar and MacConkey's agar are used and incubated at 37°C for 24 hours. Next day, the number of colonies obtained is counted and the total count per ml is calculated.

The fixed volume loop is 4mm in diameter and can hold 0.005 ml urine (i.e. 200 loopfuls make one ml), the total bacterial count per ml will be number of colonies multiplied by 200. Single bacterium would form a single colony, therefore, the number of colonies shall be equal to number of bacteria present.

Interpretation of results

Kass (1956) gave a criterion of active bacterial infection of urinary tract as follows:

(*i*) Count more than 10^5 bacteria of single species per ml : *significant bacteriuria* which indicates active UTI.

(*ii*) Between 10^4 to 10^5 bacteria per ml is of *doubtful significance*, specimen should be repeated for culture.

(*iii*) Less than 10^4 bacteria per ml : no *significant growth* but regarded as contaminant. Contamination is also considered when three or more bacteria are isolated.

Identification of the organisms

The organisms are identified by colony characteristics, Gram staining, motility, biochemical reactions and serological tests.

3. Other Methods

(i) Dip slides

Commercially available plastic slides coated with CLED (Cysteine lactose electrolyte deficient) agar on one side and MacConkey's agar on the other side is dipped into the urine and replaced in the sterile container and incubated at 37°C. Viable count is obtained by comparing growth with the manufacturer's chart. This method is relatively expensive.

(ii) Pour plate method

It is a quantitative method but it is too cumbersome for routine diagnostic work.

(iii) Griess nitrite test

It is based on nitrate-reducing enzymes produced by bacteria present in urine. Gram negative bacteria reduce nitrates to nitrites. The presence of nitrite detected by the test indicates UTI. Normal urine does not contain nitrite.

(iv) Triphenyltetrazolium chloride (TTC) test

TTC is reduced by several frequently encountered urinary tract pathogens and produce a pink red precipitate.

(v) Catalase test

The presence of catalase is evident by bubbles formation on addition of hydrogen peroxide. It indicates bacteriuria, though this test will be positive in catalase producing bacteria only.

(vi) Gram staining

Presence of at least one bacteria per oil immersion field (examining 20 fields) correlates with significant bacteriuria ($>10^5$ bacteria/ml).

(vii) Glucose test paper

It is based on utilisation of the minute amounts of glucose present in normal urine, by bacteria causing the infection. Hence, it indicates bacteriuria.

(viii) Polymorphonuclear neutrophils (PMNs)

PMNs are counted in uncentrifuged urine specimen with help of haemocytometer. 8 PMN/mm^3 is indicative of infection.

(ix) Leucocyte esterase

Presence of this enzyme in urine is indication of bacteriuria.

(x) Detection of lipopolysaccharide (endotoxin) in urine specimen

It is useful mainly in Gram negative bacilli.

4. Differentiation of Upper UTI and Lower UTI

Antibody coated bacteria detected by immunofluorescence test indicates upper UTI. This is based on the assumption that bacteria coated with antibodies are present in urine only when the kidneys are infected (upper UTI). These antibody coated bacteria are absent in lower UTI.

D. Antibiotic Susceptibility Testing

It is done by Stokes disc diffusion method to find out suitable antibiotics for treatment.

VI. TUBERCULOSIS OF KIDNEY AND URINARY TRACT

Tuberculosis of kidney is a blood-borne infection. The patient presents with frequency and painless haematuria and routine urine culture does not show any pathogen. Tuberculosis must be considered in cases where pyuria is present without bacteriuria.

Laboratory Diagnosis

Specimen

Excretion of *M. tuberculosis* from kidney is intermittent, hence, mid-stream urine specimen is not useful. Early morning urine specimens should be collected in sterile container on three consecutive days.

Direct Ziehl-Neelsen Staining

Smear made from centrifuged deposit of urine is stained with Ziehl-Neelsen staining and may reveal acid-fast bacilli. Saprophytic mycobacteria (e.g. *M. smegmatis*) may be present in normal urine which may be excluded by using acid-alcohol as decolourising agent in staining procedure. *M. tuberculosis* is acid-alcohol-fast while *M. smegmatis* is only acid-fast.

Culture

Culture is performed on Lowenstein-Jensen medium and incubated for 6-8 weeks. Growth is identified by Ziehl-Neelsen staining and biochemical tests.

KEY POINTS

1. *Urinary tract infection* (*UTI*) is defined as a disease caused by microbial invasion of the genitourinary tract that extends from the renal cortex of the kidney to the urethral meatus.
2. The presence of detectable bacteria in the urine is named as *bacteriuria*.
3. Lower UTI includes *urethritis*, *cystitis*, or *prostatitis* while upper UTI includes *acute pyelitis* or *acute pyelonephritis*.
4. *Escherichia coli* is the *commonest cause* of UTI.
5. *Midstream urine specimen* (*MSU*) is collected for laboratory diagnosis of UTI.
6. Culture of the urine is necessary for the identification of organism. *Standard loop technique* is used to culture a fixed volume of *uncentrifuged* urine.
7. Count *more than* 10^5 *bacteria* of single species per ml is called *significant bacteriuria*. It indicates active UTI.

YOU MUST KNOW

1. Causative organisms of urinary tract infection.
2. Laboratory diagnosis of urinary tract infection.

STUDY QUESTION

1. Name various organisms causing urinary tract infection. Discuss the laboratory diagnosis of this condition.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Which of the following conditions is/are called lower urinary tract infection?
   (a) Cystitis (b) Urethritis (c) Prostatitis (d) All of the above
2. What is the commonest cause of urinary tract infection?
   (a) *Escherichia coli* (b) *Proteus mirabilis* (c) *Pseudomonas aeruginosa* (d) *Serratia* species
3. Which of the following cocci may cause urinary tract infection?
   (a) *Enterococcus faecalis* (b) *Staphylococcus aureus* (c) *Staphylococcus saprophyticus* (d) All of the above
4. What is significant bacteriuria?
   (a) Bacterial count > 10^5 per mL (b) Bacterial count between 10^4 to 10^5 per mL
   (c) Bacterial count < 10^4 per mL (d) None of the above
5. Which of the following methods may be used for diagnosis of urinary tract infection?
   (a) Griess nitrate test (b) Triphenyl tetrazolium chloride test
   (c) Detection of leucocyte esterase enzyme (d) All of the above

Answers (MCQs):

1. (d) 2. (a) 3. (d) 4. (a) 5. (d)

Chapter 70

DIARRHOEAL DISEASES

I. DEFINITIONS

A. Diarrhoea

Diarrhoea is defined as an increase in the frequency, fluidity or volume of bowel movements, relative to the usual habits of an individual. Passage of three or more motions a day can be taken as diarrhoea.

B. Gastroenteritis

It is often used as synonym for acute diarrhoea, especially when associated with vomiting. Gastroenteritis may be defined as inflammation of the mucous membrane of stomach and intestine resulting in frequent loose motions with or without mucous and with or without blood, pain abdomen and with or without fever.

C. Dysentery

Dysentery means passage of blood and mucous with motion, often associated with tenesmus.

For all practical purposes the terms diarrhoea, gastroenteritis and dysentery are collectively included as *diarrhoeal diseases*.

D. Traveller's Diarrhoea

It is an acute diarrhoeal illness that sometimes occurs in visitors from foreign countries, within a week or two of arrival in a developing country.

E. Food Poisoning

The term food poisoning means an illness acquired through consumption of food or drink contaminated either with microorganisms, their toxins or chemical poisons. But traditionally, it is restricted to acute gastroenteritis caused by microbial contamination of food.

II. AETIOLOGY OF DIARRHOEAL DISEASES

A. Bacteria

1. *Vibrios*
   (*i*) *Vibrio cholerae*
   (*ii*) *V. parahaemolyticus*
   (*iii*) Other *halophilic vibrios*
2. *Esch. coli* (ETEC, EPEC)
3. *Salmonellae*
   (*i*) *Salmonella enteritidis*
   (*ii*) *S. typhimurium*
   (*iii*) Other Salmonella spp.
4. *Shigella* spp.
5. *Campylobacter jejuni*
6. *Yersinia enterocolitica*
7. *Clostridium perfringens*
8. *C. difficile*
9. *Staph. aureus*
10. *Bacillus cereus*
11. *Aeromonas hydrophila*
12. *Plesiomonas shigelloides*

B. Viruses

1. *Rotavirus*
2. *Norwalk virus*
3. *Adenovirus*
4. *Astrovirus*
5. *Calicivirus*

C. Protozoa

1. *Entamoeba histolytica*
2. *Giardia lamblia*
3. *Cryptosporidium parvum*

D. Fungus

1. *Candida albicans*

A. Bacteria

1. Vibrios

(i) Vibrio cholerae

V. cholerae, *classical* as well as *ElTor* biotypes, cause cholera. The vibrio enters the intestine through food or drink. *Vibrio cholerae* produces cholera toxin (CT) which is very similar to the heat labile toxin (LT) of *Esch. coli*.

(ii) Vibrio parahaemolyticus

It causes invasive diarrhoea and has been responsible for the majority of food poisoning cases in Japan. It occurs due to consumption of raw seafood (fish and shell fish). The mechanism of *V. parahaemolyticus* diarrhoea is not well understood. They produce a heat labile enterotoxin like that of *Esch. coli*.

(iii) Other halophilic vibrios

V. mimicus and V. vulnificus cause sporadic cases of diarrhoeal disease.

LABORATORY DIAGNOSIS

(a) Specimen

- Faeces

(b) Culture

Specimen is cultured on selective media such as TCBS or bile salt agar. Culture plates are incubated at 37°C for 24-48 hours. As *Vibrio parahaemolyticus* is a halophilic vibrio, it will grow only in media containing sodium chloride.

(c) Identification

Identification of isolates depends on colony morphology, biochemical reactions and slide agglutination test.

2. Esch. coli

(i) ETEC

Enterotoxigenic *Esch. coli* (ETEC) produce a heat-labile enterotoxin (LT), heat stable enterotoxin (ST) or both. ETEC causes mild to severe diarrhoea in the developing countries. This is the most important cause of traveller's diarrhoea.

(ii) EPEC

Enteropathogenic *Esch. coli* (EPEC) have been recognised as responsible for diarrhoea in infants. They neither produce toxin nor invade gut mucosa. The exact mechanism of their action is not known. They adhere to mucosal cells of small intestine and multiply.

(iii) EIEC

Enteroinvasive *Esch. coli* (EIEC) may cause watery diarrhoea or typical dysentery. These strains are invasive and resemble shigellae in pathogenicity.

(iv) EHEC

Enterohaemorrhagic *Esch. coli* (EHEC) is associated with haemorrhagic colitis and haemolytic uraemic syndrome. EHEC is, nowadays, called verocytotoxigenic *Esch. coli* (VTEC). In haemorrhagic colitis there is a blood stained diarrhoea. All age groups are affected.

It forms a verocytotoxin (VT) resembling the toxin of *Sh. shiga*. *Esch. coli* serogroup O157 is the verocytotoxigenic strain. Food is the important source of infection.

(v) EAEC

Enteroaggregative *Esch. coli* (EAEC) has been associated with persistent diarrhoea, especially in developing countries. It forms a heat stable enterotoxin called EAST 1 ('enteroaggregative heat stable enterotoxin-1').

LABORATORY DIAGNOSIS

(a) Specimen

- Faeces

(b) Culture

Faeces is inoculated on blood agar and MacConkey's agar. These media are incubated at 37°C for 24 hours.

(c) Identification

Identification of isolated organism is made by colony morphology, biochemical reactions, slide agglutination with antisera.

Sereny test is used for the identification of EIEC strains. Invasion of cultured HeLa cells is another method for identification of these strains.

Production of verocytotoxin (VT) is confirmed by testing the strains on Vero cells, in which they cause cytopathic effects.

3. Salmonellae

Acute gastroenteritis due to salmonellae is characterised by self-limited fever and diarrhoea. Incubation period is about 12-36 hours. The large majority of outbreaks are caused by *S. typhimurium, S. enteritidis, S. dublin, S. choleraesuis, S. heidelberg* and *S. thompson*. Human infection is usually caused by consumption of animal foods or food products. Infection leads to salmonella food poisoning. Sometimes septicaemia may develop.

LABORATORY DIAGNOSIS

(i) Specimen

- Faeces or any suspected food-stuff

(ii) Culture

Specimen is inoculated on an enrichment medium and the selective media such as MacConkey's agar, deoxycholate agar (DCA) and XLD agar. Wilson and Blair's medium may also be used. Selective media are incubated at 37°C for 24 hours. After 6 hours incubation of enrichment medium, subculture is made onto selective medium. Pale non-lactose fermenting colonies develop on MacConkey's agar and DCA or black shiny colonies on Wilson and Blair's medium. On XLD medium, colonies are red in colour.

(iii) Identification

It depends on colony morphology and biochemical reactions. The species is identified by agglutination test with polyvalent antisera using that of H and O.

4. Shigellae

All four serogroups of shigellae (*Shigella dysenteriae, S. flexneri, S. boydii* and *S. sonnei*) cause bacillary dysentery. In addition, shigellae may also produce a mild watery diarrhoea. All of them are invasive pathogens but *S. dysenteriae* type 1 also produces an enterotoxin and a cytotoxin. The infection is spread by faeces of patients and convalescent carriers via food.

LABORATORY DIAGNOSIS

(i) Specimens

- Faeces

(ii) Culture

Culture is done in the same way as that for salmonellae. Colonies are non-lactose fermenting on MacConkey's agar and DCA medium. *S. sonnei* is a late lactose fermenter.

(iii) Identification

It depends on colony morphology, biochemical reactions and agglutination test by group specific polyvalent antisera.

5. Campylobacter jejuni

C. jejuni occurs in intestinal flora of many animals, especially poultry which probably serves as the major source of human infection. Milk and waterborne outbreaks of diarrhoea have been reported. Incubation period varies from 3 to 10 days. The disease is sometimes associated with vomiting and bloody mucoid stools.

LABORATORY DIAGNOSIS

(i) Specimen

- Faeces

(ii) Culture

Faeces is inoculated on a selective medium containing vancomycin and polymyxin. The inoculated culture medium is incubated at 43°C under microaerophilic conditions. *C. jejuni* and *C. coli* selectively grow against other faecal bacteria.

(iii) Identification

The organisms are curved, Gram negative bacilli exhibiting darting motility. They are oxidase positive.

6. *Yersinia enterocolitica*

Y. enterocolitica has been identified as an important cause of diarrhoea. Sources of infection are birds and animals. Foodborne outbreaks have been reported. The symptoms appear to be related to the age of the patient. Predominant symptom in young children is acute watery diarrhoea. In older children and young adults, there is pain in abdomen, and fever which mimics appendicitis.

LABORATORY DIAGNOSIS

(i) Specimens

- Faeces
- Blood

(ii) Culture

It can be isolated from faeces or blood cultures. It produces non-lactose fermenting (NLF) colonies. It is Gram negative aerobic bacillus.

7. *Clostridium perfringens*

The organism is widely distributed in soil and is commonly found in faeces of man and animals. *Cl. perfringens* gastroenteritis or food poisoning is characterised by diarrhoea and abdominal pain following ingestion of contaminated food (meat, poultry products). Most cases of human clostridial gastroenteritis are caused by type A strains. The incubation period is 6-12 hours. Type A strains produce a heat-labile enterotoxin which acts on the membrane permeability of the small intestine. Enterotoxin production has also been reported for type C and type D strains.

LABORATORY DIAGNOSIS

Since *Cl. perfringens* type A forms a part of normal flora in 5-30% of the population, it is difficult to interpret its causative role. When the same serotype is isolated from large number of the victims of an outbreak and from the suspected food, a presumptive diagnosis can be made.

(i) Specimens

- Faeces
- Food

(ii) Culture

The specimen is inoculated on blood agar and incubated anaerobically at 37°C for 24 hours.

(iii) Identification

Colonies are either β-haemolytic or non-haemolytic. Gram staining shows Gram positive bacillus with subterminal spore. The organisms are further identified by Nagler reaction and serotyped by agglutination test.

8. *Clostridium difficile*

Cl. difficile is associated with antibiotic-associated diarrhoea and pseudomembranous colitis. Most common antibiotics involved are lincomycin, clindamycin, ampicillin and cephalosporins. There is an overgrowth of antibiotic-resistant *Cl. difficile* due to suppression of gut flora by antimicrobial therapy. *Cl. difficile* produces an enterotoxin (toxin A) and a cytotoxin (toxin B).

LABORATORY DIAGNOSIS

(i) Specimen

- Faeces

(ii) Culture

Faeces is cultured on selective media with subsequent toxigenicity test.

(iii) Demonstration of toxin

Toxin can be demonstrated in the faeces of patients by its characteristic effects on HEp-2 and human diploid cell cultures, or by ELISA. Toxin is specifically neutralised by antiserum of *Cl. sordelli*.

9. *Staphylococcus aureus*

Certain strains of *Staph. aureus* produce enterotoxins. There are six types of enterotoxins (A to F). Of these, enterotoxin type A is most often incriminated in outbreaks of staphylococcal food poisoning. These enterotoxins are heat-stable. Acute staphylococcal food poisoning is due to ingestion of preformed enterotoxin in contaminated food, and the causative organisms are often absent from the stool during the acute illness. The incubation period is 2 to 6 hours. Usually, there is an acute onset of nausea and vomiting, sometimes followed by diarrhoea. It is a self-limiting disease.

LABORATORY DIAGNOSIS

(i) Specimens

- Vomit
- Faeces
- Suspected food.

(ii) Culture

Specimen is inoculated on a selective medium (mannitol salt agar) or on ordinary media. Selective medium is preferred to isolate the organism from stool as it contains mixed flora.

(iii) Identification

The organism is identified by colony morphology, Gram staining, catalase test and coagulase test. Phage typing may be done for epidemiological purposes.

(iv) Demonstration of enterotoxin

Reverse passive latex agglutination assay (RPLA) or ELISA can be used to detect enterotoxin in food, vomit or culture filtrate of isolated strain. These kits are available commercially.

10. Bacillus cereus

It is ubiquitous aerobic spore bearing bacillus. It is capable of producing two discrete enterotoxins, one resembling LT of *Esch. coli* and other to that of staphylococcal enterotoxin. *B. cereus* can cause two distinct clinical syndromes, one characterised by diarrhoea and abdominal cramps (incubation period 6-16 hours), similar to gastroenteritis caused by *Esch. coli* and the other by nausea and vomiting (incubation period 1–2 hours), resembling staphylococcal food poisoning. The short incubation type of *B. cereus* food poisoning is invariably associated with contaminated fried rice. Brief rewarming of the contaminated food does not destroy heat stable enterotoxin. Soups and sauces are commonly implicated in long incubation type of *B. cereus* food poisoning.

LABORATORY DIAGNOSIS

(i) Specimens

- Vomit
- Faeces
- Suspected food

(ii) Culture

Specimen is cultured on ordinary media (nutrient agar or blood agar) and incubated at 37°C for 24 hours. A special mannitol-egg yolk-phenol red-polymyxin agar (MYPA) medium may also be used.

(iii) Identification

Colonies have *curled hair* appearance. Gram staining shows Gram positive spore bearing bacilli.

11. Other Bacteria

Aeromonas hydrophila and *Plesiomonas shigelloides* have been reported to cause diarrhoeal diseases.

12. Types of Bacterial Diarrhoea

Bacterial diarrhoea may be divided into two groups, those caused by invasive bacterial pathogens and those caused by non-invasive pathogens.

(i) Invasive bacterial pathogens

Salmonella species
Shigella species
Enteroinvasive Esch. coli (EIEC)
Enterohaemorrhagic Esch. coli (EHEC)
Vibrio parahaemolytius
Campylobacter jejuni
Yersinia enterocolitica

(ii) Non-invasive bacterial pathogens

These organisms cause gastroenteritis or food poisoning by production of toxin.

Enterotoxigenic *Esch. coli* (ETEC)
Enteropathogenic *Esch. coli* (EPEC)
Vibrio cholerae
Shigella dysentriae type 1
Staphylococcus aureus
Bacillus cereus
Clostridium perfringens
Clostridium difficile

B. Viruses

1. Rotavirus

This virus is now considered the most common cause of diarrhoea in infants and young children. The disease peaks from the age of six months to two years. The mode of infection is believed to be faeco-oral. The incubation period is 2 to 4 days. Vomiting is a prominent early symptom, often preceding diarrhoea. The stools are watery. Mild fever and respiratory symptoms may occur in some cases. Rotavirus may cause sporadic diarrhoea as well as large epidemics. The average duration of illness is 5 to 7 days.

2. Norwalk Virus

In addition to rotaviruses, *Norovirus* and *Sapovirus* genera cause mainly gastroenteritis in humans. They belong to family *Calciviridae*. They were also named "Norwalk-like viruses" and "Sapporo-like viruses" after the prototype viruses, the Norwalk and Sapporo virus respectively. Norwalk virus has been described here.

The Norwalk virus and related viruses appear to cause diarrhoea, mainly in older children and adults. Foodborne outbreaks have been reported. The incubation period is about 12 to 48 hours. Symptoms include nausea, vomiting, diarrhoea and mild fever. Prevalence of infection appears to be much less than that with rotavirus.

3. Adenoviruses

Adenoviruses have been reported to cause diarrhoeal disease in children. Adenovirus type 40 and 41 have been identified in such cases.

4. Other Viruses

Astroviruses, Caliciviruses, Torovirus, Picobirnavirus and Bocavirus have been reported to be associated with diarrhoeal illness.

5. Laboratory Diagnosis of Viral Diarrhoeas

(i) Specimen

- Faeces

(ii) Electron microscopy

Electron microscopy of faeces may demonstrate virus particles as in rotavirus and adenoviruses.

(iii) Fluorescent antibody test and ELISA test

Viral antigens in faeces can be detected by these tests.

C. Protozoa

1. Entamoeba histolytica

It causes amoebic dysentery. Human cyst passers are the source of infection. Food and drinks are the usual vehicles. It can also lead to extraintestinal manifestations.

2. Giardia lamblia

Infection with *Giardia lamblia*, may lead to explosive watery diarrhoea, foul smelling stools, and steatorrhoea. Asymptomatic carriers constitute the source of infection. Infection may take place through food and drink.

3. Cryptosporidium parvum

This parasite causes enteritis in calves and a variety of other animals and birds. *Cryptosporidium parvum* has been found to cause diarrhoea in man, particularly in the immunodeficient and those in close contact with animals.

4. Balantidium coli

This is a rare cause of chronic recurrent diarrhoea, with dysenteric episodes in some.

5. Laboratory Diagnosis

(i) Specimen

- Faeces

(ii) Microscopy

(a) Saline preparation and Iodine mount

Motility of trophozoites can be observed in saline preparation while cysts take up iodine and appear distinct in iodine mount. Cysts and motile trophozoites of *E. histolytica* can be observed in faeces of amoebic dysentery. *Giardia lamblia* cysts in formed stools, or trophozoites in fresh diarrhoeal stools can be seen in giardiasis. Trophozoites of *Balantidium coli* are found in liquid stool of this parasitic infection. Rarely cysts may be seen in formed stools.

(b) Acid-fast staining

Faeces smear shows acid-fast oocyst of *Cryptosporidium parvum.*

(iii) Serological tests

Indirect haemagglutination assay (IHA) and ELISA are used to detect antibody titre in sera of patients with amoebiasis.

D. Fungus

There have been reports of diarrhoea associated with *Candida albicans.*

III. DYSENTERY

Microorganisms causing dysentery are listed as follows:

A. Bacteria

1. *Shigella spp.* (*Sh. dysentriae, Sh. flexneri, Sh. boydii* and *Sh. sonnei*)
2. *Esch. coli* (EIEC, EPEC and EHEC)

B. Protozoa

1. *Entamoeba histolytica*
2. *Balantidium coli*

IV. FOOD POISONING

The term bacterial food poisoning is conventionally restricted to acute gastroenteritis due to the presence of bacteria, usually in large numbers or their products (toxin) in food. It may be divided into three types:

Infective type: Infective doses of microorganisms are ingested with food. Multiplication of bacteria occurs in-vivo. The typical example is food poisoning by salmonellae. Incubation period is generally 8-24 hours.

Toxic type: Preformed bacterial toxin is ingested with food, as in staphylococcal food poisoning. Incubation period is short (2 to 6 hours).

Intermediate type: Bacteria ingested with food release the toxin in the gut. The typical example is *Cl. perfringens* food poisoning. The incubation period is 6 to 12 hours.

A. Causes

1. Infective Type

Salmonella spp. (*S. typhimurium, S. enteritidis, S. newport, S. thompson, S. indiana*)
Vibrio parahaemolyticus
Campylobacter jejuni

2. Toxic Type

Staphylococcus aureus
Bacillus cereus
Clostridium botulinum

3. Intermediate Type

Clostridium perfringens

B. Food-Borne Botulism

It is a severe, often fatal, form of food-poisoning caused by ingestion of preformed toxin in food contaminated with *Cl. botulinum*. Incubation period is 1 to 2 days, but may be longer. The disease is characterised by pronounced neurotoxic effects. Symptoms may be nausea, vomiting or abdominal pain but no diarrhoea. Food-borne botulism has been assoicated with canned food, particularly vegetables and fruits. Spores of *Cl. botulinum* can withstand boiling in water (100°C) for several hours. There are seven main types of toxins (A to G) produced by *Cl. botulinum*. Toxin types A,B,E are the most frequently associated with human disease. Botulinum toxin (neurotoxin) is absorbed from the intestinal tract and enters the vascular system. It is then transported to peripheral cholinergic nerve terminals where it blocks the release of acetylcholine.

1. Clinical Features

Cranial nerve involvement usually affects the occulomotor muscles leading to diplopia and drooping eyelids with a squint. There may be vertigo, blurred vision and dry mouth. There is progressive symmetric descending flaccid paralysis which can lead to respiratory failure and death.

2. Laboratory Diagnosis

(i) Specimens

- Food
- Vomitus
- Faeces
- Blood

(ii) Demonstration of toxin

Toxin can be demonstrated in specimens by toxin-antitoxin neutralisation tests in mice.

(iii) Demonstration of the organisms

Smears made from suspected food and faeces are examined by Gram staining. Gram positive sporing bacilli can be seen. Culture may be done on blood agar or cooked meat broth (CMB) under anaerobic conditions. Detection of toxin in culture fluid is done by toxigenicity test in mice. Mere presence of bacilli in food or faeces in absence of toxin is of no significance.

C. Laboratory Diagnosis of Other Organisms

To diagnose a case of food poisoning, the aetiological agent should be demonstrated in the stool of patient and also in the food consumed. Laboratory diagnosis of other microorganisms responsible for food poisoning has been described earlier in this chapter under 'diarrhoeal diseases'.

For details of various organisms described in this chapter, refer to concerned chapters.

KEY POINTS

1. Diarrhoea is defined as an increase in the frequency, fluidity or volume of bowel movements, relative to the usual habits of an individual. Passage of three or more motions a day can be taken as diarrohea.
2. *Gastroenteritis* may be defined as inflammation of the mucous membrane of stomach and intestine resulting in frequent loose motions with or without mucous and with or without blood, pain abdomen and with or without fever.
3. *Dysentery* means passage of *blood* and mucous with motion, often associated with tenesmus.
4. *Traveller's diarrhea* is an acute diarrhoeal illness that sometimes occurs in visitors from foreign countries within a week or two of arrival in a developing country.
5. The term *food poisoning* means an illness acquired through consumption of food or drink contaminated either with microorganisms, their toxins or chemical poisons. But traditionally, it is restricted to acute gastroenteritis caused by microbial contamination of food.
6. *Vibrio cholerae, Esch. coli, Salmonellae* are some important bacterial causes of diarrhoeal diseases. Rotavirus is the most important viral etiology of diarrhoea.
7. *Shigella spp.* and *Entamoeba histolytica* cause *bacillary dysentery* and *amoebic dysentery* respectively.
8. *Salmonella typhimurium* and *Vibrio parahaemolyticus* cause infective type of food poisoning while *Staphylococcus aureus* is an example of toxic type of food poisoning.
9. Laboratory diagnosis of diarrhoea, dysentery and food poisoning depends on *isolation of organism* from the relevant specimen.

YOU MUST KNOW

1. Definitions of diarrhoea, gastroenteritis, dysentery and food poisoning.
2. Aetiology of diarrhoeal diseases.
3. Laboratory diagnosis of diarrhoea due to *Esch. coli*.
4. Laboratory diagnosis of bacillary dysentery.
5. Laboratory diagnosis of food poisoning.

STUDY QUESTIONS

1. Enumerate the different causes of diarrhoea. How will you diagnose it in the laboratory?
2. Discuss in detail the laboratory diagnosis of dysentery.
3. Name various organisms causing food poisoning. Describe briefly the laboratory diagnosis of this condition.
4. Write short notes on:
   (a) Traveller's diarrhoea (b) Viral diarrhoeas (c) Food-borne botulism

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Which of the following bacteria can cause diarrhoea?
   (a) *Vibrio cholerae* (b) *Escherichia coli*
   (c) *Yersinia enterocolitica* (d) All of the above
2. Which of the following viruses can cause diarrhoea?
   (a) Rotavirus (b) Norwalk virus
   (c) Calcivirus (d) All of the above
3. Which of the following protozoa can cause diarrhoea?
   (a) *Entamoeba histolytica* (b) *Giardia lamblia*
   (c) *Cryptosporidium parvum* (d) All of the above
4. Which of the following is the most important cause of traveller's diarrhoea?
   (a) Enterotoxigenic *Escherichia coli* (b) Enteroinvasive *Esch. coli*
   (c) Enterohaemorrhagic *Esch. coli* (d) Enteropathogenic *Esch. coli*
5. Which of the following organisms can cause dysentery?
   (a) *Shigella dysenteriae* (b) *Enteroinvasive Escherichia coli*
   (c) *Entamoeba histolytica* (d) All of the above
6. Infective type of food poisoning is caused by:
   (a) *Salmonella typhimurium* (b) *Salmonella enteritidis*
   (c) *Campylobacter jejuni* (d) All of the above
7. Toxic type of food poisoning is caused by:
   (a) *Staphylococcus aureus* (b) *Bacillus cereus*
   (c) *Clostridium botulinum* (d) All of the above
8. What is the incubation period in *Staphylococcus aureus* food poisoning?
   (a) 2 to 6 hours (b) 6 to 12 hours
   (c) 8 to 24 hours (d) More than 24 hours

Answers (MCQs):

1. (d) 2. (d) 3. (d) 4. (a) 5. (d)
6. (d) 7. (d) 8. (a)

Chapter 71

MENINGITIS

Meningitis is an inflammation of the membranes surrounding the brain and spinal cord. It may be caused by viruses, bacteria, fungi or protozoa. Meningitis of bacterial origin may be caused by pyogenic organisms (acute pyogenic meningitis) or by *M. tuberculosis* (tuberculous meningitis). Aseptic meningitis is a term used for meningitis caused by viruses and a few cases caused by bacteria (other than those responsible for acute pyogenic meningitis), fungi and protozoa. Tuberculous meningitis is also included as a part of aseptic meningitis.

I. ACUTE PYOGENIC MENINGITIS

Diagnosis of acute pyogenic meningitis depends on direct examination of cerebrospinal fluid (CSF) and its bacteriological examination (Table 71.1).

A. Causative Organisms

In children and adults

Neisseria meningitidis (meningococcus)
Streptococcus pneumoniae (pneumococcus)
Haemophilus influenzae
Staphylococcus aureus
Listeria monocytogenes
Esch. coli
Proteus spp.
Klebsiella spp.
Citrobacter spp.
Enterobacter spp.
Serratia
} organisms occasionally encountered

In neonates and infants

Esch. coli
Group B streptococci
Staph. aureus
} important causes in first few months
H. influenzae
Listeria monocytogenes
Streptococcus pneumoniae
Klebsiella spp.

Meningococcal and pneumococcal meningitis can occur at any age. *H. influenzae* is common in meningitis cases in children from 3 months to 5 years. *Listeria monocytogenes* is very important causative agent in immunocompromised hosts.

B. Laboratory Diagnosis

1. Specimen

CSF is obtained by lumbar puncture under strict aseptic conditions. It is collected in three sterile containers, one each for cell count, biochemical analysis and bacteriological examination. The fluid should be examined immediately after collection or placed in the incubator at 37°C (never refrigerate the CSF as *H. influenzae* may die). Biochemical analysis and cell count give an indication of the type of meningitis (Table 71.1). In acute pyogenic meningitis, the CSF usually contains more than 1000 leucocytes per cu.mm and as many as 90-95% of which are neutrophils. The total protein content

Table 71.1 Cerebrospinal Fluid (CSF) in Normal Individuals and in Different Types of Meningitis.

Characteristic	Normal CSF	CSF in		
		Acute pyogenic meningitis	Tuberculous meningitis	Viral meningitis
I. *Pressure*	Normal	Highly increased	Moderately increased	Slightly increased
II. *Direct examination*				
A. Cell count				
1. Total cell (per cu.mm)	1-3	1,000-20,000	50-500	10-500
2. Predominant cell	Lymphocytes	Neutrophils (90-95%)	Lymphocytes (90%)	Lymphocytes
B. Biochemical analysis				
1. Total proteins (mg%)	30-45	100-600 (Highly increased)	80-120 (moderately increased)	60-80 (Slightly increased)
2. Sugars (mg%)	40-80	Diminished or absent (10-20)	Diminished (30-50)	Normal
III. *Bacteriological examination*				
A. Microscopy				
1. Gram staining	Nil	Gram negative cocci, Gram positive cocci, Gram negative bacilli or Gram positive bacilli may be found depending upon the causative agent responsible. Nil	—	—
2. Ziehl Neelsen staining	Nil		Acid-fast bacilli (AFB) may be found	
B. Culture	Nil	According to the causative agent, specific organism may grow on appropriate media.	*M. tuberculosis* may grow on LJ media.	Viruses may be grown on cell cultures.

is elevated and the glucose level is markedly diminished or even absent.

2. Bacteriological Examination

The CSF is divided into three portions. One portion is centrifuged and microscopy is performed from the deposit. The supernatant may be used for antigen detection. The second portion of the CSF is inoculated on culture media. The third portion is incubated overnight at 37°C, either as such or after adding an equal volume of glucose broth.

(i) Microscopy

Gram staining of centrifuged deposit will reveal plenty of pus cells and few Gram positive or Gram negative organisms.

(ii) Antigen detection

The supernatant part of CSF contains antigen, which may be demonstrated by latex agglutination or countercurrent-immunoelectrophoresis (CIEP). These tests are used for rapid diagnosis of meningitis and are particularly useful in partially treated patients in whom smear and culture may be negative. These are available for *N. meningitidis*, *Str. pneumoniae*, *H. influenzae* type b and Group *B streptococcus*.

(iii) Culture

(a) CSF culture

CSF is inoculated onto blood agar, chocolate agar and cooked meat broth (CMB). The culture plates are incubated at 37°C for 24 hours in an atmosphere of 5-10% CO_2.

The isolated organisms are identified by colony morphology, Gram staining from colonies, biochemical reactions and/or serological tests.

(b) Blood culture

Blood culture is particularly useful in meningitis due to *N. meningitidis*, *H. influenzae* and *Str. pneumoniae*. About 50% of these cases have positive blood culture.

(iv) Agglutination

The isolated organisms may be grouped by agglutination with appropriate antisera.

(v) Demonstration of bacterial endotoxin

This is especially useful when a patient has been partially treated and culture shows no growth. Bacterial endotoxin in blood can be detected by the limulus lysate test. The principle of the test is that extract prepared from the amoebocytes (blood cells) of the horse shoe crab (*Limulus polyphemus*) is coagulated when mixed with blood containing endotoxin. It is extremely sensitive test to detect bacterial endotoxin.

For detail identification of different organisms, refer to the respective chapters.

II. TUBERCULOUS MENINGITIS

The CSF in tuberculous meningitis shows moderate rise in cell count (50-500/cu.mm) and predominant cells are lymphocytes. There is moderate rise of total protein and sugar is slightly reduced (Table 71.1).

Laboratory Diagnosis

1. Specimen

CSF is collected by lumbar puncture in a sterile container under aseptic conditions. When CSF is allowed to stand, a fibrin web (*cobweb*) often develops. Cell count and biochemical analysis can be done as described earlier.

2. Microscopy

Ziehl-Neelsen (ZN) staining of the smear prepared from centrifuged deposit shows a fair number of lymphocytes along with few acid-fast bacilli (AFB).

3. Culture

Centrifuged deposit of CSF is inoculated on Lowenstein-Jensen (LJ) media and incubated at 37°C for 6-8 weeks. Identification of *M. tuberculosis* depends on colony morphology, ZN staining from colonies and biochemical reactions.

III. ASEPTIC MENINGITIS

In aseptic meningitis CSF is slightly turbid. It contains 10-500 leucocytes per cu. mm, most of which are lymphocytes. Causative agents are mostly viruses and a few cases are caused by bacteria, fungi and protozoa (Table 71.2). However, tuberculous meningitis has been described separately in this chapter. Aseptic meningitis caused by fungi is found in immunocompromised patients e.g. those with leukaemia, lymphoma, AIDS or on immunosuppressive drugs. *C. neoformans* is particularly seen in AIDS cases.

Table 71.2 Causative Agents of Aseptic Meningitis

Viruses
Enteroviruses (echo, coxsackie and polioviruses)
Mumps virus
Herpes simplex virus
Varicella zoster virus
Measles virus
Adenoviruses
Arboviruses
Bacteria
M. tuberculosis
Leptospira interrogans
Treponema pallidum
Fungi
Cryptococcus neoformans
Candida albicans
Protozoa
Acanthamoeba
Naegleria
Toxoplasma gondii

Laboratory Diagnosis

CSF is collected in the same manner as described earlier. Cell count and biochemical analysis is performed. Microscopy, culture and other tests may be performed according to the suspected causative agent (viruses, fungi or protozoa).

KEY POINTS

1. *Meningitis* is an inflammation of the membranes surrounding the brain and spinal cord. Meningitis of bacterial origin may be caused by pyogenic organisms (*acute pyogenic meningitis*) or by *M. tuberculosis* (*tuberculous meningitis*).
2. *Aseptic meningitis* is a term used for meningitis caused by *viruses* and a few causes caused by bacteria (other than those responsible for acute pyogenic meningitis), *fungi* and protozoa.
3. *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Haemophilus influenzae* are important bacteria responsible for acute pyogenic meningitis.

4. *Direct microscopy* by Gram staining, *antigen detection* and *culture* are key methods in laboratory diagnosis of acute pyogenic meningitis. These tests are done on a CSF specimen.
5. For laboratory diagnosis of tuberculous meningitis, direct microscopy by *Ziehl-Neelsen* (ZN) staining and culture on *Lowerstein-Jensen* (LJ) medium are useful.

YOU MUST KNOW

1. Causative organisms of acute pyogenic meningitis.
2. Laboratory diagnosis of acute pyogenic meningitis.
3. Characteristic features of cerebrospinal fluid (CSF) in different types of meningitis.
4. Causative agents of aseptic meningitis.

STUDY QUESTIONS

1. Name various organisms causing meningitis. Discuss the laboratory diagnosis of acute pyogenic meningitis.
2. Write short notes on:
   (a) Tuberculous meningitis (b) Aseptic meningitis

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Which of the following bacteria can cause acute pyogenic meningitis?
   (a) *Neisseria meningitidis* (b) *Streptococcus pneumoniae*
   (c) *Haemophilus influenzae* (d) All of the above
2. Which of the following viruses may cause aseptic meningitis?
   (a) *Enteroviruses* (b) *Herpes simplex virus*
   (c) Arboviruses (d) All of the above
3. Which of the following may cause aseptic meningitis in immunocompromised patients?
   (a) *Neisseria meningitidis* (b) *Streptococcus pneumoniae*
   (c) *Haemophilus influenzae* (d) *Cryptococcus neoformans*
4. Which of the following parasites can cause aseptic meningitis?
   (a) *Acanthamoeba* (b) *Naegleria*
   (c) *Toxoplasma gondii* (d) All of the above

Answers (MCQs):

1. (d) 2. (d) 3. (d) 4. (d)

Chapter 72

BACTERAEMIA, SEPTICAEMIA AND INFECTIVE ENDOCARDITIS

I. BACTERAEMIA AND SEPTICAEMIA

1. *Bacteraemia* is defined as presence of bacteria in blood without any multiplication.
2. *Septicaemia* is a condition in which bacteria circulate and actively multiply in the bloodstream.
3. *Pyaemia* is essentially septicaemia with metastatic infection.
4. *Endotoxaemia* is a condition when bacterial endotoxin circulates in the blood.

Bacteria may enter the blood:

(*i*) from an infective focus or from breakage of blood vessels adjacent to skin (normal flora), or

(*ii*) by introduction of contaminated material into the vascular system directly.

When hypotension and signs of inadequate perfusion develops, it is called *septic shock*. It is mediated by various bacterial products in the blood, particularly Gram negative endotoxins. About 40% cases of Gram negative bacteraemia are complicated by shock.

Organisms most commonly isolated from cases of septicaemia are listed in Table 72.1.

II. INFECTIVE ENDOCARDITIS

A. Clinical Types

1. Subacute endocarditis
2. Acute endocarditis
3. Postoperative endocarditis
4. Endocarditis associated with intravenous drug abuse

Table 72.1 Causative Organisms of Septicaemia

Gram negative bacilli (60-70% cases)
Salmonella typhi
S. paratyphi A
S. paratyphi B
S. paratyphi C
Brucella spp.
Haemophilus influenzae
Escherichia coli
Klebsiella pneumoniae
Proteus spp.
Enterobacter spp.
Bacteroides spp.
Pseudomonas spp.
Gram positive cocci (20-40% cases)
Staphylococcus aureus
Staph. epidermidis
Streptococcus pyogenes
Str. pneumoniae
Gram positive bacilli
Listeria monocytogenes
Gram negative cocci
Neisseria meningitidis

1. Subacute endocarditis

Subacute endocarditis is more common and comprises of almost 70% cases of bacterial endocarditis. It runs a chronic course. Organisms of relatively low virulence cause infection on damaged or defective valve cusps, and large firm vegetations comprising of dense fibrin, platelet aggregates with bacterial colonies are formed. Causative agents of subacute bacterial endocarditis are shown in Table 72.2.

Table 72.2 Causative Agents of Subacute Endocarditis

Bacteria	
Viridans group of streptococci	
Streptococcus sanguis	
Str. mutans	responsible for 60-80% of cases
Str. mitis	
Enterococcus faecalis	
Staph. epidermidis	
Coxiella burnetii	
Chlamydia psittaci	
Fungi	
Candida albicans	
Aspergillus spp.	

2. Acute endocarditis

This is usually due to highly virulent pyogenic bacteria which affect both normal or abnormal (damaged, defective) valves. Vegetations are large and valve destruction is greater than that in subacute endocarditis. It is rapidly progressive disease. *Staph. aureus* is the most common causative agent and responsible for about 25 per cent of cases. Some of the causative agents of acute endocarditis are shown in Table 72.3.

Table 72.3 Causative Agents of Acute Endocarditis

Staphylococcus aureus
Streptococcus pneumoniae
Other pyogenic cocci
Str. pyogenes
Str. agalactiae (group B)

3. Post-operative endocarditis

This is endocarditis following cardiac surgery particularly in prosthetic valve replacement. Causative agents include:

(*i*) *Staphylococcus epidermidis*—It is the commonest aetiologic agent.
(*ii*) *Staph. aureus.*
(*iii*) *Candida albicans.*

4. Endocarditis associated with intravenous drug abuse

Drug abusers with endocarditis are often young males. They have normal tricuspid valves. The skin is the commonest source of infection.

Causative agents

(*i*) *Staphylococcus aureus*—It is the commonest aetiological agent.
(*ii*) Other organisms
Viridans group of streptococci
Candida spp.
Pseudomonas spp.

B. Pathogenesis

Although patients at risk are those who have pre-existing cardiac disease, but about one third of cases occur in previously normal hearts. About 70% patients have one or other predisposing cardiac lesion and other abnormalities that predispose to endocarditis. These include:

1. rheumatic valvular disease
2. congenital valve deformities
3. cardiac valve prosthesis
4. degenerative cardiac disease
5. drug abuse

C. Laboratory Diagnosis

Diagnosis of infective endocarditis depends on isolation of the causative agent from blood. Blood culture is the main diagnostic procedure.

1. Specimen

Three to six samples of blood, 10 ml each should be collected over 24 hours. Samples should be collected before starting antimicrobial therapy. Blood is collected from antecubital vein under all aseptic conditions using sterile disposable syringe. Each sample should be directly inoculated into 50-100 ml of glucose broth. Large amount of blood and media are required because:

(*i*) the number of organisms in the blood may be very few and
(*ii*) the blood of patients contain bactericidal substances which are diluted by large volume of medium.

Repeated blood cultures are made because the bacteraemia is intermittent in cases of infective endocarditis.

2. Culture

Cultures (glucose broths) are incubated at 37°C for at

least 3 weeks and are to be observed daily for early signs of growth. Subcultures are made on solid media such as blood agar and MacConkey agar after 24 hours, 48 hours and once a week thereafter. These solid media are incubated at 37°C for 24 hours.

3. *Identification*

The isolated organism is identified by colony morphology, Gram staining, biochemical reactions and serological tests. For details of these agents, refer to the corresponding chapters.

4. *Antibiotic Sensitivity Tests*

The routine disc diffusion method is not adequate for guiding treatment of infective endocarditis. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the antimicrobial agent for the isolated organism must be determined. The measurement of MIC and MBC helps to determine the adequate dose of the antibiotic to be used for ensuring the serum levels that can penetrate the valves and kill the organisms.

5. *Culture Negative Endocarditis*

In about 10-20% cases, blood cultures are persistently negative. It may be due to following reasons:

(*i*) Recent antibiotic therapy: In such cases, repeated blood cultures done after the stoppage of antibiotics may give positive results.

(*ii*) Inadequate number of specimens: As bacteraemia is intermittent, repeated blood cultures are necessary.

(*iii*) Infection with *Coxiella burnetii* or *Chlamydia* spp.: Blood cultures may be negative because *Coxiella burnetii* and *Chlamydia* spp. cannot grow on cell free media.

6. *Other Tests for Diagnosis*

(*i*) Total leucocyte count (TLC): Leucocytosis is common.

(*ii*) Erythocyte sedimentation rate (ESR): It is elevated.

(*iii*) Echocardiogram: It demonstrates the vegetations and valve changes.

D. Treatment

Viridans group of streptococci are highly sensitive to penicillin. Other antibiotics used are gentamicin and ampicillin. Use of two bactericidal drugs in combination is preferred. Infecting organisms are very sensitive to antibiotics, but the drugs cannot reach the organisms within the vegetation in adequate concentration, where these organisms are present in large numbers. Hence antibiotics should be given in high doses and preferably intravenously to eliminate the bacteria from these sites. Treatment should be given for a minimum period of 4 weeks. Certain organisms may require treatment for 4 to 8 weeks. Vancomycin is the antimicrobial of choice for endocarditis caused by methicillin resistant *Staph. aureus* (MRSA).

E. Prevention

Preventive measures include:

1. Good dental hygiene because causative organisms of infective endocarditis are part of normal flora of mouth.
2. Prophylactic antibiotic should be given before dental extraction and other surgical procedures.

KEY POINTS

1. *Bacteraemia* is defined as presence of bacteria in blood without any multiplication.
2. *Septicaemia* is a condition in which bacteria circulate and actively multiply in the blood stream.
3. *Pyaemia* is essentially septicaemia with metastatic infection.
4. *Endotoxaemia* is a condition when bacterial endotoxin circulates in the blood.
5. *Gram negative bacilli* are responsible in majority of septicaemia cases.
6. *Viridans group of streptococci* are responsible for *subacute endocarditis*. *Staph. aureus* is the most common causative agent for *acute endocarditis*.
7. Diagnosis of infective endocarditis depends on *isolation* of causative agent from blood. *Blood culture* is the main diagnostic procedure. *Three to six* samples of blood should be collected over 24 hours.

YOU MUST KNOW

1. Definitions of bacteraemia, septicaemia, pyaemia and endotoxaemia.
2. Causative organisms of septicaemia.
3. Causative organisms of subacute endocarditis and acute endocarditis.
4. Laboratory diagnosis of subacute bacterial endocarditis.

STUDY QUESTIONS

1. Define bacteraemia, septicaemia, pyaemia and endotoxaemia. Name various organisms causing septicaemia.
2. Name the various organisms causing infective endocarditis. Discuss the laboratory diagnosis of subacute bacterial endocarditis.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Which of the following is the commonest cause of subacute endocarditis?
   (a) *Staphylococcus epidermidis* (b) Viridans group of streptococci
   (c) *Candida albicans* (d) *Aspergillus fumigatus*
2. Which is the commonest aetiologial agent of endocarditis associated with intravenous drug abusers?
   (a) *Staphylococcus aureus* (b) *Staphylococcus epidermidis*
   (c) *Aspergillus fumigatus* (d) *Streptococcus agalactiae*

Answers (MCQs):

1. (b) 2. (a)

Chapter 73

FEVER OF UNKNOWN ORIGIN (FUO)

Fever of unknown origin (FUO) may be defined as any febrile illness (temperature greater than 38°C) lasting 3 weeks or longer, without any obvious cause and failure to reach a diagnosis despite one week of inpatient investigation.

I. CAUSES OF FUO

The causes of FUO include infections, neoplasms, connective tissue disorders, granulomatous diseases and drug reactions.

A. Infections

Infections account for about one third cases of FUO.

1. Bacterial Infections

- Enteric fever
- Urinary tract infection
- Lung abscess and other deep abscesses
- Septicaemia associated with pneumonia, infective endocarditis etc.
- Tuberculosis
- Brucellosis
- Rheumatic fever
- Relapsing fever
- Leptospirosis
- Typhus fever
- Q fever

2. Parasitic Infections

- Malaria
- Hepatic amoebiasis or liver abscess
- Visceral leishmaniasis (Kala-azar)
- Filariasis
- Toxoplasmosis
- Trypanosomiasis (only in tropical Africa)

3. Viral Infections

- Infectious mononucleosis
- Cytomegalovirus infection
- Hepatitis A virus infection
- Hepatitis B virus infection
- Rubella and other infectious fevers without typical rash
- HIV infection

4. Fungal Infections
- Histoplasmosis
- Coccidioidomycosis

B. Neoplasms
- Hodgkin's lymphoma
- Non-Hodgkin's lymphoma
- Leukaemia
- Hypernephroma
- Hepatoma
- Disseminated malignancy

C. Connective Tissue Disorders
- Systemic lupus erythematosus (SLE)
- Polyarteritis nodosa

D. Granulomatous Diseases
- Sarcoidosis
- Crohn's disease

E. Drug Reactions
- Drug induced fevers

II. LABORATORY DIAGNOSIS

A. Bacterial Infections

1. Specimens

Blood	:	for blood culture, peripheral blood smear, haematology, serology and other tests
Urine	:	for UTI
Sputum	:	in cases of lung infections
Pus	:	in localised abscesses

2. Collection
All the specimens should be collected preferably prior to antimicrobial therapy. These specimens must be collected in sterile containers under aseptic conditions. Blood is collected in blood culture bottles for culture and in a sterile vial for serology. Mid-stream urine specimen should be collected in a sterile universal container.

3. Culture
Culture of these specimens may be performed for isolating the causative organism.

(i) Blood culture
5 ml of blood is collected in each bottle of 50 ml glucose broth and 50 ml taurocholate broth. These broths are incubated at 37°C for 24 hours and then subcultures are made on blood agar (from glucose broth) and MacConkey agar (from taurocholate broth). Blood agar and MacConkey agar plates are incubated at 37°C for 24 hours.

(ii) Urine culture
A calibrated volume of midstream urine specimen is inoculated on blood agar and MacConkey agar. These media are incubated at 37°C for 24 hours. In case of renal tuberculosis, culture should be performed on Lowenstein-Jensen (LJ) medium.

(iii) Sputum culture
Specimen is inoculated on blood agar and MacConkey agar plates and incubated at 37°C for 24 hours. In case of tuberculosis, specimen should be cultured on Lowenstein-Jensen (LJ) medium and incubated at 37°C for 6 weeks.

(iv) Pus culture
Pus is inoculated in glucose broth, blood agar and MacConkey agar. These media are incubated at 37°C for 24 hours. For *M. tuberculosis*, pus should be cultured on LJ media. When suspecting anaerobic organisms, culture of pus should be performed under anaerobic conditions.

4. Identification
Organisms may be identified on the basis of colony morphology, Gram staining, biochemical reactions and agglutination etc. For *M. tuberculosis*, Ziehl-Neelsen (ZN) staining is performed to detect acid-fast bacilli (AFB). This is further confirmed by culture and biochemical reactions. For details of individual organism, refer to corresponding chapters.

5. Serology
Serology is useful in infectious mononucleosis (Paul-Bunnell test), enteric fever, hepatitis A, B infections, CMV infections and sometimes in amoebiasis.

B. Parasitic Infections
Stained peripheral blood smears (thin and thick) will help in diagnosis of malaria, leishmaniasis, filariasis and toxoplasmosis. Wet blood film may show microfilaria in cases of filariasis. Serology is useful in amoebiasis.

C. Viral Infections
Peripheral blood smear may be helpful in infectious mononucleosis. Viral infections may be detected either by tissue culture or by serology. Paul-Bunnel test is useful in infectious mononucleosis.

D. Fungal Infections
Specimens may be cultured on Sabouraud's dextrose agar or Brain-heart infusion agar.

E. Other Tests for Diagnosis

1. Skin Tests

- Mantoux test
- Skin tests for histoplasmosis, coccidioidomycosis, sarcoidosis

2. Haematology

- Total leucocyte count (TLC)
- Differential leucocyte count (DLC)

These are non-specific tests.

3. Immunologic Tests

- LE cell phenomenon and antinuclear antibody test in SLE

4. Biopsy

- Biopsy of lymph node or other tissues.

KEY POINTS

1. *Fever of unknown origin* (*FUO*) may be defined as any febrile illness (temperature greater than 38°C) lasting 3 weeks or longer, without any obvious cause and failure to reach a diagnosis despite one week of inpatient investigation.
2. The causes of FUO include *infections*, neoplasms, connective tissue disorders, granulomatous diseases and drug reactions.
3. *Enteric fever* is the most common bacterial infection as cause of FUO.
4. Culture of different specimens may be performed for isolating the causative organism. *Blood culture* is the most important investigation.

YOU MUST KNOW

1. Definition of Fever of unknown orgin (FUO).
2. Causes of FUO.
3. Laboratory diagnosis of FUO.

STUDY QUESTIONS

Q. Define and enumerate the causes of Fever of unknown origin (FUO). Discuss the laboratory diagnosis of FUO.

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Which of the following bacterial infections can result in fever of unkown origin?
   (a) Enteric fever
   (b) Brucellosis
   (c) Urinary tract infection
   (d) All of the above
2. Which of the following parasitic infections may present as fever of unknown origin?
   (a) Malaria
   (b) Hepatic amoebiasis
   (c) Visceral leishmaniasis
   (d) All of the above

Answers (MCQs):

1. (d) 2. (d)

Chapter 74

SEXUALLY TRANSMITTED DISEASES

The sexually transmitted diseases (STDs) are a group of communicable diseases which are transmitted predominantly or entirely by sexual contact. The causative organisms include a wide range of bacterial, viral. protozoal and fungal agents. STD may present as genital ulcers, genital discharge without any genital lesion or only as systemic manifestations.

I. CAUSATIVE ORGANISMS

Organisms responsible for sexually transmitted diseases (STDs) and their clinical presentations are shown in Table 74.1.

II. LABORATORY DIAGNOSIS

Laboratory diagnosis of these diseases have already been described in corresponding chapters. However, salient features for laboratory diagnosis of important STDs are mentioned here.

A. Gonorrhoea

It is caused by *Neisseria gonorrhoeae*.

1. Specimens

(*i*) Urethral discharge
(*ii*) Cervical swab
(*iii*) In chronic cases : Discharge after prostatic massage in males
(*iv*) Rectal swab

2. Direct Gram Staining

Intracellular Gram-negative diplococci in smears is diagnostic in males (95% cases). It is sometimes difficult to diagnose gonorrhoea in females on direct Gram staining due to the presence of mixed normal flora.

3. Culture

(*i*) Chocolate agar
(*ii*) Thayer-Martin medium

The inoculated plates are incubated at 37°C for 24-48 hours in the presence of CO_2.

4. Identification

Identification of organism is based on colony morphology, Gram staining from colonies and biochemical reactions.

(i) Colony morphology

Translucent colonies are formed.

Table 74.1 Organisms Responsible for Sexually Transmitted Diseases

STDs	Organisms
A. *Painless genital ulcers*	
• Syphilis	*Treponema pallidum*
• Lymphogranuloma venereum (LGV)	*Chlamydia trachomatis*
• Donovanosis	*Klebsiella granulomatis* (formerly named as *Calymmatobacterium granulomatis*)
B. *Painful genital ulcers*	
• Chancroid	*Haemophilus ducreyi*
• Herpes genitalis	Herpes simplex viruses (HSV) type 2 and 1
C. *Urethral discharge*	
• Gonorrhoea	*Neisseria gonorrhoeae*
• Non-gonococcal urethritis (NGU)	*Chlamydia trachomatis* (types D—K)
	Ureaplasma urealyticum
	Mycoplasma genitalium
	M. hominis
D. *Vaginal discharge*	
• Gonorrhoea	*N. gonorrhoeae*
• NGU	*C. trachomatis*
	M. hominis
• Trichomoniasis	*Trichomonas vaginalis*
• Vaginitis	*Gardnerella vaginalis*
	Mobiluncus spp.
• Vulvo-vaginal candidiasis	*Candida albicans*
E. *Genital warts*	Human papilloma viruses
F. *No genital lesions but only systemic manifestations*	HIV-1 and HIV-2
	Hepatitis B virus (HBV)
	Hepatitis C virus (HCV)
G. *Miscellaneous*	Group B streptococci
	Molluscum contagiosum virus
	Cytomegalovirus
	Phthirus pubis
	Sarcoptes scabei
	Shigella spp.
	Campylobacter spp.
	Giardia lamblia
	Entamoeba histolytica

(ii) Gram staining

Gram negative diplococci

(iii) Biochemical

They are oxidase positive and produce acid from glucose but not lactose, maltose or sucrose

B. Non-Gonococcal Urethritis (NGU)

Symptoms of discharge and dysuria clinically indistinguishable from gonorrhoea caused by organisms other than *N. gonorrhoeae* is called *non-gonococcal urethritis* (NGU). Causative agents are shown in Table 74.1. Laboratory diagnosis of NGU due to *C. trachomatis* is described here but for the diagnosis of other aetiological agents, refer to the respective chapters.

LABORATORY DIAGNOSIS

1. Specimens

(*i*) Swabs from exudate of urethra
(*ii*) Cervical discharge

2. Direct examination

(i) Giemsa stain

It shows intracytoplasmic inclusion bodies suggestive of *C. trachomatis.*

(ii) Detection of antigen

Smears made from exudate are examined by immunofluorescence test with a monoclonal antibody or by ELISA for detection of elementary bodies of *C. trachomatis.*

3. Culture
The exudate is inoculated on McCoy or HeLa cell cultures treated with cycloheximide. Intracytoplasmic glycogen-rich inclusions are detected by Giemsa stain or by immunofluorescence. These are suggestive of *C. trachomatis*.

4. Serology
(*i*) Complement fixation test (CFT)
(*ii*) Micro-immunofluorescence or ELISA is useful for detection of serovar-specific antibody.

C. Syphilis
Syphilis is caused by *Treponema pallidum*.

1. Specimens
(*i*) Fluid from chancre
(*ii*) Scrapings from ulcerated secondary lesions
(*iii*) Blood for serology

2. Direct Microscopy
T. pallidum can be demonstrated in exudate by dark ground microscopy or phase contrast microscopy. Motile organisms may be seen.

3. Serological tests
(i) Non specific tests
(a) VDRL test
(b) Rapid plasma reagin (RPR) test

(ii) Specific tests
(a) TPHA (Treponema pallidum haemagglutination assay)
(b) FTA-ABS (Fluorescent treponemal antibody absorption test)
(c) TPI (Treponema pallidum immobilisation test)

VDRL and TPHA are two most commonly used tests.

D. Chancroid
Chancroid or soft chancre is caused by *H. ducreyi*.

1. Specimen
Exudate

2. Gram Staining
Smear shows Gram negative coccobacilli.

3. Culture
Exudate is cultured onto chocolate agar enriched with isovitalex and foetal calf serum, and containing vancomycin as selective agent. Culture plates are incubated at 35°C under 10% CO_2 and high humidity.

4. Identification
Colony morphology and Gram staining are useful in identification of the organism.

(i) Colony morphology
H. ducreyi forms small, grey, translucent colonies.

(ii) Gram staining
It shows Gram negative coccobacilli.

E. Lymphogranuloma Venereum (LGV)
It is caused by *C. trachomatis* serotypes L1, L2 and L3.

1. Serological Tests
(*i*) Microimmunofluorescence
(*ii*) CFT
} for antibody detection

2. Frei Test
It is a skin test using LGV antigen and shows delayed type of hypersensitivity.

3. Isolation
Chlamydiae can be cultivated in variety of cells from chick embryos and mammals. They also grow in yolk sac of eggs. The inclusions in the cell cultures may be stained with Giemsa stain.

4. Direct Microscopy
Smears of material aspirated from the bubos may show the elementary bodies. The sensitivity of microscopy is very low.

F. Donovanosis
Donovanosis is caused by *Klebsiella (Calymmatobacterium) granulomatis*.

1. Specimen
Tissue smear from the ulcer

2. Staining
Giemsa staining shows Donovan bodies. The Donovan bodies are rounded coccobacilli, 1–2 mm, which lie within cystic spaces in large mononuclear cells. They show bipolar condensation of chromatin, giving a closed safety pin appearance in stained smears.

G. Herpes Genitalis

Aetiological agent is *Herpes simplex virus* (HSV), types 1 and 2 but type 2 strains are more commonly associated.

1. Specimens

(*i*) Scrapings from base of the lesions
(*ii*) Blood for serology

2. Direct Microscopy

Giemsa or Wright staining shows characteristic intranuclear inclusions.

3. Culture

Diagnosis is confirmed by tissue culture in human diploid fibroblast cells. Cytopathogenic effect (CPE) occurs within 24 hours.

4. Serology

Antibody detection by complement fixation test (CFT) is useful in diagnosis of primary infection.

H. Trichomoniasis

It is caused by *Trichomonas vaginalis*.

1. Specimen

Swab of vaginal discharge is examined freshly. If delay in transport is inevitable, specimen should be collected in stuart's transport medium.

2. Direct Microscopy

Direct wet film shows motile trichomonads. Direct microscopy is at least 80% as positive as culture.

3. Culture

Specimen is cultured in Fineberg's medium and incubated for 5 days and examined for motile protozoa.

I. Vulvovaginal Candidiasis

It is caused by various species of *Candida* but *C. albicans* accounts for 80% of cases.

1. Specimen

Swab from vaginal secretions

2. Direct Microscopy

(*i*) *KOH mount*
It shows yeast cells.
(*ii*) *Gram staining*
Characteristic Gram positive budding yeast cells and pseudohyphae may be seen.

3. Culture

The specimen is inoculated on Sabouraud's dextrose agar (SDA) and incubated at 37°C for 48 hours.

4. Identification

(*i*) *Colony morphology*
Colonies are creamy white and smooth.
(*ii*) *Gram staining*
Gram stained smear shows budding Gram positive yeast cells.
(*iii*) *Germ tube formation*
C. albicans forms germ tube within two hours when incubated in human serum at 37°C.
(*iv*) *Chlamydospores formation*
C. albicans forms chlamydospores on cornmeal agar

J. Genital Warts

Genital warts, also known as *condyloma acuminata* are common in sexually active adults. These are usually due to human papillomavirus (HPV) types 6 and 11.

Cytological or histological examination of cells in urine is used for detection of inclusion bodies of HPV.

KEY POINTS

1. The *sexually transmitted diseases* (*STDs*) are a group of communicable diseases which are transmitted predominantly or entirely by *sexual contact*.
2. The causative organisms include a wide range of bacterial, viral, protozoal and fungal agents.
3. STD may present as *genital ulcers*, *genital discharge* without any genital lesion or only as systemic manifestations.
4. Painless genital ulcers are present in syphilis (*Treponema pallidum*), lymphogranuloma venereum (*Chlamydia trachomatis*) and donovanosis (*Klebsiella granulomatis*).
5. Painful genital ulcers are present in chancroid (*Haemophilus ducreyi*) and herpes genitalis (*Herpes simplex viruses* type 2 and 1)
6. Genital discharge is present in gonorrhoea (*N. gonorrhoeae*), non-gonococcal urethritis (*C. trachomatis*) and trichomoniasis (*Trichomonas vaginalis*).
7. Systemic manifestations without any genital lesion are present in *HIV*, *hepatitis B virus* (*HBV*) and *hepatitis C virus* (*HCV*).
8. Laboratory diagnosis is according to the suspected organism responsible for the manifestations of that particular STD.

YOU MUST KNOW

1. List of causative organisms of sexually transmitted diseases (STDs).
2. Causative agents of painless genital ulcers.
3. Causative agents of painful genital ulcers.
4. Causative agents of urethral discharge.
5. Laboratory diagnosis of gonorrhoea.
6. Non-gonococcal urethritis (NGU).
7. Laboratory diagnosis of syphilis.

STUDY QUESTIONS

1. Name various organisms causing sexually transmitted diseases. Discuss the laboratory diagnosis of syphilis.
2. Write short notes on :
   (a) Laboratory diagnosis of gonorrhoea (b) NGU (c) Chancroid
   (d) Vulvovaginal candidiasis (e) Donovanosis (f) LGV

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Which of the following organisms can cause painless genital ulcers?
   (a) *Treponema pallidum* (b) *Chlamydia trachomatis*
   (c) *Klebsiella granulomatis* (d) All of the above
2. Painful genital ulcers may occur in:
   (a) Syphilis (b) Chancroid
   (c) Donovanosis (d) Lymphogranuloma venereum
3. Urethral discharge is present in:
   (a) Syphilis (b) Gonorrhoea (c) Chancroid (d) Herpes genitalis
4. Causative agents of non-gonococcal urethritis may be:
   (a) *Chlamydia trachomatis* (b) *Ureaplasma urealyticum*
   (c) *Mycoplasma genitalium* (d) All of the above
5. Which of the following agents can cause genital warts?
   (a) *Chlamydia trachomatis* (b) *Herpes simplex virus* (c) *Human papilloma viruses* (d) *Mycoplasma hominis*
6. Which of the following agents can cause sexually transmitted disease without any genital lesions but with only systemic manifestations?
   (a) HIV (b) Hepatitis B virus (c) Hepatitis C virus (d) All of the above
7. Which of the following agents can cause sexually transmitted disease?
   (a) *Mycoplasma hominis* (b) *Entamoeba histolytica* (c) *Sarcoptes scabei* (d) All of the above
8. What is the causative agent of chancroid?
   (a) *Haemophilus ducreyi* (b) *Klebsiella granulomatis*
   (c) *Mycoplasma hominis* (d) *Candida albicans*
9. What is the causative agent of donovanosis?
   (a) *Haemophilus ducreyi* (b) *Klebsiella granulomatis*
   (c) *Mycoplasma hominis* (d) *Candida albicans*
10. What is the causative agent of lymphogranuloma venereum?
    (a) *Chlamydia trachomatis* serotypes A to C (b) *C. trachomatis* serotypes D to K
    (c) *C. trachomatis* serotypes L1 to L3 (d) *Chlamydophila pneumoniae*

Answers (MCQs):

1. (d)	2. (b)	3. (b)	4. (d)	5. (c)
6. (d)	7. (d)	8. (a)	9. (b)	10. (c)

Chapter 75

HEALTH ASSOCIATED INFECTION

Health Associated Infection

A. Definition
B. Factors Influencing Infection
C. Sources of Infection
D. Microorganisms
E. Modes of Transmission
F. Common Health Associated Infections
G. Diagnosis and Control
H. Prevention
I. Infection Control Policy

HEALTH ASSOCIATED INFECTION

A. Definition

The terms *health associated infection, health care associated infection, hospital acquired infection, hospital-associated infection, hospital infection or nosocomial infection* (*nosocomion*, meaning hospital) is defined as infection developing in patients after admission to the hospital, which was neither present nor in the incubation period at the time of hospitalisation. Such infections may become evident during their stay in the hospital or, sometimes, after their discharge.

B. Factors Influencing Infection

Health associated infection is probably a great problem today despite advances in hygiene and treatment of infecting organisms. Several factors contribute to the occurrence and severity of these infections. These include:

1. Susceptible Patients

Many patients in hospitals have impaired defence mechanisms due to pre-existing diseases, such as diabetes, immunosuppression and patients with prosthetic implants. They are, therefore, more susceptible to infection.

2. Hospital Environment

The hospital environment is heavily laden with a wide variety of pathogens. These pathogens may be present in air, dust, water, food or antiseptic lotions. Equipment may be contaminated. Bedding, linen and utensils may act as fomites. Patients shed the organisms from their bodies while hospital personnel spread these organisms through their hands and clothes.

3. Diagnostic or Therapeutic Procedures

During diagnostic or therapeutic procedures such as insertion of urethral or intravenous catheters, the slightest lapse in asepsis may lead to infection.

4. Drug Resistance

Hospital infections are generally refractory to treatment as the infecting agents are usually multidrug resistant.

5. Transfusion

Blood, blood products and intravenous fluids used for transfusion, if not properly screened, can transmit many infections.

6. Advances in Medical Progress

Advances in treatment of cancer, organ transplantation, implanted prostheses and other sophisticated technologies enhance the risk of infection.

C. Sources of Infection

1. Exogenous

Hospital-infection is mostly exogenous from another patient or member of the staff or from the environment in the hospital. Patients and hospital personnel suffering from infection, or asymptomatic carriers are the most important sources. Environmental sources include inanimate objects, air, water and food in the hospital. Inanimate objects in the hospital are medical equipments (endoscopes, cystoscopes, catheters etc.), bed pans, surfaces contaminated by patients secretions, excretions, blood and body fluids.

2. Endogenous

Patients own flora may invade the patient's tissue during some surgical operation or instrumental manipulations.

D. Microorganisms

Almost any microorganism can, on occasion, cause hospital acquired infection but those that survive in the hospital environment for long periods and develop resistance to antibiotics and disinfectants are particularly important. *Staph. aureus* strains, resistant to multiple antibiotics spread globally in the 1950's and 1960's, colonising hospitals and continue to be very common agents in nosocomial infection. *Staph. epidermidis* and Group D streptococci also are sometimes responsible for health-associated infections.

In recent decades, *Esch. coli, Klebsiella, Enterobacter, Proteus* and *Serratia* have become the most important hospital pathogens, particularly because of dissemination among them of R factor conferring multiple drug resistance.

Pseudomonas aeruginosa and other *Pseudomonas* species have always been important hospital pathogens because of their intrinsic resistance to most antibiotics and ability to survive and even multiply in disinfectants solutions.

Tetanus spores can survive in dust and may sometimes contaminate items used in hospitals. Hospital tetanus is usually due to faulty sterilisation techniques or other lapses in asepsis.

HIV and hepatitis B and C viruses are transmitted by contaminated blood or blood products. Screening of blood donors has reduced the risk to a large extent.However, HIV escapes detection during the window period.

Viral diarrhoea and chickenpox may spread in hospitals. Cytomegalovirus, influenza, herpesvirus, enteroviruses and arenaviruses are some other viruses which may cause hospital infection.

Candida albicans, Aspergillus, Mucor, Entamoeba histolytica, Pneumocystis carinii and *Toxoplasma gondii* may also sometimes cause hospital infection.

E. Modes of Transmission

There are four main routes of transmission of infection:

1. Contact

It is the principal route of transmission of nosocomial pathogens.

(i) Hands or clothing

The hands of hospital staff are an important vehicle of spread of infection. There is adequate scope of transmission of microorganisms from one person to another by contact of hands and clothings of attendants. *Staph. aureus* and *Str. pyogenes* are two important pathogens spread by hand contact.

(ii) Inanimate objects

Certain instruments (endoscope, bronchoscope, cystoscope), if not properly disinfected, may transmit pathogenic organisms (e.g. *Pseudomonas aeruginosa*).

2. Airborne

(i) Droplets

Droplets of respiratory infection is transmitted by inhalation.

(ii) Dust

Dust from bedding, floors, exudates dispersed from a wound during dressing and from skin by natural shedding of skin scales (measles, staphylococcal sepsis), may contribute in spread of infections e.g. *Pseudomonas aeruginosa, Staph. aureus.*

(iii) Aerosols

Aerosols produced by nebulizers, humidifiers and air conditioning apparatus transmit certain pathogens to the respiratory tract. Occurrence of legionellae in hospital water supply has led to outbreaks of infections mainly with *Legionella pneumophila.*

3. Oral Route

Hospital food may contain antibiotic-resistant bacteria (*Pseudomonas aeruginosa, Esch. coli*, Klebsiella spp. and others), which may colonise the intestine and later cause infection in susceptible patients.

4. Parenteral Route

With the introduction of disposable syringes and needles, transmission of infection by parenteral route has been infrequent. However, certain infections may be transmitted by blood transfusion or tissue donation, contaminated blood products (factor VIII) and contaminated infusion

fluids. Hepatitis B and HIV are two viruses which may be transmitted in this way.

F. Common Health Associated Infections (Table 75.1)

Common health associated infections are as follows:

1. Urinary Tract Infection

This is usually associated with catheterisation or instrumentation of urethra, bladder or kidneys. Urinary tract infection associated with catheterisation is named as *catheter associated urinary tract infection* (CAUTI). Infection is caused by *Esch. coli, Klebsiella, Proteus, Serratia, Pseudomonas, Providencia,* coagulase negative staphylococci, enterococci and *Candida albicans*. Infection can be prevented by strict asepsis during catheterisation.

2. Respiratory Infection

Aspiration in unconscious patients and pulmonary ventilation may lead to nosocomial pneumonia. It is called *ventilator associated pneumonia* (VAP). The major pathogens include *Staph. aureus, Klebsiella spp., Enterobacter, Serratia, Proteus, Esch. coli, Pseudomonas aeruginosa, Acinetobacter*, *Legionella pneumophila* and respiratory viruses.

3. Wound and Skin Sepsis

The incidence of post-operative infection is higher in elderly patients. Most wound infections manifest within a week of surgery. *Staph. aureus* is the predominant pathogen, followed by *Pseudomonas aeruginosa* and then *Esch. coli, Proteus,* enterococci and coagulase negative staphylococci.

4. Gastrointestinal Infections

Food poisoning and neonatal septicaemia in hospitals have been reported. *Salmonella* and *Shigella sonnei* are mainly associated with these infections.

5. Burns

Staph. aureus, Pseudomonas aeruginosa, Acinetobacter and *Str. pyogenes* are responsible for hospital associated infections in cases of burns.

6. Bacteraemia and Septicaemia

These may be consequences of infection at any site but are generally caused by infected intravenous cannulae. Gram negative bacilli are common pathogens. Infection can be prevented by proper skin toilet before 'cut-down'. Intravenous rehydration in diarrhoea should be replaced by oral fluids as early as possible.

Staph. epidermidis bacteraemia is found commonly in patients with artificial heart valves. Bacteraemia in those with valvular defects may lead to endocarditis.

G. Diagnosis and Control

Health associated infection may occur sporadically or as outbreaks. Diagnosis is by the routine bacteriological methods such as direct smear examination, culture and sensitivity testing. This requires the samples from possible sources of infection such as hospital personnel, inanimate objects, water, air or food. Typing of isolate (phage typing, bacteriocin typing, biotyping or antibiogram) may indicate a causal connection.

Control of hospital infection should be a permanent ongoing activity. Examples of sources of hospital outbreaks are nasal carriage of staphylococci in hospital staff or Pseudomonas growing in lotions. Carriers should be suitably treated.

The cause of infection may be a defective autoclave, therefore, sterilisation techniques have to be tested.

Unfortunately, in many hospitals, infection control is attempted by use of more and more antibiotics. This may lead to selective colonisation by multiresistant pathogens and has a harmful effect.

Table 75.1 Common Health Associated Infections

Health associated infection	Causative organisms
Urinary tract infection	*Esch. coli, Klebsiella, Proteus, Serratia, Pseudomonas, Providencia,* coagulase negative staphylococci, enterococci and *Candida albicans.*
Respiratory infection	*Staph. aureus, Klebsiella, Enterobacter, Serratia, Proteus, Esch. coli, Pseudomonas aeruginosa, Acinetobacter, Legionella pneumophila and respiratory viruses*
Wound and skin sepsis	*Staph. aureus, Pseudomonas aeruginosa, Esch. coli, Proteus,* enterococci and coagulase negative staphylococci
Gastrointestinal infection	*Salmonella, Shigella sonnei* and *viruses*
Burns	*Staph. aureus, Pseudomonas aeruginosa, Acinetobacter and Str. pyogenes*

H. Prevention

The health associated infections can be prevented by following means:

1. Transmission of infection can be controlled by regular proper washing of hands, disinfection of equipments and change of working clothes.
2. Administration of antibiotic therapy to the carrier staff or source patient to destroy the pathogenic agents.
3. Proper sterilisation and disinfection of the inanimate objects should be done. This helps to control the source of infection.
4. Disinfection of excreta and infected material is necessary to control the exit point of infection.
5. The use of sterile dressings, surgical gloves, face-masks and I/V fluids further contribute in control of infection.
6. Preoperative disinfection of the patient's skin.
7. Rational antibiotic prophylaxis.
8. Proper investigation of hospital-associated infection and the treatment of such cases.

I. Infection Control Policy

Every hospital must have an effective infection control committee (ICC) which should be responsible for the control of health associated infection (HAI). The committee should be chaired by medical superintendent and should have a microbiologist as infection control officer and heads of all clinical departments, blood bank, microbiologist, medical record officer, chief of nursing services and infection control sister as members. Chief of all the supportive services (O.T., dietetics, laundry, house keeping etc.) should be included as invited members.

ICC must meet at least once every month to formulate and update policy on matters related to hospital infection and to manage outbreaks of health associated infection. The Committee will review infection control activities of the hospital, emergence of drug resistance, use of different antimicrobial agents, sterilisation and disinfection procedures, hospital environment, incidence and types of infections and antimicrobial sensitivity patterns of the prevalent pathogens.

KEY POINTS

1. The terms *health associated infection, hospital acquired infection* or *nosocomial infection* is defined as infection developing in patients after admission to the hospital, which was neither present nor in the incubation period at the time of hospitalisation.
2. Health associated infection is mostly exogenous from another patient or member of the staff or from the environment in the hospital.
3. Almost any microorganism can, on occasion, cause health associated infection but those that survive in the hospital environment for long periods and develop resistance to antibiotics and disinfectants are particularly important.
4. *Staph. aureus*, *Pseudomonas aeruginosa*, *Esch. coli*, *Klebsiella* are some important agents responsible for health associated infections.
5. *Diagnosis* of hospital associated infection is done by the *routine bacteriological methods*. This requires the samples from possible sources of infection such as *hospital personnel*, *inanimate objects*, *water*, *air* or *food*.
6. Every hospital must have an effective infection control committee (ICC) which should be responsible for the control of health associated infection (HAI).

YOU MUST KNOW

1. Definition of health associated infection.
2. List of modes of transmission of health associated infection.
3. Common health associated infections and causative organisms responsible for these conditions.
4. Diagnosis, control and prevention of health associated infection.

STUDY QUESTIONS

1. Define health associated infection. Enumerate the microorganisms causing it. What are the factors which contribute in development of this infection?
2. Write short notes on:
   (a) Modes of transmission of health associated infection
   (b) Diagnosis and control of health associated infection
   (c) Prevention of health associated infection
   (d) Infection control policy

MULTIPLE CHOICE QUESTIONS (MCQs)

1. What are diifferent modes of transmission of health assiciated infections?
   (a) Through contact
   (b) Airborne
   (c) Parenteral route
   (d) All of the above
2. Which of the following bacteria can cause hospital acquired urinary tract infection?
   (a) *Escherichia coli*
   (b) *Klebsiella species*
   (c) *Proteus species*
   (d) All of the above
3. Which of the following bacteria can cause infection in hospitalised burn patients?
   (a) *Pseudomonas aeruginosa*
   (b) *Staphylococcus aureus*
   (c) *Acinetobacter species*
   (d) All of the above
4. Which of the following bacteria can cause hospital acquired respiratory infections?
   (a) *Staphylococcus aureus*
   (b) *Klebsiella species*
   (c) *Enterobacter species*
   (d) All of the above

Answers (MCQs):

1. (d) 2. (d) 3. (d) 4. (d)

Chapter 76

PROPHYLACTIC IMMUNISATION

Immunisation against infectious diseases has been an important contribution of microbiology to medicine. Aim of immunisation is to produce a degree of resistance sufficient to prevent a clinical attack of the natural infection. It may be active or passive immunisation.

I. ACTIVE IMMUNISATION

Active immunisation is done by use of vaccines. It works through two key elements of adaptive immunity i.e. specificity and memory.

Specificity: Vaccine induces production of specific antibody.

Memory: Due to presence of memory cells, the secondary response is prompt, powerful and prolonged with production of higher level of antibodies than that in primary response.

Vaccines may be *live attenuated, killed* or in the form of *toxoids* (Table 76.1).

A new type of vaccine, *subunit vaccine*, prepared by purifying the fragments of major immunogenic components of microorganisms has also been developed.

Table 76.1 Vaccines and Toxoids

Live attenuated vaccines	Killed vaccines	Toxoids
• BCG vaccine • OPV • Measles vaccine • Mumps vaccine • Rubella vaccine • Oral typhoid fever vaccine • Varicella vaccine • Yellow fever	• Pertussis vaccine • Typhoid fever vaccine • Cholera vaccine • Rabies vaccine • Japanese encephalitis vaccine • Killed polio vaccine • Influenza vaccine • Hepatitis B vaccine • Plague vaccine • Pneumococcal vaccine • Meningococcal vaccine	• Diphtheria toxoid • Tetanus toxoid

A. Live Attenuated Vaccines

A single dose of live vaccine is sufficient for immunisation.

The *attenuated organism* can multiply in the body to provide a continuous antigenic stimulus and thus serves both as primary and booster dose. The attenuated organisms are the suspensions of living organisms with

reduced virulence. These mimic natural infection with antibody production but without symptoms. Examples of some live vaccines are : oral polio vaccine (OPV); mumps, measles and rubella (MMR) and yellow fever.

Sometimes related organisms with shared antigens are used for preparation of live vaccine e.g. a bovine tubercle bacillus for BCG vaccine and vaccinia virus for small pox vaccine.

Live vaccines should not be administered in immunocompromised hosts (leukaemia, malignancy, AIDS etc.) in whom there is an increased incidence of severe complications.

B. Killed (inactivated) Vaccines

These vaccines possess antigens common to the original pathogen but do not replicate. With killed vaccine, usually three doses of vaccines are required to have effective immune response. Booster doses are necessary. Killed vaccines include typhoid, cholera, rabies, hepatitis B, influenza, pertussis and pneumococcal vaccines.

C. Toxoids

Toxoids are modified toxins which have lost toxigenicity but retained the antigenicity. These are usually prepared by treating the toxins with formalin (formol toxoids). Toxoids are used for prophylaxis against those infections in which pathogenesis is attributable to a toxin.

Antigenicity of toxoids is enhanced by adsorption on to a mineral carrier such as aluminium hydroxide or aluminium phosphate. Such adsorbed toxoids remain longer in a depot after injection and serves as a better antigen. In DPT (diphtheria, pertussis, tetanus) vaccine, tetanus toxoid and diphtheria toxoid are mixed with a pertussis vaccine. Pertussis vaccine acts as an adjuvant. Tetanus toxoid (TT) and diphtheria toxoid (DT) are two widely used toxoids for immunisation.

D. Special Types of Vaccines

1. Vaccines produced by cloning the surface antigen in yeast cells e.g. hepatitis B vaccine.
2. Subunit vaccines consist of purified fragments of the major immunogenic components of microorganisms produced by recombinant DNA technology.

E. Immunisation Schedule

1. National Immunisation Schedule

The national immunisation schedule is shown in Table 76.2.

2. WHO Universal Immunisation Programme (UIP)

In 1974, WHO launched a global immunisation programme known as expanded programme of immunisation (EPI) to protect all children against six diseases namely, poliomyelitis, diphtheria, whooping cough, tetanus, tuberculosis and measles by the year 2000. EPI was launched in India in 1978. The programme is now called Universal Child Immunisation, 1990. In India it is named as the Universal Immunisation Programme (UIP).

II. PASSIVE IMMUNISATION

Passive immunisation is used when it is considered necessary to give immediate protection to an anticipated infection. Immunity produced is short-lasting. Human sera and animal sera are used for passive immunisation.

A. Human Sera

Two types of normal human immunoglobulins are available, *pooled immunoglobulins* and *specific (hyperimmune) immunoglobulins.*

1. Pooled Immunoglobulins

It is prepared from pooled normal human serum containing high levels of appropriate antibody. Human normal immunoglobulin is used for short-term prophylaxis of hepatitis A and measles after contact with a case.

2. Specific (hyperimmune) Immunoglobulins

It is prepared from sera of patients who are recovering from infection (convalescent sera) or from persons who have been actively immunised against a specific infection. Specific immunoglobulins are available for passive immunisation against

(*i*) tetanus (human tetanus immunoglobulin, HTIG)
(*ii*) hepatitis B (hepatitis B immunoglobulin, HBIG)
(*iii*) rabies (HRIG)
(*iv*) varicella-zoster (ZIG)
(*v*) vaccinia (AVIG)

ADMINISTRATION OF HUMAN SERA

(*a*) Human sera are administered by intramuscular injection. However, in case of rabies, half dose is given around the bite wound and the other half is administered intramuscularly.
(*b*) Generally these sera should not be administered shortly before or after active immunisation to avoid inhibition of immune response.

B. Animal Sera

The term antiserum is applied to antibodies prepared in animals. These sera are raised in horses by active immunisation. The equine antisera were previously used widely but current trend is in favour of using human sera as far as possible. The animal sera contain foreign protein

Table 76.2 National Immunisation Schedule

Age	Vaccine	Dose	Route of administration
Infants and children At birth (for institutional deliveries)	BCG•	0.1 mL (0.05 mL until one month of age)	Intradermal
	OPV-zero dose	Two drops	Oral
	Hepatitis B vaccine	0.5 mL	Intramuscular
6 weeks	DPT-1	0.5 mL	Intramuscular
	OPV-1	Two drops	Oral
	Hepatitis B vaccine	0.5 mL	Intramuscular
10 Weeks	DPT-2	0.5 mL	Intramuscular
	OPV-2	Two drops	Oral
	Hepatitis B vaccine	0.5 mL	Intramuscular
14 weeks	DPT-3	0.5 mL	Intramuscular
	OPV-3	Two drops	Oral
	Hepatitis B vaccine	0.5 mL	Intramuscular
9-12 months	Measles vaccine	0.5 mL	Subcutaneous
	Vitamin A (1st dose) at 9 months	1 ml (One lakh IU)	Oral
16 to 24 months	DPT••	0.5 mL	Intramuscular
	OPV••	Two drops	Oral
	Measles••• (2nd dose)	0.5 mL	Subcutaneous
	Vitamin A•••• (2nd to 9th dose)	2 mL (Two lakh IU)	Oral
5-6 years	DPT••	0.5 mL	Intramuscular
10 years	TT••	0.5 mL	Intramuscular
16 years	TT••	0.5 mL	Intramuscular
For pregnant women As early as possible during pregnancy (first contact)	TT•••••	0.5 mL	Intramuscular

Hib (given as pentavalent containing Hib + DPT + hepatitis B). Three doses given intramuscular 6, 10 and 14 weeks of age. It is given only in 8 states of India.

• At birth or as early as possible till one year of age.

•• Booster dose

••• Given as MR (Measles, rubella)/MMR (Measles, mumps, rubella) vaccine

•••• Vitamin A 2nd to 9th dose are given at interval of one month up to 5 years of age.

••••• Two doses should be given at an interval of one month, if not vaccinated previously. If vaccinated with 2TT doses in a pregnancy within last three years then only single dose is given which acts as a booster dose.

DPT-1 = first dose of DPT OPV-1 = first dose of OPV

DPT-2 = second dose of DPT OPV-2 = second dose of OPV

DPT-3 = third dose of DPT OPV-3 = third dose of OPV

to which recipient forms antibody resulting in:

1. rapid elimination, much faster than human immunoglobulin,
2. hypersensitivity reaction (serum sickness and anaphylaxis) in some cases.

III. COMBINED PASSIVE AND ACTIVE IMMUNISATION

In some diseases (tetanus, diphtheria, rabies) passive immunisation is often undertaken in conjunction with inactivated vaccines, to provide both immediate (but short lasting) passive immunity and slowly developing active immunity. Both injections should be administered at separate sites.

IV. INDIVIDUAL IMMUNISATION

Vaccines offered under national immunisation schedule are limited by economic considerations. Some important vaccines are omitted. These include varicella vaccine and typhoid vaccine. These may be supplemented by individual initiative, whenever possible.

1. Varicella Vaccine

The vaccine is given as a single subcutaneous dose in children 9 months to 12 years of age, and as two doses at an interval of at least 6 weeks, in those older. It is live attenuated vaccine. It is contraindicated in pregnancy.

2. Typhoid Vaccine

Two recent typhoid vaccines, the live oral Gal-E mutant vaccine and the injectable purified Vi polysaccharide vaccine are recommended for immunisation of those five years old or above.

KEY POINTS

1. Aim of *immunisation* is to produce a degree of resistance sufficient to prevent a clinical attack of the natural infection. It may be active or passive immunisation.
2. *Active* immunisation is done by use of *vaccines*.
3. Vaccines may be *live attenuated*, *killed* or in the term of *toxoids*.
4. *BCG* vaccine, *oral polio* vaccine, *measles* vaccine, *mumps* vaccine and *rubella* vaccine are some important live attenuated vaccines.
5. Killed vaccines include *pertussis* vaccine, *cholera* vaccine, *rabies* vaccine, *killed polio vaccine* and *hepatitis B* vaccine.
6. *Diphtheria toxoid* (*DT*) and *tetanus toxoid* (*TT*) are two examples of toxoids.
7. Passive immunisation is used when it is considered necessary to give immediate protection to an anticipated infection.
8. *Human sera* and *animal sera* are used for passive immunisation. *Specific immunoglobulins* are available for passive immunisation against *tetanus*, *hepatitis B* and *rabies*.

YOU MUST KNOW

1. List of live attenuated vaccines.
2. List of killed vaccines.
3. List of toxoids.
4. How toxoid differs from toxin?
5. Immunisation schedule.
6. Passive immunisation.

STUDY QUESTIONS

Q. Write short notes on:
(a) Live attenuated vaccines
(b) Killed vaccines
(c) Toxoids
(d) Passive immunisation

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Which of the following vaccines is/are live attenuated vaccine/s?
(a) BCG vaccine
(b) Measles vaccine
(c) MMR vaccine
(d) All of the above
2. Which of the following vaccines is/are killed vaccine/s?
(a) Pertussis vaccine
(b) Rabies vaccine
(c) Japanese encephalitis vaccine
(d) All of the above
3. Which of the following vaccines is/are prepared from bacterial exotoxin/s?
(a) Vaccine for diphtheria
(b) Vaccine for tetanus
(c) Both of the above
(d) None of the above

4. Which of the following vaccines should be preferably given at the birth of a child?
   (a) BCG vaccine (b) DPT vaccine
   (c) MMR vaccine (d) All of the above
5. What is the route of administration of BCG vaccine?
   (a) Intramuscular (b) Intradermal
   (c) Subcutaneous (d) Oral
6. Measles vaccine should be administered at the age of:
   (a) 6 weeks (b) 10 weeks
   (c) 9 months (d) 12 months
7. What is the route of administration of measles vaccine?
   (a) Intramuscular (b) Intradermal
   (c) Subcutaneous (d) Oral
8. At what age first dose of vitamin A is given?
   (a) 6 weeks (b) 6 months
   (c) 7 months (d) 9 months
9. What is the amount second dose of vitamin A given to children?
   (a) 1 lakh IU (b) 2 lakh IU
   (c) 5 lakh IU (d) 6 lakh IU
10. How many doses of tetanus toxoid should be given to a pregnant woman who is not vaccinated for tetanus previously?
    (a) One (b) Two
    (c) Three (d) Four
11. Specific (hyperimmune) immunoglobulins are available for passive immunisation against:
    (a) Tetanus (b) Hepatitis B infection
    (c) Rabies (d) All of the above
12. Combined passive and active immunisation is often undertaken in:
    (a) Tetanus (b) Diphtheria
    (c) Rabies (d) All of the above

Answers (MCQs):

1. (a) 2. (d) 3. (c) 4. (a) 5. (b) 6. (c)
7. (c) 8. (b) 9. (d) 10. (b) 11. (d) 12. (d)

Chapter 77

ANTIMICROBIAL THERAPY

Antimicrobial agent is a chemical substance that inhibits the growth or causes the death of a microorganism. The term antibiotic is defined as a substance produced by a microorganism or a similar substance produced by chemical synthesis and is capable of inhibiting the growth or causing death of other microorganisms in low concentrations. Chloramphenicol was originally obtained from *Streptomyces venezuelae* but now is entirely synthesised by chemical methods.

I. MECHANISMS OF ACTION OF ANTIBIOTICS

The antimicrobial drugs may act by killing the bacteria (*bactericidal*) or by inhibiting the growth of bacteria (*bacteriostatic*). The mechanisms of action of these drugs can be divided into five groups.

A. Interfering with Cell Wall Synthesis

The antibiotics which interfere with cell-wall synthesis are penicillins, cephalosporins, bacitracin, vancomycin and cycloserine. All β-lactam antibiotics (penicillins, cephalosporins) bind to receptors (penicillin binding proteins present on the inner layer of cytoplasmic membrane) and this leads to interference with the synthesis of peptidoglycan of cell wall. This makes the cell membrance vulnerable to damage by solutes of the plasma. Penicillins and cephalosporins are non-toxic to mammalian cells due to absence of peptidoglycan. The cell-walls of Gram negative bacteria is more complex, therefore, these drugs cannot penetrate the cell in adequate concentration.

B. Acting on Cytoplasmic Membrane

Polymyxin, nystatin and amphotericin B bind with the cytoplasmic membrane. As a result of this, the semipermeability of membrance is lost and essential low molecular weight intermediates and coenzymes pass into the environment causing cell death.

C. Inhibiting Protein Synthesis

Transcription and *translation* are two essential steps in the process of protein synthesis. An antibiotic that inhibits either of these will inhibit protein synthesis.

1. *Inhibitors of Transcription*

Rifampicin inactivates DNA-dependent RNA polymerase thus inhibiting transcription.

2. *Inhibitors of Translation*

Some antibiotics combine with 30S and 50S components of ribosomes and lead to malfunctioning of ribosomes. It may affect initiation, elongation or termination of peptide chain leading to inhibition of protein synthesis and the cell eventually dies.

Antibiotics that inhibit 30S ribosomes include aminoglycosides (streptomycin, kanamycin, amikacin, neomycin and tobramycin) and tetracyclines (tetracycline, doxycycline and minocycline). Antibiotics that inhibit 50S ribosomes include erythromycin, chloramphenicol and lincomycin.

D. Inhibiting DNA Function

Novobiocin, metronidazole, mitomycin and quinolones (nalidixic acid, norfloxacin and ciprofloxacin) alter the functioning of DNA. Novobiocin inhibits the replication of DNA. Metronidazole damages DNA and inhibits its replication. Quinolones block the DNA gyrase and thus they inhibit DNA synthesis of bacteria.

E. Metabolic Antagonists

Sulphonamides, sulphones (dapsone), PAS, INH and trimethoprim act as metabolic antagonists. Para-aminobenzoic acid (PABA) is an essential metabolite in many organisms. It gets attached to the active site of the enzyme produced by bacterium and is converted into dihydrofolic acid and then to tetrahydrofolic acid. This acts as an essential co-factor for bacterial cell growth. Sulphonamides have a chemical structure similar to that of PABA, and thus competes with latter in the bacterial metabolism. Sulphonamide resistant bacteria as well as host cells also require folic acid for cell growth but they are capable of taking up preformed folic acid from the environment and thus their growth is independent of the conversion of PABA to tetrahydrofolic acid. Trimethoprim is a selective inhibitor of bacterial dihydrofolate reductase enzyme which is essential for conversion of dihydrofolic acid to tetrahydrofolic acid.

Mechanisms of action of different antibiotics are shown in Table 77.1.

II. ANTIMICROBIAL DRUGS

A. Penicillins

The penicillins possess a common chemical nucleus (6-aminopenicillanic acid) which contains a β-lactam ring essential for their biological activity (Fig. 77.1). They are bactericidal and inhibit synthesis of cell wall. Benzyl penicillins, water soluble sodium and potassium salts, are mainly active against Gram positive organisms. They are susceptible to hydrolysis by β-lactamases. Some semisynthetic penicillins are β-lactamase (penicillinase) resistant e.g. methicillin and cloxacillin. Some other penicillins are effective against both Gram positive and Gram negative organisms but are destroyed by β-lactamases, e.g. ampicillin, amoxycillin, carbenicillin,

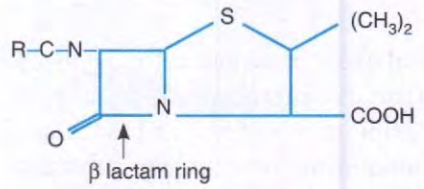


Fig. 77.1 Structure of penicillins

Table 77.1 Mechanisms of action of antibiotics

Interference of cell wall synthesis	Inhibition of cytoplasmic membrane function	Inhibition of protein synthesis	Inhibition of DNA function	Metabolic antagonists
• Penicillins • Cephalosporins • Bacitracin • Vancomycin • Cycloserine	• Polymycin • Nystatin • Amphotericin B	• Streptomyin • Kanamycin • Amikacin • Neomycin • Tobramycin • Tetracycline • Doxycycline • Minocycline • Erythromycin • Chloramphenicol • Lincomycin	• Nalidixic and • Norfloxacin • Ciprofloxacin • Novobiocin • Metronidazole	• Sulphonamides • Dapsone • PAS • Isoniazid • Trimethoprim

ticarcillin and piperacillin. Penicillins active against *Pseudomonas aeruginosa* include carbenicillin, ticarcillin and piperacillin.

B. Cephalosporins

Cephalosporins are β-lactam antibiotics related to penicillins. In place of 6–aminopenicillanic acid, they have a nucleus of 7-aminocephalosporanic acid. They are resistant to β-lactamases in varying degrees. These are bactericidal drugs and their mode of action is similar to that of penicillins. They are active against both Gram positive and Gram negative bacteria. The important cephalosporins are shown in Table 77.2.

Table 77.2 Cephalosporins

Cephalosporins	Antibacterial spectrum
First generation	
Cephalothin Cephalexin Cephaloridine Cephazolin Cephradine Cefadroxil Cephradine	*Staph. aureus,* streptococci (other than enterococci), *Esch. coli, Klebsiella, Proteus mirabilis* and *H. influenzae*
Second generation	
Cefamandole Cefoxitin Cefuroxime Cefonicid Ceforanide Cefaclor Cefprozil Cefmetazole Cefotetan Loracarbef	First generation spectrum and indole positive *Proteus, Enterobacter, Citrobacter, Serratia* and Gram negative anaerobes
Third generation	
Cefotaxime Cefoperazone Ceftizoxime Ceftazidime Ceftriaxone Cefixime Ceftibuten Cefdinir Cefditoren Cefpodoxime	Second generation spectrum and *N. gonorrhoeae* including β lactamase producing strains, *Ps. aeruginosa*
Fourth generation	
Cefepime Cefpirome Ceftaroline Ceftobiprole	Third generation spectrum including enhanced activity against Enterobacter and Citrobacter spp. that are resistant to third generation cephalosporins.

C. Aminoglycosides

They are used most widely against Gram negative bacteria. Mode of action is by inhibiting protein synthesis of bacteria. They are bactericidal in action. This group includes streptomycin, gentamicin, kanamycin, amikacin, neomycin and tobramycin.

Streptomycin is most active against mycobacterial infections. Kanamycin and neomycin are poorly absorbed from intestinal tract. Amikacin is semisynthetic derivative of kanamycin. Gentamicin is the most widely used aminoglycoside in various coliform infections. Other aminoglycosides used in these infections are tobramycin and netilmicin. Netilmicin is the semisynthetic derivative of tobramycin.

D. Tetracyclines

Tetracyclines are effective to a variable extent in both cocci and bacilli. They are highly effective against rickettsial and chlamydial infections. Like aminoglycosides, they act by inhibiting protein synthesis of bacteria and are bacteriostatic.

The semisynthetic tetracyclines include dimethylchlortetracycline, doxycycline and rolytetracycline.

E. Other Antibiotics

1. Macrolides

They have a macrocyclic lactone ring to which sugars are attached e.g. erythromycin. Erythromycin acts on Gram positive cocci, *Neisseria, H. influenzae* and is useful in penicillin resistant staphylococci. It acts by inhibiting protein synthesis and is bacteriostatic.

2. Clindamycin and Lincomycin

Clindamycin is a chlorine substituted derivative of lincomycin. Lincomycin acts by inhibiting protein synthesis of bacteria.

3. Vancomycin

It is bactericidal for staphylococci and *Clostridia*. Mode of action is by inhibition of cell wall synthesis. It is used orally in antibiotic associated colitis. It is also used in methicillin resistant *Staph. aureus* (MRSA) infections.

4. Quinolones

They are effective in infections caused by Gram positive and Gram negative bacteria. They are synthetic analogues of nalidixic acid. These include norfloxacin and ciprofloxacin. They inhibit bacterial DNA synthesis and are bactericidal drugs.

5. Bacitracin

It is an extract of *Bacillus subtilis* (Tracy strain). It is

poorly absorbed from intestine or from wounds. It is bactericidal for staphylococci. It also acts against penicillin resistant strains of staphylococci.

6. Chloramphenicol

It is a broad spectrum antibiotic and is used in enteric fever, meningitis and locally as eye drops in conjunctivitis. It acts by inhibiting protein synthesis and is bacteriostatic. It sometimes causes aplastic anaemia.

F. Sulphonamides and Trimethoprim

A combination of sulphonamide and trimethoprim shows synergistic action *in-vitro*. The combination is widely used in urinary and respiratory tract infections, salmonellosis and other infections. They are effective against a variety of Gram positive and Gram negative organisms and certain *Chlamydiae*. Sulphonamides are bacteriostatic while trimethoprim may be bactericidal. Due to a structural similarity with PABA, sulphonamides compete with the latter in bacterial metabolism.

G. Antituberculous Drugs

Isoniazid is specifically active at low concentration in mycobacteria. It is bacteriostatic agent. It penetrates well into tissues and fluids and acts on intracellular organisms. Ethambutol interferes with RNA metabolism while PAS (para-amino salicylic acid) acts as analogue of PABA. Rifampicin inhibits RNA synthesis by combining with DNA-dependant RNA polymerase. Cycloserine inhibits cell wall synthesis.

Combination of antituberculous drugs is essential in treatment of tuberculosis to prevent emergence of drug resistance.

H. Metronidazole

Metronidazole is effective against anaerobes and certain protozoa. It inhibits DNA synthesis and is bactericidal.

III. ANTIBIOTIC RESISTANCE

The emergence of antibiotic resistance is a major problem in antimicrobial therapy. At first, there is an emergence of a small number of resistant bacteria which soon multiply selectively in the presence of antibiotic at the cost of sensitive bacteria.

A. Mechanisms

1. Permeability

Some microorganisms change their cell wall permeability to the drug, possibly by alteration in the chemical nature of outer membrane. Example is tetracycline resistance by *Pseudomonas aeruginosa*.

2. Production of Enzymes

Penicillin resistant *Staphylococcus aureus* produces an enzyme β-lactamase that destroys the penicillin.

3. Altered Structural Target

Aminoglycosides attach with 30S subunit of ribosome but resistant bacteria develop an altered receptor. Same mechanism also applies to erythromycin which attaches with 50S subunit of ribosome.

4. Altered Metabolic Pathway

By altering metabolic pathway, bacteria bypass the reaction inhibited by the drug. Sulphonamide resistant bacteria utilise preformed folic acid and do not require the conversion of PABA to folic acid (a reaction inhibited by sulphonamides).

B. Genetic Basis of Resistance

1. Chromosomal Resistance

This occurs as a result of spontaneous mutation. In clinical practice, mutational resistance is of great importance in tuberculosis. The antimicrobial drugs selectively suppress susceptible organisms but resistant mutants will multiply unchecked. If two or more antituberculous drugs are used for treatment, resistant mutant does not occur, as a mutant resistant to one drug will be destroyed by the other drug. This is the rationale behind multiple drug therapy used in tuberculosis.

2. Extrachromosomal Resistance

This occurs by transfer of plasmids and genetic material. Transferable drug resistance mediated by the R factor is the most important. R factors are plasmids that contain genes that code for drug resistance against one and often several antimicrobial drugs. Inactivation of drugs occur by plasmid coded enzymes e.g. β-lactamases destroy the β-lactam ring which is responsible for the antibacterial action of β-lactam antibiotics (penicillins and cephalosporins)). Chloramphenicol is inactivated by different acetyltransferases encoded by resistance determinants on plasmids.

METHODS OF TRANSFER OF PLASMID AND GENETIC MATERIAL

(a) Transduction

Acquisition of resistance by transduction is common in staphylococci. Plasmid (carrying gene for β-lactamase production) enclosed in a bacteriophage is transferred from a penicillin-resistant staphylococcus to a susceptible staphylococcus.

(b) Conjugation

R factors carrying genes for drug resistance are transferred by conjugation. This is the most common method of spread of multiple drug resistance among different genera of Gram negative bacteria.

(c) Transformation

Transfer of naked DNA carrying the genes for drug resistance may occur by this method.

(d) Transposition

A transposon is a segment of DNA that has the ability to move around between chromosomal and extrachromosomal DNA molecules within cells. This mode of genetic transfer is called transposition. These transposons may encode antibiotic resistance.

KEY POINTS

1. *Antimicrobial agent* is a chemical substance that inhibits the growth or causes the death of a microorganism.
2. The term *antibiotic* is defined as a substance produced by a microorganism or a similar substance produced by chemical synthesis and is capable of inhibiting the growth or causing death of other microorganisms in low concentrations.
3. The antimicrobial drugs may act by killing the bacteria (*bactericidal*) or by inhibiting the growth of bacteria (*bacteriostatic*).
4. The mechanism of action of these drugs can be due to *interfering* with *cell wall synthesis*, *acting on cytoplasmic membrane*, *inhibiting protein synthesis*, *inhibiting DNA function* or, acting as *metabolic antagonist*.
5. The emergence of antibiotic resistance is a major problem in antimicrobial therapy.
6. Genetic basis of resistance may be *chromosomal* resistance or *extrachromosomal* resistance.
7. Chromosomal resistance occurs as a result of spontaneous *mutation*. This type of resistance is found in *M. tuberculosis*.
8. Extrachromosomal resistance occurs by *transfer of plasmids* and *genetic material*. Transferable drug resistance mediated by the *R factor* is the most important.
9. *Transduction* and *conjugation* are two methods of transfer of plasmid and genetic material.
10. Acquisition of resistance by transduction is common in *Staphylococci*. Plasmid (carrying gene for β-lactamase production) is transferred from a penicillin-resistant staphylococcus to a susceptible staphylococcus.

YOU MUST KNOW

1. Mechanisms of antibiotic resistance.
2. List of cephalosporins of first, second, third and fourth generation.

STUDY QUESTIONS

Q. Write short notes on:
   (a) Live attenuated vaccines
   (b) Killed vaccines
   (c) Toxoids
   (d) Passive immunisation

MULTIPLE CHOICE QUESTIONS (MCQs)

1. What is the mechanism of action of antibiotic cephalosporin?
   (a) Interfering with cell wall synthesis
   (b) Inhibiting protein synthesis
   (c) Inhibiting DNA function
   (d) Acting on cytoplasmic membrane
2. Which of the following antibiotics act/s by interfering with cell wall synthesis?
   (a) Penicillins
   (b) Bacitracin
   (c) Vancomycin
   (d) All of the above

3. Which of the following antibiotics act/s on cytoplasmic membrane
   (a) Polymyxin (b) Nystatin
   (c) Amphotericin B (d) All of the above
4. Which of the following antibiotics act/s by inhibiting protein synthesis?
   (a) Rifampicin (b) Kanamycin
   (c) Minocycline (d) All of the above
5. Which antibiotic/s act/s by inhibiting DNA function?
   (a) Metronidazole (b) Nalidixic acid
   (c) Norfloxacin (d) All of the above
6. Which antibiotic/s act/s as metabolic antagonists for their action?
   (a) Sulphones (b) Isoniazid
   (c) Trimethoprim (d) All of the above
7. Which of the following is third generation cephalosporin?
   (a) Cephalexin (b) Cefoxitin
   (c) Cefotaxime (d) Cefaclor
8. Which of the following is fourth generation cephalosporin?
   (a) Cephazolin (b) Cefprozil
   (c) Cefmetazole (d) Cefepime
9. All of the following are third generation cephalosporins except:
   (a) Cefoperazone (b) Ceftozidime
   (c) Ceftriaxone (d) Cephradine
10. What is the genetic basis of drug resistance in *Mycobacterium tuberculosis*?
    (a) Transformation (b) Transduction
    (c) Mutation (d) Conjugation
11. How does *Staphylococcus aureus* acquire drug resistance?
    (a) Transformation (b) Transduction
    (c) Mutation (d) Conjugation
12. Which of the following bacteria acquire/s drug resistance by conjugation?
    (a) *Staphylococcus aureus* (b) *Mycobacterium tuberculosis*
    (c) *Shigella dysenteriae* (d) All of the above

Answers (MCQs):

1. (a)	2. (d)	3. (d)	4. (d)	5. (d)
6. (d)	7. (c)	8. (d)	9. (d)	10. (c)
11. (b)	12. (c)			

◆◆◆

Chapter 78

ANTIMICROBIAL SENSITIVITY TESTING

Pathogenic bacteria exhibit great strain variations in susceptibility to antibiotics. It is, therefore, essential to determine the susceptibility of isolates to antibiotics that are likely to be used in the treatment.

I. ANTIMICROBIAL SENSITIVITY TESTS

Antimicrobial sensitivity tests are of two types:

A. Diffusion tests
   Stokes disc diffusion method; Kirby-Bauer disc diffusion method
B. Dilution tests
   Broth dilution method; Agar dilution method

A. Diffusion Tests

Disc diffusion tests are most widely used to determine the susceptibility of isolates of pathogenic bacteria to antibiotics that are likely to be used in the treatment. Here, the antibiotic is allowed to diffuse through a solid medium so that the concentration is highest near the site of application of the antibiotic disc and decreases with the distance. These tests have, however, been found to be unsuitable for slow growing microbes.

The 'disc diffusion' method uses filter paper discs charged with appropriate concentration of the drugs. The test bacterium is inoculated on the medium and these antibiotic discs are applied. Sensitivity to the drug is determined from the inhibition of bacterial growth around the disc.

Medium

The medium should support good overnight growth of both the test and the control organisms. Mueller-Hinton agar may be used for testing aerobes and facultative anaerobes. Nutrient agar is another alternative medium used. The medium is prepared in the petri dish (100 mm in diameter). The depth of the medium should be 4 mm (around 25 ml of medium). The plates may be stored at 4°C for up to one week. The pH of the medium should be between 7.2 and 7.4. A more acidic pH decreases the activity of aminoglycosides and macrolide antibiotics such as erythromycin. On the other hand, a more alkaline pH favours the action of tetracycline, novobiocin and fusidic acid.

Some drugs, such as sulphonamides and trimethoprim, are inhibited by thymidine present in the culture medium. 5% lysed horse blood is added to the medium to neutralise the inhibitory effect of thymidine. Lysed horse blood is also needed to support the growth of fastidious organisms such as *H. influenzae*. The addition of 5% NaCl to the medium is required in one of the methods for detecting resistance to methicillin in strains of staphylococci

Inoculum

The organisms are isolated in pure culture on a solid medium. Isolated colonies are inoculated in a suitable broth medium and incubated at 35-37°C for 4-6 hours. The density of the organisms in broth is adjusted to approximately 10^7 cfu/ml by comparing its turbidity with

Table 78.1 Control Strains of Stokes and Kirby-Bauer Disc Diffusion Methods

Test bacteria	Control strain	
	Stokes	Kirby-Bauer
Coliforms	*Esch. coli* NCTC 10418	*Esch. coli* ATCC 25922
Pseudomonas	*Ps. aeruginosa* NCTC 10662	*Ps. aeruginosa* ATCC 27853
Enterococci	*E. faecalis* NCTC 12697	*E. faecalis* ATCC 29212
Haemophilus spp.	*H. influenzae* NCTC 11931	*H. influenzae* ATCC 49247
N. gonorrhoeae	*N. gonorrhoeae* (sensitive strain)	*N. gonorrhoea* ATCC 49226
Other organisms that can grow aerobically	*Staph. aureus* NCTC 6571	*Staph. aureus* ATCC 25923

that of 0.5 Mc Farland opacity standard tube. This broth is inoculated on the medium by spreading with sterile swabs. The ideal inoculum after overnight incubation gives even semi-confluent growth. Too heavy an inoculum reduces the size of inhibition zones. Similarly, inoculum of control strain should also be prepared as in the case of test strain.

Control Strains

Control strains for Stokes and Kirby-Bauer disc diffusion methods are shown in Table 78.1.

Antibiotic Discs

Antibiotic discs may be prepared in the laboratory or purchased commercially. Filter paper discs of 6 mm in diameter are used. For preparation of discs in the laboratory, pure antimicrobial agents should be used and not those used for clinical practice. Distilled water serves to dissolve most antibiotic powders but some antibiotics may require different diluents (Table 78.2). Disc contents of antimicrobial agents are shown in Table 78.3. The discs are stored dry in the cold. These should be warmed slowly to room temperature to overcome hydrolysis. Hence, discs should be taken out from refrigerator 1-2 hours before applying on the medium.

Antibiotics should be chosen with discrimination. Only those clinically relevant should be tested.

Table 78.2 Diluents for Various Antibiotics

Antibiotic	Diluent
Chloramphenicol	Ethanol
Rifampicin	Ethanol
Erythromycin	Ethanol
Nitrofurantoin	NaOH solution
Sulphonamides	NaOH solution
Trimethoprim	Acetic acid
Amoxycillin	$NaHCO_3$ (Saturated)
Ceftazidime	$NaHCO_3$ (Saturated)

1. Stokes Disc Diffusion Method

The test bacterium is inoculated on the central one third and control on upper and lower thirds of the plate. However, in modified Stokes disc diffusion method, the test bacterium is inoculated over the upper and lower thirds of the plate and control on central one third. Antibiotic discs are applied between the standard and test inocula (Fig. 78.1). A maximum of six antibiotic discs can be applied on a 100 mm diameter plate (petridish). The plates are then incubated at 37°C for 16-18 hours.

REPORTING

Plates in which growth is not semiconfluent should not be reported unless the test is repeated. The reporting of results is done by comparing the zones of inhibition of control and test bacterium. Measure the zone size i.e. the

Table 78.3 Disc Concentrations of Commonly used Antibiotics

Antibiotic	Disc concentration
Benzyl penicillin (for staphylococci)	1.2 µg
Ampicillin (for enterobacteriaceae and enterococci)	10 µg
Amoxycillin	20 µg
Cephalexin	30 µg
Methicillin	5 µg
Carbenicillin	100 µg
Gentamicin	10 µg
Amikacin	30 µg
Erythromycin	15 µg
Tetracycline	30 µg
Chloramphenicol (for enterobacteriaceae)	30 µg
Nalidixic acid	30 µg
Trimethoprim	5 µg
Ciprofloxacin	1 µg

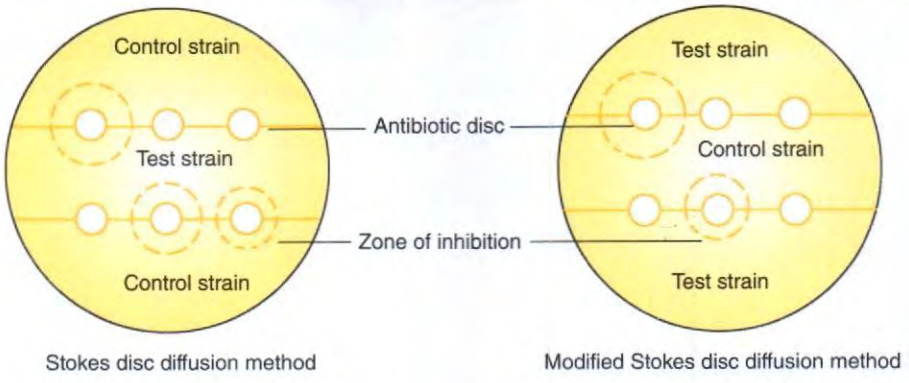


Fig. 78.1 Stokes disc diffusion method and modified Stokes disc diffusion method

distance in mm from edge of the disc to the zone edge. It is interpreted as follows:

Sensitive

- The zone of test bacterium is equal to, or larger than that of control strain.
- If zone size of test bacterium is smaller than that of control, the difference between the two should not be more than 3 mm.

Intermediate sensitive

The zone size of the test bacterium should be at least 2 mm and the difference between the zone of test and control strain should be at least 3 mm.

Resistant

The zone size of the test bacterium is smaller than 2 mm.

2. *Kirby-Bauer Disc Diffusion Method*

Dip a cotton swab into inoculum. Inoculate the Mueller-Hinton agar plate by streaking the swab three times over the entire agar surface. Allow 3-5 minutes for the surface of agar to dry before applying the antibiotic discs, using either sterile forceps or multidisc dispenser. On a plate of 100 mm diameter, seven discs may be applied, one in the centre and six in the periphery (Fig. 78.2). The plates are then incubated at 37°C for 16-18 hours. The zones of complete growth inhibition around each of the discs are measured. The diameter of the disc is included in this measurement. The interpretation of zone size into sensitive, intermediate or resistant is based on interpretation chart (Table 78.4). Control strains of *Staph. aureus, Esch. coli, Ps. aeruginosa* etc. should be tested each time when a new batch of discs or agar is used.

Interpretation

For interpretation of the results of Stokes and Kirby-Bauer diffusion methods, the following additional points should be considered in certain conditions.

(*i*) Penicillinase producing strains of *Staphylococcus* sometimes fail to form enough enzyme to neutralise penicillin close to the disc, it will show a zone of inhibition but colonies at the edge are large and there is not gradual fading away of growth towards the disc. It should be reported resistant, irrespective of the zone size.

(*ii*) Methicillin resistant *Staphylococcus aureus* (MRSA) will often appear fully sensitive when tested in ordinary way. Many of these organisms grow slowly in the presence of methicillin. The growth will only appear within the zone when the incubation is continued for 48 hours. This problem can be overcome either by incubating the plates at 30°C or by using 5% salt agar and incubating at 37°C.

(*iii*) *Proteus mirabilis* and *Proteus vulgaris* may swarm on agar surface resulting in a thin veil that may penetrate into the zones of inhibition. The zones of swarming should be ignored and the outer margin, which is clearly outlined, should be measured.

(*iv*) When trimethoprim and sulphamethoxazole discs containing both drugs are used, it is impossible to know whether the bacterium is sensitive to both or

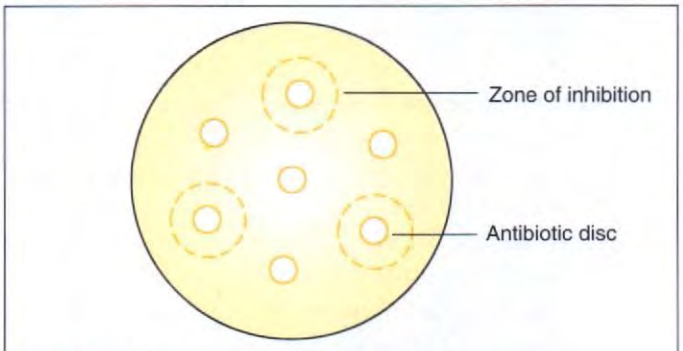


Fig. 78.2 Kirby-Bauer disc diffusion method

Table 78.4 Interpretation Chart used in Kirby-Bauer Disc Diffusion Method

Antibiotic*	Diameter of zone of inhibition (in mm)		
	Resistant	Intermediate sensitive	Sensitive
Benzyl penicillin	≤ 28	—	≥ 29
Ampicillin Enterobacteriaceae	≤ 13	14-16	≥ 17
Methicillin	≤ 9	10-13	≥14
Carbenicillin *Esch. coli* and *Proteus sp.* *Ps. aeruginosa*	 ≤ 17 ≤ 13	 18-20 14-17	 ≥ 23 ≥ 18
Gentamicin	≤ 12	—	≥ 13
Amikacin	≤ 14	15-16	≥ 17
Erythromycin	≤ 13	14-17	≥ 18
Tetracycline	≤ 14	15-18	≥ 19
Chloramphenicol	≤ 12	13-17	≥ 18
Nalidixic acid	≤ 13	14-18	≥ 19
Trimethoprim	≤ 10	11-15	≥ 16
Ciprofloxacin	≤ 15	16-20	≥ 21

* Only limited antibiotics have been shown in the table.

only to one of these. To overcome this problem, each drug should be tested separately.

(*v*) Polymyxin diffuses poorly in agar hence these zones are small. Interpretation is as follows:
Sensitive—Same as described earlier
Resistant—The zone size of the test bacterium is smaller than that of the control strain. Difference between the two should be more than 3 mm.

(*vi*) Large zones are seen around ciprofloxacin discs with the sensitive control strains. Interpretation is as follows:

(a) When *Staph. aureus* or *Ps. aeruginosa* is used as sensitive control.
Sensitive—Inhibition zone of the test bacterium is equal to, greater than, or not more than 7 mm smaller than that of control.
Intermediate sensitive—Inhibition zone of the test bacterium is more than 2 mm but is smaller than that of the control by more than 7 mm.
Resistant—Inhibition zone of the test bacterium is 2 mm or less.

(b) When *Esch. coli* or *H. Influenzae* is used as sensitive control.
Sensitive—Inhibition zone of the test bacterium is equal to, greater than, or not more than 10 mm smaller than that of the control.
Intermediate sensitive—Inhibition zone of the test bacterium is more than 2 mm but is smaller than that of the control by more than 10 mm.
Resistant—Inhibition zone of the test bacterium is 2 mm or less.

3. Primary Disc Diffusion Test

The disc diffusion methods, as described above, are done after the pathogenic bacteria are isolated from the clinical specimens. When results are required urgently, the 'primary disc diffusion test' may be performed. Here, the clinical specimen is directly inoculated uniformly on the surface of a plate and the antibiotic discs are applied. The results of the primary test should be verified by testing the isolates subsequently.

4. Epsilometer or E-Test

Epsilometer or E-test is antimicrobial sensitivity test to detect minimum inhibitory concentration (MIC) of antibiotic (Fig. 78.3). It is a recent modification of the agar diffusion sensitivity test. It uses an absorbent strip with a known gradient of antibiotic concentration along its length. When the strip is placed on the agar plate inoculated with the test organism, the antibiotic diffuses into the medium. The minimum inhibitory concentration

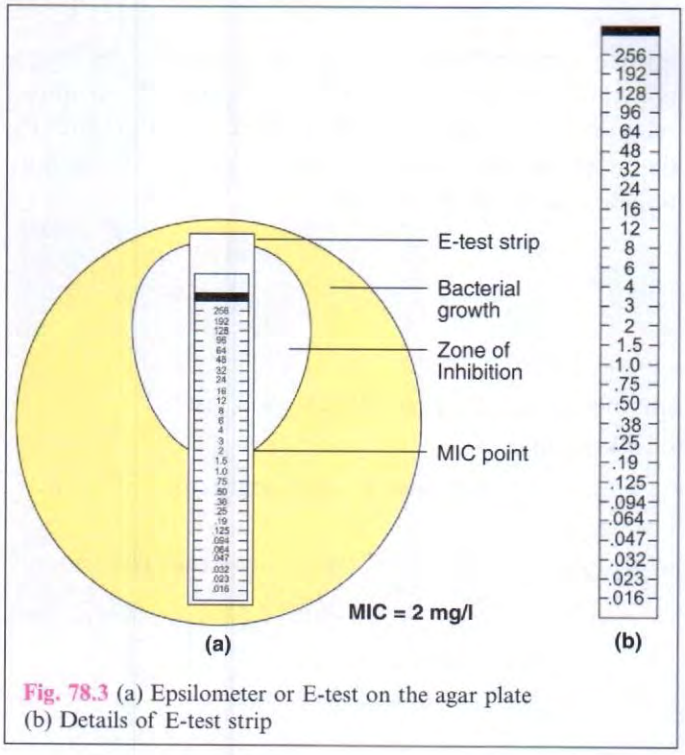


Fig. 78.3 (a) Epsilometer or E-test on the agar plate (b) Details of E-test strip

(MIC) is recorded as the lowest concentration of the gradient which inhibits the growth of the organism.

B. Dilution Tests

1. Broth Dilution Method

Serial dilutions of the drug in Mueller-Hinton broth are taken in tubes and a standardised suspension of the test bacterium inoculated. An organism of known sensitivity should also be titrated. Incubate at 37°C for 16-18 hours. For determination of MIC of methicillin incubate at 30°C. The minimum inhibitory concentration (MIC) is read by noting the lowest concentration of the drug at which there is no visible growth (Fig. 78.4). The minimum bactericidal concentration (MBC) is determined by subculturing from each tube showing no growth on a nutrient agar plate without any antimicrobial agent. Incubate the plates and examine them for growth, if any. The tube containing the lowest concentration of the drug that fails to show growth, on subculture, is the MBC of the drug for that test strain (Fig. 78.4). MIC inhibits the bacterial growth while MBC kills the bacterium.

The uses of determination of MIC are:

(*i*) When the therapeutic dose is to be regulated accurately as in the treatment of bacterial endocarditis.

(*ii*) For testing antimicrobial sensitivities of slow growing bacteria such as tubercle bacilli.

(*iii*) When small degrees of resistance are to be demonstrated.

2. Agar Dilution Method

Serial dilutions of the drug are prepared in agar and poured into plates. Many strains can be inoculated on each plate containing an antibiotic dilution. This method is more convenient when several strains are to be tested at the same time.

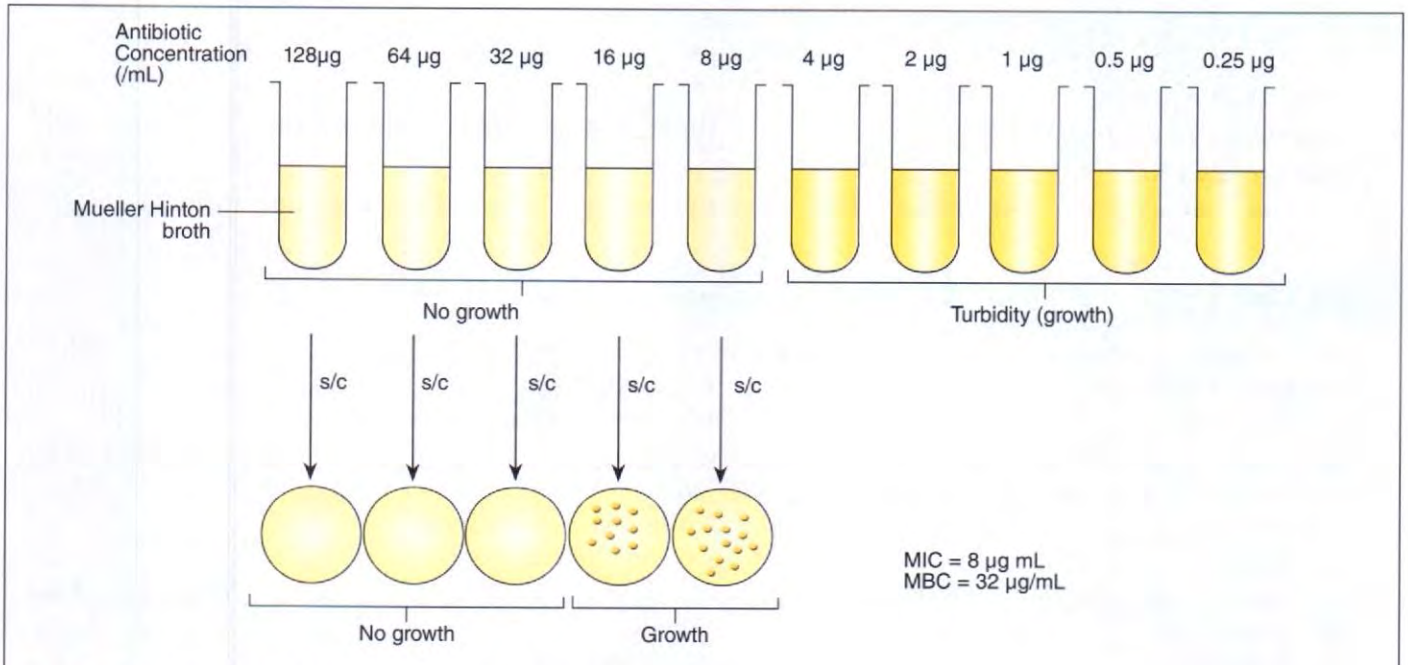


Fig. 78.4 Broth dilution methods showing MIC and MBC

II. ANTIBIOTIC ASSAYS IN BODY FLUIDS

These are required to assess the toxic level and therapeutic level of the drug in blood and other body fluids. These assays are generally done by making serial dilutions of the specimen and inoculating standard suspensions of bacteria of known MIC. By inhibition of bacterial growth, drug levels can be estimated. Another technique is by agar diffusion method. This depends on the direct relationship between the drug concentration and the diameter of the zone of inhibition, with a standard sensitive strain of bacterium.

KEY POINTS

1. Antimicrobial sensitivity tests are of two types: *diffusion tests* and *dilution tests.*
2. *Stokes* disc diffusion method and *Kirby–Bauer* disc diffusion method are examples of diffusion tests.
3. Dilution tests include *broth dilution* method and *agar dilution* method.
4. *Epsilometer* or *E-test* is a modification of the agar diffusion sensitivity test. It uses an absorbent strip with a known gradient of antibiotic concentration along its length.
5. The *minimum inhibitory concentration* (*MIC*) *inhibits* the bacterial growth while the *minimum bactericidal concentration* (*MBC*) *kills* the bacterium.

YOU MUST KNOW

1. List of antimicrobial sensitivity tests.
2. List of diffusion tests.
3. Stokes disc diffusion method and its reporting.
4. Difference between stokes disc diffusion and modified stokes disc diffusion method.
5. Kirby-Bauer disc diffusion method and how it differs from stokes disc diffusion method?
6. Epsilometer (E) test.
7. Minimum inhibitory concentration (MIC).
8. Minimum bactericidal concentration (MBC).

STUDY QUESTIONS

1. Write short notes on:
   (a) Stokes disc diffusion method
   (b) Kirby-Bauer disc diffusion method
2. Write briefly about:
   (a) Minimum inhibitory concentration (MIC)
   (b) Minimum bactericidal concentration (MBC)

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Which medium is the most ideal for antibiotic sensitivity testing of bacterial isolates?
   (a) Mueller-Hinton agar
   (b) Nutrient agar
   (c) Blood agar
   (d) MacConkey agar
2. In Stokes disc diffusion method, if the size of zone of inhibition of test bacterium is equal to, larger than or not more than 3 mm smaller than the control strain zone, bacterial strain is considered as :
   (a) Sensitive
   (b) Intermediate sensitive
   (c) Resistant
   (d) None of the above
3. In Stokes disc diffusion method, if the zone size of inhibition of test bacterium is smaller than 2 mm, bacterial strain is considered as :
   (a) Sensitive
   (b) Intermediate sensitive
   (c) Resistant
   (d) None of the above

4. In which method of antibiotic sensitivity testing both control and test strains are inoculated on the same culture plate?
   (a) Stokes disc diffusion method (b) Kirby-Bauer disc diffusion method
   (c) Epsilometer test (d) None of the above
5. In which method of antibiotic sensitivity testing; control strain is not inoculated on the same culture plate on which test strain is inoculated?
   (a) Stokes disc diffusion method (b) Kirby-Bauer disc diffusion method
   (c) Both of the above (d) None of the above
6. In which method, an absorbent strip with a known gradient of antibiotic concentration along its length is used for antibiotic sensitivity testing?
   (a) Kirby-Bauer disc diffusion method (b) Epsilometer test
   (c) Primary disc diffusion test (d) Agar dilution method
7. Which is the method used for determining minimum inhibitory concentration of an antibiotic?
   (a) Kirby-Bauer disc diffusion method (b) Primary disc diffusion test
   (c) Broth dilution method (d) None of the above
8. Minimum inhibitory concentration of an antibiotic can be detected by:
   (a) Broth dilution method (b) Epsilometer test
   (c) Both of the above (d) None of the above

Answers (MCQs):

1. (c) 2. (a) 3. (c) 4. (a) 5. (b)
6. (b) 7. (c) 8. (c)

Chapter 79

MOLECULAR DETECTION OF MICROORGANISMS

I. INTRODUCTION

Molecular microbiology has emerged as the leading area in clinical microbiology laboratory and created new opportunities for laboratory diagnosis to affect patient care. It also helps in disease prognosis and monitoring the response to treatment. However, in most cases the new molecular methods supplement rather than replace the conventional laboratory tests for diagnosis. Culture has long been the 'gold standard' for infectious disease diagnosis but for several diseases molecular methods have replaced the culture as the 'gold standard'. Hepatitis C, enteroviral meningitis, herpes simplex virus (HSV) encephalitis and genital infections with *C. trachomatis* are some examples for which nucleic acid-based tests are the new gold standards. Molecular methods have been found to be advantageous in situations in which conventional methods are slow, insensitive, expensive or not available.

II. MOLECULAR METHODS

Molecular methods are mainly of two major types, amplified methods and non-amplified methods as follows:

Amplified methods

1. Polymerase chain reaction (PCR)
2. Transcription mediated amplification (TMA)
3. Nucleic acid sequence based amplification (NASBA)
4. Ligase chain reaction (LCR)

Non-amplified method

Nucleic acid probes

A. Amplified Methods

Amplification of nucleic acid is the basis of all the amplified methods. Amplified nucleic acid may be either DNA or RNA.

1. Polymerase Chain Reaction (PCR)

The basic principle of Polymerase chain reaction (PCR) has been described in Chapter 9. It is the target amplification system. PCR permits the synthesis of essentially limitless quantities of a target nucleic acid sequence. All the target amplification techniques are sensitive to contamination and can lead to false positive reactions. This drawback must be taken care of before any of these techniques are used in the clinical laboratory. However, false positive reactions can be reduced by special laboratory design practices. Other target amplification techniques include TMA and NASBA.

Besides originally described PCR, other types of PCR include reverse-transcriptase PCR (RT-PCR), nested PCR, multiplex PCR and real-time PCR

(i) RT-PCR

As originally described, PCR was a technique for DNA amplification. Reverse-transcriptase PCR (RT-PCR) was developed to amplify RNA. In this technique target is

RNA instead of DNA. In this process complementary DNA (cDNA) is first produced from RNA with the help of enzyme reverse transcriptase and then cDNA is amplified by PCR.

(ii) Nested PCR

Nested PCR was developed to increase the sensitivity and specificity of PCR technique. It uses two pairs of amplification primers. One primer pair is used in the first round of PCR to amplify the desired sequence. The amplified product of the first round is then subjected to second round of PCR with the second set of primers which anneal to the sequences found only in the first round products.

(iii) Multiplex PCR

Two or more primer sets designed for amplification of different targets are included in this technique. This will help in amplification of more than one target sequence in a clinical specimen. Mutliplex PCRs are usually less sensitive than PCRs with single set of primers.

PCR has been applied in clinical laboratory for diagnosis of various infectious agents (Table 79.1).

Table 79.1 PCR in Diagnosis of Infectious Agents

Bacteria
M. tuberculosis, Legionella pneumophila, Helicobacter pylori, Chlamydia trachomatis, Mycoplasma pneumoniae
Viruses
Cytomegalovirus, Herpes simplex virus, Hepatitis B virus, Hepatitis C virus, Coxsackie virus, Measles virus, Human immunodeficiency virus (HIV-1 and HIV-2), Human papilloma virus, Rotavirus, Rubella virus, Human herpes virus-6 (HHV-6), rhinovirus, parvovirus, adenovirus.
Fungi
Candida albicans, Cryptococcus neoformans, Pneumocystis jiroveci.
Protozoa
Toxoplasma gondii, Trypanosoma cruzi, Plasmodium sp.

(iv) Real Time PCR

Real-time PCR is a major breakthrough for detection of PCR products. Other names for real-time PCR include *kinetic PCR* and *homogeneous PCR*. In real-time PCR, amplified products (amplicons) are detected as they accumulate after each cycle, in contrast to standard PCR where amplicons are detected at the end of the procedure. Thus, a positive result can be obtained quickly, often while the assay is still running. This technique does not use an agarose gel for detecting amplicons. The imaging system for detection of amplicons is a part of the real time instrument. Another major benefit is that the reactions occur in closed tubes which do not have to be opened for detection. Hence there is very small chance that amplicon from a real-time PCR will contaminate equipment, reagents, and work spaces. Real-time PCR can also be used to quantitate nucleic acids, which is useful for monitoring the certain diseases, such as HIV and hepatitis. Real-time PCR uses a fluorescent reporter dye. Real-time PCR instrument has the ability to measure the increase in reporter fluorescence as PCR product accumulates. Fluorescent peaks are recorded by the computer system as fluorescence intensity versus PCR cycle number.

2. Transcription Mediated Amplification (TMA)

Transcription mediated amplification (TMA) is an isothermal RNA amplification method. RNA target is reverse transcribed into cDNA and then RNA copies are synthesised with the help of RNA polymerase. A 10^9 fold amplification of the target RNA can be achieved in about 2 hours.

Advantages of TMA include no requirement for a thermal cycler, and contamination risk is minimised. TMA-based assays are available for detection of *M. tuberculosis, C. trachomatis, N. gonorrhoeae,* HCV and HIV-1.

3. Nucleic Acid Sequence-Based Amplification (NASBA)

Like TMA, it is also an isothermal RNA amplification method. The method is similar to TMA, RNA target is reverse transcribed into cDNA and then RNA copies are synthesised with the help of RNA polymerase. It also does not require thermal cycler. AMVRT, RNaseH and T7 bacteriophage RNA polymerase are used in NASBA, while TMA uses an RT enzyme with endogenous RNaseH activity and T7 RNA polymerase. NASBA based kits for detection and quantitation of HIV-1 RNA and CMV RNA are available.

4. Ligase Chain Reaction (LCR)

Ligase chain reaction (LCR) is a probe amplification method in contrast to above mentioned methods which are target amplification methods. Probe amplification method differs from target amplification method in that amplified products contain only a sequence present in the initial probes.

In LCR, two oligonucleotide probes hybridise adjacent to one another on each of the denatured target DNA strands in such a way that a 'nick' is formed. Enzyme DNA ligase then seals the nick by joining the 3' end

of one probe and the 5' end of the other. Each ligated product, as well as the original target, serve as a template in subsequent rounds of denaturation, annealing and ligation. It results in an exponential increase of ligated products. Like PCR, LCR also requires thermal cycler. LCR based amplification has been used for *M. tuberculosis, N. gonorrhoeae* and *C. trachomatis*.

B. Non-Amplified Method

Nucleic Acid Probes

Nucleic acid probes are non-amplified methods. Principle of nucleic acid probes is described in Chapter 9 under heading 'DNA probes'. Nucleic acid probes are segments of DNA or RNA labelled with radioisotopes or enzymes that can hybridise to complementary nucleic acid with high degree of specificity. A number of DNA probes have been developed for direct detection of microorganisms in clinical specimens and for identification of organisms after isolation of culture. Applications of DNA probe technology in microbiology are shown in Table 79.2.

Table 79.2 Applications of DNA probe technology

- In clinical microbiology:
  - Direct detection of microbes in specimens.
  - To detect microbes which are either difficult or impossible to culture.
- Identification of culture isolates
- Strain identification
- To identify toxins, virulence factors
- Identification of resistant markers

Nucleic acid probes for direct detection of group A streptococci, *Chlamydia trachomatis* and *Neisseria gonorrhoeae* are available. Probes for identification of group A streptococci, group B streptococci, enterococci, *Haemophilus influenzae,* mycobacteria, *N. gonorrhoeae, Staphylococcus aureus, Streptococcus pneumoniae, Campylobacter sp., Histoplasma capsulatum, Blastomyces dermatidis* and *Coccidioides immitis* isolated in culture are also available. DNA probes for detection of LT and ST toxins of *Esch. coli* are also available.

III. SIGNIFICANCE IN CLINICAL LABORATORY

Molecular methods have a significant role in the following situations in clinical microbiology laboratory.

1. Detection of uncultivable and slow growing microorganisms.
2. Role in clinical virology
3. Disease prognosis
4. Response to treatment

1. Detection of Uncultivable and Slow Growing Microorganisms

The greatest advantage of molecular methods has been in the discovery of previously unrecognised or uncultivable organisms. A number of microorganisms have been first identified directly from clinical specimens by molecular methods. HCV, *Bartonella henselae,* Sin nobre virus and Human herpes virus 8 (HHV-8) are some examples of human pathogens first identified from clinical specimens using molecular methods.

These molecular methods are also useful for fastidious microorganisms which may die in transit or may be overgrown by contaminants when cultured. *N. gonorrhoeae* is one such example whose nucleic acid can be detected under circumstances in which it cannot be cultured. The use of improper collection, inappropriate transport conditions or delay in transport can reduce the viability of the organism but do not affect the nucleic acid detection.

2. Role in Clinical Virology

Molecular methods to replace culture for detection of bacteria in routine practice are limited because of need to isolate the organisms for antibiotic sensitivity testing. These methods can actually replace the culture only in those microorganisms which have predictable antibiotic susceptibility, and consequently, routine susceptibility testing is not performed.

Culture-based methods in virology are costly and antiviral susceptibility testing is not routinely done. Molecular approaches are often faster, more sensitive, and more cost-effective than the conventional approaches in clinical virology. Enteroviral meningitis, HSV encephalitis and CMV infections in immunocompromised patients are examples for which nucleic acid based tests are relevant and cost-effective for diagnosis.

3. Disease Prognosis

Molecular methods provide important information which may predict disease progression. HIV-1 viral load as a predictor of progression to AIDS is probably the best example.

Subtyping of certain viruses by molecular methods may also provide information about the severity of infection. HPV causes dysplasia, neoplasia and carcinoma of cervix in women. HPV types 16 and 18 are associated with a high risk of progression to neoplasia, whereas HPV types 6 and 11 have a low risk.

4. Response to Treatment

Molecular methods have been developed to detect the genes responsible for drug resistance. These methods

have been used to supplement conventional antimicrobial susceptibility testing for the detection of methicillin resistance in staphylococci, vancomycin resistance in enterococci and rifampicin resistance in *Mycobacterium tuberculosis*.

Molecular techniques have a significant role in predicting and monitoring patient responses to antiviral therapy. HIV-1 viral load assays have been developed to monitor the response of antiretroviral therapy. Viral load assays have also been used in monitoring the response to therapy in patients who are chronically infected with HBV and HCV.

Drug resistance mutations in RT and protease genes of HIV-1 can be detected by molecular methods. These mutations lead to lower levels of sensitivity to antiretroviral drugs and are important causes of treatment failure. This helps to determine an appropriate treatment in patients who do not respond to therapy.

KEY POINTS

1. Molecular methods are mainly of two major types, *amplified methods* and *non-amplified methods*.
2. *Polymerase chain reaction* (*PCR*) is an example of amplified methods.
3. *Nucleic acid probes* are non-amplified methods.
4. Molecular methods have a significant role in detection of *uncultivable* and *slow* growing microorganisms.

YOU MUST KNOW

1. Polymerase chain reaction (PCR).
2. PCR in diagnosis of infectious agents.
3. Nucleic acid probes.

STUDY QUESTIONS

Q. Write short notes on:
(a) Detection of microorganisms by molecular methods
(b) Nucleic acid probes
(c) Significance of molecular methods in clinical microbiology
(d) RT-PCR

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Which of the following molecular methods is/are amplified methods for detection of microorganisms?
   (a) Polymerase chain reaction (PCR)
   (b) Ligase chain reaction (LCR)
   (c) RT-PCR
   (d) All of the above
2. What is amplified by reverse-transcriptase PCR?
   (a) DNA (b) RNA (c) Protein (d) None of the above
3. How many pairs of amplification primers are used in nested PCR?
   (a) One (b) Two (c) Three (d) Five
4. Which of the following is/are isothermal RNA amplification method/s?
   (a) Transcription mediated amplification
   (b) Nucleic acid sequence based amplification
   (c) Both of the above
   (d) None of the above
5. Polymerase chain reaction can be used for :
   (a) *Legionella pneumophila*
   (b) HIV
   (c) *Mycoplasma pneumoniae*
   (d) All of the above
6. DNA probes can be applied for :
   (a) Detection of microbes in specimens
   (b) Identification of culture isolates
   (c) Identification of resistant markers
   (d) All of the above

Answers (MCQs):

1. (d) 2. (b) 3. (b) 4. (c) 5. (d) 6. (d)

Chapter 80

BACTERIOLOGY OF WATER, MILK AND AIR

I. BACTERIOLOGY OF WATER

Water for human consumption, must be free from chemical substances and pathogenic microorganisms. Drinking water should not only be safe but also pleasant to drink i.e. clear, colourless and devoid of disagreeable taste or smell.

Drinking water is liable to be contaminated with sewage or other excreted material which may cause intestinal or other systemic infections. The hazards of water pollution are classified into two broad groups i.e. *biological* and *chemical*. Biological hazards are due to infective agents which may lead to water-borne diseases. Chemical pollutants include poisonous chemical substances, industrial or agricultural wastes.

Faecal pollution of water supplies may lead to various water-borne diseases. The object of microbiological study of water supplies is to detect whether pollution of the water by pathogenic organisms has occurred or not. Though it would be ideal to demonstrate the possible pathogens in water, but it is not practicable as they are usually few and far outnumbered by non-pathogenic organisms. Hence, we rely on tests that detect indicators of human/animal faecal pollution i.e. intestinal organisms. The organisms most commonly used as indicators of pollution are *Esch. coli* and the coliform group. In certain conditions, *Streptococcus faecalis* and *Clostridium perfringens* are also searched for.

A. Bacterial Flora in Water

The bacteria found in water can be divided into three groups:

1. Natural water bacteria

The organisms that are commonly found in water free from gross pollution are included in this group.

2. Soil bacteria

These organisms are frequently washed into the water during heavy rains. These are not normal inhabitants of water.

3. Sewage bacteria

Many of the organisms included in this group are normal inhabitants of the intestine of man and animals. Others live mainly on decomposing organic matter.

Bacterial flora in water is shown in Table 80.1.

Table 80.1 Bacterial Flora in Water

1. Natural water bacteria	*Micrococcus, Serratia, Flavobacterium, Pseudomonas, Alcaligenes* and *Acinetobacter*
2. Soil bacteria (washed into water)	*Enterobacter aerogenes, E. cloacae* and *Bacillus subtilis*
3. Sewage bacteria	
(*a*) Intestinal bacteria (through sewage)	*Escherichia coli, Enterococcus faecalis, Clostridium perfringens, S. typhi* and *V. cholerae*
(*b*) Sewage bacteria proper	*Proteus* and *Clostridium sporogenes*

B. Factors Determining the Number of Bacteria in Water

1. *Type of water:* Surface water is more likely to be contaminated. Deep waters are generally pure.
2. *Temperature:* When organic matter content is high, rise in temperature leads to multiplication of bacteria. Low temperature favours the survival of bacteria.
3. *Organic matter:* When organic matter is plentiful, organisms are abundant.
4. *Light:* Sunrays can be bactericidal, provided the water is clear and static. These can penetrate only to a depth of five feet.
5. *Acidity:* Acidity of water has a bactericidal action and plays a considerable part in the purification of some waters.
6. *Salinity:* A high salinity has a deleterious effect on most bacteria, however halophilic bacteria can survive in saline water.
7. *Protozoal content:* Protozoa, by ingesting and destroying bacteria, play a part in bringing down the bacterial number.
8. *Storage:* Storage of water decreases bacterial count due to sedimentation and devitalisation.

C. Water-Borne Pathogens

Pollution of drinking water may introduce a variety of pathogens (Table 80.2).

Table 80.2 Water-Borne Pathogens

Bacterial	*Vibrio cholerae, Escherichia coli, Salmonella typhi, S. paratyphi A, B and C, Shigella spp., Yersinia enterocolitica, Campylobacter jejuni and C. coli.*
Viral	Hepatitis A virus, hepatitis E virus, poliomyelitis virus, rotavirus
Protozoal	*Entamoeba histolytica, Giardia lamblia, Balantidium coli, Cryptosporidium, Isospora*
Helminthic	*Ascaris lumbricoides, Enterobius vermicularis, Trichuris trichura*
Pathogens transmitted through aquatic hosts	
(a) Cyclops	*Dracunculus medinensis and Diphyllobothrium latum*
(b) Snail	Schistosomes

D. Bacteriological Examination

1. Collection of Water Samples

Water samples are to be collected in heat sterilised glass bottles of 230 ml with ground glass stoppers protected by Kraft paper. To neutralise the bactericidal effect of chlorine in water, a crystal of sodium thiosulphate is introduced into the bottle prior to sterilisation.

(i) Sampling from a tap or pump outlet

Clean the tap or pump outlet from outside. A tap is turned at maximum flow rate and let the water flow for 5 minutes. Open the stopper, fill the bottle and replace the stopper.

(ii) Sampling of water from a reservoir (streams, rivers, lakes and tanks)

The stopper is removed and the bottle is submerged to a depth of about 20 cm with mouth facing upwards. If there is a current, the bottle should face the water current.

(iii) Sampling from a dug well

A stone is tied with the sampling bottle. Then a clean cord of suitable length is tied with the bottle and lowered to the required depth. When the bottle is filled, pull it out and stopper it. The bottle should not touch the sides of the well at any time.

2. Transport

Water bottles should be wrapped in a Kraft paper. The water samples should be properly labelled with details of the source, time and date of collection. These should be delivered to the laboratory as quickly as possible. Where delay is anticipated, the bottles should be kept on ice, preferably in an ice box and protected from light. These samples should be processed immediately.

3. Methods of Analysis

The standard tests usually employed for water bacteriology are:

(*i*) Presumptive coliform count
(*ii*) Differential coliform count
(*iii*) Detection of faecal streptococci and *Clostridium perfringens*
(*iv*) Membrane filtration test

(i) Presumptive coliform count

Multiple tube method is generally used for estimation of probable number of coliform bacilli in water. By this method the most probable number (MPN) of coliform organisms are detected in 100 ml water.

(a) Media

Double strength and single strength MacConkey broths in bottles or tubes containing Durham's tube for indication of gas production are used. These media contain bromocresol blue as indicator.

(b) Procedure

Measured amounts of water samples are added by sterile graduated pipettes as under:

- 50 ml of water to one bottle of 50 ml double strength medium.
- 10 ml of water each to five tubes of 10 ml double strength medium.
- 1 ml of water each to five tubes of 5 ml single strength medium.
- 0.1 ml of water each of five tubes of 5 ml single strength medium.

The inoculated tubes/bottles are incubated at 37°C for 48 hours. An estimate of coliform count per 100 ml is made from the tubes/.bottles showing acid and gas production using the probability table (See Appendix). The presumptive coliform count of 0, 1-3, 4-10 and >10 per 100 ml is interpreted as excellent, satisfactory, suspicious and unsatisfactory respectively.

(ii) Differential coliform count

Eijkman test

Some spore-bearing organisms give false positive reactions in the presumptive coliform test. The Eijkman test is done to confirm that the coliform bacilli detected in the presumptive test are *Esch. coli*. After the usual presumptive test, subcultures are made from all the positive tubes/bottles to fresh tubes of single strength MacConkey broth with Durham's tube. These are incubated at 44°C in water bath and examined after 24 hours. Those tubes showing gas in Durham's tube contain *Esch. coli*. Confirmation of *Esch. coli* is made by plating on solid media and indole production.

(iii) Detection of faecal streptococci and Clostridium perfringens

Their presence in water provides useful confirmatory evidence of the faecal pollution in doubtful cases.

(a) Faecal streptococci

Subcultures are made from positive tubes/bottles in presumptive coliform test into tubes containing 5 ml of glucose azide broth and incubated at 45°C for 18 hours. Presence of acid in the medium indicates the presence of *Enterococcus faecalis*. Further confirmation can be done by plating onto MacConkey agar medium.

(b) Clostridium perfringens

The water sample is inoculated in litmus milk medium and incubated anaerobically at 37°C for 5 days. A typical stormy clot reaction with acidity confirms the presence of *Cl. perfringens*.

(iv) Membrane filtration method

A measured volume of water is filtered through a membrane which retains the bacteria on its surface. The membrane is then placed on a suitable medium and incubated, so that bacteria grows into colonies on its upper surface. The number of colonies is counted and the bacterial content of the water is calculated.

4. Examination for Specific Pathogens

Specific pathogens may be isolated from water by employing enrichment and selective media. For isolation of *S. typhi*, equal volume of water is added to the double strength selenite broth followed by incubation and then subcultured on selective medium. For *V. cholerae*, alkaline peptone water is mixed with nine times its volume of water, incubated and subcultured on selective medium. Isolated organisms are identified by biochemical tests and serotyping.

Pathogenic organisms may also be isolated by 'membrane filtration method' described earlier.

II. BACTERIOLOGY OF MILK

Milk contains some bacteria derived from various sources; including the commensal and pathogenic flora of the udder, teat canals and skin, milker's hands or milking equipment and water used for cleaning the udders. Bacterial spores and some preformed bacterial or fungal toxins are not destroyed by pasteurisation temperatures.

A. Milk-Borne Diseases

Milk is a good medium for bacteria and a good vehicle for many pathogens. Milk-borne diseases (Table 80.3) can be of three types:

1. Infections primarily of animals, which can be transmitted to humans.
2. Milk contaminated by excreta of animals and other sources.
3. Infections primarily of humans but transmitted through milk.

Table 80.3 Milk-Borne Diseases

Source	Diseases and organisms
Infection primarily of animals	Tuberculosis, brucellosis, salmonellosis fever, streptococcal infections, staphylococcal infections, cow-pox, anthrax, leptospirosis, *Campylobacter fetus, Y. enterocolitica*
Contamination of milk by ticks and rats	Tick-borne encephalitis, *Streptobacillus moniliformis* (rat-borne)
Infections primarily of humans	Enteric fever, cholera, shigellosis, staphylococcal food poisoning, streptococcal infections, tuberculosis, enteropathogenic *Esch. coli, hepatitis*

B. Bacteriological Examination

In can be carried out by following methods:

1. Viable count
2. Coliform count
3. Chemical tests
   (*i*) Methylene blue reduction test
   (*ii*) Phosphatase test
   (*iii*) Turbidity test
4. Detection of specific pathogens

1. Viable Count

This is done by plate dilution method. Serial dilutions of milk sample are incorporated in yeast extract milk agar and then incubated at 30-31°C for 72 hours. Number of colonies multiplied by dilution factor gives the colony count in the fixed amount of milk.

2. Coliform Count

Varying dilutions of milk are inoculated into three tubes of MacConkey's broth with inverted Durham's tube and incubated at 37°C for 48 hours. The production of acid and gas is the evidence of presence of coliform bacilli. All coliforms are killed by pasteurisation. The presence of coliform bacilli in pasteurised milk indicates improper pasteurisation or post-pasteurisation contamination.

3. Chemical Tests

(i) Methylene blue reduction test

This test is an economical substitute for viable count. Viable bacteria reduce the methylene blue in milk when kept in a dark place. The test is performed by mixing 1 ml of methylene blue solution to 10 ml of milk in a test tube. The tube is incubated in dark at 37°C. The milk is considered satisfactory if it fails to decolourise methylene blue in 30 minutes.

(ii) Phosphatase test

The enzyme phosphatase is normally present in milk and is inactivated during pasteurisation. The test depends upon the ability of this enzyme to breakdown disodium p-nitrophenyl phosphate and liberate p-nitrophenyl phosphate.

One ml of milk is added to 5 ml buffer-substrate solution (buffer + disodium p-nitrophenyl phosphate) in a test tube and incubated in water bath at 37°C for 2 hours. A yellow colour is produced if milk contains phosphatase. The colour is quantitated by a colorimeter.

(iii) Turbidity test

This test is used to check the sterilisation of milk. When milk is heated to at least 100°C for 5 minutes, all heat coagulable proteins are precipitated. If ammonium sulphate is then added to this milk, no turbidity results. Absence of turbidity indicates that the milk has been boiled or heated to at least 100°C for five minutes.

4. Detection of Specific Pathogens

(i) Tubercle bacilli

The milk is centrifuged at 3,000 rpm for 30 minutes. The part of deposit is inoculated on Lowenstein-Jensen (LJ) medium and other part into guinea pigs. If the milk contains tubercle bacilli, growth of these organisms can be seen. The animals are observed for a period of three months for tuberculosis.

(ii) Brucella

Isolation of *Brucella* is attempted by inoculating cream from the milk sample on serum dextrose agar. It may also be injected into guinea pigs. The animals are killed after six weeks and the serum is examined for agglutinins and the spleen used for culture of brucellae.

Diagnosis of brucellosis in animals can be made by demonstrating antibodies in milk by the *milk ring test* and the *Whey agglutination test* (refer to Chapter 41 on Brucella).

III. BACTERIOLOGY OF AIR

Since a man respires about 15 cubic meters of air in a day, therefore, the bacterial content of air is important particularly so when the air contains pathogenic organisms. Man is an important source of spreading bacteria in the environment during coughing and sneezing. The bacterial content in air depends on increased density of human and animal population, nature of soil, amount of vegetation and atmospheric conditions.

A. Bacteriological Examination

Bacteriological examination of air is necessary for surgical operation theatres, hospital wards, store house of food and pharmacy. There are two types of method for bacteriological examination of air:

1. Settle plate method
2. Slit sampler method

1. Settle Plate Method

Petri dishes containing nutrient agar and blood agar are left open for half to one hour. Large bacteria carrying dust particles settle onto the medium. The plates are incubated at 37°C for 24 hours. Colonies are then counted. For pathogenic staphylococci and streptococci, blood agar plates are used. This method is used for testing air in surgical operation theatres and hospital wards.

2. Slit Sampler Method

The number of bacteria in a measured volume of air is determined by slit sampler method. One cubic foot volume of air is directed onto a plate containing culture medium through a slit of 0.25 mm wide. The plate is rotated mechanically so as to allow the organisms to spread out evenly on the medium. The culture media are incubated and the colonies are counted. The number of colonies gives the number of bacteria present in the air.

B. Acceptable Limit of Air Pollution

Bacterial count should not exceed the upper limit of 50 per cubic foot in factories, offices and homes; 10 per cubic foot in operation theatre and 1 per cubic foot in operation theatre for neurosurgery.

KEY POINTS

1. The standard tests usually employed for water bacteriology include *presumptive coliform count*, *differential coliform count*, *detection of faecal streptococci* and *membrane filtration test*.
2. *Multiple tube method* is generally used for presumptive coliform count.
3. The *Eijkman test* is done to confirm that the coliform bacilli detected in the presumptive test are *Esch. coli*.
4. Bacteriological examination of milk can be carried out by *viable count*, *coliform count*, *methylene blue reduction test*, *phosphatase test* and *turbidity test*.
5. Bacteriological examination of air is done by *settle plate method* and *slit sampler method*.

YOU MUST KNOW

1. The tests usually employed for water bacteriology.
2. The methods used for bacteriological examination of milk.
3. Settle plate method and slit sampler method used for bacteriology of air.

STUDY QUESTIONS

1. Name various water-borne pathogens. Discuss the bacteriological examination of water.
2. Write short notes on:
   (a) Bacterial flora in water (b) Bacteriology of milk (c) Bacteriology of air

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Which of the following is/are water-borne pathogen/s?
   (a) Hepatitis E virus (b) *Salmonella typhi*
   (c) *Entamoeba histolytica* (d) All of the above
2. Which of the following parasites can be water-borne pathogens?
   (a) *Cryptosporidum* (b) *Isospora*
   (c) *Giardia lamblia* (d) All of the above

3. *Diphyllobothrium latum* is transmitted through :
   (a) Cyclops (b) Snail
   (c) Both of the above (d) None of the above
4. Schistomes are transmitted through :
   (a) Cyclops (b) Snail
   (c) Both of the above (d) None of the above
5. Which of the following bacteria can act as indicator/s of faecal pollution of drinking water?
   (a) *Escherichia coli* (b) *Bacillus subtilis*
   (c) Diphtheroids (d) All of the above
6. Drinking water is considered satisfactory if the presumptiv coliform count of water is :
   (a) 0/100 mL (b) 1-3/100 mL
   (c) 4-10/100 mL (d) > 10/100 mL
7. Which test is done to confirm that the coliform bacilli detected in the presumptive test of water are *Escherichia coli*?
   (a) Eijkman test (b) Membrane filtration method
   (c) Turbidity test (d) Phosphatase test
8. In Eijkman test, MacConkey broth tubes are incubated at :
   (a) 25 °C (b) 37 °C
   (c) 44 °C (d) 52 °C
9. Which of the following bacteria can act as indicator for faecal pollution of water?
   (a) Coliforms (b) *Enterococcus faecalis*
   (c) *Clostridium perfringens* (d) All of the above
10. Which of the following can be milk-borne diseases in humans?
    (a) Enteric fever (b) Cholera
    (c) Staphylococcal food poisoning (d) All of the above
11. Which of the following chemical tests can be used for bacteriological examination of milk?
    (a) Methylene blue reduction test (b) Phosphatase test
    (c) Turbidity test (d) All of the above
12. Which of the following tests can be used for diagnosis of brucellosis in animals?
    (a) Milk ring test (b) Whey agglutination test
    (c) Both of the above (d) None of the above
13. What is the acceptable limit of bacterial count in air in operation theatre?
    (a) 50 per cubic feet (b) 10 per cubic feet
    (c) 4 per cubic feet (d) 1 per cubic feet
14. What is the acceptable limit of bacterial count in air in operation theatre for neurosurgery?
    (a) 50 per cubic feet (b) 10 per cubic feet
    (c) 4 per cubic feet (d) 1 per cubic feet

Answers (MCQs):

1. (d)	2. (d)	3. (a)	4. (b)	5. (a)
6. (b)	7. (a)	8. (c)	9. (d)	10. (d)
11. (d)	12. (c)	13. (b)	14. (d)	

Chapter 81

HAND HYGIENE

I. INTRODUCTION

Hands have been known to be involved in many infectious disease outbreaks especially in nurseries, neonatal units, intensive care units and other hospital settings. This happens because hands of medical personnel may be transiently infected with pathogenic organisms from infected patients or environment, and thus these can be transmitted to other patients. Therefore, hand hygiene is very important in keeping the health care associated infections at minimum.

II. TYPES OF HAND HYGIENE TECHNIQUES

1. Routine hand washing
2. Hygienic hand care
3. Surgical hand washing or hand scrub

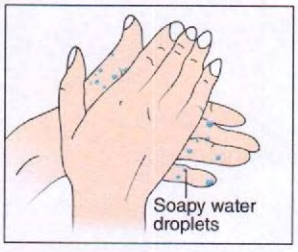
1. Routine Hand Washing

It involves washing of hands with soap and water. It removes most transient microorganisms from the hands. Routine hand washing should be performed in the following conditions.

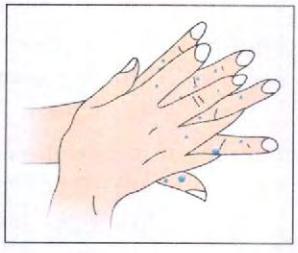
(a) Before and after contact with patients,
(b) Before wearing and after removing gloves,
(c) After wound dressing,
(d) After contact with body fluids of patients,
(e) After handling devices for patient care or having contact with inanimate objects in and around patient.

2. Hygienic Hand Care

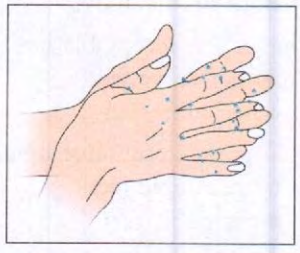
- Wash hands with soap and water by rubbing hands in the six movements by covering all surfaces of hands as shown in Fig. 81.1.



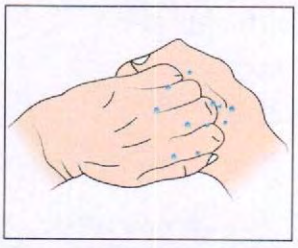
Step 1: Wet hands and apply soap. Rub palms together until soap is bubbly.



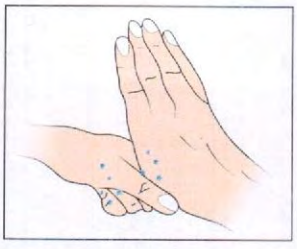
Step 2: Rub each palm over the back of the other palm.



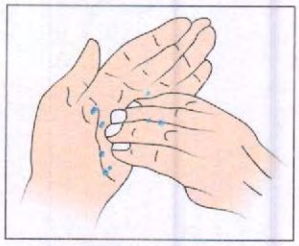
Step 3: Rub between your fingers on both hands.



Step 4: Rub both hands with your fingers.



Step 5: Rub around both your thumbs.



Step 6: Rub in circles on both your palms and then rinse and dry your hands.

Fig. 81.1 Hygiene hand care–standard method

- Rinse hands with water.
- Dry hands with single-use towel.

Alcohol based method

- When alcohol-based hand rub solution is used instead of soap, dry hands are required. Rest of the procedure is same as above with the difference that no towel is required for drying as alcohol evaporates in the environment.

3. Surgical Hand Washing or Hand Scrub

- It requires more time for washing than hygiene hand care.
- Besides hands, wrists and forearms are also included for washing.
- For drying, sterile towels are used.

KEY POINTS

1. Hands have been known to be involved in many infections disease outbreaks.
2. Hand hygiene is very important in keeping the health care associated infections at minimum.
3. There are three types of hand hygiene techniques namely routine hand washing, hygienic hand care, surgical hand washing or hand scrub.
4. Six steps are involved in standard method of hygiene hand care.
5. When alcohol-based hand rub solution is used instead of soap, dry hands are required. Rest of the procedure is same except that no towel is required for drying as alcohol evaporates in the environment.

STUDY QUESTION

1. Write briefly above hand hygiene.

Chapter 82

BIOMEDICAL WASTE MANAGEMENT

I. INTRODUCTION

Hospital waste is defined as all waste generated from medical facilities including office and kitchen wastes. Only a small fraction of the total waste generated by health care institutions is actually infectious. In contrast to radioactive or chemical wastes, infectious waste cannot be identified objectively. It can be defined as the waste that is capable of causing an infectious disease. All generators of infectious waste must have proper waste management programme for its disposal. Hospital waste management is of utmost importance for the safety of laboratory personnel as well as for the community. Biomedical waste is a broader term which includes waste generated in the diagnosis, treatment and immunisation of humans or animals or in research activities or in the production of biologicals.

Concerns about transmission of the hepatitis B virus (HBV) and human immunodeficiency virus (HIV) led to the introduction of 'universal precautions', to minimise the infections in medical laboratory workers and health care personnel. These universal precautions include:

1. Assume that all specimens/patients are potentially infectious for HIV and other blood borne pathogens.
2. All blood specimens or body fluids should be placed in a leak-proof impervious bags for transportation to the laboratory.
3. Use gloves while handling blood and body fluid specimens and other objects exposed to them. If there is a likelihood of spattering, use face masks with glasses or goggles.
4. Wear laboratory coats or gowns while working in the laboratory. Wrap-around gowns should be preferred. These should not be taken outside.
5. Never pipette by mouth. Mechanical pipetting devices should be used.
6. Decontaminate the laboratory work surfaces with an appropriate disinfectant after the spillage of blood or other body fluids and when the procedures are completed.
7. Limit use of needles and syringes to situations for which there are no other alternatives.
8. Biological safety hoods should be used for laboratory work.
9. All the potentially contaminated materials of the laboratory should be decontaminated before disposal or reprocessing.
10. Always wash hands after completing laboratory work and remove all protective clothings before leaving the laboratory.

Most common agents which are associated with laboratory acquired infections include hepatitis B virus, *Coccidioides immitis, Bacillus anthracis,* Brucella species, *Mycobacterium tuberculosis, Francisella tularensis* and shigella species.

II. CATEGORIES OF BIOMEDICAL WASTE

The new Biomedical waste rules 2011 has been notified to replace the earlier rules (1998) and the amendments thereof. According to new rules, eight categories of biomedical waste have been described instead of ten categories under the earlier rules (1998).

Category No. 1
Human anatomical waste
Category No. 2
Animal Waste
Category No. 3
Microbiology & biotechnology waste and other laboratory waste
Category No. 4
Waste Sharps
Category No. 5
Discarded medicines and cytotoxic drugs
Category No. 6
Soiled waste
Category No. 7
Infectious solid waste
Category No. 8
Chemical waste

III. SEGREGATION OF WASTE

Prior to treatment and disposal of waste, it is important to segregate the infectious waste from non-infectious waste and to collect these in appropriate receptacles. The wastes are segregated preferably at the point of generation. This is the most important step to safeguard the occupational health of health care personnel.

Infectious waste includes microbiological wastes (cultures etc.), blood and body fluids, contaminated laboratory wastes, used sharps, pathological wastes (samples, tissues etc.), bedding and other wastes like bandages and used cotton swabs; and animal carcasses used in laboratory experiments. Besides infectious waste, chemical waste is also a hazardous waste.

Covered containers should be used for waste segregation. Black plastic bags are used for general or noninfectious waste while yellow plastic bags for infectious substances. For used sharps (needles, syringes, lancets and other invasive tools), use blue or white coloured rigid, puncture proof containers. Proper segregation will minimise the waste stream needing special treatment i.e. infectious waste. This practice also helps in safe handling and transportation of waste. Segregation of waste in bags of different colours is shown in Table 82.1.

IV. WASTE TREATMENT AND DISPOSAL

A. Waste Treatment

Following techniques are in use for treatment of infected material.

1. Double chambered incineration
2. Autoclaving
3. Microwaving
4. Hydroclaving
5. Plasma torch
6. Chemical treatment

1. Double Chambered Incineration

Incineration burns waste at very high temperatures and thus reduces its volume. The double chambered incinerator contains two chambers. Waste is burnt in one chamber (primary chamber) at 800°C. Combustion of gases emitted from the first chamber, occurs in the second or secondary chamber. This chamber has a high temperature of 1000°C. The negative pressure is maintained inside the incinerator by the system, thereby forcing the end-gases out of the chimney.

The incinerator has an advantage of dealing with all pathological and cytotoxic wastes. Body parts, animal waste, microbiological waste and soiled dressings can be treated with this technique. The disadvantage of incinerator is that it generates highly toxic gases (e.g. dioxins and furans, if PVC plastics are present). It

Table 82.1 Colour coding of bags for segregation of biomedical waste

Colour	Type of container	Category of Waste	Waste treatment
Yellow	Non-chlorinated plastic bag	1, 2, 5, 6	Incineration
Red	Plastic bag or puncture proof container for sharps	3, 4, 7	Autoclaving/microwaving/chemical treatment
Blue	Non-chlorinated plastic bag/container	8	Chemical treatment
Black	Non-chlorinated plastic bag	Municipal waste	Disposal in Muncipal dump sites

adversely affects the health of the community. Another disadvantage is that recycling and reprocessing of materials cannot be done. Burning of plastic waste or sharps is also not recommended.

2. *Autoclaving*

Autoclave relies on the circulation of steam through the infectious waste to decontaminate it.

There are two kinds of autoclaves: the Gravity autoclave and the Prevacuum type. In the Prevacuum type, steam is created outside the chamber loaded with waste. Air in the chamber is then gradually removed and steam is injected in. This type of autoclave eliminate 'cold spots' and 'air pockets' (where the steam is unable to penetrate) by creating this vacuum. This ensures quicker heating. A temperature of 121°C and pressure of 15 pounds per square inch is used. For gravity autoclave, the waste material should be subjected to autoclave residence time of not less than 60 minutes, while in a vacuum autoclave time period should not be less than 45 minutes. Biological (Bacillus stearothermophilus spores) or chemical indicators (strips/tapes) should be used for validation test of autoclave.

Autoclaving is used for microbiological waste, blood and blood products, body fluids and used sharps. It is not recommended for pathological waste. Autoclaved material is typically land-filled, therefore, it has a large strain on land fill capacity.

3. *Microwaving*

Radiations produced by the microwave are involved to break apart molecular chemical bonds and thus disinfect infectious waste. The microwaves heat the waste to temperatures of 97° to 100°C. Cycle time is 40-45 minutes. It has advantage of disinfecting the waste and there are no hazardous emissions. However, it cannot be used to treat body parts and tissues.

4. *Hydroclaving*

The hydroclave is an expansion of the autoclave technology. Unlike in the autoclave, steam is introduced into the hollow walls of the hydroclave. The steam does not come in direct contact with the waste. Volume reduction of waste is much more than autoclave. Cycle time is one hour.

Hydroclave contains a series of large rotating rods, which spin slowly, putting the material into steam chamber as it is loaded. The rods continue to spin and rupture the waste bags and ensure complete exposure to the heat. After the sterilisation, the material is passed into a shredder. The waste can then be safely recycled or land filled. All items including pathological waste can be treated.

5. *Plasma Torch*

It has a very high temperature. It is very costly but safe. The advantage is that there is no need of segregation.

6. *Chemical Treatment*

Chemical treatment ensures disinfection. 1% hypochlorite solution or any other equivalent chemical reagent may be used.

B. Disposal

Land filling, deep burial and sewage are used for disposal. Infectious waste after treatment can be disposed of by land-filling or deep burial. Liquid waste can be disposed in sewage drains. Besides treatment, incineration is also a method of disposal.

Treatment methods used for different types of infectious wastes are shown in Table 82.2.

V. WASTE MANAGEMENT PROGRAMME

All laboratories should develop waste management programme according to the specific needs of the individual laboratory. The policies and procedures should

Table 82.2 Treatment Methods used for Infectious Waste

Type of infectious waste	Treatment method		
	Steam (Autoclave)	Incineration	Chemical
Microbiological waste	+	–	+
Human blood, blood products, body fluids	+	+	–
Pathological waste	–	+	–
Used sharps	+	–	+
Clean up of infectious waste spill	+	+	–
Animal carcasses	–	+	–

be incorporated in the laboratory's operating manuals. Emphasis should be on waste minimisation (by reducing waste, reuse and recycling), proper segregation, and health and safety of the workers. All personnel generating, collecting, transporting and storing infectious waste must be trained under the programme.

KEY POINTS

1. *Hospital waste* is defined as all waste generated from medical facilities including office and kitchen wastes. Only a small fraction of the total waste generated by health care institutions is actually *infectious*.
2. Concerns about transmission of the hepatitis B virus (HBV) and human immunodeficiency virus (HIV) led to the introduction of 'universal precautions', to minimise the infections in medical laboratory workers and health care personnel.
3. Prior to treatment and disposal of waste, it is important to *segregate* the infectious waste from non-infectious waste and to collect these in appropriate receptacles. The wastes are segregated preferably at the point of generation.
4. *Infectious waste* includes *microbiological wastes*, *blood* and *body fluids*, *contaminated laboratory wastes*, *used sharps*, *pathological wastes*, *bedding* and other wastes like *bandages* and *used cotton swabs*, and animal carcasses used in laboratory experiments.
5. Double chambered *incineration*, *autoclaving*, *microwaving*, *hydroclaving*, *plasma torch* and *chemical treatment* are the various techniques used in treatment of infectious waste.
6. Infectious waste after treatment can be *disposed of* by *land-filling* or *deep burial*.

YOU MUST KNOW

1. Universal precautions.
2. Segregation of waste and its importance.
3. List of treatment methods used for infectious waste.

STUDY QUESTIONS

1. Describe various techniques used for the treatment and disposal of biomedical waste.
2. Write short notes on:
   (a) Universal precautions (b) Segregation of waste

MULTIPLE CHOICE QUESTIONS (MCQs)

1. How many categories of biomedical waste are there?
   (a) Five (b) Seven (c) Eight (d) Ten
2. What colour code/s is/are used for plastic bag/s for segregation of biomedical waste?
   (a) Yellow (b) Red (c) Black (d) All of the above
3. Which colour plastic bag is used for non-infectious waste?
   (a) Yellow (b) Red (c) Black (d) Blue
4. Which of the following methods can be used for treatment of biomedical waste?
   (a) Incineration (b) Autoclaving (c) Microwaving (d) All of the above
5. Which is the most commonly used method for the treatment of microbiological waste?
   (a) Autoclaving (b) Microwaving (c) Plasma torch (d) None of the above

Answers (MCQs):

1. (c) 2. (d) 3. (c) 4. (d) 5. (a)

Chapter 83

VEHICLES AND VECTORS

Infectious agent may be transmitted from the reservoir or source of infection to a susceptible individual in many different ways. Vehicles and vectors are two important ways of transporting an infectious agent to a susceptible individual.

I. VEHICLES

Various vehicles for transmission of the infectious agent include:

- water
- food (including raw vegetables, fruits, milk and milk products)
- ice
- blood
- serum
- plasma
- Other biological products e.g. tissues and organs.

Water and food are the most frequent vehicles of transmission, because they are used by everyone.

A. Diseases Transmitted by Water and Food

Acute diarrhoeas
Cholera
Typhoid fever
Poliomyelitis
Hepatitis A virus infection
Food poisoning
Intestinal parasitic infestations

B. Diseases Transmitted by Blood

Diseases caused by the following infectious agents may be transmitted by blood.

1. Viruses

Hepatitis B and other hepatitis viruses
Human immunodeficiency viruses
Human T cell lymphotropic viruses
Cytomegaloviruses

2. Bacteria

Treponema pallidum

3. Parasites

Plasmodia
Trypanosoma cruzi

II. VECTORS

Vector is defined as an arthropod or any living carrier that transports an infectious agent to a susceptible individual. Vector transmission may be mechanical or biological. In biological transmission, the disease agent has a developmental cycle or multiplication in the vector.

A. Mechanical Transmission

The infectious agent is only mechanically transported and there is no development or multiplication of the agent on or within the vector.

B. Biological Transmission

The infectious agent undergoes replication or development or both in vector and requires an incubation period before its transmission. Biological transmission is of three types:

Propagative

The agent only multiplies in the vector, but there is no change in its form e.g. plague bacilli in rat fleas.

Cyclopropagative

The agent changes its form and number e.g. malarial parasites in mosquito.

Cyclodevelopment

The agent undergoes only development but no multiplication, e.g. microfilaria in mosquito.

Vertical transmission of the infectious agent from the infected female to her progeny in the vector is known as *transovarial transmission*.

MEDICAL ENTOMOLOGY

A study of the arthropods of medical importance is known as medical entomology. Arthropods act as vectors or carriers of diseases (Table 83.1).

Table 83.1 Diseases Transmitted by Arthropods as Vectors

Mosquito	:	Malaria, filaria, Japanese encephalitis, dengue, West-Nile, yellow fever, dengue haemorrhagic fever
Sandfly	:	Kala-azar, oriental sore, sandfly fever, oroya fever, Chandipura
Rat flea	:	Bubonic plague, endemic typhus, chiggerosis, *Hymenolepis diminuta*
Hard tick	:	Tick typhus, Kyasanur forest disease (KFD), tularaemia, human babesiosis
Soft Tick	:	Q fever, endemic relapsing fever
Trombiculid mite	:	Scrub typhus, Rickettsial-pox
Cyclops	:	Guinea-worm disease, fish tapeworm
Louse	:	Epidemic typhus, epidemic relapsing fever, trench fever
Reduviid bug	:	Chagas disease
Tsetse fly	:	Sleeping sickness
House fly	:	Typhoid and paratyphoid fevers, diarrhoea, cholera, dysentery, gastroenteritis.

KEY POINTS

1. *Vehicles* and *vectors* are two important ways of transporting an infectious agent to a susceptible individual.
2. *Water*, *food*, *blood*, *sera* and other biological products are various vehicles for transmission of the infectious agent.
3. *Acute diarrhoeas*, *cholera*, *typhoid fever* are the diseases transmitted by water and food.
4. Hepatitis B, human immunodeficiency viruses are the viruses transmitted by *blood* and *blood products*.
5. *Vector* is defined as an *arthropod* or *any living carrier* that transports an infectious agent to a susceptible individual.
6. Malaria, filaria, dengue, kala-azar are some diseases transmitted by arthropods as vectors.
7. A study of the arthropods of medical importance is known as *medical entomology*.

YOU MUST KNOW

1. List of diseases transmitted by water and food.
2. List of diseases transmitted by blood.
3. List of diseases transmitted by different arthropods as vectors.

STUDY QUESTIONS

1. Discuss the role of vehicles and vectors in transmission of infectious agents.
2. Write short notes on:
   (a) Water-borne diseases
   (b) Diseases transmitted by blood and blood products
   (c) Diseases transmitted by arthropods as vectors

MULTIPLE CHOICE QUESTIONS (MCQs)

1. Which of the following diseases can be transmitted by water and food?
   (a) Cholera (b) Poliomyelitis
   (c) Hepatitis A virus infection (d) All of the above
2. Which of the following diseases can be transmitted by blood?
   (a) Hepatitis B infection (b) HIV infection
   (c) Syphilis (d) All of the above
3. All of the following diseases are transmitted by mosquito except :
   (a) Japanese encephalitis (b) Yellow fever
   (c) West-Nile fever (d) Oroya fever
4. All of the following diseases are transmitted by sandfly except
   (a) Kala-azar (b) Oriental sore
   (c) Oroya fever (d) West-Nile fever
5. Which of the following diseases are transmitted by rat flea?
   (a) Plague (b) Endemic typhus
   (c) Chiggerosis (d) All of the above
6. Which of the following diseases are transmitted by hard tick?
   (a) Kyasanur Forest disease (b) Tularaemia
   (c) Human babesiosis (d) All of the above
7. Which of the following diseases are transmitted by louse?
   (a) Epidemic typhus (b) Epidemic relapsing fever
   (c) Trench fever (d) All of the above
8. Chagas disease is transmitted by
   (a) Reduviid bug (b) Louse
   (c) Tick (d) Mite
9. Sleeping sickness is transmitted by :
   (a) Tsetse fly (b) Soft tick
   (c) Rat flea (d) Louse
10. Scrab typhus is transmitted by :
    (a) Trombiculid mite (b) Soft tick
    (c) Rat flea (d) Louse
11. In which of the following diseases, housefly can act as a vector?
    (a) Typhoid fever (b) Cholera
    (c) Paratyphoid fever (d) All of the above

Answers (MCQs):

1. (d)	2. (d)	3. (d)	4. (d)	5. (d)
6. (d)	7. (d)	8. (a)	9. (a)	
10. (a)	11. (d)			

APPENDIX

Probability Table 1 (According to McCrady)

Quantity of water	10 ml	1 ml	0.1 ml	
No. of samples of each quantity tested	5	5	5	
Number of tubes giving positive reactions (acid and gas)	0	0	0	0
	0	0	1	2
	0	0	2	4
	0	1	0	2
	0	1	1	4
	0	1	2	6
	0	2	0	4
	0	2	1	6
	0	3	0	6
	1	0	0	2
	1	0	1	4
	1	0	2	6
	1	0	3	8
	1	1	0	4
	1	1	1	6
	1	1	2	8
	1	2	0	6
	1	2	1	8
	1	2	2	10
	1	3	0	8
	1	3	1	10
	1	4	0	11
	2	0	0	5
	2	0	1	7
	2	0	2	9
	2	0	3	12
	2	1	0	7
	2	1	1	9
	2	1	2	12
	2	2	0	9
	2	2	1	12
	2	2	2	14
	2	3	0	12
	2	3	1	14
	2	4	0	15
	3	0	0	8
	3	0	1	11
Probable number of coliform bacilli in 100 ml of water				

(Contd.)

Table 1 (Contd.)

Quantity of water	10 ml	1 ml	0.1 ml	
No. of samples of each quantity tested	5	5	5	
Number of tubes giving positive reactions (acid and gas)	3	0	2	13
	3	1	0	11
	3	1	1	14
	3	1	2	17
	3	1	3	20
	3	2	0	14
	3	2	1	17
	3	2	3	20
	3	3	0	17
	3	3	1	20
	3	4	0	20
	3	4	1	25
	3	5	0	25
	4	0	0	13
	4	0	1	17
	4	0	2	20
	4	0	3	25
	4	1	0	17
	4	1	1	20
	4	1	2	25
	4	2	0	20
	4	2	1	25
	4	2	2	30
	4	3	0	25
	4	3	1	35
	4	3	2	40
	4	4	0	35
	4	4	1	40
	4	4	2	45
	4	5	0	40
	4	5	1	50
	4	5	2	55
	5	0	0	25
	5	0	1	30
	5	0	2	45
	5	0	3	60
	5	0	4	75
Probable number of coliform bacilli in 100 ml of water				

(Contd.)

Quantity of water	10 ml	1 ml	0.1 ml	
No. of samples of each quantity tested	5	5	5	
Number of tubes giving positive reactions (acid and gas)	5	1	0	35
	5	1	1	45
	5	1	2	65
	5	1	3	85
	5	1	4	115
	5	2	0	50
	5	2	1	70
	5	2	2	95
	5	2	3	120
	5	2	4	150
	5	2	5	175
	5	3	0	80
	5	3	1	110
	5	3	2	140
	5	3	3	175
	5	3	4	200
	5	3	5	250
	5	4	0	130
	5	4	1	170
	5	4	2	225
	5	4	3	275
	5	4	4	350
	5	4	5	425
	5	5	0	250
	5	5	1	350
	5	5	2	550
	5	5	3	900
	5	5	4	1600
	5	5	5	1800+

Probable number of coliform bacilli in 100 ml of water

Probability Table 2 (According to McCrady)

Quantity of water	50 ml	10 ml	1 ml	
No. of samples of each quantity tested	1	5	5	
Number of tubes giving positive reactions (acid and gas)	0	0	0	0
	0	0	1	1
	0	0	2	2
	0	1	0	1
	0	1	1	2
	0	1	2	3
	0	2	0	2
	0	2	1	3
	0	2	2	4
	0	3	0	3
	0	3	1	5
	0	4	0	5
	1	0	0	1
	1	0	1	3
	1	0	2	4
	1	0	3	6
	1	1	0	3
	1	1	1	5
	1	1	2	7
	1	1	3	9
	1	2	0	5
	1	2	1	7
	1	2	2	10
	1	2	3	12
	1	3	0	18
	1	3	1	11
	1	3	2	14
	1	3	3	18
	1	3	4	20
	1	4	0	13
	1	4	1	17
	1	4	2	20
	1	4	3	30
	1	4	4	35
	1	4	5	40
	1	5	0	25
	1	5	1	35
	1	5	2	50
	1	5	3	90
	1	5	4	160
	1	5	5	180 +

Probable number of coliform bacilli in 100 ml of water

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