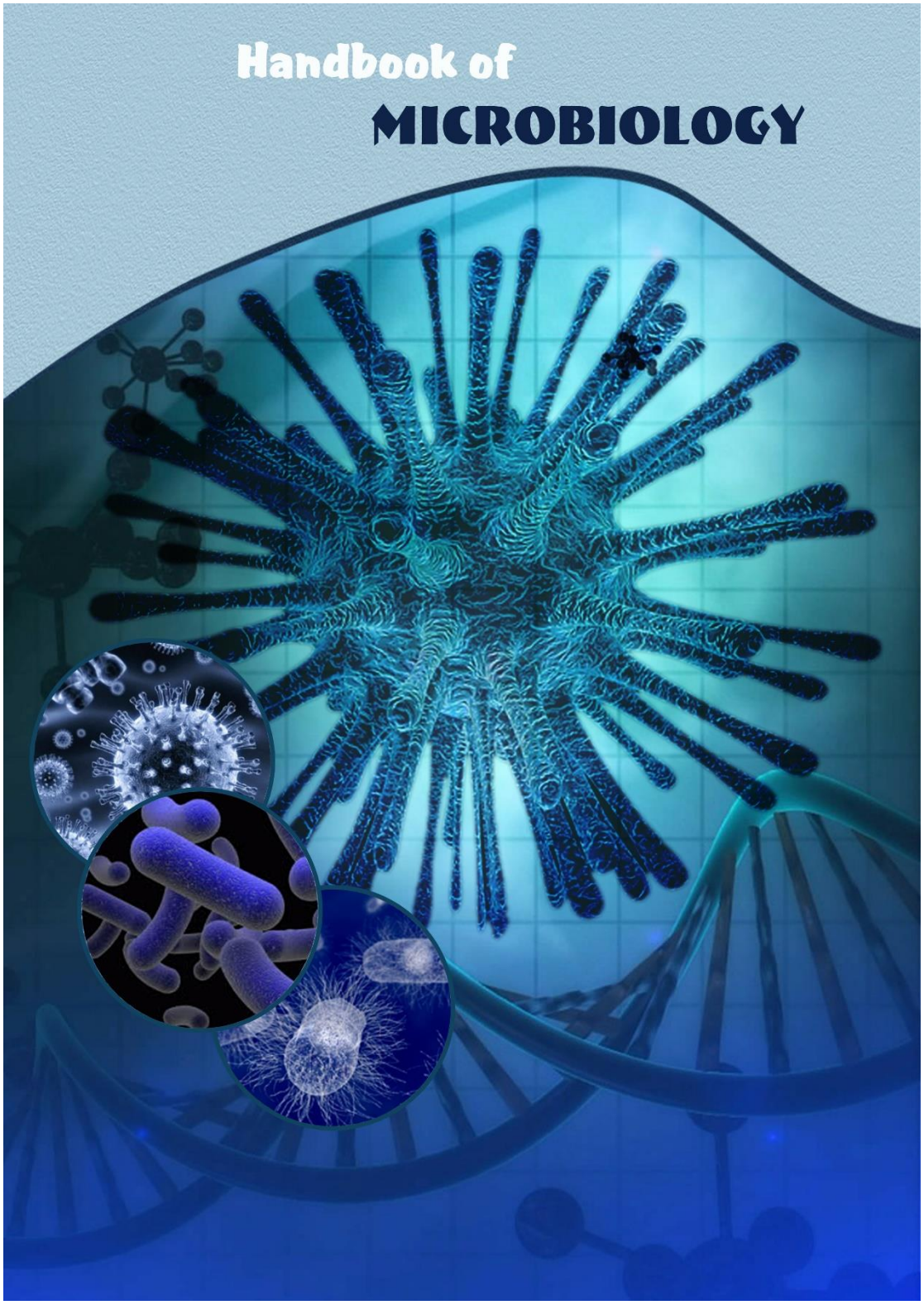
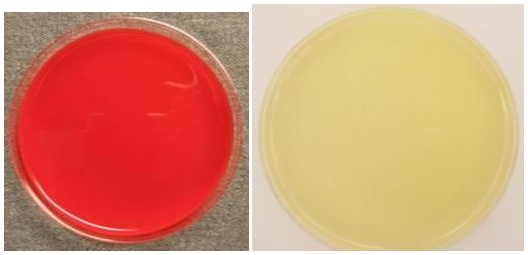

Handbook of
MICROBIOLOGY

H.S.C. (VOCATIONAL)

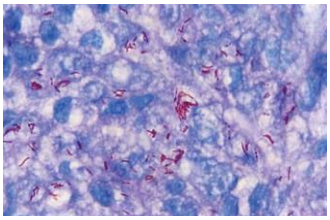
MEDICAL LABORATORY TECHNICIAN

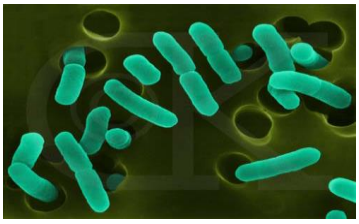
STD: XI (PAPER-3)

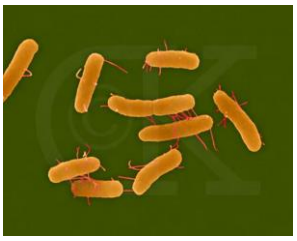


MICROBIOLOGY

THEORY HANDBOOK







INDEX

1. Introduction to Microbiology

Objectives:
After learning this lesson the student will know the contributions of different people in the field of Microbiology. Also they will learn about the inventions and discoveries in this field. They will understand what microbes are and how and why here are studied.

1.1 Historical Aspect

Disease and death have always held the attention of the human mind. Ancient humans ascribed them to divine wrath and other supernatural forces. There have been, from very early times, occasional suggestions that diseases may result from invasion of the body by external contagion.

The science of microbiology started with the invention of the microscope. The English scientist **Robert Hooke** is credited with being the first person to use a microscope for academic study. That was in the early 1660's. In 1665 Hooke published his landmark book Micrographia, which described the microscopic world for the first time.

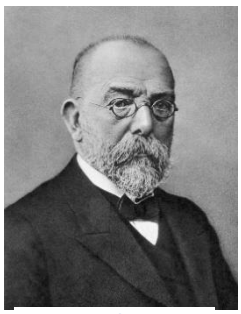


Fig. 1.1 Robert Hooke

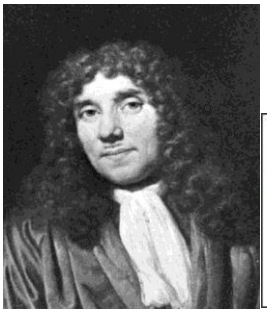


Fig.1. 2 Antonie Van Leeuwenhoek with his Microscope

Antonie Van Leeuwenhoek, A Dutch tradesman from Netherland is best known for his contribution to the improvement of the microscope and for his contributions towards the establishment of microbiology. Using his handcrafted microscopes, van Leeuwenhoek was the first to observe and describe single celled organisms that he first referred to as 'animalcules', and which we now refer to as microorganisms. Hence, Antonie Van Leeuwenhoek is called as "father of Microbiology'.

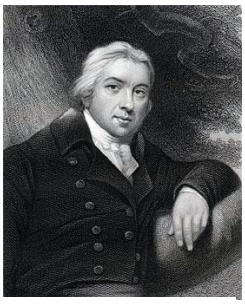


Fig. 1.3 Edward Jenner

The credit for the invention of inoculation goes to **Edward Jenner** who in 1796 injected pus taken from a cowpox pustule in to the arm of an eight-year-old boy James Phipps. Jenner subsequently proved that having been inoculated with cowpox Phipps was immune to smallpox. He submitted a paper to the Royal Society in 1797 describing his experiment. In 1798, the results were finally published and Jenner coined the word vaccine from the Latin 'vacca' for cow. Edward Jenner is called as ‘Father of Immunology’.


Fig.1. 4 Louis Pasteur

Louis Pasteur was probably the greatest biologist of the nineteenth century. He developed the ‘germ theory of disease’, which was a significant breakthrough in medicine that ultimately improved the health of everyone on the planet. He was also able to prove that life itself did not "spontaneously come into being" through a series of experiments using a sterilized flask. He successfully showed that life can only be generated from existing life. Pasteur also showed that fermentation - a process used in baking and brewing - was caused by microorganisms. As a result of this work he went on to develop the process for sterilizing milk and this was named after him - pasteurization.

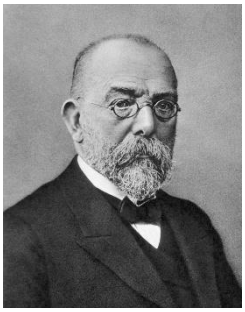


Fig. 1. 5 Robert Koch

Robert Koch was a German physician and pioneering microbiologist. The founder of modern bacteriology, he is known for his role in identifying the specific causative agents of tuberculosis, cholera, and anthrax. Koch's postulates are four criteria designed to establish a causative relationship between a microbe and a disease. The postulates were formulated by Robert Koch and Friedrich Loeffler in 1884.

Koch's postulates are the following:

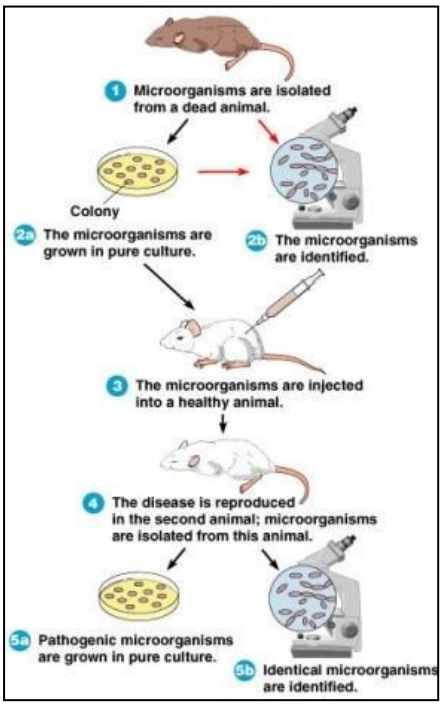


Figure 1.6 Koch's Postulates

1. The microorganism must be found in abundance in all organisms suffering from the disease, but should not be found in healthy organisms.

2. The microorganism must be isolated from a diseased organism and grown in pure culture.

3. The cultured microorganism should cause disease when introduced into a healthy organism.

4. The microorganism must be reisolated from the inoculated, diseased experimental host and identified as being identical to the original specific causative agent.

1.2 Definition

Medical microbiology is the study of microbes that infect humans, the diseases they cause, their diagnosis, prevention and treatment. It also deals with the response of the human host to microbial and other antigens.

Review Questions:

1. Define microbiology.
2. Who is called as ‘Father of Microbilogy’? Why?
3. Who invented Vaccines? How?
4. What is the contribution of Louis Pasteur in the field of Microbiology?
5. Enumerate the components of Koch’s' postulates.

Chapter 2 Sterilization and Disinfection

Objective-

To make students aware of various methods/techniques of sterilization and disinfection.

2.1 Definitions:

- **Sterilisation** is defined as the process by which an article, surface, medium is freed of microorganisms either in vegetative or spore form.
- **Disinfection** is destruction of all pathogenic organisms or organisms capable of giving rise to infection.
- **Antisepsis** is the prevention of infection by inhibiting the growth of bacteria.

2.2 Methods of sterilization:

Sterilising agents are classified as **physical** and **chemical** agents.

Different physical methods are as follows:

1. Sunlight
2. Drying
3. Dry heat
4. Moist heat
5. Filtration
6. Radiation
7. Sonic and Ultra sonic vibration

Different chemicals agents are as follows:

1. Aldehydes
2. Alcohols
3. Dyes
4. Halogens
5. Phenols
6. Surface active agents
7. Metallic salts
8. Gases

2.2.1 Physical agents

Among the different agents heat is most commonly used method. Sterilisation by heat depends on number of factors.

- Type of heat - Dry or Moist
- Temperature and time
- The number of microorganisms
- Type of microorganisms - vegetative or spore bearing.
- The type of material from which organisms have to be removed.

The time required for sterilization is inversely proportional to temperature and directly proportional to number of microorganisms in suspension.

2.2.1a Dry heat

Flamming - It is commonly used method for inoculating loops, wires, points of Forceps and spatulas which are held in Bunsen burner till they become red hot. Needles, mouths of culture tubes, glass slides are passed through flame without allowing them to become red hot.

Hot air oven -The Hot air oven works on dry heat sterilization process. It is used for sterilization of articles made up of glass or metal such as syringes, needles, scalpels, pipettes, foreceps, scissors, swabs etc. It can not be used for liquid media. This method is more effective than the boiling water method.

Construction-

Hot air oven is like a cupboard. Its inner chamber has adjustable shelves. It has a fan inside for even distribution of hot air and elimination of air pockets. The oven is heated by electricity. Heating elements are present in the wall of chamber. Thermostat is used to adjust required temperature. The material should be arranged in such a way that it should allow free circulation of air in between. It should not be overloaded. Glasswares

should be perfectly dry before keeping inside. Holding temperature is 160^0C for one hour.

Figure 2.1 Hot Air oven

Sterilization technique-

- Wrap the glassware like petridishes, pipettes, specimen tubes, syringes in white paper sheet and tie with string.
- For syringes separate barrel and piston. Needles should well protected.
- The sucking end of pipette can be plugged with nonabsorbent cotton wool and put into metal container with loose lid.
- Pasteur pipettes are put into large size tubes and then plugged with cotton.
- After keeping all material inside the oven close the door.
- Switch on the oven. Set the temperature at 160^0C and leave it for one hour. Switch off the oven and allow it cool slowly for 2 hours.
- Open the door after cooling and take out material and store.

2.2.1b Moist heat

Temperature below 100^0C - Pasteurisation used for milk. Temperature is either 63^0C for 30 min or 72^0C for 15-20 sec followed by cooling to 13^0C. L-J medium, Loeffler's serum slope 80-85^0C for half an hour on three successive days in an inspissator.

Temperature at 100^0C - Boiling should be done atleast for the period of 10-30 mins. Lid of steriliser should not be opened during process. This is not used for sterilization of instruments used for surgery.

Temperature above 100^0 C (Steam under pressure)

Autoclave-This is most effective and commonly used method of Sterilization.It kills all vegetative and spore bearing organisms.It sterilizes all material which can be sterilized with hot air oven. Also liquid media can be sterilized. For liquid media do not fill tube fully. Keep one quarter space for the liquid to expand.

Figure 2.2 Autoclave

Principle- Steam put under pressure will reach temp above boiling water temp. Temperature of steam under pressure is 121^0C. This saturated steam has high penetrating power. It penetrates wrapping more rapidly and reach surface of atoms to be sterilized.

Construction and working –

Laboratory autoclave consists of vertical or horizontal cylinder of stainless steel in a supporting sheet-iron case. Lid is fastened by screw clamps and made air tight by an asbestos washer. On lid on upper side there is discharge tap for air and steam, a pressure gauge and a safety valve. Heating is done by gas or electricity. Sterilisation is carried out at the temperature between 108^0C - 147^0C. A variety of materials like dressings, media, pharmaceutical agents, instruments, laboratoryware, etc can be sterilized.

Heat is conducted through walls of sealed containers till the temperature of fluid inside is in equilibrium with the steam outside. Sufficient water is put into cylinder i.e. up to the basket support. Materials to be sterilized are kept on the tray and close the lid. The lid is screwed tight with discharge tap open. Heating is started. The safety valve is adjusted to required pressure. The steam and air mixture is allowed to escape freely till all air has been displaced. This can be tested by allowing the escaping steam to pass into pail of water through rubber tubing. When no more air bubbles come out, the discharge tap is closed.The steam pressure increases inside and when it reaches the desired set level, the safety valve opens and excess steam escapes. From this point holding period is calculated.Temperature at this time is 121^0C. Continue sterilization for 20-30min. After this turn off heat. Allow it to cool till pressuregauge indicates that it is atmospheric pressure. The discharge tap is opened slowly. Unscrew the clamps when hissing sound stops. Take off the lid and leave the material to cool.

2.2.1c Filtration

Filtration is an important method of sterilization without heating. Filters work by allowing solution to pass through filter pores having diameter too small for microbes to pass.

This is useful for antibiotics, sera and carbohydrate solutions used in preparation of culture media. This is useful when we want to isolate organisms which are scanty in sample and we have to study them.

Filters can be sintered glass funnels made from cellulose esters. For the removal of bacteria filters with average pore size of 0.2µm is commonly used but through this viruses and phage can pass, so this method is not useful for them.

There are different types of filters. They are

- Earthenware candles- Berkefield, Chamberland, Mandler
- Asbestos disc filters - Seitz
- Sintered glass filters
- Membrane filters

Membrane filtration

The Membrane filter Technique is an effective, accepted technique for testing fluid samples for microbiological contamination. It involves less preparation than many traditional methods, and is one of a few methods that will allow the isolation and enumeration of microorganisms. The membrane filtration technique also provides presence or absence information within 24 hours.

Advantages of membrane filtration

- Permits testing of large sample volumes.
- Reduces preparation time as compared to many traditional methods.
- Allows isolation and enumeration of discrete colonies of bacteria.
- Provides presence or absence information within 24 hours.

- Effective and acceptable technique. Used to monitor drinking water in government laboratories.
- Useful for bacterial monitoring in the pharmaceutical, cosmetics, electronics, and food and beverage industries.
- Allows for removal of bacteriostatic or cidal agents that would not be removed in Pour Plate, Spread Plate, or MPN techniques.

Figure 2.3 Membrane filter

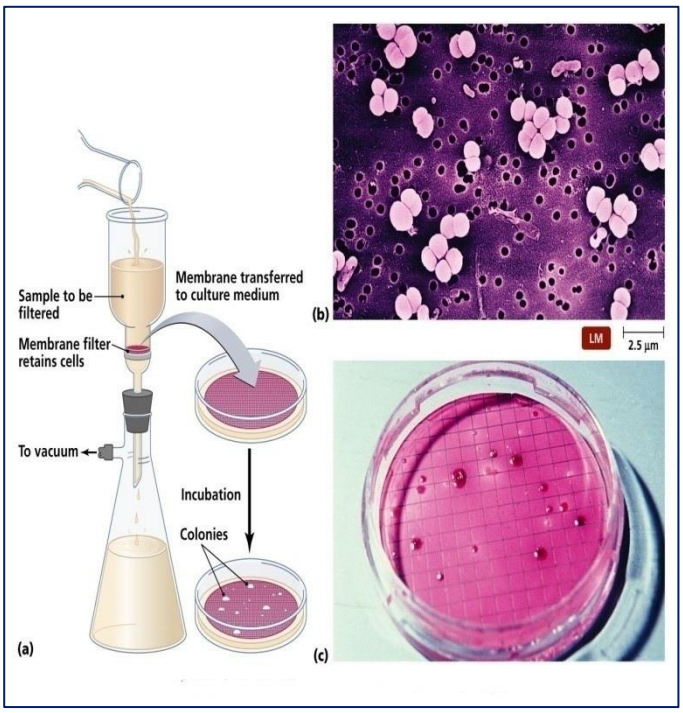


Figure 2.4 Membrane Filtration Technique

2.2.2 Chemical agents of sterilization

Disinfectants are those chemicals that destroy pathogenic bacteria from inanimate surfaces. Some chemical have very narrow spectrum of activity and some have very wide.

2.2.2a Criteria for an ideal disinfectant

An ideal disinfectant should have following criteria:

- Should have wide spectrum of activity
- Should be active in the presence of organic matter

- Should make effective contact and be wettable
- Should be active in any pH i. e. acidic or alkaline
- Should be stable
- Should have long shelf life
- Should be speedy in action.
- Should have high penetrating power
- Should be non-toxic, non-allergic, non-irritative or non-corrosive
- Should be compatible with other disinfectant
- Should not interfere with healing.
- Should not leave non-volatile residue or stain
- Efficacy should not be lost on reasonable dilution
- Should not be expensive and must be available easily
- Should be safe and easy to use

There is no single disinfectant which is an ideal.

Factors determining potency of disinfectant:

- Concentration of the substance
- Nature of the microorganisms
- Presence of extraneous material
- Time of action
- pH of the medium
- Temperature

Figure 2.5 Common Chemical Disinfectant

2.2.2b Important chemical disinfectants:

- **Aldehydes-**

  Formaldehyde is used to preserve anatomical specimens. Formaldehyde gas is used to sterilize heat sensitive catheters and instruments. It is used for fumigation of wards, sick rooms and laboratories.

  Glutaraldehyde- effective against TB bacilli, fungi and viruses.

- **Alcohols-**

  Commonly ethyl and isopropyl alcohol is used. 70% aqeous solution of ethyl alcohol is more effective. It is used as skin antiseptic.

  Isopropyl alcohol is used for disinfection of clinical thermometers.

  Methyl alcohol is fungicidal, so used for treating cabinets affected by them.

- **Dyes-**

  Aniline and Acridine dyes for skin and wound antisepsis. They are more active against Gram positive bacteria than Gram negative bacteria.

- **Halogens**-

  Iodine - aqueous and alcoholic solution is used as antiseptic.

  Povidone-iodine (Betadine) - most commonly used antiseptic and disinfectant.

  Chlorine is used in water supplies, swimming pools, food and dairy industries.

- **Phenols –**

  They act as disinfectant in high concentration and antiseptic in low concentration. Various combinations are used to control pyogenic cocci in surgical and neonatal units.

- **Gases-**

  Ethylene oxide is highly explosive and inflammable.

Formaldehyde gas is used for fumigation of operation theatres.

Betapropiolactone (BPL) is more effective than formaldehyde for fumigation purposes. It is used for sterilization of biological products.

- **Surface active agents-**

  They reduce surface tension. They are active against vegetative cells, Mycobacteria and enveloped viruses. They are widely used as disinfectants at dilution of 1-2% for domestic use and in hospitals.

- **Metallic salts-**

  The salts of silver, copper, mercury are used as disinfectant. Copper salts are fungicides.

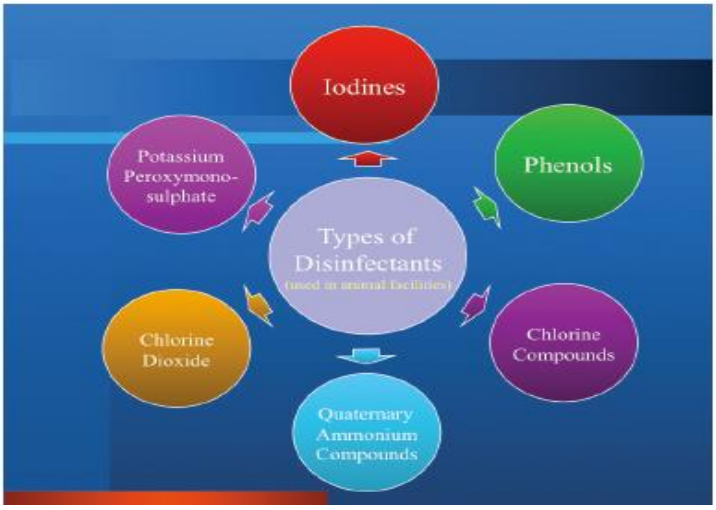


Figure 2.6Chemical disinfectant

Figure 2.7 Potency of disinfectant

Review Questions-

1. Define sterilization, disinfection and antisepsis**.**
2. Enumerate physical and chemical agents of sterilization.
3. What is holding period for an autoclave and hot air oven?
4. On what principle does an autoclave works?
5. How will you prepare material for sterilization by an autoclave/hot air oven?
6. What are criteria for an ideal disinfectant?
7. Name the chemical agents used for fumigation purpose.
8. Describe structure and working of hot air oven/autoclave.
9. What is flamming?
10. At what temperature milk is sterilized?
11. How sterilization is carried out by boiling?

****

3. Requirment and use of Laboratory instruments

Objective - Student should know commonly used instruments, their construction and uses in microbiology.

3.1 Introduction

Proper knowledge and proper use of laboratory instruments is must for obtaining accurate results. Different instruments having different functions are used in microbiology e.g. autoclave is used for sterilization, microscope for studying morphology of bacteria, etc.

3.2 Microscope and its types

Microscope is an essential instrument for microbiologist. It is a combinations of lenses so adjusted that minute objects invisible to naked eyes are magnified made visible.

There are different types of microscopes. They are -

Light microscope. It is also called as **optical microscope.** It is a type of Compound microscope used to visualize various microorganisms. This type uses visible light and system of lenses to magnify the images to manyfold. Thus light microscopes are divided in to different types.

- **Simple microscope** - It is a simple single lens microscope which is the original light microscope. It is considered as primitive most.
- **Compound microscope** - It is most commonly used microscope. It is an integral part of pathological laboratory, school and college laboratory. It uses two optical parts ocular lens and objective lens. With the help of this image is magnified and can be easily observed. These microscopes are heavier than simple microscope. These microscopes collect the light from the sample with the help

of multiple lenses. There are different types of compound microscopes.

1. **Standard compound light microscop**e - This microscope consists of an eyepiece which is in line with revolving nose piece. The nose piece consists of two or more objective lenses.The light passes from the stage through a hole into the sample and from sample it passes to the lenses.Then images enlarge 4X, 10X, 40X or 100X as per the objective lenses.

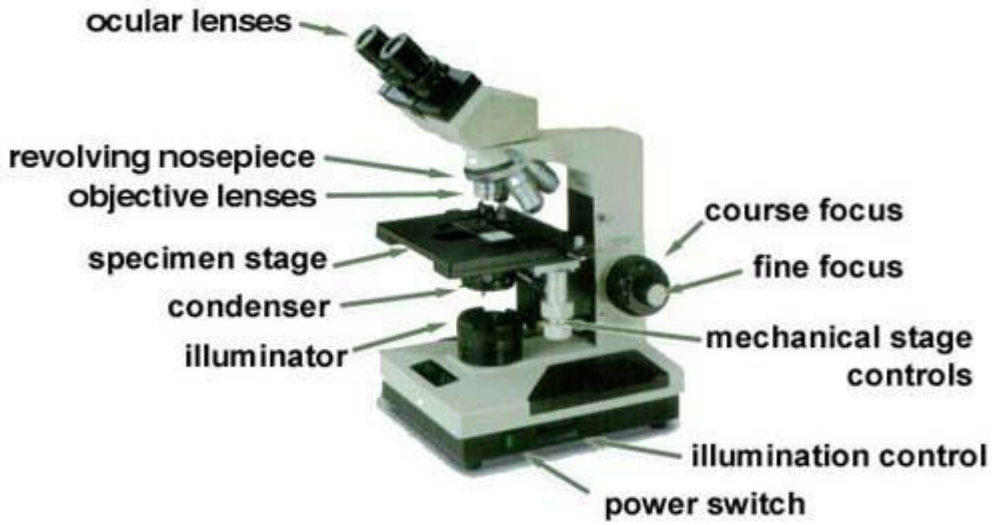


Figure 3.1 Microscope

Standard compound light microscope

2. **Stereo microscope -** Also called dissecting microscope. It has low magnification power. They are used to observe specimens that are a bit larger in size. It consists of two optical paths which are at different angles. This helps to see the specimen in three dimensions. It is used in anatomy and physiology for carrying out microsurgery, dissection, fine repair, etc.
3. **UV microscope -** A UV microscope uses UV light to produce an image which has resolution twice that is seen with visible light microscope. As UV light is

harmful to eye a digital sensor is used to observe image.

4. **Fluorescence microscope** - It uses high energy short wavelength light that excites the electrons of certain molecules present in the sample. This high energy is converted into low energy long wavelength light which is within visible spectrum which helps in the formation of image.
5. **Dark-field microscope** - The microscope uses special condenser lens that helps scattering light. This is used to observe spirochetes
6. **Phase contrast microscope** - It uses special condenser that helps in throwing light out of phase. This helps the light to pass through the object at different speeds. It is used to observe unstained specimens. It is used to study different parts of cell.

Electron microscope- It is one of the most advanced microscopes used today. This microscope is powered by a beam of electrons with very short wavelength. This microscope is used to study tiny cells as well as large molecules.There are different types electron microscopes. They are

- Transmission Electron Microscope (TEM) - is used to study cells.
- Scanning electron Microscope (SEM) - It has low magnification power than TEM but it demonstrates three dimensional images.
- Reflection Electron Microscope (REM) - It detects elastically scattered electrons.

Figure 3.2Scanning electron Microscope

3.3 Incubator

An incubator is a device which is used to grow and maintain microbiological cultures. The incubators maintains optimal temperature, humidity and other conditions such as oxygen and carbon dioxide content of atmosphere inside. They are temperature controlled chambers which are insulated. The most commonly used temperature is 37^0C. When hot air oven is set at lower temperature i.e. 37^0C it can be used as an incubator.

Figure 3.3**Laboratory incubator**

3.4 Autoclave

Refer to page number ------ in topic sterilization and Disinfection.

3.5 Hot Air oven

Refer to page number ----- in topic sterilization and Disinfection.

3.6 Anaerobic jar

McIntosh Fildes Jar

It is one of the physical methods to produce anaerobiasis. It is used to produce anaerobic environment during the incubation of anaerobic cultures in the laboratory. It works on the principle of evacuation and replacement, where the air inside the chamber is evacuated and replaced with hydrogen or a mixture of gases.

Figure 3.4 McIntosh Filde's Jar

McIntosh and Fildes jar consists of a stout glass or metal (8x5") jar with a lid that can be tightly clamped with a screw to make it airtight. The lid has two taps, one acts as outlet that is connected to a vacuum pump and the othe as inlet through which hydrogen gas is supplied. The lid also consists of two electrical terminals, which is connected on the underside to a small porcelain spool wrapped by a layer of palladinised asbestos. Subsequent models didnot have electrical terminals as cold catalyst was being used. The presence of air is deleterious for many anaerobic bacteria and must be incubated in its absence. The inoculated culture plates are placed inside a metal jar and the lid clamped tight. The air inside is removed using a vacuum pump. The pressure inside the chamber is reduced to 100 mm below mercury.

It is not practically possible to evacuate all the air since it will cause boiling in the liquid or detachment of the medium. Despite evacuation of air, some amount of oxygenwill still be left behind. Hence the air is replaced with

hydrogen gas from a cylinder. The pressure inside the chamber is brought backto atmospheric pressure (760mm of Hg). Using palladium catalyst residual oxygen can be made to react with hydrogen to form water, but this reaction is not spontaneous. This reaction is catalysed by palladium catalyst that is heated using electricity. As the reaction continues more hydrogen is used up. This process is allowed to continue for 20 minutes. Use of hydrogen gas and use of electricity can lead to explosions. Hence hydrogen gas has been substituted by a mixture of gases (consisting of 5%CO_2, 10%H_2 and 85%N_2) and a cold catalyst consisting of an alumina tablet coated with palladium. The jar is then placed inside an incubator at 37^oC for 48hours.

Disadvantages: Palladium catalyst is inactivated by excess moisture and has to be rejuvenated by heating them at 160^oC for two hours. Subsequently, introduction of silica gel absorbent solved this problem. This system is excellent but requires skill to operate and special apparatus that are costly. Requirements of vacuum pump and supply of gas is a major drawback of this system and hence it is being replaced by more convenient GasPak system.

The **Gaspak** system is simple and effective. It does not require need for drawing a vacuum and adding hydrogen. The GasPak is commercially available as a disposable envelope containing chemicals which release Co_2 and H_2 on addition of water. Once the inoculated plates are kept inside a jar, the GasPak envelope with water added is kept inside and lid is made tight. Hydrogen and carbon dioxide are liberated and the presence of cold catalyst in the envelope allows the combination of hydrogen and oxygen to produce anaerobic condition.

Figure 3.6 Gas Pack Method

3.7 Water bath

There are two types of waterbaths – (a) incubator waterbath and (b) boiling waterbath.

(a) Incubator waterbath - It has constant temperature device which is electrically operated, and it is controlled by thermostat. Due to thermostat temperature is maintained within range. Generally it is at 37^0C. In case of inactivation of complement temperature is kept at 56^0C.

(b) Boiling waterbath - It is required in some chemical reactions and for preparing solutions. It does not require any setting of temperature which is there in incubator waterbath. As long as the water is boiling temperature is at 100^0C.

Figure 3.5 Incubator waterbath

3.8 ELISA reader

An ELISA reader measures and quantitates the color differences in the 12 wells of the plate. ELISA readers do spectrophotometry. They emit light of one wavelength and measure the amount of light absorbed and reflected by an object. ELISA reader can also measure fluorescence and luminescence. An ELISA plate reader requires less quantity of sample to get a result. ELISA plate reader measures more samples in a short period of time.

Applications

ELISA readers are used to measure antibody but now they are used for protein and enzyme assay. They are also used for detection of HIV infection and measuring nucleic acids.

Figure 3.6 ELISA Reader

3.9 Laminar air flow

Laminar air flow maintains a sterile working area which is required in medical and research laboratories. Laminar air flow cabinets create particle free environment to carry out specialized work.

Laminar flow cabinets are made up of stainless steel with no gaps so that there is no built up of bacteria. The cabinets can be vertical or horizontal. They are known as clean benches as the air required for the working is thoroughly cleaned by the precision filtration process. The filtration system consists of pre-filters and a HEPA filter. The laminar flow cabinet is enclosed on the sides and constant positive pressure is maintained to prevent intrusion of contaminated room air.

Figure 3.7 Laminar air flow

Review questions:

1) What is the principle of ELISA?
2) Desribe the uses of Hot air oven and incubator.
3) What are types of waterbath?
4) What is laminar air flow?
5) Write a note on ELISA reader.
6) What is the principle of autoclave?
7) What is GasPak system?

3. Bacterial Anatomy

Objectives:
After reading this chapter the student will be able to:
- Describe bacterial size, shape& arrangement.
- Describe the structures found in bacterial cell and the function of each structure.

Introduction

Bacteria are unicellular, prokaryotic microorganisms without chlorophyll.

4.1 Size of bacteria.

The unit used to measure the size of bacteria is micron (micrometre µm)

1 µ or 1 µm = 1/1000th of 1 millimetre.

1 millimicron (mµ) or nanometre (nm) = 1/1000th of 1 micron.

Bacteria of medical importance generally measure 0.2 – 1.5 µ in diameter and 3 – 5 µ in length. We cannot see the objects less than 200 µ in size with naked eyes. Hence, bacteria are magnified using microscopes and observed.

4.2 Shape of bacteria

Classification of bacteria depending on shape -

1. Cocci – (From Kokkos meaning berry) are spherical or oval in shape. e.g.Staphylococci, Streptococci.
2. Bacilli – (From baculus meaning rod) are rod shaped. e.g. E.coli, K. Pneumoniae.
3. Vibrios – Comma shaped. e. g. Vibrio Cholera.
4. Spirilla – Rigid spiral forms. e. g. Spirillum.
5. Spirochaetes – Slender, flexous, spiral forms. e. g. Treponema.
6. Actinomycetes – Branching, filamentous bacteria.
7. Mycoplasma – Cell wall deficient bacteria and hence do not possess stable shape.
8. Rickettsiae and Chlamydiae – These are very small, unable to grow outside living cells, and previously considered as viruses but as they are having typical bacterial cell wall and bacterial enzymes are considered as bacteria

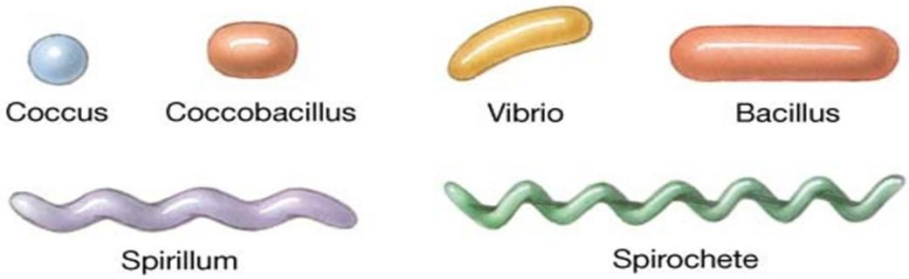


Figure 4.1 Different shapes of bacteria

4.3 Arrangement of bacteria -

Bacteria sometimes show characteristic cellular arrangement or grouping.

1. Cocci arranged in clusters – e.g. Staphylococci.
2. Cocci arranged in chains – e.g. Streptococci.
3. Cocci in group of four – e.g. Tetrads.
4. Cocci in groups of eight – e.g. Sarcina.
5. Cocci in pairs (Diplococci) – e.g. Pneumococci.
   Some bacilli show following arrangement –
6. Chains – e.g. Streptobacilli – Bacillus anthracis
7. Pairs – e.g. K. Pneumoniae.
8. Cuneiform or Chinese letter arrangement – (Bacteria arranged at angles to each other.) e.g. Corynebacteria.

Diagram showing arrangement of bacteria

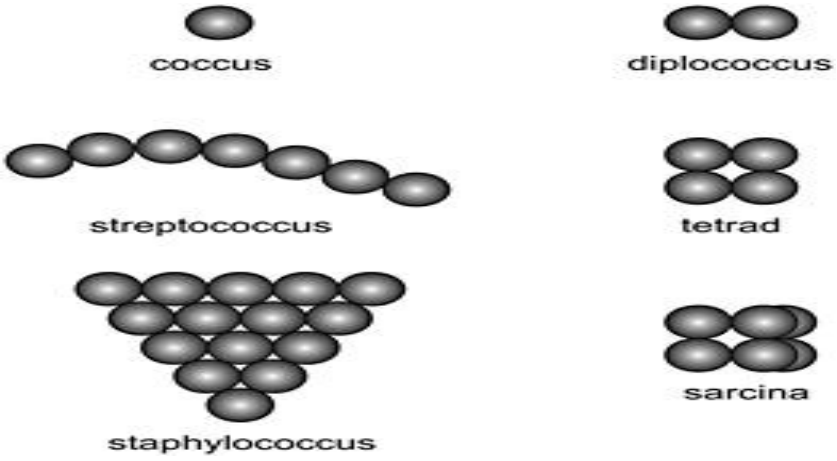


Figure 4.2 Arrangement of bacteria

4.4 Bacterial cell

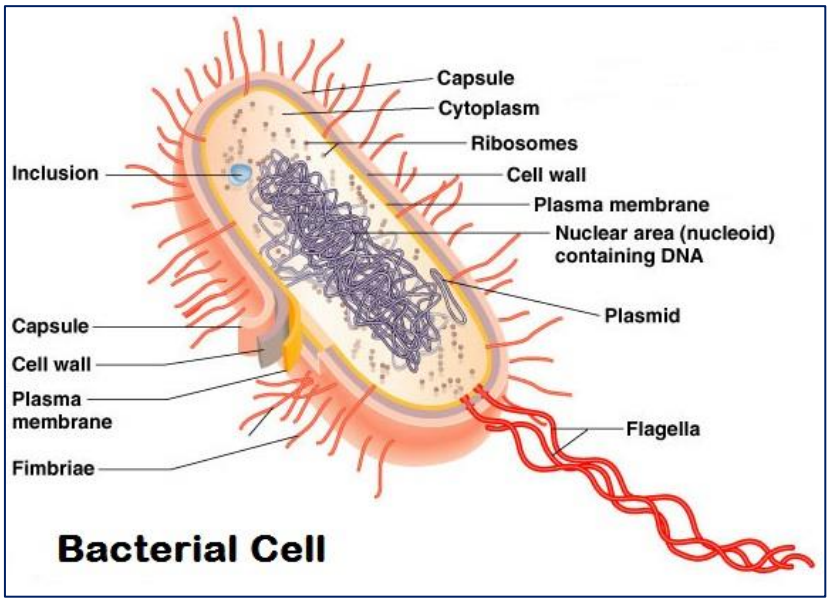


Figure 4.3 Bacterial cell

A typical bacterial cell consist of

1. Outer envelope and
2. Cytoplasm with cytoplasmic inclusions.

- Outer envelope – It consist of two components
  a) A rigid cell wall.
  b) Underlying cell cytoplasmic or plasma membrane.

4.5 Cell wall Structure – It is tough and surrounds bacteria like a shell. It is 10 – 25 nm in thickness. It contains peptidoglycans (mucopeptide) composed of N-acetyl muramic acid and N-acetyl glucosamine molecules arranged alternately in chains cross linked by peptide subunits.

Functions –

1. Gives rigidity and shape to cell.
2. Helps to maintain osmotic pressure and protects cell against osmotic damage.
3. It takes part in cell division.
4. It carries bacterial antigens that are important in virulence and immunity.

5. It provides site for phage absorption, antibiotics and lysozymes.
6. It takes part in cell division.

Cytoplasmic membrane -

Structure – It is thin (5 – 10 nm in thickness), elastic, semipermeable membrane, lining the inner surface of cell wall. It is trilamillar structure containing lipids, proteins and small amounts of carbohydrates.

Functions –

1 It selectively allows passage of nutrients inside and waste products outside the cell.

2. It contains enzymes of TCA cycle, cytochrome oxidase, enzymes required for cell respiration and cell wall synthesis.

4.6 Cytoplasm with cytoplasmic inclusions -

Cytoplasm is viscous watery solution containing variety of organic and inorganic solutes. It contains inclusions like ribosomes, mesosomes, granules, vacuoles and nuclear body.

Besides these essential components, some bacteria may possess additional structures. The bacteria may be enclosed in a viscid layer, which may be loose slime layer or organized as a capsule. Some bacteria carry filamentous appendages protruding from cell surface that are flagella which are organ of locomotion and fimbriae which are organ of adhesion.

Ribosomes – Tiny granules 10 – 15 nm in size and are scattered in the cytoplasm. They are composed of RNA and proteins.

Function – Protein synthesis.

Mesosomes – Mesosomes are complex infoldings of the cell membrane.

Function – Center for respiratory activity. It takes part in cell division.

Granules – They are not permanent and essential structures. e.g. Lipid granules, Volutin granules and Sulphur granules. They are source of stored energy.

4.7 Nucleus – The nucleus in bacteria is not well developed and is without nuclear membrane and nucleolus. It contains single, circular molecule of double stranded deoxyribonucleic acid (DNA) and about 1 mm in length.

Functions – It controls growth and metabolism of cell, multiplication of cell, and hereditary transmission of characters.

4.8 Slime layer and capsule – Many bacteria secrete a viscid material around the cell surface. When it is loose, undemarcated secretion, it is called slime layer. When this is organized into sharply defined structure, it is known as capsule.

Functions –Enhances bacterial virulence by inhibiting phagocytosis.Acts as protective covering against antibacterial action of substances such as lysozyme.Acts as antigen.

Demonstration of capsule -

1. Negative staining – Using India ink or nigrosine. Capsules are seen as clear halos around bacteria, against a black background.

2. Quellung reaction – When a suspension of capsulated bacteria is mixed with its specific anticapsular serum and examined under the microscope, the capsule becomes very prominent and appears 'swollen'.

4.9 Flagella – These are thread like structures arising from cytoplasm and extending out through cell wall. They are contractile, about 5 – 20 µ in length and 0.01 – 0.02 µin diameter. All motile bacteria except spirochaetes possess flagella. Chemically they are composed of protein known as flagellin.

Structure – It consist of three parts –

1. Fillament 2. Hook 3. Basal body.

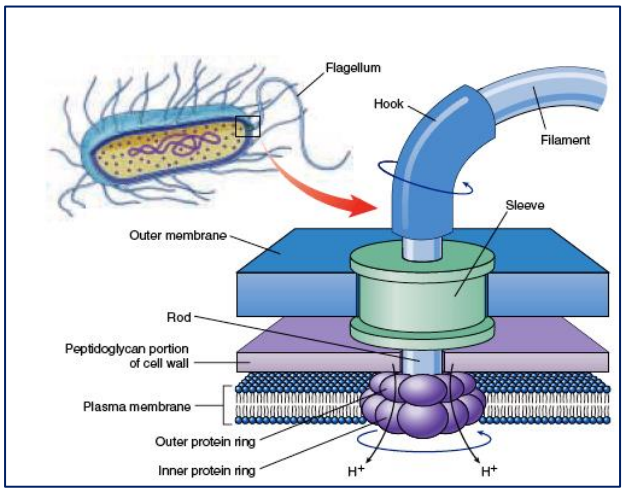


Figure 4.4 Structure of Flagella

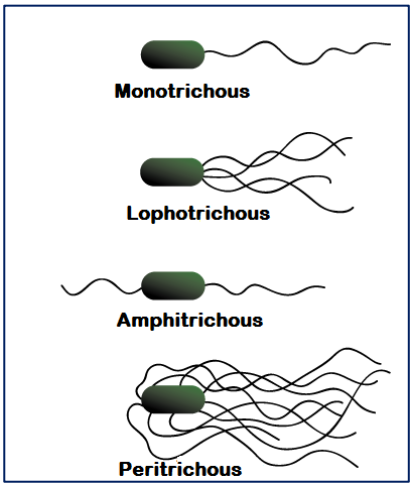


Figure 4.5 Types of Flagella

Types of Flagella

There are 4 different types of flagella as per the arrngements of flagella.

A. Monotrichous – Single flagella at one end. e. g. V. Cholera.
B. Lophotrichous – Tuft of flagella at one or both ends. e. g. Spirilla.
C. Amphitrichous – Single flagella at both ends.e.g. A. faecalis.
D. Peritrichous – Flagella all around the cell. e.g. S. typhi.

Function – Organ of locomotion, responsible for motility. Constitutes the flagellar (H) antigen. Specific flagellar antibodies are produced in high titres in response to antigenic stimulation of flagella. These antibodies are useful in serodiagnosis.

Demonstration –

- Electron microscopy.
- Dark ground illumination.
- Impregnation technique.
- Indirect methods by which motility of bacteria can be seen or demonstrated.

- Spreading type of growth on medium e.g. swarming growth of Proteus species.
- Hanging drop preparation.
- Spreading of bacteria in semisolid agar.e.g. By using Craigie's tube method.

4.10 Pili or Fimbriae - These are hair like appendages projecting from cell surface as straight filaments. They are 0.1 – 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 is having 100 – 500 fimbriae arranged peritrichously. They are antigenic. They are composed of protein called pilin. They are not related to motility and are found on motile as well as non-motile bacteria.

Functions –

- Fimbriae are organ of adhesion.This property enhances the virulence ofbacteria.
- Transfer of genetic material by sex pili.
- Help to form pellicle in liquid media.
- Participate in haemagglutination reaction.

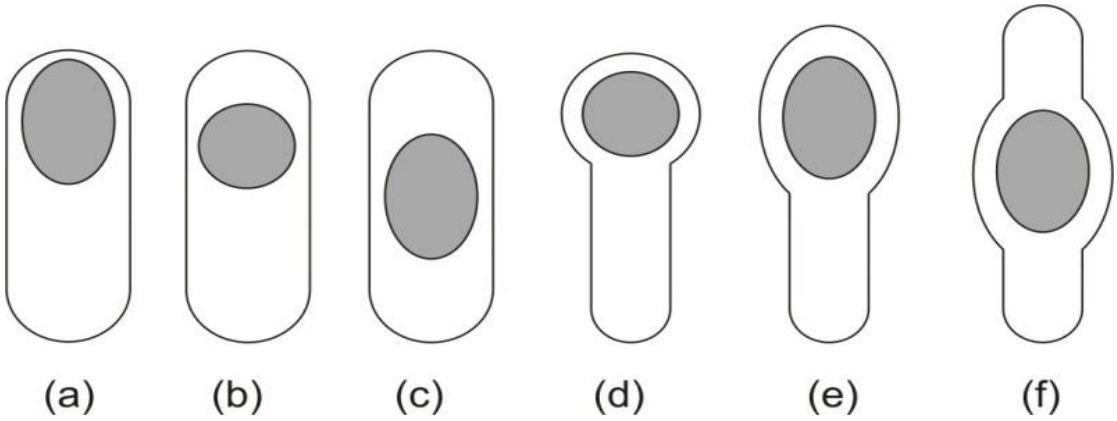
Demonstration

1. Electron microscopy.
2. Haemagglutination.

4.11 Spore- Spores are highly resistant resting phase of bacteria formed during abnormal environmental conditions like depletion of nutrients. As bacterial spores are formed within the parent cell, these are called endospores. Each bacterium forms one spore which on germination forms a single vegetative cell; hence it is not a method of reproduction.

Resistance – They are resistant to boiling, disinfectants and heating. Spores of all medically important bacteria are destroyed by autoclaving at 121˚c for 15 minutes.

Shape and position of spores - Spore may be oval or spherical in shape. Spore may be central, sub-terminal or terminal. Spore may be bulging or nonbulging.



(a)Terminal Oval (b) Subterminal round (c) Central Oval (d) Terminal round Bulging (e) Terminal Oval Bulging (f) Central Oval Bulging

Figure 4.6 Types of spores

Spore forming bacteria -

GenusBacillus – B.anthracis and B.subtilis.

Genus Clostridia – Cl.tetani, Cl.welchii, Cl.botulinum.

Use – Spores of B.stearothermophilus are used as indicator for proper sterilization in process of autoclaving.

Demonstration –

1. Gram Staining – Spores appear as an unstained refractile body within the cell.
2. Modified Ziehl-Neelsen (Z-N) staining - Z.N. stains with 0.25 - 0.5% sulphuric acid as decolouring agent is used for spore staining.

Review Questions:

1. Describe Quellang reaction.
2. Name different types of spores.
3. Acid fast bacilli are detected by which stain?
4. How motility of a bacterium is tested?
5. Note on flagella.

5. Staining of bacteria

Objectives – After reading this chapter, the student will know different staining techniques used to stain bacteria.

5.1 Introduction

Bacteria have nearly the same refractive index as water, therefore when they are observed under a microscope they are opaque or nearly invisible to the naked eye. Different types of staining methods are used to make the cells and their internal structures more visible under the light microscope.

A stain is a substance that adheres to a cell, giving the cell color. The presence of color gives the cells significant contrast so are much more visible. Different stains have different affinities for different organisms, or different parts of organisms. They are used to differentiate different types of organisms or to view specific parts of organisms.

5.2 Staining methods –

a) **Simple staining** (Monochrome staining) – Contains single staining reagent. Dyes such as methylene blue, basic fuchsin are used for staining. They provide colour contrast, but impart the same colour to all bacteria in the smear.

b) **Negative staining -** In this, the background is stained and organisms appear as colourless objects against a dark background. This method is used for demonstration of capsule and spirochaetes. E.g. India ink or nigrosine.

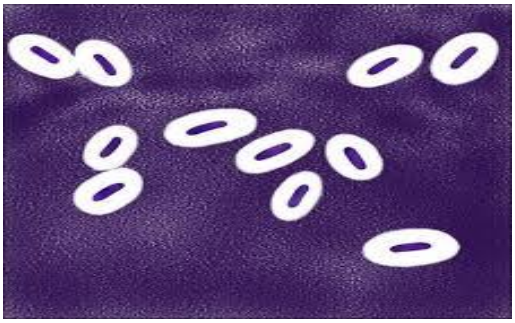


Figure 5.1 Negative Staining

c) **Impregnation technique -** Cells or structures too thin to be seen under the ordinary microscope are made visible by increasing their thickness by impregnating silver on the surface. Such methods are used for the demonstration of spirochetes and bacterial flagella.

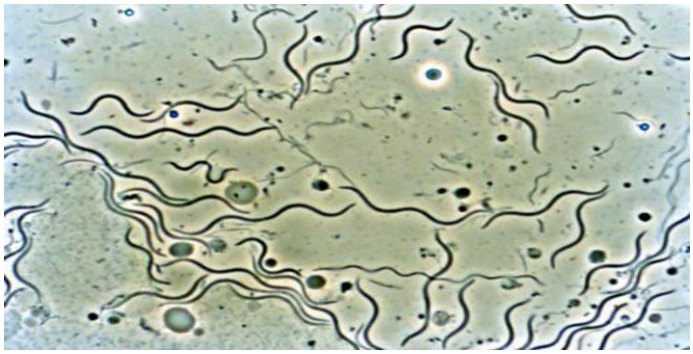


Figure 5.2 Spirochaetes stained with silver impregnation technique

d) Differential staining –These methods impart different colours to different bacteria. The two most widely used differential stains are the Gram stain and Acid Fast stain.

5.3 Preparation and fixation of smear - Prepare smear from clinical specimen, culture colony or broth culture on clean glass slide, allow it to air dry and fix it by flaming.

Precautions –

- Label the slide with grease pencil.
- Ifspecimen is thick, put drop of the sterile distilled water or normal saline on the slide with the help of sterile loop and then transfer specimen in it to make smear.
- Specimens taken on cotton swab are directly smeared on slide.
- If specimen material is poor in cells e.g. C.S.F. it is first centrifuged and smearis prepared from sediment.
- Smears are first left to air dry and then they are fixed by passing the slide quickly through a flame 2 – 3 times only.

5.4 Gram staining - It is the most commonly used method devised by the histologist Christian Gram (1884) to stain bacteria in tissue.

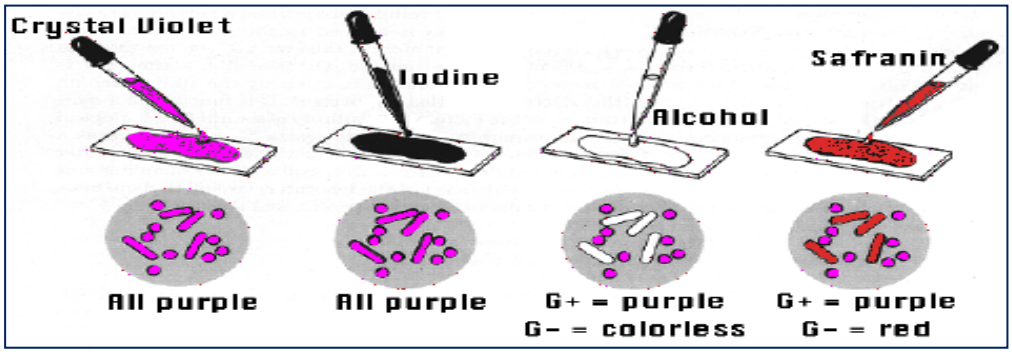


Figure 5.3 Gram Staining Technique

Procedure –

Prepare smear from clinical specimen, culture smear of colony or broth culture on a clean glass slide, allow it to air dry and fix it by flaming.

- **Staining –** Cover smear with dyes such as crystal violet, methyl violet or gentian violet to smear. Allow it to act for 30 – 60 seconds.
- **Application of mordant** – Pour of the primary stain& apply Gram's iodine for 1 minute and wash with water.

- **Decolourization** – Decolourise the smear with an organic solvent-absolute alcohol, acetone or aniline for 10 –30 seconds , until the colour oozes from slide.
- Wash with water.
- **Counterstaining** – Counter stain with a dye of contrasting colour-safranin, carbol fuchsin or neutral red for 15 – 30 seconds.
- Wash with water and blot dry.
- Observe the slide under 100 X after putting a drop of oil.

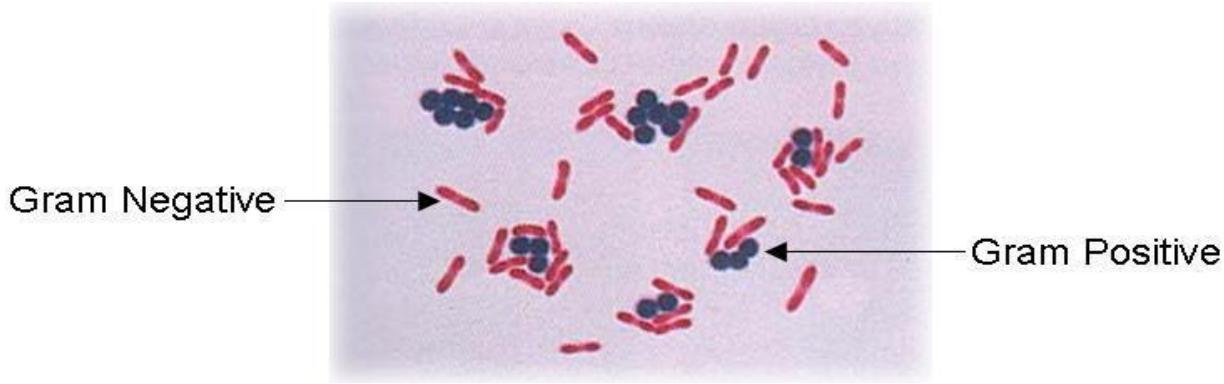


Figure 5.4 Gram staining

Observation - Gram positive- Voilet colour

Gram negative- Pink colour

The Gram positive may appear Gram negative because of

- Prolonged decolourization.
- Damage to cell wall.
- Death of bacteria.
- The Gram negative may appear Gram positive because of inadequate decolourization.

Mechanism of Gram reaction -

- **Acidic protoplasm theory** – According to this theory, as compaired to Gram negative bacteria, Gram positive bacteria have more acidic protoplasm and the primary stain used is basic in nature. Hence, Gram positive bacteria retain the primary stain more strongly than

Gram negative bacteria. Also the iodine makes the cytoplasm more acidic and acts as a mordant, increasing the attraction of the primary stain to the cell cytoplasm. It thus helps to fix the stain in bacterial stain.

- **Lipid content theory** – According to this theory, the lipid content of cell wall is more in Gram negative bacteria and less in Gram positive bacteria. During Gram reaction there is a formation of primary stain- iodine complex in both Gram positive and Gram negative bacteria. However, when alcohol is applied, the lipid in the cell wall of Gram negative bacteria gets dissolved in alcohol, leading to an increase in the pore size through which the dye- iodine complex diffuses out during the process of decolourization. On the other hand, the dye – iodine complex gets trapped within the Gram positive cells because of fewer lipids and they thus retain the violet colour.
- **Cell wall permeability** – The cell wall of Gram positive bacteria contain more mucopeptide because of which it is thicker and stronger, hence dye – iodine complex does not come out of the Gram positive cell. However, in Gram negative bacteria mucopeptide is less and therefore their cell wall is relatively less strong and thin, hence dye – iodine complex diffuses out of cell freely and they take up the colour of the counter stain.
- **Magnesium ribonucleate theory** – According to this theory magnesium ribonucleate is present in Gram positive bacteria which help to retain primary stain. This magnesium ribonucleate is absent in Gram negative bacteria.

Uses – Gram staining is an essential procedure used in identification of bacteria. Gram positive and Gram negative bacteria differ in their growth requirements, susceptibility to antibiotics and pathogenicity.

5.5 Acid Fast Stain (Ziehl-Neelsen Stain) - **The** acid fast stain was discovered by Ehrlich andsubsequentlymodified by Ziehl and Neelsen. Staining of Mycobacteria (usuallyTubercle& lepra bacilli) is done by this technique. These bacteria resist decolourisation with acid. They is relatively impermeable and resistant to simple stains.

Procedure –

- Prepare smear on the clean glass slide, allow it to air dry and fix it by flaming.
- **Primary staining** – Cover the smear with filtered carbol fuchsin and heat until steam rises. Do not boil. Heating is necessary for penetration of stain into the cell wall.
- Wash with water.
- **Decolourization -** Cover the slide with 20% sulphuric acid (H_2SO_4). The red colour of the smear changes to yellowish brown. In case of lepra bacilli 5 % H_2SO_4 is used as M.Leprae are less acid fast.
- Wash with water and observe the colour of the smear, if it is red, repeat decolourization till it becomes yellowish brown.
- Wash with water.
- Apply 90 % alcohol for 2 minutes for decolourization (this step is optional and may be omitted, if specimen is not urine).
- Wash with water.
- **Counter staining** – Counter stain with methylene blue or malachite green for 1 – 2 minutes.
- Wash, blot dry and observe under 100 X after putting a drop of oil.

Observations –

- Acid fast bacilli appear bright red.
- Pus cells, epithelial cells and other organisms appear blue or green.

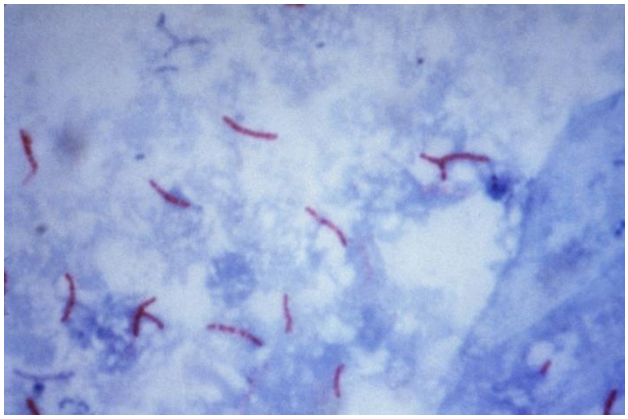


Figure 5.5 M. Tuberculosis in sputum

Mechanism of acid-fast-stain

- Acid-fast organisms are not easily stained because they are coated with lipids, fatty acids and higher alcohols in their cell wall.
- Mycolic acid (waxy substance) present in the cell wall does not allow the stain to penetrate easily inside these organisms.
- In Ziehl-Neelsen's (Z-N) staining, carbol fuchsin (basic fuchsin + phenol), a phenolic solution of basic fuchsin, is applied with heat. Heat and phenol facilitate penetration of the dye.
- Subsequently, when decolourised by acid, the dye does not come out because it is soluble in phenol and phenol is more soluble in lipid substances (mycolicacid) hence, there is no decolourization and they retain the colour of the basic fuchsin (primarystain).

  The positive findings are reported as follows-

Number of acid fast Bacilli in 10 fields	Report
None	Absent
1 – 2	Positive +
2 – 10	Positive ++
10 – 100	Positive +++
Above 100	Positive ++++

5.6 Albert staining - This staining technique is specifically used for the identification of Corynebacterium diphtheriae. It demonstrates the presence of metachromatic granules found in C.diphtheriae which provides provisional diagnosis for diphtheria. Smears of throat swabs and occasionally nasal secretions are taken for examination.

Reagents –

- **Solution A** : Albert's stain

  Toluidine blue 0.15 g

  Malachite green 0.20 g

  Glacial acetic acid 1 ml

  Alcohol (95 %) 2 ml

  Distilled water 100 ml

- **Solution B** : Albert's Iodine stain

  Potassium iodide 3 g

  Iodine 2 g

  Distilled water 300 ml

Procedure –

- Cover the heat fixed smear with solution A (Albert's stain) for 5 minutes.
- Drain the stain but do not wash.
- Flood with solution B (Albert's Iodine stain) and keep for 1 to 2 minutes.
- Wash carefully under running tap water, drain, blot dry and examine under oil immersion objective.

Result:

- Metachromatic granules: bluish black.
- Bacillary body : green or bluish green

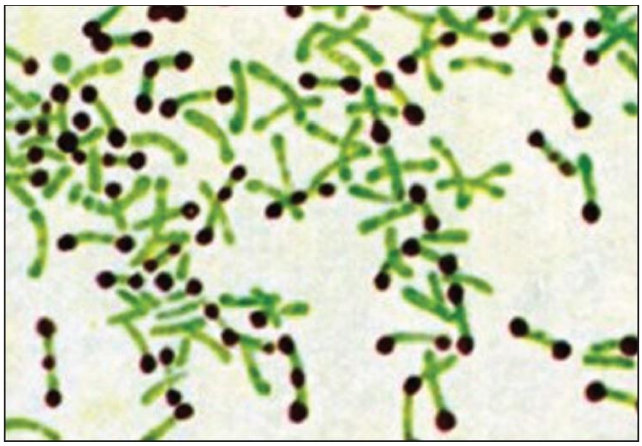


Figure 5.6 Albert's staining

5.7 Staining of flagella or Spirochaetes

Principle: Since heat denatures the spirochetes, a chemical fixative such as absolute alcohol is used as a fixative. Afterwards silver impregnation method of Fontana is used. Deposition of silver salt in the organism makes them visible microscopically.

Reagents –Fixative

- Glacial acetic acid: 1 ml
- Formalin : 2 ml
- Distilled water to : 100 ml

Mordant

- Phenol : 1 g.
- Tannic acid : 5 g.
- Distilled water : 100 ml.

Ammoniated silver nitrate –

a) 10 % (v/v) ammonia.

b) 0.5 % (w/v) silver nitrate.

Add solution (a) to solution (b) until the precipitate formed just dissolves. Add solution (b) dropwise till the precipitate returns. The precipitate should not redissolve again.

Procedure –

- Make a smear of bacterial suspension or tooth tarter in a drop of normal saline.
- Treat the film three times (30 seconds each) with the fixative.
- Wash off with absolute alcohol and flood with alcohol for about 3 minutes.
- Drain alcohol completely and air dry the smear.
- Flood the smear with mordant. Heat it till steam rises for 30 seconds.
- Wash the smear under running tap water. Dry the smear completely.
- Treat with ammoniated silver nitrate. Heat till steam rises for 30 seconds (The film should appear brown).
- Wash in distilled water and dry in air.
- Mount &observe under oil immersion lens,

Result –

a) Spirochaetes : Brownish black.

b) Background : Brownish yellow.

5.8 **Staining of bacterial spore**

Method - Schuffer and Fulton's method.

Requirements –

- 5 % (w/v) malachite green.
- 0.5 % (w/v) safranin.
- A given culture.

Procedure –

- Prepare a smear on glass slide, dry it in air and fix it by heat.

- Cover the smear with malachite green stain and heat gently (till steam rises).
- Wash under running tap water.
- Cover the smear with safranin for 1 – 2 minutes.
- Wash under running tap water. Dry the smear in air and observe under oil immersion lens.

Results –

- Spores : Green colour
- Cells : Red colour.

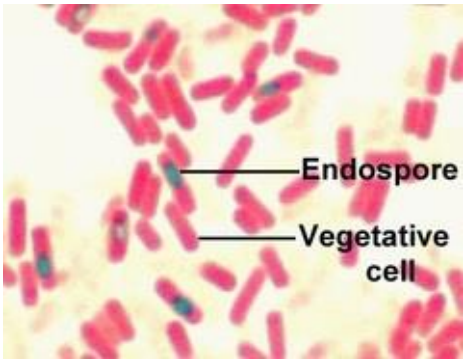


Figure 5.6 Spore staining - Schuffer and Fulton's method

Review Questions

1. Describe Gram staining.
2. Desribe the teories of Gram's staining?
3. Describe acid fast staining.
4. Composition of solution A and B of Albert stain.
5. Describe flagellar staining.
6. How will you gradeAFB positive sputum sample?

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6. Bacterial Physiology

Objectives –After reading this chapter student will understand about bacterial nutrition, gaseous requirements, temperature requirement & other physiological requirements of bacteria. Student will have knowledge ofsalient features of the bacterial growth curve. This knowledge is essential for growing bacteria in the laboratory.

6.1 Introduction – For the study of a bacterial species it is necessary to grow the bacteria and for that we must know its growth requirements. Two important conditions must be fulfilled.

a) Suitable nutrition should be supplied.
b) Physical environmental conditions should be optimum.

6.2 Bacterial nutrition – The principal constituent of bacterial cells is water which forms 80 % of the total weight of the cells. Proteins, polysaccharides, lipids, nucleic acids, mucopeptides and low molecular weight compounds make up the rest.

For growth and multiplication of bacteria the minimum nutritional requirements are-

- Water.
- Source of carbon.
- Source of nitrogen.
- Inorganic salts - sulphur, phosphorus, sodium, potassium, magnesium, iron, manganese, calcium and cobalt.

Bacteria are classified into two major groups depending on growth requirements –

1. Autotrophs – Bacteria that can synthesize all their organic compounds. They are medically less important.
2. Heterotrophs – Nutritional requirements of bacteria vary widely and are medically important.

These are again divided into two groups-

(i) Nonexacting bacteria or Nonfastidious – These are bacteria whose nutrition requirements are minimum or basic and are able to synthesize all their structural units from carbon, nitrogen and inorganic salts. Examples are Pseudomonas and Staphylococcus aureus.

(ii) Exacting bacteria or Fastidious – Bacteria which require more than the basic nutrition are called Exacting bacteria or fastidious bacteria. Examples are Genus Streptococcus and Neisseria.

6.3 Basic nutritional requirements –

1. **Water**

   It is the most important requirement because it is the principal constituent of a bacterial cell. It constitutes about 80 % of the total weight.

   It is vehicle for the entry of all nutrients into the cells and for the elimination of all waste products.

   It participates in metabolic reaction.

   It forms an integral part of the protoplasm

2. **Inorganic salt**-These are required for osmotic regulation and to provide traceelements essential certain enzyme systems.

3. **Growth factors**

   These include vitamins, purines, pyrimidines, amino acids, etc.

4. **Moisture and desiccate-** Moisture is an absolute requirement for growth. The capacity to survive in dry environment varies from organism to organism. Some bacteria like Gonococci and T.pallidium die quickly in dry conditions, where as Staphylococcus aureus and tubercle bacilli can survive drying for weeks and months.

5. **Gaseous requirements** –

(a) Oxygen – Depending upon the influence of oxygen on growth viability, bacteria are divided into aerobes, anaerobes and microaerophilic bacteria.

- **Aerobic:** These bacteria require oxygen for their growth. They may be divided into-
  (i) Obligate aerobes- Obligate aerobes like, Vibrio cholera grow only in the presence of oxygen. They will not grow if oxygen is not provided.
  (ii) Facultative anaerobes- They are ordinarily aerobic but can grow in the absence of oxygen though less abundantly. Most bacteria of medical importance belong to this group.
- **Anaerobic bacteria**: These bacteria grow only in the absence of oxygen. They cannot grow if oxygen is present. Obligate anaerobes like, Genus Clostridia die on exposure to oxygen.
- **Microaerophilic** – These bacteria are those that grow in the presence oflow oxygen tension.

(b) Carbon Dioxide –

- Bacteria require small amount of carbon dioxide for their growth.It is obtained from atmosphere or CO2 is produced endogenously by bacteria during metabolism.
- A few bacteria require additional carbon dioxide (5 – 10 %) for their growth, e.g. Brucella abortus, Neisseria sp., Pneumococci etc.
- These bacteria which require additional carbon dioxide are known as capnophilic bacteria.

6. **Temperature -** Bacteria vary in their requirement of temperature for growth. For each species, there is a ‘temperature range’, and

growth does not occur above the maximum or below the minimu of this range. The temperature at which growth occurs best is known as the optimum temperature, which in the case of most pathogenic bacteria is 37˚C. Bacteria which grow best at temperatures of 25ºC - 40˚C are called mesophilic. All parasites of warm blooded animals are **mesophilic.** Within the group of mesophilic bacteria, some like Pseudomonas aeruginosa have a wider range (5ºC - 43˚C), while others like the gonococcus have a restricted range (30ºC - 39˚C). **Psychrophilic** bacteria are those that grow best at temperatures below 20˚C. They are soil and water saprophytes. Another group of nonpathogenic bacteria, the **thermophiles** grow best at high temperatures, 55ºC - 80˚C.

7. **PH -** The growth and multiplication of bacteria is affected by pH of the medium. Most pathogenic bacteria grow best (optimum pH) at a neutral or slightly alkaline pH (7.2 – 7.6). Some bacteria grow at acidic pH, e.g. Lactobacillus sp. and are known as acidophilic. Vibrio cholera grows best in alkaline medium.
8. **Light –** Bacteria grow well in dark. They are sensitive to ultraviolet rays and other radiations. Photosynthetic bacteria require light and photochromogenic mycobacteria produce pigment only when exposed to light.
9. **Osmotic pressure –** Bacteria are very tolerant of changes in the osmotic pressure of their environment because of the strength of their cell wall. If a bacteria is suddenly exposed to solutions of higher salt concentration there is shrinkage of the protoplasm and water travels outwards. This happens more readily in Gram negative than in Gram positive bacteria. This is called Plasmolysis. Sudden transfer of a bacteria from a concentrated to a weak solution or to distilled water may cause Plasmoptysis.

This means swelling and bursting of the cell as a result of excessive Osmotic taking in of water.

6.4 Bacterial Growth Curve

When a bacterium is grown in a suitable liquid medium and Incubated, the growth follows a definite pattern. Ifbacterial counts are made at intervalsafter inoculation and plotted in relation to time, a growth curve is obtained. Two types of growthcurve can be drawn according to the measurement of cell numbers that are used.

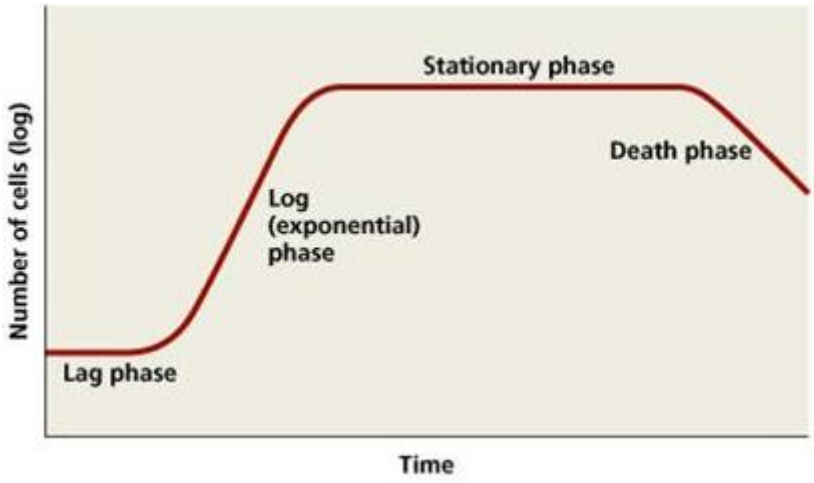


Figure 7.1 Bacterial growth curve

Total Count –This is based on the number of cells present, whether living or dead. Living cells are those that divide. Dead cells are those that do not divide.

Viable Count –This measures only those cells capable of growing and henceproducing a colony. Four main phases are recongnized in the growthcurve:

- Lag.
- Log.
- Stationary.

- Death/Decline.

Lag Phase –

- There is no multiplication of cells.
- There is increase in size of the cell.
- There is more metabolic activity.
- The duration of this phase is dependent on the time taken for the organism to adapt itself to the new growth environment. In this period the bacteria builds up necessary enzymes and metabolic intermediates in adequate quantities for multiplication to proceed. Maximum cell size is achieved towards the end of the lag phase.

Log Phase –

- The cells divide at a constant rate.
- The cell numbers increase exponentially and a straight line is obtained in the graph.

Stationary Phase –

- After some time, exponential growth is no longer possible and the rate of multiplication decreases and then stops when the cells pass into the stationary phase. This is because of exhaustion of nutrients or accumulation of toxic waste end products.

Phase of Decline –

- This is the phase when the population decreases due to death of cells.
- In the death or decline phase, the total count remains the same but the viable count decreases.

Review Questions

1. Differentiate between autotroph and heterotrophs.
2. What are fastidious and nonfastidious bacteria?
3. Describe bacterial growth curve.

4. What is total and viable count?
5. Note on nutritional requirement of bacteria.

*****

7. Cultivation of Microorganisms

Objectives: Students get an idea about different types of culture media and how to prepare them.

7.1 Introduction-To identify bacteria which give rise to a disease it is essential to grow the bacteria from the infected material. Only after growing them and isolating them they can be identified. If ideal conditions

are provided the bacteria can grow very rapidly and the growth can beobserved in a laboratory. The substances which are used for the bacterial growth are called as “Culture Media.”

7.2 Need for culturing bacteria

1) Isolating a bacterium from sites in body normally known to be sterile is an indication of its role in the disease process.
2) Culturing bacteria is also the initial step in studying its morphology and its identification.
3) Bacteria have to be cultured in order to obtain antigens from developing serological assays and vaccines.
4) Certain genetic studies and manipulations of the cells also need that bacteria be cultured in vitro.
5) Culturing on solid media is another convenient way of separating bacteria in mixtures.

7.3 Basic Requirements of a Culture Medium

Since most of the bacteria or pathogenic, the culture media should have composition and reaction similar to that of the tissues and body fluids. The bacteria vary in their nutritional requirements. The basic requirements of a culture media are –

1. Nutrients
    - Energy Source
    - Carbon Source
    - Nitrogen Source
2. Mineral Salts – Sulphate, Phosphate, Chlorides and Carbonates of K, Na, Mg, Fe and Ca.
3. A suitable pH, usually 7.2 to 7.6
4. Adequate Oxidation-Reduction potential (Eh)

5. Accessory Growth Factors
    - Tryptophan for S. Typhi
    - Glutathione for Gonococci
    - X & V factors for H. influenza

7.4 The Characteristics of an ideal culture medium are

1. It must give a satisfactory growth from a small inoculums;
2. It should give a rapid growth;
3. It should be easy to prepare;
4. It should be reasonably cheap;
5. It should be readily reproducible;
6. It should be able to demonstrate all the characteristics of the bacteria from the culture medium.

7.5 Classification Culture media

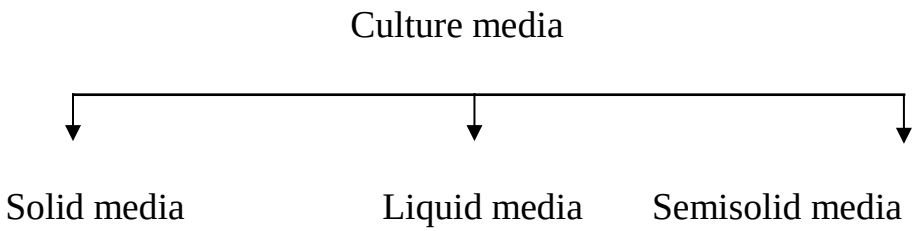
Numerous culture media have been devised. The original media used by Louis Pasture were liquids such urine or meat broth. However, liquid media have their uses, and disadvantages, too. For example, for obtaining bacterial growth from blood or water when large volumes have to be tested, and for preparing bulk cultures of antigens or vaccines.

While bacteria grow diffusely in liquids, they produce discrete visible growth on solid media. On solid media, bacteria have distinct colony morphology and exhibit many other characteristic features such as pigmentation or haemolysis, making identification easy. The earliest solid medium was cooked-cut-potato used by Robert Koch. Later he introduced gelatin to solidify liquid media but it was not satisfactory as gelatin is liquefied at 24^0C and also many proteolytic bacteria. The use of agar to

solidify culture media was suggested by Frau Hesse, the wife of one of lab assistant.

Culture media classification:

(1) Classification based on the consistency:

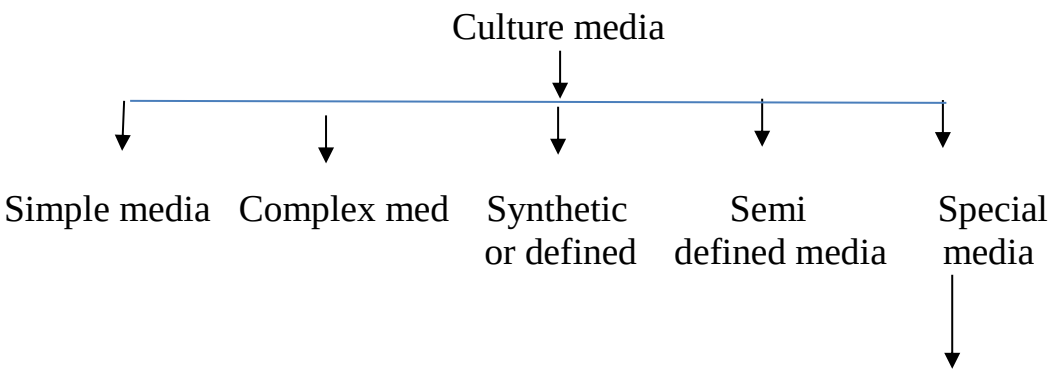


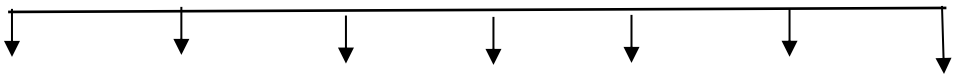
(a) Liquid Media: They are fluid in nature, usually placed in test tubes. For example nutrient broth. In liquid medium, bacteria grow uniformly producing general turbidity.

(b) Solid Media: These are prepared by adding solidifying agents like gelatin and agar to the liquid medium. For example, nutrient agar.

(c) Semi solid Media: Such media are fairly soft and are useful in demonstrating bacterial motility and separating motile from non-motile strains.

(2) **Classification based on Nutritional Component.**





Enriched Enrichment Selective Indicator Transport Differential Sugar

Classification based on Oxygen Requirement

- Aerobic Media
- Anaerobic Media

1. **Simple media [Basal media]:** These are simple media contains only basic substances i.e CO_2, N & minerals. An example is nutrient broth. It consists of peptone, meat extract, sodium chloride and water. Nutrient agar prepared by adding 2% agar to nutrient broth is the simplest and most common medium in routine diagnostic laboratories.
2. **Complex Media:** Exact chemical composition of ingredients of culture medium is not known. Widely used for the cultivation of bacteria for diagnostic purposes.
3. **Synthetic or Defined media:** These media are prepared from pure chemical substances and hence exact composition of the medium is known. These media are used for various special studies such as metabolic requirements. e.g. Dubo's medium
4. **Semidefined media:** The Composition is approximately known. Simple peptone water medium, 1% peptone with 0.5% NaCl in water are few examples.
5. **Enriched Media:** Substances such as blood, egg, serum are added to the basal medium.Used to grow bacteria which are more exacting in their nutritional needs.e.g. Blood agar, Chcolate agar.
6. **Enrichment Media:** Used for obtaining the desired bacteria in more numbers from the mixed culture. They containsubstances that stimulates the desired bacteria or has inhibitory substances that inhibits

the growth of unwanted bacteria. E.g. Selenite F broth and Tetrathionate broth for S. Typhi.

7. **Selective Media:** This media enables a greater number of the required bacteria to form colonies than the other bacteria. E.g. L-J medium for the selective isolation of M. tuberculosis or Desoxy cholate agar for Salmonella and Shigella.
8. **Indicator Media:** this media contains an indicator which changes colour when a bacterium grows in them. E.g. Wilson and Blair medium for S. Typhi.
9. **Differential Media**: A medium which has substances incorporated in it, enabling it to bring out differing characteristics of bacteria and thus helping to distinguish between them. E.g. MacConkey agar – consists of peptone, lactose, agar, neutral red and taurocholate – differentiates between lactose fermenters and non-lactose fermenters.
10. **Sugar Media:** they contain 1% of the sugar in peptone water along with an appropriate indicator. A small tube called Durham's tube is kept inverted in the sugar tube to detect gas production.
11. **Transport Media:** This media is used in case of delicate organisms which may not survive the time taken for transporting the specimen to the lab or may be overgrown by nonpathogens E.g. Stuart's medium.

7.6 Commonly used laboratory culture media:

1. Nutrient Agar:

It is prepared by adding 2% agar to nutrient broth is the simplest and most common medium in routine diagnostic laboratories. If the concentration of agar is reduced to 0.2-0.5%; Semisolid or sloppy agar is obtained which enables motile organism to spread. Increasing

Figure7.1

the concentration of agar to 6% prevents spreading or swarming by organism such as proteus.

2. **Mac Conkey's Agar :**

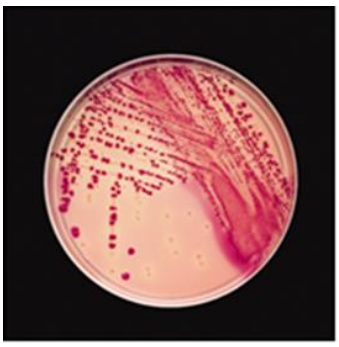


Figure 7.2

Mac Conkey's medium is an example of differential medium Mac Conkey's medium has different substances incorporated in it, enabling it to bring out different characteristics of bacteria and thus helping to distinguish between them. The medium is composed of peptone lactose, agar, netural red and taurocholate shows up lactose fermenters as pink colonies, while non lactose fermenters are colourless or pale. This medium is also termed as indicator medium.

3. **Blood Agar :**

Figure 7.3

Blood agar is an example of enriched media. In enriched media, substances such as blood, serum, or egg are added to a basal medium. These media are used to grow bacteria which are more exacting in their nutritional needs. Other examples of enriched media are chocolate agar and egg media.

<u>Special media:</u>

a. **Desoxycholate Citrate Agar [DCA]** is an example of selective medium. In selective media, substances which have a stimulating effect on the bacteria to be grown or an inhibitory effect on those to be suppressed

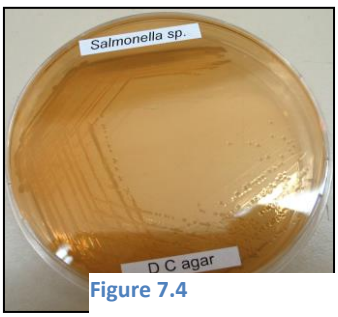


Figure 7.4

are incorporated. For example, deoxycholate citrate medium for dysentery & salmonella bacilli.

b. **Lowenstein Jensen [L-J] medium:** Lowenstein Jensen Medium (L. J. Medium) is used for the isolation and cultivation of Mycobacterium species. It was originally formulated by Lowenstein, containing congo red and malachite green dyes. Jensen modified Lowensteins medium by altering the citrate and phosphate contents, eliminating the congo red dye and by increasing the malachite green concentration. Penicillin and Nalidixic acid along with malachite green prevents growth of the gram positive and gram negative bacteria.

Figure 7.5

c. **Eosin methylene blue agar (EMB):**
It is a selective and differential medium used to isolate fecal coliforms. Eosin Y and methylene blue are pH indicator dyes which combine to form a dark purple precipitate at low pH; they also serve to inhibit the growth of most Gram positive organisms. Sucrose and lactose serve as fermentable carbohydrate sources which encourage the growth of fecal coliforms and provide a means of differentiating them.

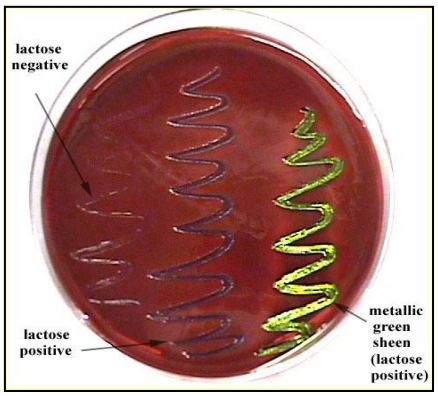


Figure 7.6

Transport Media

Stuart's medium: It is an example of transport medium. In case of delicate organism [for Example, gonococci] which may not survive the time taker for transporting the specimen to the laboratory or may be overgrown by non pathogens [such as dysentery or cholera organism in faeces], special media are devised for transporting such specimens. These are termed as transport media, for example, stuart's medium a non-nutrient soft agar gel containing a reducing agent to prevent oxidation, and charcoal to neutralise certain bacterial inhibitors for gonococci and buffered glycerol saline for enteric bacilli.

Anaerobic media

Robertson's cooked meat medium: It is an example of anaerobic medium. Place meat in 1 ounce bottles to the depth of 2.5 cms and cover it with 15 ml of broth. Autoclave at 120^0C for 20 min. After sterilization, adjust the pH to 7.5. It is used to cultivate the anaerobic bacteria, for example, Clostridium - Cl. Tetani, Cl. perfringes.

Figure 7.7 Robertson's cooked meat medium:

7.7 Quality control:

Culture media used to be prepared in laboratories themselves starting with basic ingredients. Not only is this Laborious but it also leads to considerable batch variation in quality of media with the ready availability of commercially dehydrated culture media. The process of media making has become simpler and its quality control move uniform.

Review Questions:

1. What are the uses of liquid media?
2. Why gelatin is not useful for preparing solid media?

3. What to you understand by synthetic or defined media?
4. What is the chief constituent of agar?
5. What is the nutrient value of agar?
6. What are the contents of peptone?
7. Why blood agar is an enriched media?
8. Desoxycholate citrate agar [DCA] is an example of?
9. Enumerate any 3-4 transport media.
10. What are the requirements culture media?
11. Write about Lowensten-Jensen medium?
12. What do you understand by the term 'Quality Control'?

****

8. Various Culture Methods

Objectives – After reading this chapter, the student will know different culture methods including anaerobic culture methods of cultivating bacteria.

8.1 Introduction: Culture methods employed depend on the purpose for which they are intended. The methods of culture used ordinarily in the laboratory are:

1. Streak
2. Lawn;
3. Stroke
4. Stab;
5. Pour plate
6. Liquid culture;
7. Anaerobic culture method.

8.2Different culture methods:

1. **The streak culture** [surface plating]: This method is routinely employed for the isolation of bacteria in pure culture from clinical specimens. One loopful of culture is made as a primary inoculum and is then distributed thinly over the plate by streaking it with the loop in a series of parallel lines in different segments of the plate. Loop flamed and cooled between the different sets of streaks. On incubation growth may be confluent at the site of the original inoculation but becomes progressively thinner and well separated colonies are obtained over the final series of streaks. A platinum loop is charged with the specimen to be cultured. Loops for routine work are made of Nichrome resistance wire [24 SWG size] is used in place of platinum loop, owing to the high cost of platinum.

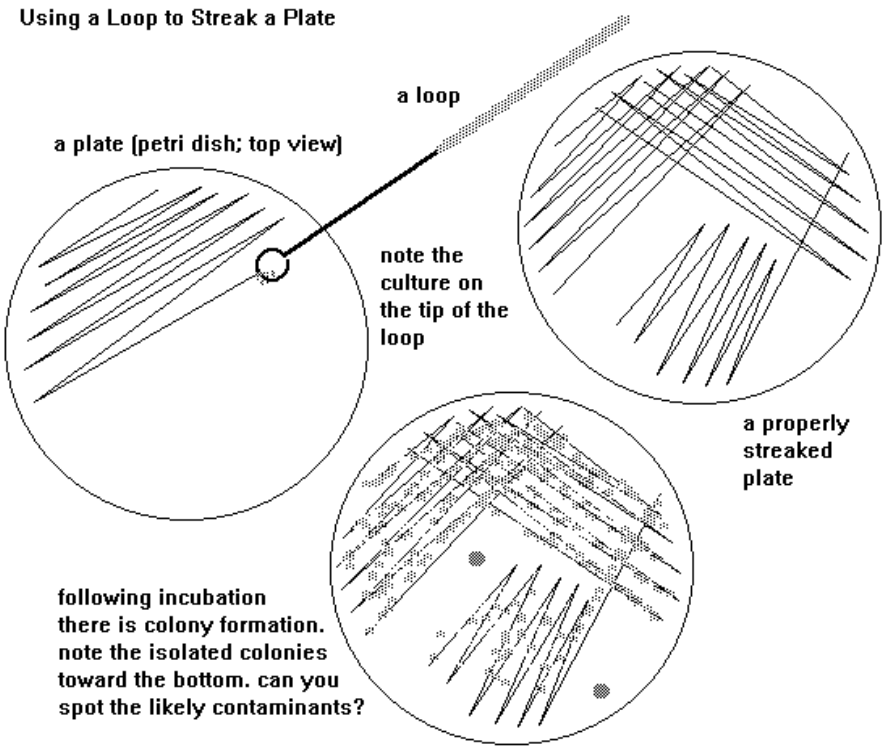


Figure8.1

2. The lawn Culture:

Figure 8.1 Lawn Culture

Also called as carpet culture. Itprovides a uniform surface growth of the bacterium and is useful for bacteriophage typing and antibiotics sensitivity testing [disc method]. Lawn cultures are prepared by flooding the surface of the plate with a liquid culture or suspension of the bacterium and incubating the plate. Alternatively, the surface of the plate may be inoculated by applying a swab soaked in the bacterial culture or suspension. Lawn or carpet cultures are employed when a large amount of growth is required on solid media, for example, in the preparation of bacterial antigens and vaccines.

3. **The stroke culture:** The mediumin tubes contains agar slope [Slant] and is employed for providing a pure growth of the bacterium for slide agglutination and other diagnostic tests.

4. **Stab cultures:** Stab cultures are prepared by puncturing a suitable medium such as nutrient gelatin or glucose agar with long, straight charged wire. The medium is allowed to set, with the tube in the upright position, providing a flat surface at the top of the medium. Stab cultures are employed mainly for demonstration of gelatin liquefaction and oxygen requirement of the bacterium under study. They are also used in the maintenance of stock cultures.

Figure 8.2

5. **Pour plate culture:** For preparing pour plate culture, tubes containing 15 ml of the agar medium are melted and left to cool in a water bath at 45-50^{0}C. Appropriate dilution of the inoculum [1ml] is added to molten agar, mixed well and the contents of the tubes poured into sterile Petri dishes and allowed to set. After incubation, colonies will be seen well distributed throughout the depth of the medium and can be enumerated using colony counters.

   The pour plate method gives an estimate of the viable bacterial count in a suspension and is the recommended method for quantitative urine culture.

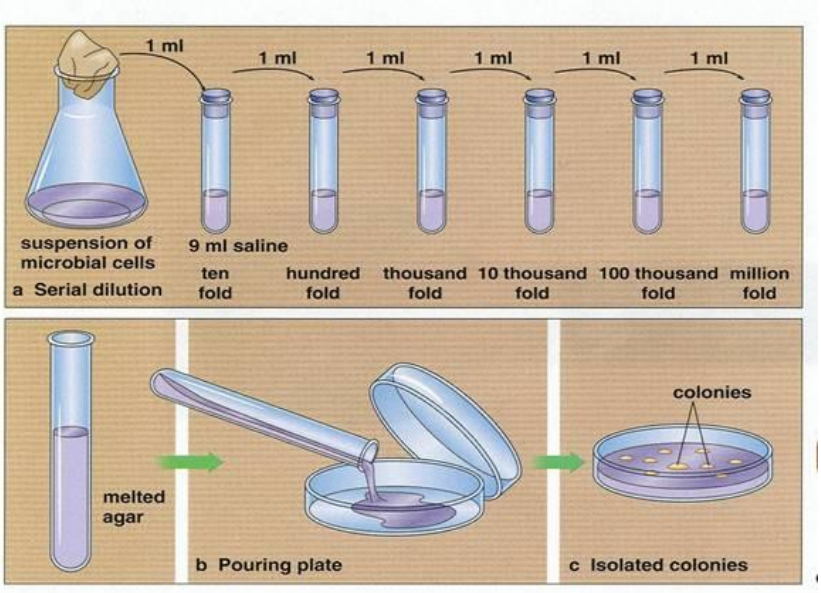


Figure 8.3

6. **Sweep plate method:** In the sweep plate method, the edges of the Petri dishes containing the culture medium are rubbed over the fabric, with the medium facing it. The dust particles stirred up from the cloth plate settle on the culture medium, and colonies develop on incubation. They can be counted and estimate made.

7. **Liquid cultures:** Liquid cultures in tubes, bottles or flasks may be inoculated by touching with a charged loop or by adding the inoculums with pipettes has or syringes. Large inocula can be employed in liquid cultures and hence this method is adopted for blood culture and for sterility tests, where, the concentration of bacteria in the inocula is expected to be small. Liquid cultures are preferable for inocula containing antibiotics and other inhibitory substances, as these are rendered ineffective by dilution in the medium. Liquid cultures are also prepared when large yields

Figure 8.4

are desired the yield being enhanced by agitation, aeration addition of nutrients and removal of toxic metabolites [continuous culture methods].

The major disadvantage of liquid culture is that it does not provide a pure culture from mixed inocula.

8.3 Anaerobic Culture Methods

Anaerobic bacteria differ in their requirement of and sensitivity to oxygen. Some, such as clostridium [Cl.] histolyticum, are aero tolerant and may produce some growth on the surface of aerobic plates, while others such as Cl. tetani, are strict anaerobes and form surface growth only if the oxygen tension is less than 2 mm Hg. A number of methods have been described for achieving anaerobiosis; some of which are listed and described:

1] Exclusion of oxygen or production of vacuum;

2] Displacement of oxygen with other gases;

3] Absorption of oxygen by chemical or biological method; and

4] Reduction of oxygen.

1] Displacement of oxygen: Displacement of oxygen with gases such as Hydrogen, Nitrogen, Helium or Carbon-di-oxide is sometime employed, but this method rarely produces complete anaerobiosis.

Figure 8.5

A popular, but ineffective method is the candle jar. Inoculated plates are placed inside a large airtight container and a lighted candle kept in it before the lid is sealed. The burring candle is expected to use up all the oxygen inside before it is extinguished, but some oxygen is always left behind. The candle jar provides a concentration of carbon dioxide which stimulates the growth of most bacteria.

2] **Chemical method:** In chemical method alkaline pyrogallol absorbs oxygen. This method, first introduced by Buchner [1888], has been employed with different modification for producing anaerobiosis. Pyrogallic acid added to a solution of sodium hydroxide in a large test tube placed inside an airtight jar provides anaerobiosis but a small amount of carbon monoxide, which is formed during the reaction, may be inhibitory to some bacteria. The method has been applied to single tube and plate cultures.

3] **Biological methods:** Absorption of oxygen from small closed system has been attempted by incubating along with aerobic bacteria, germinating seeds or chopped vegetables. Anaerobiosis produced by such biological methods is slow and ineffective.

4-a] McIntosh-fields'anaerobic Jar:

This is the most reliable and widely used anaerobic method. It ensures complete anaerobiosis but carries the risk of explosion, which may rarely occur. Risk can be eliminated by modification the catalyst, for example, Allumina pellets coated with palladium in a gauge sachet.

Figure 8.6

4-b] Gaspak method: This is the method of choice for preparing anaerobic jars at present. The gaspaks are commercially available as disposable envelopes, containing chemicals which generate hydrogen and carbon dioxide on the addition of water. After the

Figure 8.7

inoculated plates are kept in the jar, the gaspak envelope, with water added is placed inside, and the lide screwed tightly. Hydrogen and carbon-di-oxide are liberated and the presence of a cold catalyst in the envelope permits the combination of hydrogen and oxygen to produce an anaerobic environment. The gaspak is simple and effective, eliminating the need for drawing vacuum and adding hydrogen.

Figure 8.7

An indicator is employed for verifying the anaerobic condition in the jars. Reduced methylene blue is generally used for this purpose. Methylene blue remains colorless anaerobically but turns blue on exposure to oxygen.

Reduction of oxygen in the medium is also achieved by the use of various agents' such as:

1] a. 1% glucose b. 0.1% thioglycolate c. 0.1% ascorbic acid d.0.05% cysteine.

2] **Broth:** It is an easily prepared anaerobic medium into which piece of red hot metallic irons are introduced. Broth containing fresh animal tissue such as rabbit kidneys, spleen, testes or heart [Smith-Noguchi medium], Supports the growth of many anaerobes. Thioglycolate broth with hemin and vitamin K, Robertson's cooked meat medium are widely used fluid media for the culture of anaerobes.

3] **Pre-reduced Media** and **Anaerobic chamber** [glove box] are in use for fastidious anaerobes, particularly for quantitative cultures.

Techniques of Isolation The following methods are in use for obtaining pure cultures frommixture:

- Surface plating;
- Enrichment, selective, and indicator media;
- Pre-treatment of specimen;
- Incubation at different temperature;
- Selective filters;
- By heating a mixture.
- Cragines' tube;
- Inoculation into appropriate animals;

1] Surface plating: This method of isolation is routinely employed in clinical bacteriology and enables isolation of distinct colonies.

2] Enrichment, **selective** and**indicator media:** These are widely used for the isolation of pathogens from specimens such as faeces, with varied flora.

3] Pre-treatment of specimen: Pre-treatment of specimen with appropriate bactericidal substances which destroy the unwanted bacteria by treating acid, alkali or other substances. To which most commensals are susceptible, this method being the standard practice for the isolation of tubercle bacilli from sputum and other clinical specimens.

4] Incubation at different temperature: Separation of bacteria with different optima can be effected by this method. A mixture containing N. Meningitides and N. Catarrhal can be purified & incubation at 22^0C, when only the N. Catarrhal grow.

5] Selective filters: Bacteria of different sizes may be separated by the use of selective filters. Fillers are widely used for separating viruses from bacteria.

6] By heating a mixture: The method is useful for the isolation of tetanus bacilli from dust and similar sources.

7] Craigies' tube: This consists of a tube of semisolid agar, with a narrow tube open at both ends placed is the centre of the medium in such a way that it projects above the level of the medium. The mixture is incubated in the central tube.

8] By Inoculation into appropriate animals: Anthrax bacilli into mice or guinea pigs produces a fatal septicemia and may be cultured from the heart blood.

Review Questions:

1. What decides the employment of culture media?
2. State the reason for flaming and cooling of platinum loop during its use?
3. What are the indications for employing lawn or carpet culture?
4. Write uses of the stroke culture [Any two].
5. Mention any two uses of pour plate culture.
6. What are the gases used to displace oxygen? Can candle jar be effective in displacement of oxygen? State the reason.
7. What is the role of alkaline pyrogallol in chemical method?
8. What is the main a disadvantage of Buchner's method?
9. How can you eliminate /Remove the Risk of explosion, In Mc-Intosh fields'anaerobic Jar?
10. What is the special advantage of gaspak method?
11. What is an indicator employed in gaspak method?
12. Enumerate culture method used ordinarily in the laboratory.
13. Write in detail about the lawn or carpet culture.
14. Write in detail liquid culture.
15. Described in detail about gaspak method?
16. Name the chemical used to reduce oxygen in medium?
17. Write in short about broth.

9. Identification of Bacteria

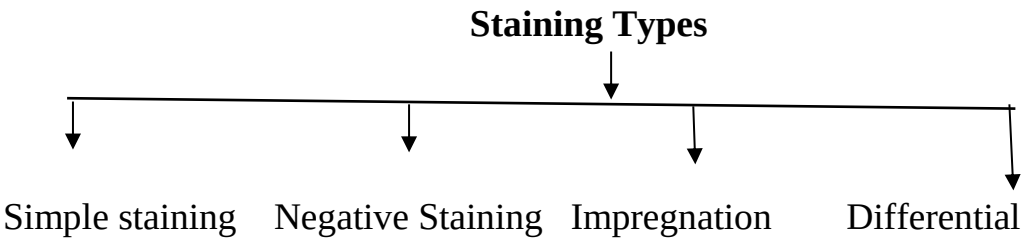
9.1 Introduction: The study of morphology and staining characteristics helps in preliminary identification of the isolate. Robert koch [1843-1910] is credited with staining techniques and methods of obtaining bacteria in pure culture, using solid medium. Once a bacterium has been obtained in pure culture, it has to be identified. The age of the culture is important. In older cultures staining characteristics either vary or are not brought out well. Simple staining brings out the morphology best. Differential and special stains are necessary to bring out characteristics like flagella, capsules, spores and metachromatic granules.

The Gram (1884) stain divides bacteria into Gram positive and Gram negative; the Ziehl-Neelsen (Z.N.) stains into 'acid fast' and ‘non-acid fast’. The fluorescent antibody technique enables one to identify them according to their surface antigens.

9.2Methods of identification of bacteria:

A. Staining: Live bacteria do not show much structural details under the light microscope due to lack of contrast. Hence it is necessary to use staining techniques to produce colour contrast. Bacteria may be stained in the living state, but this type of staining is employed only for special purposes.

Routine methods for staining of bacteria involve drying and fixing smears, procedure that kills them. Bacteria have an affinity for basic dyes due to the acidic nature of their protoplasm.



1. **Simple Stains:** Dyes such as methylene blue or basic fuchsin are used for simple staining. They provide colour contrast, but impart the same colour to all bacteria.
2. **Negative staining:** Here, bacteria are mixed with dyes such as Indian ink or Nigrosin that provide a uniformly coloured background against which the unstained bacteria stand out in contrast. This is particularly useful in the demonstration of bacterial capsule which do not take simple stains. Very slender bacteria such as spirochaetes that are not demonstrable by simple staining methods can be viewed by negative staining.
3. **Impregnation methods:** Cells and structures too thin to be seen under the ordinary microscope may be rendered visible if they are thickened by impregnation of silver on the surface. Such methods are used for the demonstration of spirochetes and bacterial flagella.
4. **Differential Stains:** These stains impart different colour to different bacteria or bacterial structures. Two most widely used differential stains are the Gram stain and the acid fast stain. The Gram stain was originally devised by the histologist Christian Gram [1884] as a method of staining bacteria in tissue.The staining techniques consists of foursteps:

   Step I - Primary staining with a pararosaniline dye such as crystal violet, methylene- violet or gentian violet;

   Step II - Application of a dilute solution of iodine;

   Step III - Decolourisation with an organic solvent such as ethanol, acetone or aniline; and

   Step IV - Counterstaining with a dye of contrasting colour, such as carbol fuchin, safranine or neutral red.

   The Gram stain differentiates bacteria into two broad groups. Gram positive bacteria are those that resist decolourisation and retain the

primary stain, appearing violet. Gram negative bacteria are decolourised by organic solvents and, therefore, take the counter stain, appearing red.

The exact mechanism of Gram reaction is not understood. The Gram positive cells have more acidic protoplasm, which may account for their retaining the basic primary dye more strongly than the Gram negative bacteria.

Decolourisation is not all or none phenomenon even Gram positive cells may be decolourized by prolonged treatment with the organic solvent, conversely, inadequate decolourisation may cause all cells to appear Gram positive. The Gram positive bacteria become Gram negative when the cell wall is damaged. Gram staining is an essential procedure used in the identification of bacteria and frequently is the only method required for studying bacterial morphology. Gram reactivity is of considerable importance as the Gram positive and negative bacteria differ not merely in staining characteristics and in structure but also in several other properties such as growth requirement, susceptibility to antibiotics and pathogenicity.

B. **Motility:** Bacterial motility except spirochaetes possesses one or more unbranched, long, sinuous filament called flagella, which are the organs of locomotion. Though flagella of different genera of bacteria have the same chemical composition, they are antigenically different. Flagellar antibodies are not protective but useful in serodiagnosis.

Motility can be observed by noting the spreading type of growth on a semisolid agar medium. Under the microscope, active motility has to be differentiated from the passive movement of the cell, either due to air currents or due to Brownian movement.

Bacterial motility may range from the slow steadely motion of peritrichate bacteria [for example, bacillus] to the darting movement of polar flagellated vibrio. The cholera vibrio may move as fast as 200 μm per second.

C. **Culturalcharacteristics:** These provide additional information for the identification of the bacterium. The characteristics revealed in different types of media are noted. While studying colonies on solid media, the following feature should be noted:

- Shape: circular, irregular or rhizoid;
- Size: in millimeters; Micrometers; etc.
- Elevation: effuse, elevated, convex, concave, umbonate or umbilicate;
- Margin: bevelled or otherwise;
- Surface: Smoth, wavy, rough, granular, papillate or glistening;
- Edges: entire, undulate, crenated, fimbriate or curled;
- Colour;
- Structure: opaque, translucent or transparent;
- Consistency: membranous, friable, butyrous or viscid; and
- Emulsifiability; whether colonies are differentiated into a central and a peripheral portion.

In a **stroke culture** following characters is to be noted:

- The degree of growth-scanty, moderate, or profuse; and
- Nature – discrete or confluent, filiform, spreading or rhizoid.

In a **fluid medium**, the degree of growth, presence of turbidity and its nature, presence of deposit and its character, nature of surface growth such pellicle and its quality and ease of disintegration and colour are noted.

D. **BiochemicalReactions :**The more important and widely used biochemical tests are :

1. **Catalase test:** In order to test for catalse production place a loop full of Hydrogen peroxide (H_2O_2) on colonies, on nutrient agar. Prompt effervescencesindicate catalase production. Culture media containing blood are unsuitable for the test as blood contains catalase.
2. **Oxidase test:** This reaction is due to a cytochrome oxidase which catalyses oxidation of reduced cytochrome by oxygen. The colonies for oxidase positive are tested by **'kovacs'** method. A strip of filter paper soaked in the oxidase reagent is placed in a Petri dish and the colony to be tested is smeared on a paper in a line about 5mm long. In a reaction the smeared area turns dark in 10 seconds. The solution [oxidase reagent] should be freshly prepared.

   The oxidase positive reaction is also performed by using a 1.0 – 1.5% solution of tetramethyl p-phenylene diamine hydrochloride is poured over the colonies. Oxidase positive colonies become maroon, purple and black in 10-30 minutes.
3. **Urease test:** Urease producing bacteria reduce urea to ammonia which is responsible for the colour. To test the urease production, 'Christensens' urease medium is used. In this test inoculate the slope heavily and incubate at 37^0C. Examine after four hours and after overnight incubation. Urease positive cultures produce a purple pink colour. The test should not be considered negative till after four days of incubation.
4. **Triple Sugar Iron [TSI]:** TSI is an example of composite medium. Composite media are being used increasingly for the

identification of isolates. These are convenient and economical, as a single composite medium indicates different properties of the bacterium which otherwise would require the use of many separate media.

TSI is a popular composite medium which indicates whether a bacterium ferments glucose only, or lactose and sucrose also, with or without gas formation besides indicating Hydrogen Sulphide [H_2S] gas production as well. The medium is distributed in tubes, with a butt and slant. The TSI medium facilitates preliminary identification of Gram negative bacilli.

Other tests such as fermentation of organic acids oxidation of gluconate, amino acid decarboxylation, and hydrolysis of sodium hippurate are sometime used.

Review Questions:

1. How does the study of morphology and staining characters help?
2. Why the age of culture is important?
3. Why differential and special stains are are necessary?
4. Write the importance of fluorescent antibody technique.
5. Why it is necessary to produce contract in staining technique?
6. What dyes are used in simple stains?
7. When negative staining is used?
8. Are the flagella antibodies helpful in diagnosis?
9. What characters are noted in a stroke culture?
10. 'Kovacs' method is useful for?
11. What is the use of 'Christiensens' urease medium?
12. Describe negative staining.
13. Write about differential stain.

14. Differentiate between Gram positive & Gram negative staining.
15. Decolourisation is not all or none phenomena, explain.
16. Write in detail about cultural characteristics.
17. How would you test for urease producing bacteria?
18. Write in detail about Triple Sugar Iron [TSI] medium.

10. Corynebacterium diphtheria

Objective - Student should know about an infectious disease diphtheria and its diagnosis.

10.1 Introduction

Corynebacteria are Gram positive bacteria, nonacid fast.nonmotile rods. They show club shaped swelling so called corynebacteria.The most important member of this is *C.diphtheriae*. It is responsible for causing diphtheria.The diphtheria bacillus first observed by Klebs and cultured by Loeffler. So it is known as Klebs-Loeffler bacillus.

10.2 Morphology

These are gram positive bacilli with tendency to clubbing at one or both ends. The bacteria group together in a characteristic way, which has been described as the form of a "V", "palisades", or "Chinese letters" or Cuneiform arrangement. Nonsporing, noncapsulated and nonmotile. Granules are present called metachromatic granules (Volutin or Babes Ernst granules). They are situated at the poles of bacilli and are called polar bodies.

When they are stained by Loeffler's methylene blue take bluish purple colour. Special stain like **Albert stain** can be used to detect granules.

Fig. 10.1Metachromatic granules

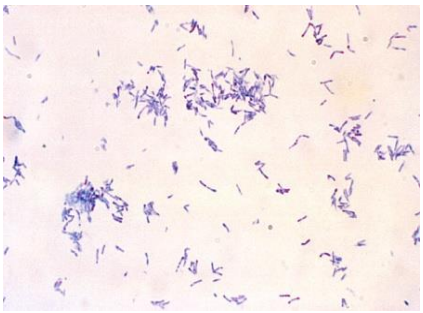


Fig. 10.2 Gram stain *C.diphtheriae*

10.3 Cultural characteristics and Biochemical reactions

Cultural characteristics - It is aerobe and facultative anaerobe. Optimum temperature is 37^0C and OptimumpH is 7.2. Grow well on enriched media. Commonly used media are - Loeffler serum slope and tellurite blood agar. On Loeffler's serum slope colonies are small, circular, white, opaque discs which later on becomes large with distinct yellow tint.

On tellurite blood agar- grey or black colour colonies.

Depending on colonies on tellurite medium and other properties like haemolysis, they are divided into three types- gravis, intermedius and mitis.

The gravis and intermedius cause more severe disease and mitis mild. Fatality rate is more with gravis and intermedius. Paralytic complications are with gravis, haemorragic in gravis and intermedius and obstructive lesions in air passages with mitis strain. Gravis and intermedius cause epidemic while mitis is endemic.

Biochemical reactions - Diphtheria bacilli ferment glucose, maltose and galactose with production of acid only. Lactose, sucrose and mannitol is not fermented. No hydrolysis of urea. Do not form phosphates.

10.4 Toxins and Resistance

Toxins - The pathogenic effects of bacteria are due to toxins. It is an exotoxin. There is variation in amount of toxins produced by each strain. The diphtheria toxin is a protein having molecular weight 62000. The toxin is labile. It can be converted in to toxoid by its prolonged storage, incubation at 37^0C for 4-6 weeks, treatment with 0.2 - 0.4% formalin or by acid pH.

Toxoid is toxin whose toxicity is lost but antigenicity is preserved. It can produce antitoxin and reacts with it specifically.

Resistance - Diphtheria bacilli are destroyed by heat in 10 minutes at 58^0C and in a minute at 100^0C. Cultures can be preserved for two or more weeks at 25 - 30^0C. It is more resistant to light, desiccation and freezing. It remains virulent in blankets and floor dust for 5 weeks. It is easily destroyed by antiseptics. It is susceptible to antibiotics.

10.6 Pathogenicity

Bacilli cause Diphtheria. It is acute infectious disease. The incubation period is commonly 3-4 days. It is divided in to three major clinical types (i) Anterior nasal, (ii) Faucial and (iii) laryngeal. But other sites like the skin, conjunctiva, vulva and other parts can also get affected. The bacilli multiply usually in the throat and release exotoxin which leads to the formation of a greyish or yellowish membrane (false membrane) commonly over tonsils, pharynx or larynx. The membrane can not be wiped away. There is congestion, edema or local tissue destruction, enlargement of the regional lymph nodes and signs of toxaemia. Depending on severity diphtheria may be classified as i) Malignant ii) septic iii) haemorragic.

The complications are-

a) Asphyxia due to mechanical obstruction to respiratory passage by false membrane.

b) Acute circulatory failure.

c) Postdiphtheric paralysis

d) Septic such as pneumonia and otitis media.

10.6 Laboratory diagnosis

1. Isolation of diphtheria bacillus

   Specimen - Swabs from lesion

   Staining - Gram stainingand metachromatic granule staining

   Immunofluorescence technique can be used to identify bacilli.

   Culture-Media used Loeffler's serum slope, Tellurite blood agar

   Blood agar-This is to differentiate diphtheria from streptococcal or staphylococcal pharyngitis.

2. Demonstration of its toxicity.

   Virulence test - It is carried out by *in vivo* and *in vitro* methods.*In vivo* is by subcutaneous or intradermal test which is not carried out now and *in vitro* by Elek's gel precipitation test and tissue culture test.

Prophylaxis

Diphtheria can be controlled by mass immunization programme. It includes active, passive or combined immunization. Susceptibility to diphtheria can be detected by Schick test.

Schick test- When the diphtheria toxin is injected intradermally into a susceptible person it causes a local reaction, while in an immune person there is no reaction as toxin is neutralized by antitoxin in circulation.

Review Questions:

1. What is diphtheria?
2. Describe morphology of diphtheria bacilli.
3. Name media used for culturing C.diphtheriae.
4. What is false membrane?
5. What are complications of diphtheria?
6. What are types of diphtheria bacilli?
7. How will you isolate and identify Corynebacterium diphtheriae?

11. Gram negative bacilli

Objectives : After reading this chapter, student will gather knowledge about morphology, Classification, Cultural characteristics, Resistance, toxinproduced, pathogenicity & laboratory diagnosis of important Gram negative bacilli.

Introduction – The pathogenic capability of Gram negative bacilli is mainly associated with specific components of the cell wall e.g. the lipopolysaccharide (LPS,endotoxin)medically relevant Gram negative bacilli include a large number of species. Some Gram negative bacilli such Escherichia coli, Proteus mirabilis etc, cause primarily urinary problems. Primarily gastrointestinal problems may be caused by other Gram negative bacilli such as Helicobacter pylori, Salmonella typhi, etc. Respiratory infections are also caused by Gram negative bacilli such as Klebsiella pneumoniae, Hemophilus influenzae, Pseudomonas aeruginosa, etc.

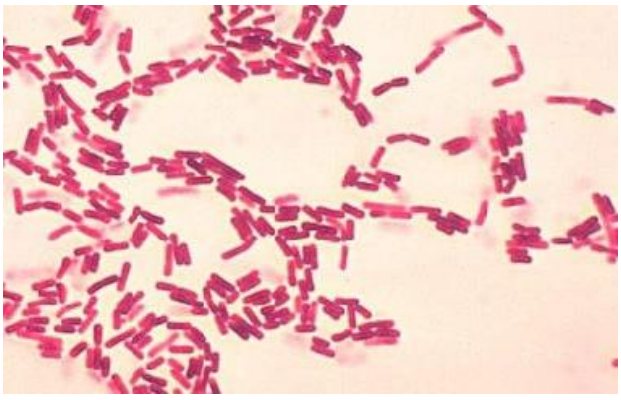


Fig 11.1**Gram negative bacilli (Gram staining)**

11.1 Escherichia coli

Morphology: Esch.coli is a Gram negative bacillus measuring 1 – 3 µ X 0.4 – 0.7 µ.

Most strains are motile by peritrichate flagella. It is non-sporing, non-capsulated.

Culture: It is aerobe & facultative anaerobe. It grows on ordinary culture medium at optimum temperature of 37˚C (temp. 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.

Resistance: Killed by chlorination of water & by moist heat at 60˚C.

Toxins: Some strains of E.coli produce enterotoxins, haemolysin & verocytotoxin.

Pathogenicity: Infections caused by E. coli –

- Urinary tract infections
- Diarrhoea
- Pyogenic Infections – Neonatal meningitis, peritonitis, abscess
- Septicemia

Figure 11.2 Lactose fermenting and nonblactose fermenting colonies

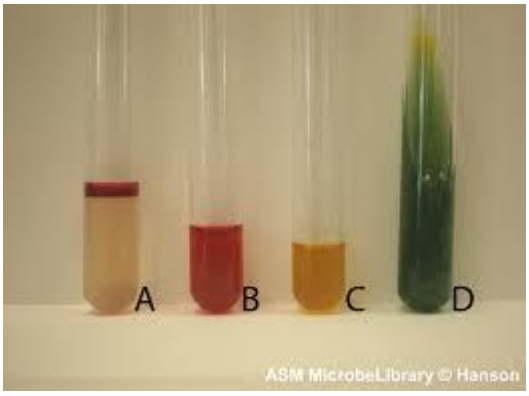


Figure 11.3 IMViC Test result of E.coli

Biochemical Reactions of E.Coli

Test	Reactions
Indole	+
MR	+
VP	--
Citrate	--
Urease	--
H2S	--
Oxidase	--
Nitrate	+
Gelatin	--
PhA (phenylalanine)	--

11.2 Klebsiella

Morphology: These are short, plump, Gram negative, capsulated, non-motile bacilli. They are about 1–2µm × 0.5 – 0.8µm in size.

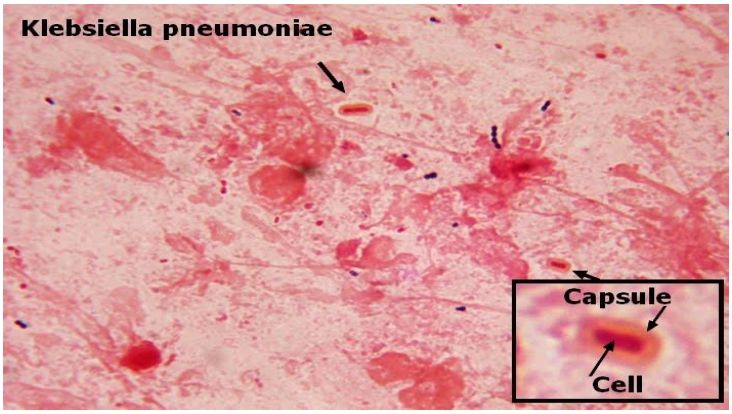


Figure 11.4

Culture:

Klebsiellae grow well on ordinary media at optimum temperature of 37˚C in 18 – 24 hrs. 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.

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 - - ++).

Pathogenesis: It is responsible for

- Severe bronchopneumonia,
- Urinary tract**i**nfections
- Nosocomial infections,
- Wound infections,
- Septicmeningitis
- Rarely diarrhoea.

Laboratory Diagnosis:

Laboratory diagnosis is done by culturing appropriatespecimens on blood and MacConkey agar. The isolate is identified by colony morphology, Gram staining, test for motility and biochemical reactions.

11.3 Proteus Species

Morphology:

These are Gram negative bacilli measuring 1- 3µm × 0.5µm in size. They are non capsulated, non-sporing and actively motile.They posses'peritrichateflagella. Many strains possess fimbriae.

Culture: They are aerobic and facultatively anaerobic.They grow on ordinary media and Cultureemits a characteristics putrefactive (fishy orseminal) odour. When grown on nutrientagar or blood agar, Pr vulgaris

and Pr.Mirabilis exhibit ‘swarming’. They form smooth, pale or colourless (NLF) colonies on MacConkey’s agar and do not swarm on this medium.

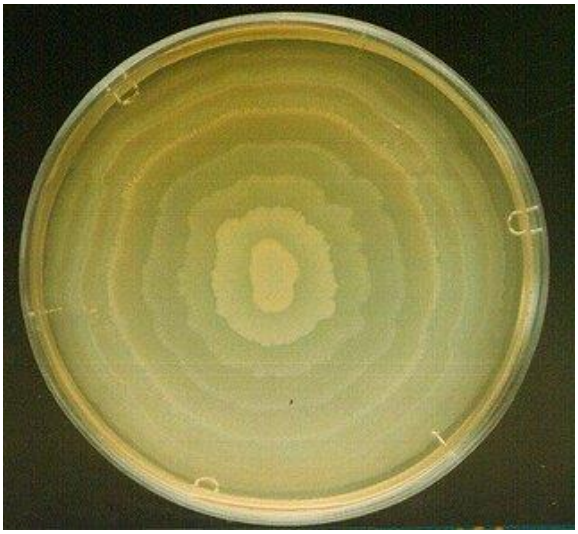


Fig. 11.5 swarming growth of Proteus

Biochemical Reactions:

The distinctive characters of this genus are:

- Deamination of phenyl alanine to phenyl pyruvic acid PPA test – It is always positive.
- Hydrolysis of urea by enzyme urease – It is another characteristic of Proteus.

Pathogenesis: They are opportunistic pathogens and may cause many types of infections such as

- Urinary tract infection (UTI),
- Pyelonephritis pyogenic lesions,
- Infection of ear,
- Respiratorytract infection
- Nosocomial infections.

Laboratory Diagnosis:

- Specimens : Mid steam urine sample in UTI; Pus in pyogenic lesions

- Culture: It is cultured on MacConkey agar or blood agar with 6 %agar toinhibit swarming.
- Gram staining- Gram negative bacilli
- Hanging drop preparation: Actively motile bacilli are observed.
- Biochemical reactions: Most important are PPA and urease tests.

	E. coli	Klebsiella	Vibrio cholera
I (Indole)	+	--	+
MR (Methyl red)	+	--	--
VP	--	+	--
Citrate utilization	--	+	

Comparative chart of important biochemical tests of few bacilli

11.4 Vibrio Cholerae

Morphology-V.cholera is a Gram negative, curved or comma-shaped rod, non-sporingnon-capsulated, about 1.5 µm × 0.2-0.4 µm in size.The organism isvery 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.

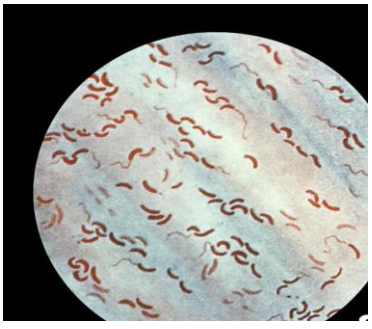


Fig. 11.6V. Cholerae

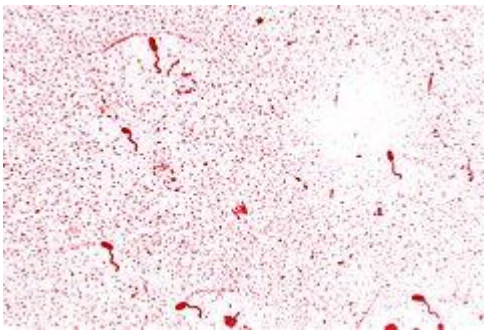


Figure 11.7

Culture –V. cholera is strongly aerobic, growth being scanty and slow anaerobically. Itgrowswithin a temperature range of 16 ˚- 40˚C but optimum temperature is 37˚C. It grows best in alkaline media, the optimum pH 8.2.

- **Ordinary Media –**
  - Nutrient agar.
  - MacConkeysagar.
  - Blood agar.
- **Special Media –**

The special media are classified as follows:

- **Transport or holding media –**
  - Venkatraman – Ramakrishnan (VR) Medium.
  - Cary – Blair Medium.
- **Enrichment media -**
  - Alkaline Paptone water (APW)
  - Monsur's taurocholate tellurite peptone water.
- **Plating media -**
  - Alkaline bile salt agar (BSA); pH8.2
  - Monsur's gelatin taurocholate trypticase tellurite agar (GTTA) medium; pH.8.5
  - Thiosulphate citrate bile sucrose (TCBS) agar; pH 8.6

Fig. 11.8Vibrio Cholerae grown on TCBS medium

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.

Catalase	Oxidase	Glucose	Lactose	Mannitol
+	+	A	_	+
Sucrose	Maltose	Mannose	Indole	NO3 reduction
+	+	+	+	+
Urease	Gelatin	MR	VP*	
_	+	_	_	

Resistance –Vibrio cholerae are 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 is survives for months insea water.

Toxins-Besides heat stable endotoxin, V. cholerae also produces exotoxi (enterotoxin).

Serotypes of Cholera Vibrios –

- *Ogawa.
- *Inaba.
- *Hikojima.

Pathogenesis – V. cholerae causes an acute diarrhoeal disease known as cholera. 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.V.cholerae produces enterotoxin and the disease.There is massive loss of water and electrolytes (sodium and bicarbonates) by action of enterotoxin.

Laboratory Diagnosis –

Specimens –

- Watery stool
- Rectal swab.

Specimens should be collected preferably prior to start of antibiotics.

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.

Culture –

- Stool sample is directly cultured on following media –

1. Selective media.
2. Enrichment media

- 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.

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 Monsurs medium the colonies has a black centre with aturbid halo around the

colony. TCBS shows yellow colonies and on BSA, translucent colonies are present.

Agglutination Test:

Colonies are picked up with a straight wire and tested with V. cholerae 01 antiserum. If positive, the test is repeated with monospecific Ogawa and Inaba sera for serotyping.

11.5 Shigella

Morphology – Shigellae are short, Gram negative bacilli measuring about 1–3 µ × 0.5µm.They are non-motile, non-capsulated and non-sporing.

Figure 11.9

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. Colonies on MacConkey's agar and deoxycholatecitrate agar (DCA) are colourless (non-lactose fermenting - NLF).

Enrichment medium–Selenite F broth.

Resistance – Shigellae are killed at 56 ˚C in one hour. Boiling or chlorination of water and pasteurization of milk are effective and destroy the bacilli

Classification – Shigellae are divided into four subgroups-

1. Sh. dysenteriae.
2. Sh. flexneri.
3. Sh. boydii.
4. Sh. sonnei.

Toxins -

1) Endotoxin-All shigellae release endotoxin after autolysis. Endotoxin has a irritating effect on intestinal wall which causes diarrhoea and subsequently intestinal ulcers.
2) Exotoxin – Enterotoxin, Neurotoxin.

Pathogenesis – Shigella cause **bacillary dysentery**. The incubation period of the disease is usually less than 48 hours. 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. 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.

Laboratory Diagnosis – Diagnosis depends upon isolating the bacillus from faeces.

1) **Specimens –** Fresh stool is collected.
2) **Transport –** Specimens should be transported immediately and inoculated without delay.
3) **Direct Microscopy –** Saline and iodine preparation of faeces show large number of pus cells, erythrocytes and macrophages.
4) **Culture –** Specimen is inoculated on selective media like MacConkey's agar, DCA or XLD agar.

11.6 Salmonella

Salmonellae produce three main types of diseases in man

- **Enteric Fever -** The causative agents of enteric fever are Salmonella typhi (causing Typhoid fever) or S.paratyphi A, B and C (causing paratyphoid fever).
- **Gastroenteritis –**These include S. typhimurium, S. enteritidis.
- **Septicaemia –** The commonly associated Salmonella is S. cholera-suis.

Morphology

Salmonellae are Gram negative bacilli measuring 1 – 3 µm × 0.5 µm. Theyare motile, non-sporing and non-capsulated. Motility is due to the presence of peritrichousflagella.

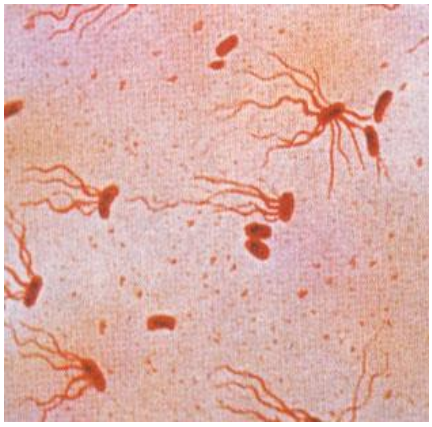


Figure 11.10 Flagellar stain for s. typhi

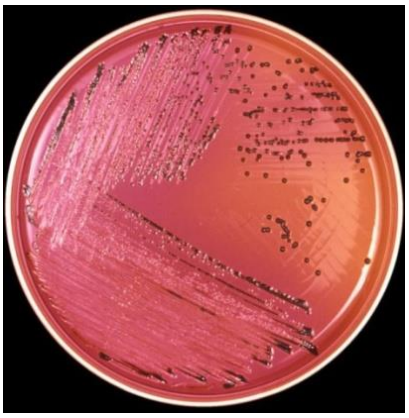


Fig.11.11S.typhi grown on XLD agar

Culture

Salmonellae grow on ordinary culture media at optimum temperature of 37^0C, pH 6 – 8 and are aerobic and facultative anaerobic. They produce colonies of 2–3 mm in diameter, circular, translucent, low convex and smooth.

On MacConkey's agarDeoxycholatecitrate agar (DCA), colonies are colourless due to non-lactose fementation (NLF)

Resistance - The Salmonellae are killed at 60 ˚C in 15 minutes. Boiling, chlorination of water and pasteurization of milk destroy the bacilli.

Antigenic structure:

Salmonellae possess three types of antigens based on which they are classified. These are

- Flagellar antigen 'H',
- Somatic antigen 'O', and
- A surface antigen 'Vi', found in some species.

Biochemical Reactions of S.typhi and S.paratyphi.

Salmonellae are catalase positive, oxidase negative & nitrate reduction positive.

Following table shows other biochemical tests:

Strain	Glucose	Mannitol	Lactose	Sucrose	indole	Citrate	MR	VP	H_2S
S. typhi	A	A	_	_	__	__	+	__	+
S. paratyphi A	AG	AG	_	_	__	__	+	__	_
S. paratyphi B	AG	AG	_	_	__	+	+	__	+
S. paratyphi C	AG	AG	_	_	__	+	+	__	+

A – Acid, AG – Acid and gas, d – delayed.

Pathogenesis

Typhoid Fever – The infection is acquired by ingestion through contaminated food and water. The incubation period is usually 7 – 14 days. Bacteria penetrate intestinal wall multiply in Payer's patches and finally enters in the circulation. Intestine gets ulcerated.

Laboratory Diagnosis – Bacteriological diagnosis of enteric fever consists of-

1. Isolation of bacilli.
2. Demonstration of antibodies.
3. Demonstration of circulating antigen.

❖ **Isolation of Bacilli –** This may be done by culture of specimens like blood, faeces, urine.

Blood Culture – 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. 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.

Fig. 11.12Blood culture bottles for adult & pediatric patient

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.

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 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.

Urine Culture - Urine culture is less frequently positive.

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.

- **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 and rise sharply during the third week of enteric fever.

Procedure - Initially Dreyer's (H) and Felix (O) tubes were used. Nowadays 5×50 mm tubes are used. Equal volumes of serial dilutions of the serum and the H and O antigens are mixed and incubated at 37 ˚C at least for 4 hours or overnight. H agglutination leads to the formation of

loose, cottonwool clumps, while O agglutination appears as a granular deposit at the bottom of the tube.

Interpretation of WidalTest

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.

- Local titre: In endemic area, low titre of agglutinins is present in the serum of normal persons.
- Immunisation– In immunization with TAB vaccine, individuals may show high titres of antibody to S. typhi, S. parayphi A and B, while in case of infection; antibodies will be seen only against the infecting organism.

11.7 Pseudomonas Aeruginosa

Morphology – It is slender, Gram negative bacillus, 1.5 – 3 µm × 0.5µm, non-capsulated, non-sporing and is actively motile by a polar flagellum. Most strains possess pili. It is non-capsulated.

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.

Growth on nutrient Agar – Colonies are smooth, large, translucent, low convex, 2 – 4 mm in diameter. The organism produces a sweetish aromatic odour. There is greenish blue pigment which diffuses into the medium.

Pigment Production – Different pigments produced by pseudomonas:

1. Pyocyanin: Bluish –green pigment
2. Flurescin (Pyoverdin): Greenish – yellow pigment
3. Pyorubin: Reddish – brown pigment
4. Pyomelanin.: Brown to black pigment

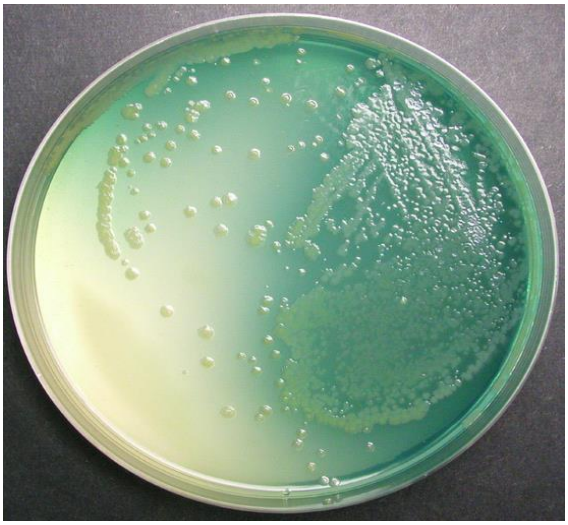


Fig. 11.13Pseudomonas grown on nutrient agar showing pigment production

Biochemical Reactions - All strains of Ps. aeruginosa are oxidase positive and utilize citrate as the sole source of carbon. They are catalase positive and indole, MR, VP and H2S tests negative.

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 resistant to commonly used antibiotics.

Toxins and Enzymes – Several toxins and enzymes produced by Ps. aeruginosa contribute to enhance its virulence.

Pathogenesis –

1. Urinary tract infections following catheterization.
2. Acute purulent meningitis following lumbar puncture.
3. Post – tracheostomy pulmonary infection.
4. Septicaemia in patients who are debilitated due to malignancy or immune – suppressive therapy.

5. Wound and burn infections.
6. Chronic otitis media and otitis externa.
7. Eye infections.
8. Acute necrotizing vasculitis which leads to haemorrhagic infection of skin and internal organs.
9. Infantile diarrhoea.

Laboratory Diagnosis –

A. Specimens **–** Pus, wound swab, urine, sputum, blood or CSF.
B. Gram Staining and Motility– Gram negative bacilli, nonmotile
C. 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.
D. Biochemical Reactions.

Review Questions:

1. Differentiate between E. coil and Klebsiella.
2. Pathogenesis of Proteus.
3. Laboratory diagnosis of urinary tract infection.
4. Special media used for V. cholera
5. What is cholera red reaction?
6. Laboratory diagnosis of bacillary dysentery.
7. Different antigens found in salmonella.
8. Laboratory diagnosis of typhoid fever.

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12 Gram Positive and Gram Negative Cocci

Gram Positive cocci

12.1 Staphylococci

Introduction: Staphylococci are Gram positive cocci that occur in grape-like Clusters. They are ubiquitous and form the most common cause of localized supportive lesions in human beings. It was Sir Alexander Ogston, a scottish surgeon, who gave it the name stophyloccocus [Staphyle, in Greek, meaning 'bunch of grapes': Kokkos, meaning a berry] due to the typical appearance of the cocci in grape like clusters in pus and in cultures.

Classification:

- ❖ Depending upon pathogenicity
  - Pathogenic- Staph. Aureus
  - Nompathogenic- Staph albus
- ❖ Depending on pigment
  - Golden yellow pigment- Staph aureus
  - White pigment- Staph. albus

Morphology:

Size: Approximately 1µm in diameter.

Shape: Spherical cocci

Arrangement: Arranged characteristically in grape like clusters. Cluster formation is due to cell division occurring in three planes. They may also be found singly, in pairs and in short chains of three or four cells, especially when examined from liquid culture. Long chain never occurs.

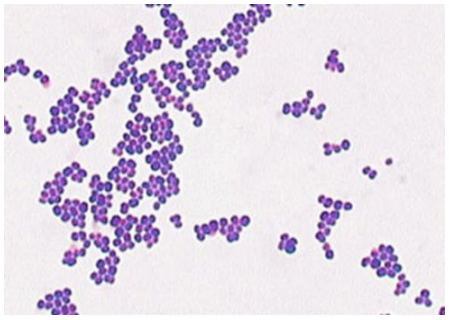

Figure 12.1

- Staining: Gram positive.
- Capsule: Non- capsulated
- Motility: Non Motile
- Spores: Non-sporing.

Cultural characteristics: Staphylococci grow readily on ordinary media within a temperature range of 10-42^{0}C, the optimum temperature being 37^{0}C and pH 7.4-7.6. They are aerobes and facultative anaerobes.

- On nutrient agar, after incubation for 24 hours, the colonies are large 2-4 mm diameter circular, convex, smooth, shiny, opaque and easily emulsifiable. Most strains produce golden yellow Pigment, though some may be white, orange or yellow. Pigment production is enhanced when 1% glycerol monoacetate or milk is incorporated in the medium. The pigment is believed to be a lipoprotein allied to carotene.
- In liquid media, uniform turbidity is produced.

Biochemical reactions:

- Staphylococci ferment a number of sugars, producing acid but no gas.
- Staph. aureus ferments mannitol not other species.
- They are catalase positive.
- Hydrolyse urea, reduce nitrates to nitrites.
- Liquefy gelatin.
- Methyl Red test [MR] and Voges-proskauer test [VP] positive but indole negative.

- Produce phosphtase.

Following criteria help to differentiate Staph.aureus and Staph.albus

- Coagulase positive,
- ferment mannitol,
- produce clear haemolysis on blood agar,
- produce a golden yellow pigment, and
- Liquefy gelatin.
- Produce phosphtase
- Tellurite reduction

Resistance: Stapholococci are more resistant organisms. They may withstand 60^0C for 30 minutes. Thermal death point is 62^0C for 30 minutes. Grow in presence of 10% NaCl. Resists 1% phenol killed by 1% mercury perchloride in 10 minutes.

Virulence: Cytolytic toxins are membrane-active substances, consisting of four haemolysin and a leucocidin.

- Alpha haemolysin is the most important among them. It is a protein inactiveated at 70^0C, but reactivated paradoxically at 100^0C.
- Beta haemolysin: It exhibits hot-cold phenomena, the haemolysis begins at 37^0C.
- Gamma haemolysin: It consists of two, separate proteins, both of which are necessary for haemolysis.
- Delta haemolysin: This has a degenerative like effect on cell membrane of RBC, WBC, Platelets & macrophages.
- Lecucocidin [Panton-Valentine Toxin]: It has two components toxin.

Pathogenicity: Staph.aureus is an important pyogenic organism and lesion is localized. Diseases are

1. Cutaneous infections- Pustules, boils, carbuncles, abscesses, styes, wound infections and burn infections.
2. Deep infections- Osteomyelitis, tonsillitis, pharyngitis, sinusitis, empyema, meningitis, pyaemia, etc.
3. Food poisoning- It occurs 2-6 hours after the ingestion of contaminated food.
4. Nosocomial infections- They are important cause of hospital acquired infections.
5. Skin exfoliative diseases- Stripping of the superficial layers of skin in various exfoliative syndromes.
6. Toxic shock syndrome (TSS)-Caused by toxic shock syndrome toxin. It shows high fever, hypotension, diarrhea, vomiting, etc.

Laboratory diagnosis:

- **Specimens**- Depend on type of infection such as
  - Pus- suppurative infections
  - Sputum- respiratory infections
  - Blood- septicemia
  - CSF- meningitis
  - Feces- food poisoning
  - Food or vomit- - food poisoning
- **Direct Microscopy**- Gram staining
- **Culture**- inoculated on Blood agar and peptone water.
- **Biochemical reactions**- Coagulase test, Mannitol fermentation Catalase test, Gelatin liquefaction, Tellurite reduction phosphtase production

Review Questions:

1. What do you understand by term 'ubiquitous'?
2. Classify staphylococci.
3. What is the selective medium for staphylococci?
4. Name the infections caused by staphylococci.
5. Describe in details about staph virulence.
6. Differentiate between stph. Aureus and staph. Albus.

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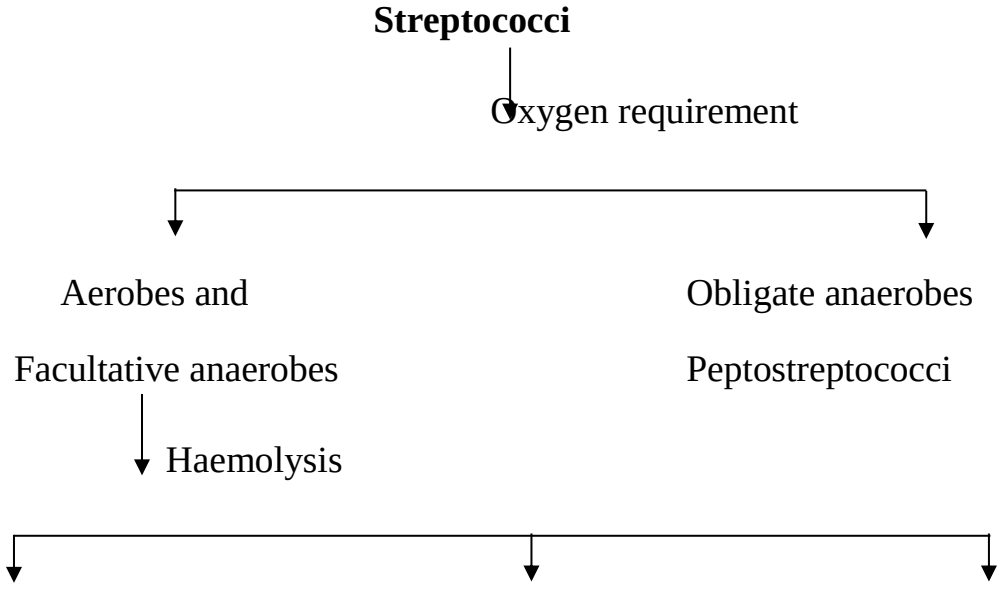
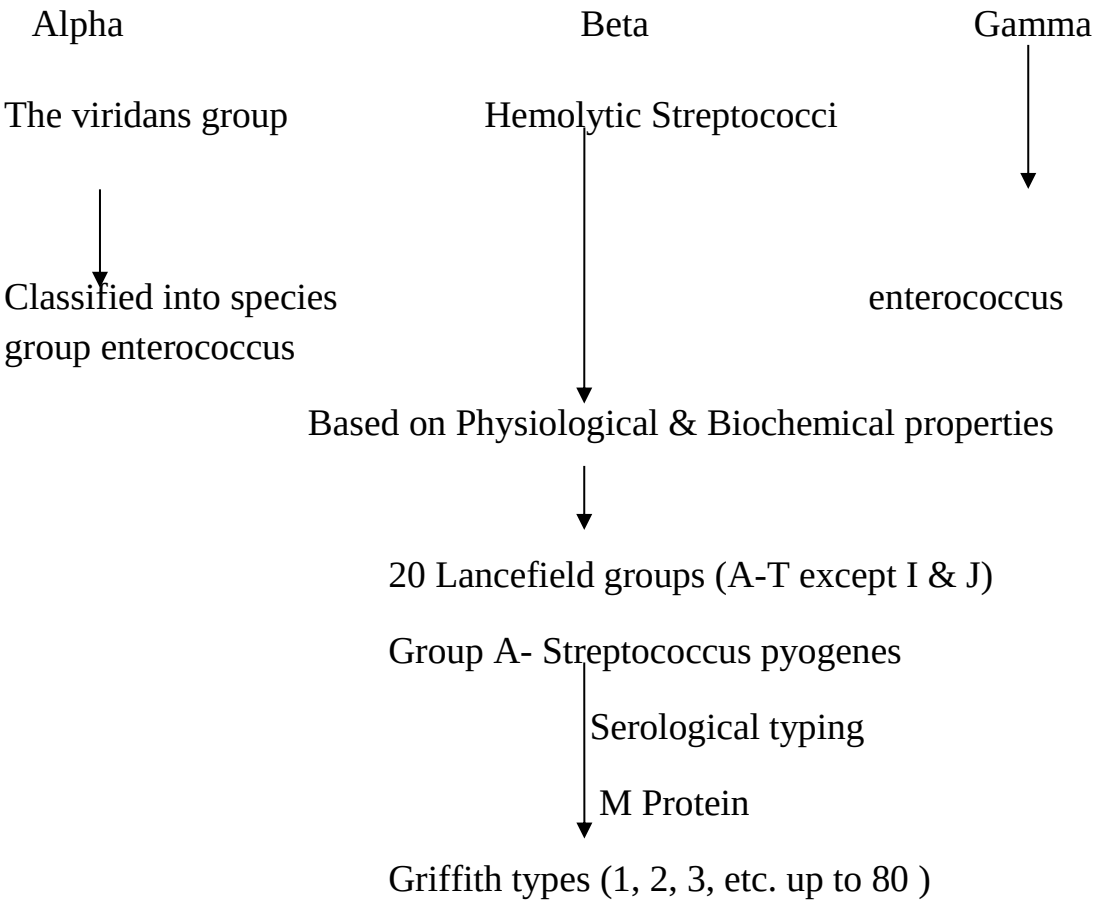
12.2 Streptococcus pyogenes

Introduction: Streptococci are Gram positive cocci arranged in chains or pairs. They are part of the normal flora of humans and animals. Some of them are human pathogens. The most important of them is streptococci [str.] pyogenes causing pyogenic infection, with a characteristic tendency to spread as opposed to staphylococcal lesions which are typically localized. Str. is also responsible for the nonsuppurative lesions, acute rheumatic fever and glomerulonephritis which occur as sequelae to infection.

Classification: Several systems of classifications have been employed but in medical bacteriology the following method is useful.





- **Alpha** hemolytic streptococci produce a greenish discoloration with partial haemolysis around the colonies. These are known as 'Viridans' [meaning, green] streptococci. The alpha streptococci are normal commensals in the throat, but may cause opportunistic infection rarely.
- **Beta:** The term 'haemolytic streptococci' strictly applies only to beta lytic strains. Most pathogenic streptococci belong to this group.
- **Gamma** or non haemolytic streptococci produce no change in the medium and so are some times referred to as,'indifferent streptococci'.They include faecal streptococci [enterococci,str. faecalis] and related species. They are called enterococcus group.

Hemolytic streptococci classified by Lancefield [1933] serologically into groups based on the nature of a Carbohydrate [C] antigen on the cell wall. They are known as 'Lancefield groups'.

Morphology:

- Size: 0.5 - 1.0 µm in diameter.
- Shape: Spherical cocci
- Arrangement: Arranged in chains. Chains are longer in liquid media than solid media. Chain formation is due to the cocci dividing in one plane only and the daughter cell failing to separate completely.
- Staining: Gram positive.
- Capsule: Non-capsulated
- Motility : Non Motile
- Spores: Nonsporing

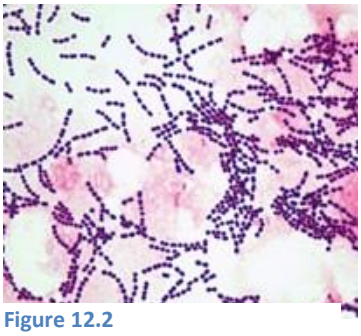


Figure 12.2

Cultural characteristics: It is an aerobe and a facultative anaerobe, growing best at temperature of 37^0C [range 22-42^0C]. It is exacting in nutritive requirement, growth occurring only in media containing fermentable carbohydrates or enriched with blood or serum. Virulent strains, on fresh isolation from lesions, produce a 'matt' [finely granular] colony, while avirulent strains form 'glossy' colonies.

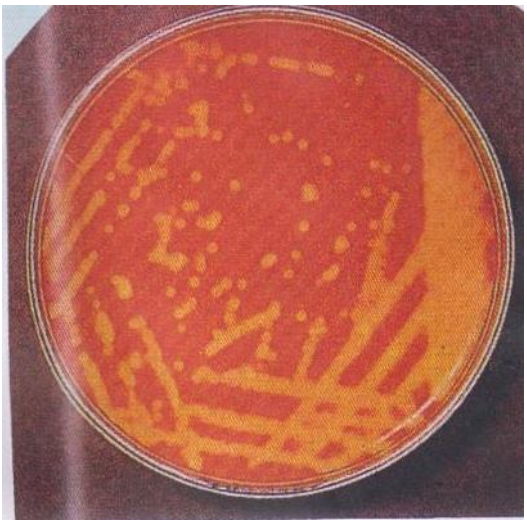


Figure 12.3

Biochemical reactions: Str. pyogenes ferment several sugars producing acid but no gas. Streptococci are catalase negative. They are not soluble in 10% bile.

Resistance: Streptococcus pyogenes is a delicate organism. It is inactivated by heat at 56^0C in 30 minutes. They can be stored in Robertson's cooked meat medium at 4^0C. It is inactivated by antiseptics.Sensitive to bacitracin. It is more resistant to crystal violet.

Toxins and Enzymes: Strepto. Pyogenes produce several exotoxins and enzymes which contribute in virulence.**Toxins** are

Haemolysins- It is of two types-'S' and 'O'

Haemolysin'O'	**Haemolysin'S'**
Oxygen and heat labile	Oxygen stable
Lyses red cells and also cytotoxic for neutrophils, platelets and cardiac tissue.	Lyses red cells and also leucocidal action.
Demonstrated in deep colonies and not in surface culture.	Demonstrated in surface culture on blood agar plates.
It is antigenic. An ASO titre > 200 units suggests either recent or recurrent streptococcal infections.	It is not antigenic.

Pyrogenic or erythrogenic toxin: It is responsible for rash of scarlet fever. The toxin is antigenic and is neutralized by antibodies present in the convalescent sera. The toxin was renamed as Streptococcal pyrogenic exotoxin (SPE).

Enzymes are

- Streptokinase(Fibrinolysin)
- Deoxyribonucleases (Streptodornase)
- Nicotinamide adenine dinucleotidase
- Hyaluronidase
- Other extracellular products.

Pathogenicity: Produces pyogenic infections with a tendency to spread locally. Different infections are as follows:-

- **Respiratory infections:** The primary site of invasion of the human body by str. pyogenes is the throat. Sore throat is the most common streptococcal disease. Tonsilitis is more common in older children and adults.Scarlet fever is a combination of sore throat and generalized erythematous rash. It is caused by a strain producing erythrogenic toxin.
- **Skin and soft tissues infections:** Str. pyogenes causes a variety of suppurative infection of the skin, including infection of wounds or burns, with a predilection to produce lymphangitis and cellulitis.
- **Genital infection:** It causes Puerperal sepsis.
- **Other suppurative infections:** Str. pyogenes may cause abscesses in internal organs such as brain, lungs, liver and kidneys, and also septicemia and pyemia.
- **Nonsuppurative complications:** Str. pyogenes infections lead to two important nonsuppurative sequelae: Acute rheumatic fever and acute glomerulonephritis.

These complications develop one to four weeks after the acute infections.

Feature	**Acute Rheumatic fever**	**Acute Glomerulonephritis**
Primary site of infection	Throat	Throat or Skin
Prior sensitization	Essential	Not necessary
Sero type responsible	Any	Pyoderma types 49, 52-55, 57-61 and Throat infection type 12, 1, 25, 4 and 3
Immune response	Marked	Moderate
Repeated attacks	Common	Absent

Course	Progressive or Static	Spontaneous Resolution
Prognosis	Variable	Good
Hereditary tendency	Present	Not known
Penicillin prophylaxis	Essential	Not indicated

Laboratory Diagnosis: It involves diagnosis of acute suppurative infections and non-suppurative complications.

Acute infections- By culture

- Specimen collection depending on type of lesion such as swab, pus, blood, or CSF
- Gram stain- Gram positive cocci in chain.
- Motility- Non-motile
- Culture- On blood agar it shows beta haemolysis. Liquid medium such as glucose broth is used.
- Biochemical reactions- Catalase negative, insoluble in 10% bile, ferment sugar with production of acid only.
- Non-suppurative infections- They are detected by demonstration of antibodies in the serum. Commonly used test is antistreptolysin O (ASO) titre. A titre more than 200 units is significant or is diagnostic in rhematic fever and it is indicative of prior streptococcal infection.

Review Questions:

1. Describe morphology of streptococci.
2. Classify streptococci.
3. Describe cultural characteristics and biochemicals of streptococci.
4. Name two factors responsible for str. pyogenes' virulence?

5. Pathogenesis of str. pyogenes?

12.3 Streptococcus pneumoniae

Introduction: St. pneumoniae a Gram positive lanceolate diplococcus, formely classified as Diplococcus pneumoniae, has been reclassified as str. pneumoniae because of its genetic relatedness to streptococcus. Pneumococcus differs from other streptococci chiefly in its morphology, bile solubility, optochin sensitivity and possession of specific polysaccharide capsule. Pneumococci are normal inhabitants of the human upper respiratory tract. They are the single most prevalent bacterial agent in pneumonia and in otitis media in children.

Classification: Str. pneumoniae are classified based on the antigenic structure of the capsular polysaccharide. Pneumococci isolated from lobar pneumonia were originally classified into three types, I, II, III, and a heterogeneous group IV.

Morphology: Pneumococci are typically small, slightly elongated cocci, with one end broad or rounded and the other pointed, presenting a flame shaped or lanceolate appearance. They occur in pairs [diplococci], with the broad ends in opposition. Pneumococci are capsulated, the capsule enclosing each pair. In culture, the typical morphology may not be apparent and the cocci are more rounded, tending to occur in short chain. They are nonmotile and non-sporing, readily stained with aniline dyes and are Gram positive.

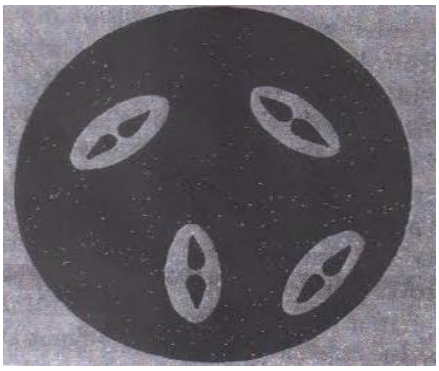


Fig. 12.4 Capsule staining

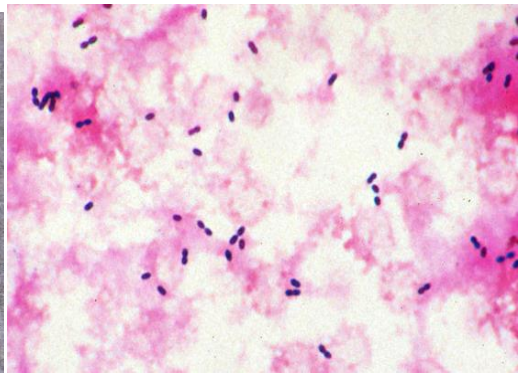


Fig. 12.5 Gram positive diplococci

Cultural characteristics: Pneumococci have complex growth requirement and grow only in enriched media. They are aerobes and facultative anaerobes, the optimum temperature being 37^0C [range 25-42^0C] and pH 7.8 [range 6.5-8.3]. Growth is improved by 5-10% carbon-dioxide [CO_2]. On blood agar, after incubation for 18 hours, the colonies are with an area of green discolouration [alpha haemolysis] around them, resembling colonies of str. viridans. Under anaerobic conditions, colonies on blood agar are surrounded by a zone of beta haemolysis due to an oxygen labile haemolysin 'O'.

Draftsmans appearance

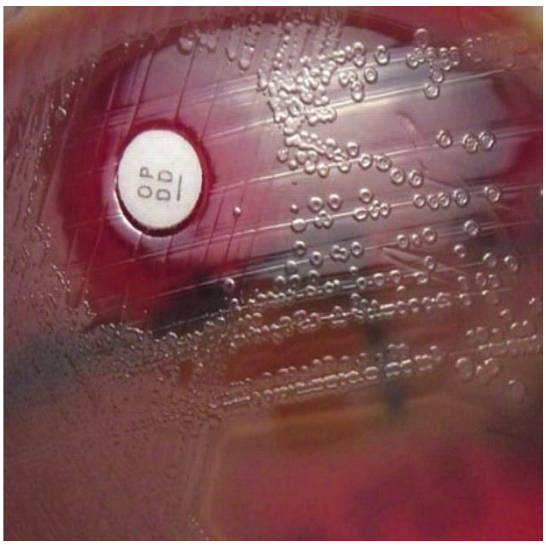


Figure 12.6

Biochemical reactions:

- Pneumococci ferment several sugars forming acid only.
- Bile soluble. Bile solubility is a constant property of pneumococci and hence is of diagnostic importance.
- Inulin fermentation.
- Pneuococococci are catalase and oxidase negative.

Resistance: Pneumococci are delicate organisms. They are destroyed at 52^0C for 15 minutes. They are sensitive to wide range of antibiotics like erythromycin, chloramphenicol, etc. They are sensitive to optochin.

Toxins: The virulence of pneumococci depends on its capsule and the production of a toxin called pneumolysin.The capsular polysaccharide, because of its acidic and hydrophilic properties, protects the cocci from phagoytosis. Pneumolysin, a membrane damaging toxin produced by pneumococci has cytotoxic and complement activating properties and so may be a virulent factor. It is immunogenic.

Pathogenicity: They are the single most prevalent bacterial agents and cause the following infections:

- Lobar pneumonia- This occurs when overall resistance get decreased. Its infective types are 1-12 in adult and 14,19 and 23 in children.
- Bronchopneumonia- It is always a secondary infection following viral infection of respiratory tract. The damage to the respiratory epithelium and excessive bronchial secretions due to primary infection facilitates the entry of pneumococci along the bronchial tree. It is frequently a terminal event in aged and debilitated patients.
- Meningitis- It is most common serious pneumococcal infection. It is second most important cause of pyogenic infection after N. meningitides. This is common in children. It is usually secondary to other pneumococcal infections such as pneumonia. Otitis media,

sinusitis, etc. Pneumococci spread from the pharynx to meninges through blood sream.

- Other infections- Pneumococci may cause empyema, pericarditis, otitis media, sinusitis, conjunctivitis, peritonitis and suppurative arthritis.

Laboratory diagnosis-

- Specimen- Depends on site of lesion. E.g. sputum, CSF, blood, pleural fluid, etc. Blood culture is useful in pneumococcal septicaemia.
- Direct microscopy- By Gram staining showing Gram positive diplococci along with large no of polymorphs.
- Capsule is demonstrated by quellung reaction.
- Culture- Inoculation on blood agar which shows haemolytic colonies.
- Biochemical reactions- Like inulin fermentataion, bile solubility, optochin sensitivity, etc.

Differences between St. Pneumoniae and st. viridans

Characteristic	**Strepto. pneumoniae (pneumococci)**	**Strepto. Viridians**
Morphology	Capsulated, lanceolate diplococci [in pairs].	Non-capsulated, oval or round cocci in chains.
Colonies on blood agar	Initially dome shaped with alpha haemolysis, later draughtsmans colonies	Dome shaped with alpha haemolysis
Colonies in liquid medium	Uniform turbidity	Granular turbidity with powdery deposit
Bile solubility	Positive	Negative
Inulin fermentation	Positive	Negative
Optochin sensitivity	Positive	Negative

Review Questions:

1. Write the difference between str. pneumoniae and str. pyogenes?
2. Write about the virulence of str. pneumococci?
3. Comment on Biochemical reactions?
4. Make list of infections caused by str. pneumococci?
5. Write in detail about cultural charactestics?

Gram Negative Cocci

12.4 Neisseria

Introduction: The genus Neisseria consists of Gram negative aerobic, nonsporulating, nonmotile, oxidase positive cocci typically arranged in pairs [diplococci]. Besides the two important pathogens, N. meningitides and N. gonorrhoeae, the genus contains many other species such as N. lactaemia that occur as commensals in the mouth or the upper respiratory tract

12.4.1 Neisseria meningitidis

Morphology: Meningococci are Gram negative oval or spherical cocci 0.6-0.8µm in size, typically arranged in pairs, with the adjacent sides flattened. In smears from lesions, the cocci are generally intracellular. They are non motile and most fresh isolates are capsulated.

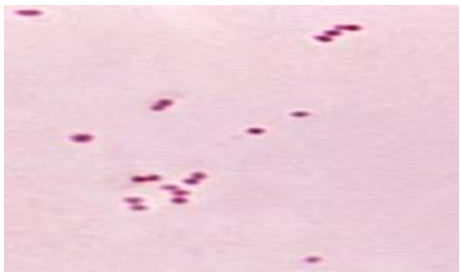


Figure 12.7 Gram negative cocci meningococci

Cultural characteristics: Meningococci have exacting growth requirement and do not grow on ordinary media. Growth occurs on media

enriched with blood, serum or ascitic fluid. Blood agar, chocolate agar and Muller- Hinton agar are commonly used media. Thayer-Martin medium with antibiotics like colistin, nystatin, vancomycin,etc act as selective medium.

They are strict aerobes, no growth occurring anaerbically. The optimum temperature for growth is 35-36^{0}C. Optimum pH is 7.4-7.6. Growth is facilitated by 5-10% CO_2 and high humidity.

On solid media colonies are small, round, convex, grey, translucent and with complete edge. They are easily emulsifiable. In liquid media it produces granular turbidity.

Biochemical reactions:

- Catalase and oxidase positive.
- Glucose and maltose are fermented, but not sucrose or lactose, producing acid but no gas.
- Indole and hydrogen sulphide [H_2S] are not produced.
- Nitrates are not reduced.

Resistance: They are very delicate organisms. They are highly susceptible to heat, dessication and disinfectant.

Pathogenicity: Meningococci cause Cerbrospinal meningitis and meningococcal septicemia. It is more common in children and young adults. They are strict human parasites. They reside in nasopharynx. Incubation period is about 3 days. The cocci spread from nasopharynx to meninges either directly along the olfactory nerve or mostly through blood stream.

Laboratory diagnosis: It involves

- Specimens- CSF, blood, nasopharyngeal swab [particularly to detect carriers] and petechial lesions can be collected. CSF is collected by lumbar puncture.
- Direct microscopy- The CSF is divided into 3 portions. The first portion is centrifuged and used for smear preparation. The smear is stained with Gram stain which shows Gram negative intracellular diplococci.

  The second portion is used for direct culture and third portion is mixed with equal volume of glucose broth. After overnight incubation it is subcultured on blood agar.
- Culture-
  - CSF- Centrifuged deposit is inoculated on blood agar or chocolate agar and incubated at 35-36^{0}C under 5-10% carbon dioxide.
  - Blood- It is positive in meningococcaemia and early cases of meningitis. The blood culture bottles are incubated at 35-36^{0}C for 24 hrs and sub-cultured on blood and chocolate agar.
  - Other specimens are inoculated on blood agar and chocolate agar.
  - Colony characteristics are studied.
- Biochemical reactions-
  - Sugar fermentation- Glucose and maltose are fermented with acid production, no gas.
  - Catalase test positive.
  - Oxidase test positive.
- Serological diagnosis-It is carried out when organism cannot be isolated in case of chronic meningococcal septicemia. Specific antibodies to capsular polysaccharide can be demonstrated by haemagglutination test.
- Molecular diagnosis- PCR can be done which is a rapid method.

Review Questions:

1. Describe morphology of meningococci.
2. What are the diseases caused by meningococci?
3. Write in detail about cultural characteristics.

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12.4.2 Neisseria Gonorrhoeae

Gonococci cause the veneral disease gonorrhoea. The gonococcus was first described by Neisser in 1879.

Morphology: Gram negative cocci arranged in pairs (diplococci) with adjacent sides concave giving typically kidney shape. They are intracellular found mainly within polymorphs. They are non-motile, non-capsulated and non-sporing. They possess hair like structures from the surface known as pili. They attach the organism to the host cells.

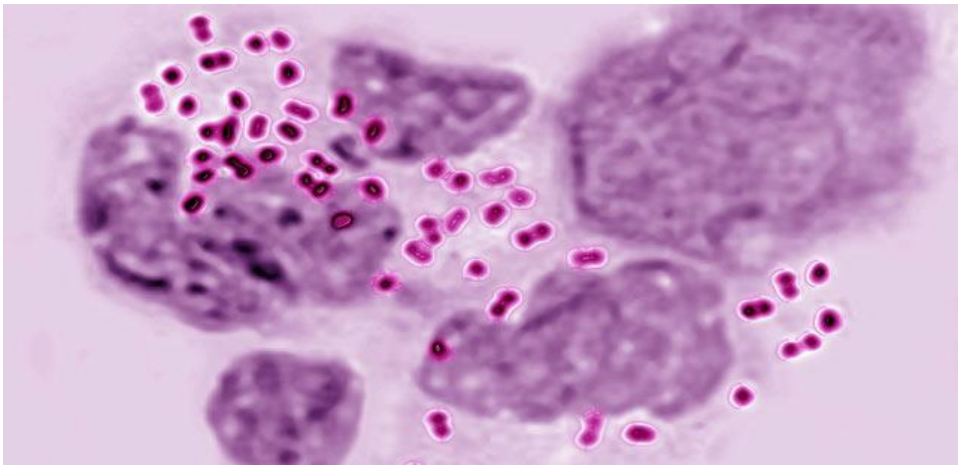


Figure 12.9 Gram negative cocci-gonococci

Cultural characteristics: Gonococci are more difficult to grow than meningococci. They are aerobic but may grow anaerobically also. Growth occurs best at pH 7.2-7.6 and at temperature of 35-36^{0}C. It is essential to provide 5-10% CO_2. They grow well on chocolate agar and Muller-Hinton agar. A popular selective medium is the Thayer-Martin mediums [containing vancomycin, colistin and Nystatin].

Colonies are small, round, translucent, convex or slightly umbonate, with finely granular surface and lobate margins. They are soft and easily emulsifiable.

Biochemical reaction:

- Gonococci ferment only glucose with the production of acid. Many resemble meningococci except in the effect on maltose.
- Oxidase positive.

Resistance: It is very delicate organism. It is a strict parasite and dies rapidly outside the human host, in 1-2 hours in exudates and 3-4 days in culture. They are readily killed by heat, drying and antiseptics.

Pathogenicity: Gonococci causes

1. Gonorrhoea- It is a sexually transmitted diseases involving urethra in both the sexes but in females, the endocervix is the primary site. Incubation period is 2-8 days. There is acute urethritis in which there is purulent urethral discharge. In males the acute urethritis may extend to prostate, testes, seminal vesicles, and epididymis.
2. In females, asymptomatic infection is common. The primary infection from urethra and cervix spread to bartholin's glands, endometrium, fallopian tubes, ovaries and lead to pelvic inflammatory disease, leading to sterility.
3. Ophthalmia neonatorum- It is non-venereal gonococcal conjunctivitis in the new born through infected birth canal.
4. Metastatic lesions such as arthritis, ulcerative endocarditis, very rarely meningitis and occasional cases of pyemia have been reported.

Laboratory diagnosis: It involves

- ❖ Specimens-

- Urethral discharge and cervical discharge (in females) in case of acute urethritis.
- In chronic urethritis- Some exudate obtain after prostatic massage or first morning drop of secretion is used.

❖ Direct microscopy- Gram staining shows Gram negative diplococcic which are intracellular.

❖ Culture-

- Direct inoculation on chocolate agar and incubated at 35-36^{0}C under 5-10% CO_2 for 48 hrs.
- Thayer –Martin medium is used as selective medium.

❖ Biochemical reactions-

- Sugar fermentation- Only glucose is fermented and acid is produced.
- Oxidase test positive.

Review Questions:

1. Describe morphology of gonococci.
2. Name the culture media used to grow gonococci?
3. Enumerate infections caused by gonococci?
4. Differentiate between gonococci and meningococci.

****

13. Mycobacteria

13.1 Introduction- Mycobacteria are slender rods that sometimes show branching filamentous forms resembling fungal mycelium. In liquid culture they form a mold-like pellicle, hence the name 'mycobacteria', meaning fungus-like bacteria. They do not stain readily, but once stained, resist decolourisation with dilute mineral acidsand therefore called acid fast bacilli or AFB.

Classification: Mycobacteria are classified in to following groups.

- **Cultivable—**
  - Tubercle Bacilli
    - Human- M. tuberculosis
    - Bovine- M. bovis
    - Murine- M. microti
    - Avain- M. avium
    - Cold blooded- M. marinum
  - Atypical Mycobacteria
    - Photochromogens
    - Scotochromogens
    - Non-photochromogens
    - Rapid growers
  - Mycobacteria causing skin ulcers
    - M. ulcerans
    - M. balnei
  - Saprophytic Mycobacteria
    - M. smegmatis
    - M. butyricum
    - M. Stercoris

- M. phlei
  - Johne's Bacillus
    - M. paratuberculosis

❖ **Non-cultivable—**

- Human- M.leprae
- Rat- M. leprae murium

13.2 Mycobacterium tuberculosis

Morphology: M. tuberculosis is a straight or slightly curved rod, about 3µm x 0.3µm, occurring singly, or in pairs or small clumps. The bacilli are non-motile, non-sporing and non-capsulated. The bacilli are Gram positive though they do not take the stain readily.

They are stained with Ziehl-Neelsen method or by fluorescent dyes (auramine-o, rhodamine). They are known as acid fast because when they are stained with carbol fuchsin of Z-N stain, they resist decolourisation by 25% sulphuric acid and absolute alcohol. The acid fastness is due to the presence of an unsaponifiable wax (mycolic acid) or to a semi-permeable membrane around the cell. With this stain the tubercle bacilli appear bright red in colour and rest of the tissue cells blue in colour. With fluorescent dyes they appear yellow luminous bacilli under the fluorescent microscope. The tubercle bacilli appear as beaded form.

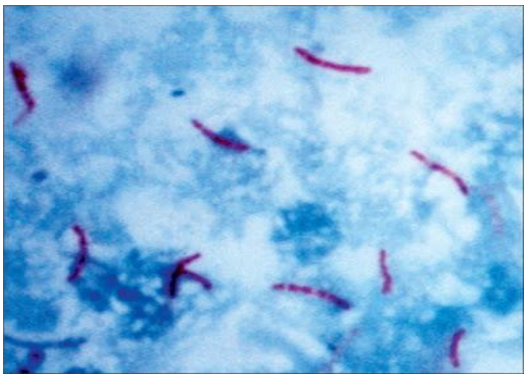


Figure 13.1 Tubercle bacilli

Mycobacterium bovis is straighter, strouter and shorter than tubercle bacilli. With Z-N they take more uniform staining than tubercle bacilli.

Cultural characteristics: The bacilli grow slowly, the generation time in vitro being 14-15 hours, colonies appear in about two weeks and may sometime take upto eight weeks, optimum temperature is 37^0C and growth does not occur below 25^0C or above 40^0C. Optimum pH is 6.4-7.0. M. tuberculosis is an obligate aerobe. Tubercle bacilli can grow on a wide range of enriched culture media Lowenstein-Jensen (L-J) medium is most commonly used. 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.

Figure 13.2 L-J medium

- Colonies of M. tuberculosis on L-J medium are rough, dry, buff coloured, raised with wrinkled surface. The growth is luxuriant (eugonic growth).
- Mycobacterium bovis shows flat, smooth, moist and white colonies which can be easily emulsified. Growth is sparsely (dysgonic growth).
- In liquid medium without dispersing agents the growth begins at the bottom creeps up at the sides and forms a prominient pellicle.

Diffuse growth is obtained in Dubos' medium containing a detergent tween-80.

Biochemical reactions: Several biochemical tests have been described for the identification of mycobacterial species.

- Niacin test: Human tubercle bacilli form niacin when grow on an egg medium. This test is positive with human type and negative with bovine type of bacilli.
- Aryl sulphatase test: This test is positive only with atypical mycobacteria.
- Neutral red test: Virulent strains of tubercle bacilli are able to bind neutral red in alkaline buffer solution, while avirulent strains are unable to do so.
- Catalase-peroxidase test: Tubercle bacilli are weakly positive for catalase and also peroxidase positive.
- Amidase test: Positive with mycobacterium tuberculosis.
- Nitrate-reduction test: Positive with M.tuberculosis and negative with M.bovis.

Resistance: Mycobacteria are killed at 60^0C in 15-20 minutes. They are sensitive to ultraviolet rays and sunlight. Cultures may be killed by exposure to direct sunlight for 2 hours but they may remain viable in sputum for 20-30 hours, in droplet nuclei for 8-10 days. They are relatively resistant to chemical disinfectant**.**

Pathogenesis: M.tuberculosis is mainly responsible for causing pulmonary tuberculosis in humans. It is divisible in to two forms- Primary and Secondary

- **Primary tuberculosis**-The infection is acquired by inhalation of infected droplets coughed or sneezed into the air by a patient with pulmonary tuberculosis. These bacilli are engulfed by alveolar macrophages in which they replicate to form a lesion called Ghon's

focus. Generally it is found in lower lobe or lower part of upper lobe. Some bacilli enter in to hilar lymph nodes. The Ghon's focus along with enlarged hilar lymph nodes is called primary complex. In case of M. bovis which enters through mouth primary complex involve the tonsil and the cervical lymph nodes or the intestine, mostly ileocaecal region and mesenteric lymph nodes.

- **Secondary tuberculosis**- It is caused by reactivation of primary lesion (endogenous) or by exogenous reinfection. Reactivation of tuberculosis likely to occur in immunocompromised individuals. The typical lesion which develops is the tubercle. It is an avascular granuloma composed of a central zone containing giant cells with or without caseation necrosis, surrounded by epithelioid cells and a peripheral zone of lymphocytes and fibroblasts. Granuloma of secondary tuberculosis most often occurs in the apex of the lungs. The necrotic element of the lesion causes tissue destruction and formation of large areas of caseation called tuberculomas. Presence of caseaous necrosis and cavities are two important features of secondary tuberculosis. Cavities may rupture into blood vessels, spreading mycobacteria throughout the body and break into airways releasing organisms in sputum (open tuberculosis).

Tuberculin [Mantoux] test- It is delayed or type IV hypersensitivity reaction. Mantoux test, 0.1 ml of Purified Protein Derivative [PPD] is injected intradermally on the flexor aspect of the forearm, with a tuberculin syringe.The site is examined after 48-72 hours and it is interpreted as follows:

Figure 13.3

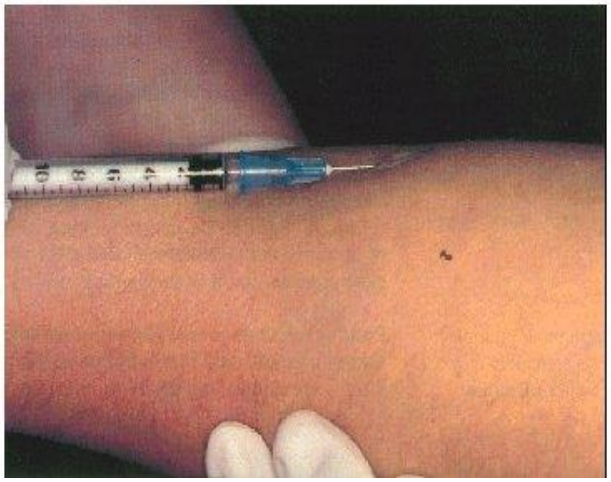


Figure 13.4

- **Positive test**-There is induration of 10mm diameter or more surrounded by erythema at the site of inoculation. Positive test indicates past infection with tubercle bacilli but does not state presence of active stage of disease.

This test is helpful in children under 5 years of age for indication of active infection.

The test becomes positive 4-6 weeks after infection or BCG vaccination.

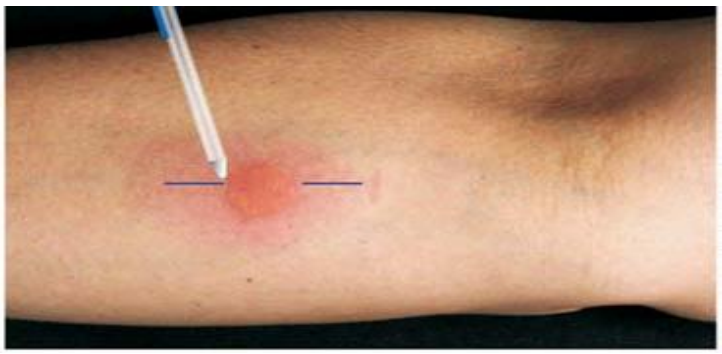


Figure 13.5

- **False negative**- The test becomes negative in
  - Miliary tuberculosis
  - When anergy develops following overwhelming infection of measles, lepromatous leprosy, malnutrition, use of corticosteroids, etc.

- **False positive**- Found with related mycobacteria like atypical mycobacteria.

Laboratory Diagnosis: Laboratory diagnosis of tuberculosis is established by demonstrating the bacillus in the lesion by

- Microscopy,
- Isolating in culture
- Molecular diagnostic methods

Specimen- Depends on site of involvement. Tuberculosis may involve lungs (pulmonary) or sites other than lungs.

- In case of pulmonary tuberculosis-
  - Sputum is the most common specimen. A morning specimen is collected on three consecutive days. If it is scanty a 24 hour sample is collected.
  - If sputum is not available laryngeal swab or bronchial washings are collected.
  - In children gastric washings are examined as children swallow the sputum.
- Tuberculosis meningitis (TBM)-
  - CSF is collected. Presence of cobweb is of diagnostic importance.
- Renal tuberculosis-
  - Morning urine sample on three consecutive days.
- Bone and joint tuberculosis-
  - Aspirated fluid
- Tissue
  - Biopsy of tissue.

- **Direct microscopy**- Preparing a smear from sample and staining with Z-N stain. Bacilli appear bright red in color against blue background. **(**New slides should be used for smears and they should not be reused as

the acid fast bacilli may not always be removed from slides by cleaning.)

At least 50,000 acid fast bacilli should be present per ml of sputum for them to be readily demonstrable in direct smears. A negative report should not be given till at least 300 fields have been examined.

Microscopy can be done on concentrated sample. More commonly Petroff's method is used.

- **Culture**-It is very sensitive method for detection of tubercle bacilli. It may detect as few as 10 -100 bacilli/ml. L-J medium is used. It is incubated at 37^0C. It is first examined after 4 days and thereafter weekly till 8 weeks.TB bacilli usually grow in 2-8 weeks. They are slow growing organisms. Negative report should be given only when there is no growth after 8 weeks.

  After isolation of colonies these are stained with Z-N stain to observe AFB.

- **Molecular methods**- Polymerase chain reaction (PCR) is a rapid method in diagnosis of tuberculosis.It is based on DNA amplification and it is carried out in clinical sample directly.

Prophylaxis: It is done by public health measures, BCG vaccination and by chemoprophylaxis. Immunoprophylaxis is by intrademal injection of the live attenuated vaccine developed by calmette and Guerin [1921] the **Bacille calmette Guerin or BCG.** The results have been varied widely, from 80% protection to a total absence of protection.

Vaccine is given intradermally in a dose of 0.1 ml BCG.

A small nodule develops at the site of vaccination 2-3 weeks after injection. Slowly it increases in size and attains a diameter of 4-8 mm after 5 weeks. It then breaks in to shallow ulcer which heals spontaneously leaving a 4-8 mm diameter permanent round scar.These individuals become tuberculin test positive after 4-6 weeks.

Figure 13.6

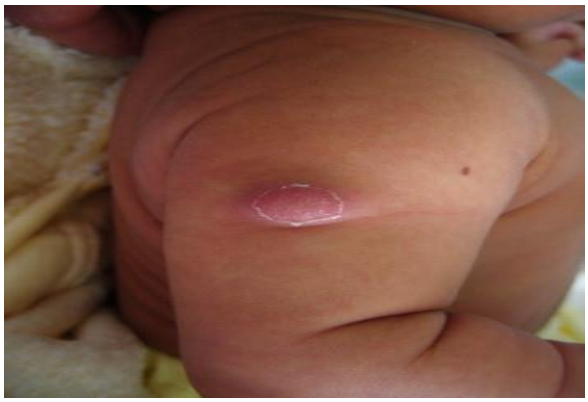


Figure 13.7

Review Questions:

1. Mention the staining technique used in tuberculosis?
2. What is the importance of Niacin test?
3. What is the host range of M. tuberculosis?
4. Write about the classification of M. tuberculosis?
5. Name the infections caused by M. tubercle bacilli?

******

13.3 Mycobacterium leprae

Introduction: Leprosy is a disease of great antiquity; having been recognized from Vedic times in India and from Biblical times in the middle east. It probably originated in the tropics and spread to the rest of the world. The lepra bacillus was the first bacterial pathogen of humans to be described it remains one of the least understood because it has not been possible to grow the bacillus in culture media.

The lepra bacillus was first observed by Hansen in 1868.So leprosy is also known as Hansen's disease. Lot of research work is done in India. ICRC Bombay grown bacilli in tissue culture. Dr. Dharmendra introduced an antigen for lepromin test.National institute of immunology; New Delhi has developed a candidate vaccine.

Morphology: M. Leprae is a straight or slightly curved rod, 1-8µm x 0.2-0.5 µm in size, showing considerable morphological variations. It is Gram positive and stains more readily than the tubercle bacillus. They are acid fast but less than tubercle bacilli. So 5% sulphuric acid is used for decolorisation in Z-N stain.

The bacilli are seen singly and in groups, intracellularly or lying free outside the cells. They frequently appear as agglomerates. These masses are known as 'globi'. The globi appear in Virchow's 'lepra cells' or 'foamy cells' which are large undifferentiated histocytes.

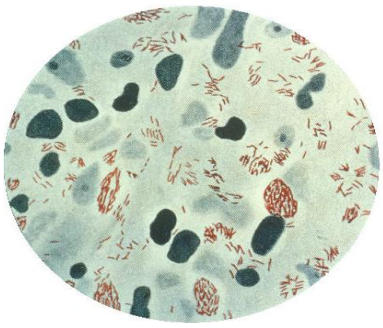


Figure 13.7 M. leprae

Cultivation: It has not so far been possible to cultivate leprae bacilli either in bacteriological media or in tissue culture. There have been several reports of successful cultivation but none has been confirmed.

Resistance: Lepra bacilli found to remain viable in warm humid environment for 9-16 days and moist soil for 46 days. They survive for two hours when get exposed to direct sunlight and 30 minutes in U-V light.

Pathogenecity: Leprosy is an exclusively human disease and the only source of infection is the patient. Leprosy is a chronic granulomatous disease of human. Following organs are primarily infected

a. Skin;
b. Peripheral nerves;
c. Nasal mucosa but capable of affecting any tissue or organ.

Leprosy is classified into five groups:

a. Tuberculoid
b. Borderline tuberculoid
c. Borderline borderline
d. Borderline lepromatous
e. Lepromatous

Lepromin Test: Lepromin test is described by Mitsuda [1919] to study the immunity in Leprosy patient. It is a skin test for delayed hypersensitivity.

Objective: The test is employed for the following purposes:

- To classify the lesion of leprosy patients
- To assess the prognosis and response to treatment.
- To assess the resistance of individual patient.
- To verify the identity of candidate of lepra bacilli.

❖ The response to injection is biphasic consists of two separate events:

- **Early reaction of Fernandez**: Consists of Erythema & induration developing in 24-48 hours and remains for 3-5 days.

- **Late reaction of Mitsuda**: In positive test there is formation of skin nodule, which is measure of cell mediated immunity induced by lepromin Injection.

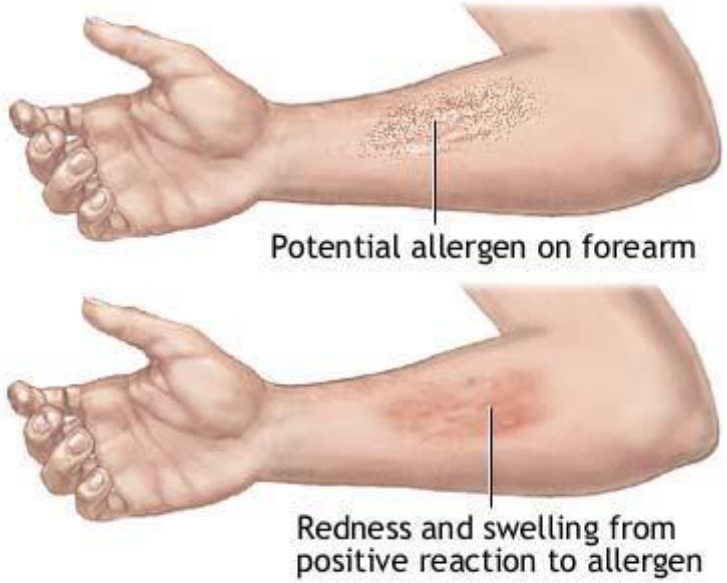
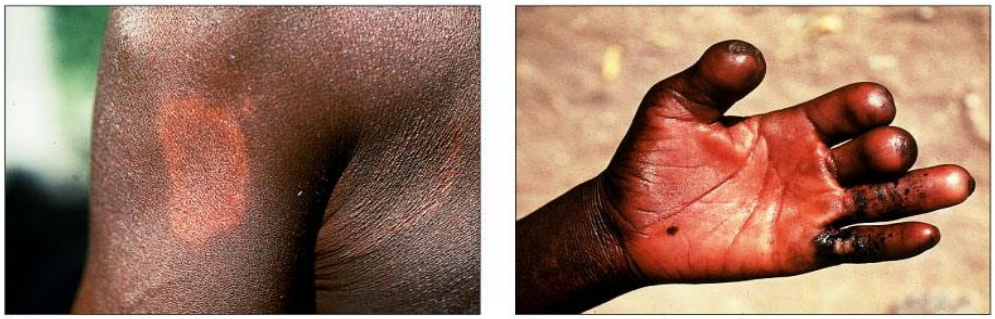


Figure 13.8

Lepromin test is positive in tuberculoid and negative in lepromatous leprosy.



(a) Tuberculoid (neural) leprosy

(b) Lepromatous (progressive) leprosy

Lab Diagnosis:

The diagnosis consists of demonstration of **acid fast bacilli** in the lesion. For routine examination **specimens** are collected from the

- Nasal mucosa

- Skin lesions
- Ear lobule
- Biopsy of the nodular lesions and thickened nerves, and lymph node puncture sometimes

A blunt narrow scalpel is introduced into the nose and internal septum scraped sufficiently to remove a piece of mucuos membrane, which is transferred to a slide to prepare uniform smear.

Sample from the skin should be obtained from the edges of the lesion rather than from the centre. About 5-6 different areas of the skin are sampled, including the skin over the buttocks, forehead, chin, cheek and ears.

Staining- The smears are stained by Ziehl-Neelson technique using 5% instead of 20% sulphuric acid for declourization.

Grading of smear is as follows-

Number of bacilli	Grade
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+
100-1000 bacilli per field	5+
More than 1000 bacilli, clumps and globi in every field	6+

Bacteriological Index (BI) - Totallying the number of pluses scored in all the smears and divided by the number of smears.

Morphological Index (MI)-It is the percentage of uniformly stained bacilli out of total number of bacilli counted.

- Lepromin test.

Prophylaxis:

- Case finding and

- Adequate therapy.

Review Questions:

1. Why M. leprosy bacilli remains to be least understood?
2. What are the 'Virchow's lepra cells'?
3. Name the organs nfected by M. leprae bacilli?
4. Describe M. Lepra bacilli's morphology.
5. Introduce M. Lepra bacilli.

******

14- Spirochaetes

Objectives: After reading this chapter, the student will be able to describe morphology & pathogenesis of T. pallidum. How to take precautions during sample collection .Laboratory tests carried out for diagnosis of syphilis with great sense of responsibility

14.1 Introduction

Spirochetes, which are helical, slender, relatively long bacteria, are widespread in nature, only a few cause diseases in humans and animals. The principal human diseases are syphilis (Treponema pallidum), lyme fever and relapsing fever (Borrelia species) and leptospirosis (Leptospira species).

Treponemes

The genus Treponema comprises spiral shaped, motile and strictly anaerobic spirochetes associated with the mucous membranes of humans and animals, they cannot be cultivated in vitro.

14.2Treponema Pallidum

Treponema pallidium is the causative agent of syphilis. The name pallidium refers to its pale staining.

Morphology:

Itis thin delicate spirochaete with tapering ends, having about ten regular spirals. It is about 10 µm long and 0.1 – 0.2 µm wide. It is actively motile, showing rotation round the long axis backward and forward movements & flexion of whole.

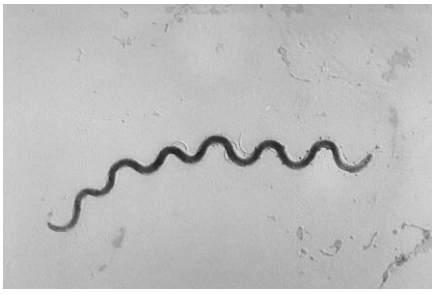


Fig. 14.1 Treponema pallidum

It does not take ordinary bacterial stains and cannot be seen under the light microscope in wet films. 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.

T. Pallidum in tissue section

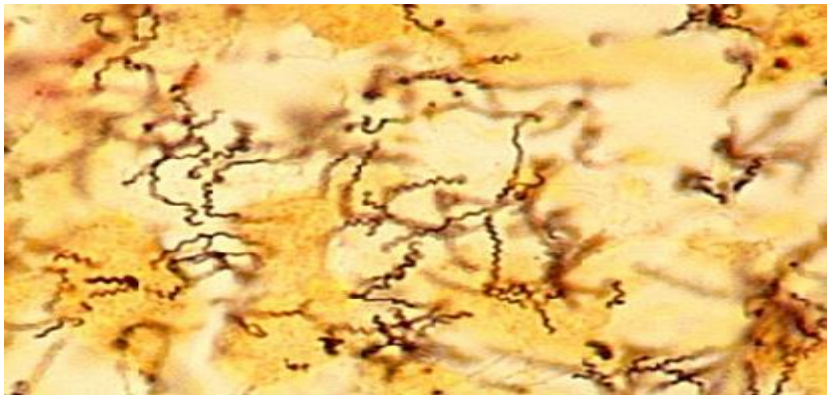


Figure 14.2

Culture – Pathogenic treponemes cannot be grown in artificial culture media in routine pathology laboratory.

14.3 Pathogenesis-

Treponemes can cross the placental barrier from the bloodstream of an infected mother and cause disease in the fetus **(congenital syphilis)** or spread from an infected individual by sexual transmission to cause **acquired syphilis**. In the latter, primary, secondary and early latent lesions constitute early manifestations while late latent, tertiary (benign gummatous syphilis) and quaternary (cardiovascular, neurological and other manifestations) lesions constitute late manifestations.

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.

14.4 Laboratory diagnosis –

Because of the social and emotional overtones of the disease, the diagnosis of syphilis should impose a great sense of responsibility on the laboratory.

Collection of specimen

Microscopy – Specimens should be collected with care as the lesions are highly infectious. The lesion is cleaned with gauze soaked in warm saline and the margins gently scraped so that the superficial epithelium is abraded. Gentle pressure is applied to the base of the lesion and the serum that exudes is collected, preventing admixture with blood. Wet films are prepared with the exudate and after applying thin coverslips, examined under the dark ground microscope

The diagnosis of syphilis consist of –

1. Demonstration of treponemes.
2. Detection of antibodies by serological tests.

Demonstration of treponem

Demonstration of treponemes by microscopy is applicable in primary and secondary stages and in cases of congenital syphilis with superficial lesions.

- **Dark ground microscopy –**

The wet film of exudate 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. Dark field examination should be repeated on three consecutive days before declaring it negative.

- **Diagnostic Methods in Syphilis-**

- 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.
- **Serological Tests –**

These tests form the mainstay of laboratory diagnosis. Depending upon the antigen used, serological tests for syphilis are divided into

- ❖ Non-treponemal-tests (cardiolipin or lipoidal antigen is used)
- ❖ 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 plasm regain (RPR), Kahn test and Wassermann reaction. All these tests are flocculation tests except Wassermann reaction which is a compliment fixation test (CFT). The Wassermann reaction is no longer in use. Similarly Kahn test is rarely done.

14.5 VDRL& RPR Tests (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.

- ➢ **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.


Fig. 14.3 V.D.R.L Slide

Results

The results of qualitative test are reported as ‘reactive’, ‘weak reactive’ or ‘non reactive’. ‘Reactive’ means positive while ‘non-reactive’ is negative.

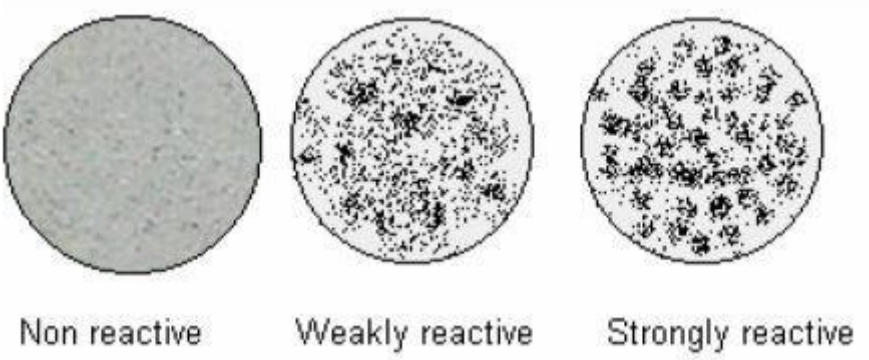


Fig. 14.4 Results of VDRL Test

- **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 cannot be used with cerebrospinal fluid (CSF)

❖ **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.

Conditions in which BFP reactions occur include:

- Leprosy (particularly lepromatous leprosy).
- Malaria.
- Relapsing fever.
- Infectious mononucleosis.
- Tropical eosinophilia.
- Hepatitis.
- Collagen diseases.

Treponemal Tests

Tests using Reiter treponeme: Reiter protein complement fixation test.

1. Tests using T.pallidum(Nichol's strain)

a) Using live T.pallidum: T.pallidum immobilization test (TPI)

b) Using killed T.pallidum :
   - T.pallidum agglutination test(TPA)
   - Treponema pallidum immune adherence(TPIA) test
   - Fluorescent treponemal antibody (FTA) test

2. Using an extract of T.pallidum
   - Treponema pallidum haemagglutination assay(TPHA) test
   - Enzyme Immunoassay (EIA).

Review questions-

1. What are spirochaetes? Which are different tyopes?
2. Describe laboratory diagnosis of syphilis.
3. Describe morphology, cultural characteristics of T. pallidum.

15. Leptospira

Objectives- Students shall understand morphology, cultural characteristics and pathogenesis of leptospira.

15.1 Introduction

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 means fine or thin). They do not stain readily. They may be observed by dark ground illumination.

15.2 Classification:

The genus Leptospira is classified into two species.

1. L. interrogans- Pathogenic.
2. L. biflexa- Saphrophytic.

15.3 Leptospira interrogans:

Morphology

They are delicate flexible helical rods about 6-20µm long and 0.1µm thick. They contain numerous coils which are set very close together that they can be distinguished only under dark ground illumination in the living state or by electron microscope. Their ends are hooked and look like umbrella handle. They are actively motile.Poor staining with aniline dyes but stained with Giemsa stain.Very well observed by silver impregnation method.

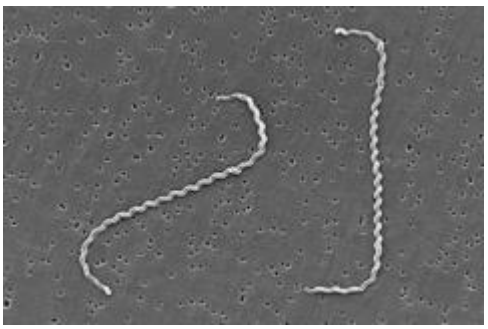


Figure 15.1

Cultural characteristics

They are aerobes and microaerophilic. Optimum temperature 28-32^{0}C and pH7.2-7.5. They can be grown on media enriched with rabbit serum. Several liquid and semisolid media, such as Korthof's, Stuart's and Fletcher's media are used. Semi-synthethic media such as EMJH (Ellinghausen, McCullough, Johnson, Harris) medium is now commonly used. Growth is detected after 6-14 days of incubation. Leptospires may be grown on chorioallantoic membrane (CAM) of chick embryo.

Resistance

They are susceptible to heat and killed in 10 mins at 50^{0}C and in 10 seconds at 60^{0}C. They are sensitive to acid and destroyed by gastric juice in 30 minutes. Bile destroys them rapidly. They are also destroyed by chlorine and other antiseptics and disinfectants.

Pathogenicity

- L.interrogans causes a zoonotic disease named leptospirosis in rodents and sometimes in domestic animals. It is transmitted to humans by direct or indirect ccontact with water contaminated by urine of carrier animals. They enter the body through cuts on the skin or through mucus membranes of the mouth, nose or conjunctiva.
- After an incubation period of 6-8 days there is onset of fever with leptospires in the blood (**septicemic phase**). This stage lasts for 3-7 days. Then the organisms disappear from the blood and enter into liver, kidney, spleen and meninges producing meningial irritation. Leptospirosis is an established cause of aseptic meningitis. They persist in the internal organs and most abundantly in the kidney. Therefore they may be demonstrsted in the urine in later stages of disease.

- Severe leptospirosis **(Weil's disease)** associated with fever, conjunctivitis, albuminuria, jaundice and haemorrhage is a fatal illness with hepatorenal damage.

Laboratory diagnosis

The diagnosis is done by demonstrstion of leptospires in blood or urine microscopically and by isolating them in culture or by serological tests.

Demonstration of leptospires in the blood or urine-

By dark-ground microscopy- Examination of blood is useful in 1st week and then from 2nd week upto 6 weeks urine is useful. Urine is centrifused and deposit is examined by dark-ground microscopy.

Culture- Sample is inoculated on Korthof's medium or Fletcher's medium.

Serological tests- It is very useful method for diagnosis of leptospirosis. Antibodies appear at the end of 1stweek and continue to rise till the 4th week and then decreases. Two types of serological tests are there.

- Screening test- These are genus specific and carried out by using a broadly reactive genus specific antigen usually nonspecific. They detect IgG and IgM antibodies.These tests are CFT, ELISA, agglutination, etc.Now a days a rapid dip stick assay is developed to detect IgM antibodies.
- Serotype specific tests- The type specific test identify the infecting serovar by demonstrating specific antibodies. For macroscopic and microscopic agglutination tests are used.
- In macroscopic agglutination test formalinised suspensions of prevalent leptospira serovars are tested with serial dilutions of test serum.
- In microscopic agglutination test (MAT) live cultures of different serotypes are used and agglutination is observed under low power

of dark-ground illumination. This test is usually done in reference laboratories.

- **Animal inoculation**-The biood or urine of the patient is inoculated intraperitoneally into guinea pigs. From third day of inoculation peritoneal fluid is examined daily for leptospires by dark-ground illumination. Heart biood is cultured and serotype is identified by agglutination.

Prophylaxis- Prevention is by

- Rodent control.
- Disinfection of water.
- The wearing of protective clothing.

Review questions:

- What are leptospira?
- How will you demonstrate leptospira?
- How will you prevent leptospirosis?
- How will you diagnosis leptospirosis?

****

16. Clostridium

Objectives- Students should know about organisms causing diseases like gas gangrene, tetanus.

16.1 Introduction

The genus Clostridium consists of Gram positive anaerobic spore forming bacilli. The spores are wider than bacillary bodies which gives bacillus swollen appearance. It resembles spindle, so called clostridium (Kloster means spindle) Clostridia are highly pleomorphic. They are rod shape usually 3-8µ×0.4-1.2µ in size. They are motile with peritrichate flagella except Cl. welchi and Cl.tetani typeVI which are nonmotile. They contain spores. Position of spores varies. They are easily stained. They are responsible for three major diseases- Gas gangrene, food poisoning and tetanus.

16.2 Classification

Classificationdepending on position of spore as

- Central or equatorial –spindle shape appearance e.g. *Cl. bifermentans*
- Subterminal-club shape e.g. *Cl. welchii*
- Oval and terminal- tennis racket shape e.g. *Cl. tertium*
- Spherical and terminal-drumstick appearance e.g. *Cl.tetani*

Classification depending on diseases produced

- The gas gangrene group- *Cl. welchii , Cl. septicum*
- Tetanus- *Cl. tetani*
- Food poisoning-
    1. Gastroenteritis- *Cl. welchii* TypeA
    2. Botulism- *Cl.botulinum*
    3. Acute colitis- *Cl. Difficile*

16.3 Clostridium welchii (Cl. Perfringens)

Clostridium perfringes is a Gram positive, rod shaped, anaerobic, spore forming bacterium of the genus Clostridium. Cl. perfringens is ever present in nature and can be found as a normal component of decaying vegetation, marine sediment, the intestinal tract of humans and other vertebrates,insects, and soil. This was first isolated by Welch and Nuttal. This is most important clostridia causing gas gangrene.

Morphology

Gram positive bacilli about 4-6 µ×1µ. It is pleomorphic, nonmotile, capsulated and sporulated. Spores are central or subterminal but spores are rarely seen in pathologic sample. Their absence is taken as characteristic morphological features of *Cl. Welchi.*

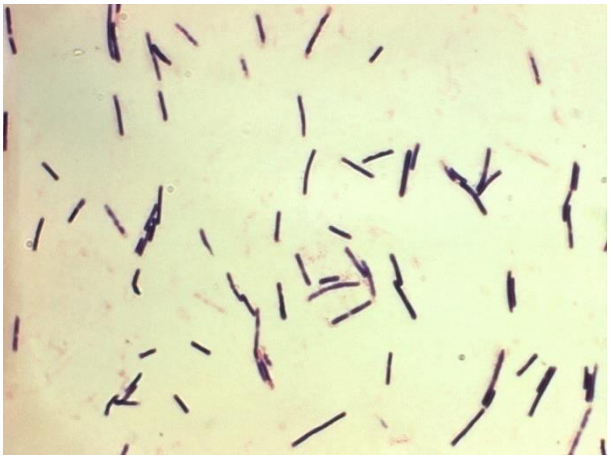


Fig. 16.1 Clostridium welchii

Cultural characteristics

- It is anaerobe but can grow under microaerophilic condition.
- Temperature required range is 20-50^{0}C. pH 5.5-8
- Well grown on enriched media.
- Commonly used medium is **Robertson' cooked meat medium**. Medium turns pink. The culture has sour odour.

- In Litmus milk-there is stormy fermentation. There is fermentation of lactose leads to acid formation which coagulates milk. The clotted milk disrupts due to gas formation.
- On Blood agar- the organism show double zone pattern of haemolysis.

Biochemicals-

- It ferments lactose, glucose, maltose and sucrose with production of acid and gas both.
- Indole negative,
- MR positive
- VP negative.
- It produces H_2S and reduces nitrates.

Resistance

Spores are destroyed in 5 minutes by boiling.Type A and Type C resists boiling for 1-3 hours. Autoclaving destroys spores. It can not be destroyed by commonly used disinfectants.

Toxins

It produces four major toxins-alha (α), beta (β), epsilon (ε) and iota (ι). Alpha toxin is responsible for pathogenic effects.Other toxins are gamma, eta, delta, theta, kappa, lambda, Mu and Nu. Depending on toxins produced *Cl. welchii* is divided into 5 types-Type A to E. Apart from these toxins it produces certain soluble substances some of them can cause pathogenic effects.

Pathogenicity

Cl. welchii produces

- Gas gangrene- Caused by *Cl. welchii* typeA mainly.They lead to wound infections or anaerobic cellulitis. All wound infections does not lead to gas gangrene. When muscle tissue gets involved then gas gangrene takes place.

- Gas gangrene is rapidly spreading, oedematous myonecrosis occurring characteristically in association with severe wounds of extensive muscle masses that have contaminated with *Cl. welchii*. Initially it was known as malignant oedema. Also called as anaerobic (clostridial) myositis and clostridial myonecrosis. Characterstically it is disease of war, in which extensive wounds with heavy contaminations are common. It is due to several species of clostridia along with anaerobic *Streptococci* and others. Clostridia enter the wounds along with implanted foreign particles like soil, road dust , bits of clothing or shrapnel.

  The incubation period is 7 hours to 6 weeks. With *Cl. Welchii* it is 10-48 hours.

  There is pain, tenderness and oedema of the affected part along with signs of toxaemia.There is discharge from the wound, later on it becomes profuse. Accumulation of gas is there. In untreated cases complications develop and it becomes fatal.
- Food poisoning- Some strains of Type A *Cl welchii* lead to food poisoning. Incubation period is 8-24 hours. There is abdominal pain, diarrhea and vomiting. Generally recovery occurs in 24-48 hours.
- Gangrenous appendicitis- caused by Type A *Cl welchii*.
- Necrotising enteritis- caused by Type C *Cl welchii*
- Necrotising colitis- Rare but fatal.
- Biliary tract infection
- Endogenous gas gangrene of intra-abdominal origin.

  Apart from these other infections like brain abscess, panophthalmitis, and thoracic infections can occur.

Laboratory diagnosis

Diagnosis should be done clinically and it is confirmed by laboratory tests. Bacteriological examination helps to differentiate it from anaerobic streptococcal myositis.

Specimen collection-

- Films from the muscles at the edges of the affected area, from the tissues in the necrotic area and from exudates in the deeper parts of wound.
- Exudate from deeper parts of wound.
- Necrotic tissue and muscle fragment.

Staining- Gram staining indicate species of clostridia and also to know if anaerobic streptococci present.

Culture-Aerobic and anaerobic culture is made on horse blood agar and heated blood agar.

Robertson's cooked meat broth are inoculated and heated at 100^0C for 5, 10, 15 and 20 minutes and subcultured on blood agar after 24-48 hours.

Nagler reaction- When Cl. welchii grown on a medium containing 6% agar, 5 % Fields' peptic digest of sheep blood and 20% of human serum with antitoxin spread on half of the plate, colonies on the half without antitoxin will show zone of opacity around them.There is no zone of opacity around the colonies on the half of plate with antitoxin due to specific neutralization of α toxin. This specific lecithinase effect is known as Nagler's reaction.

Isolates are confirmed by morphological, colony characteristics, biochemical and toxigenic characteristics.

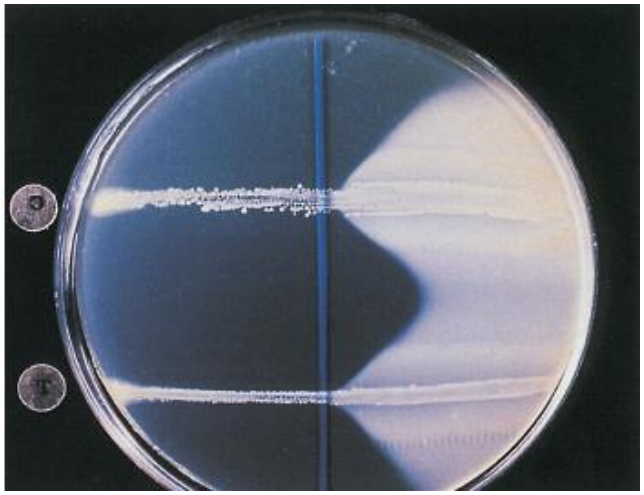


Fig. 16.2 Negler reaction

16.4 Clostridium tetani

Clostridium tetani is a causative agent of tetanus.It is widely distributed in soil and in the intestine of man and animals. It is ubiquitous.

Morphology

It is Gram positive bacilli about 4.8µ×0.5µ. It has a straight axis, parallel sides and rounded ends. The spores spherical, terminal and buldging giving organism drumstick appearance. They are motile by peritrichate flagella and noncapsulated.

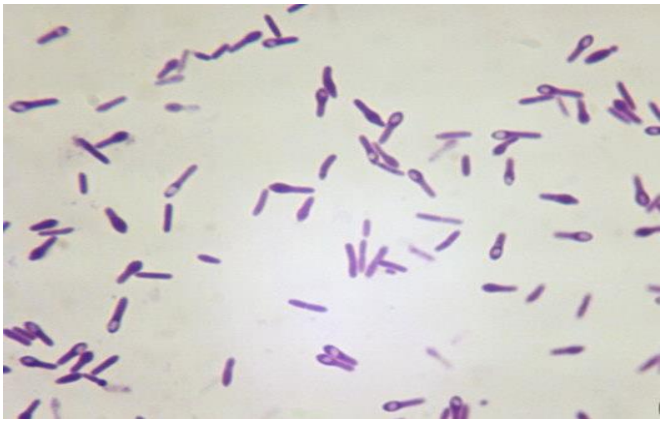


Fig. 16.3 Cl tetani

Cultural characteristics- It is an obligatory anaerobe. The optimum temperature is 37^0C and pH 7.4. Grow well on ordinary media. Growth is improved by blood and serum, not by glucose.

- On Robertson's cooked meat broth it shows turbidity with some gas formation. The meat turns black on prolong incubation.
- On horse blood agar it shows α-haemolysis.

Biochemical reactions

- Does not ferment any sugar.
- Indole positive,
- MR and VP negative.
- Does not produce H_2S
- Nitrates are not reduced.
- Greenish fluorescence on media containing neutral red.

Resistance

Majority strains of Cl. tetani are destroyed by boiling for 10-15 minutes but some require 3-4 hours. Autoclaving at 121^0C for 20 minutes destroy spores completely. Spores are resistant to antiseptics.Iodine (1% aqueous solution) and hydrogen peroxide (10 volumes) kill the spores within few hours.

Toxins

Cl.tetani produces two toxins- tetanolysin which is a haemolysin and tetanospasmin which is neurotoxin. Tetanospasmin is responsible for clinical manifestation of tetanus.

Pathogenicity

Cl. tetani is responsible for **tetanus**.Tetanus develops when wound gets infected with tetanus bacilli. It shows tonic muscular spasms which starts at the site of infection but later on spreads involving muscular system.Tetanus is an important complication of septic abortion.Only contamination with tetanus bacilli does not lead to tetanus. When all other factors like anaerobic condition, necrosis of tissue, presence of extraneous matter particularly soil,can lead to disease occurrence.

Incubation period varies from two days to several weeks but generally it is 6-12 days. The incubation period is of progressive significance, shorter the incubation period more fatal is the prognosis.The main symptom is trismus and then onset of spasm.

Laboratory diagnosis

Clinical diagnosis along with confirmation by laboratory tests involves demonstration of bacilli by microscopy, culture and animal inoculation.

Specimen- Bits of tissues from necrotic part of wound and wound swab. Microscopy is not much reliable.

Culture -On blood agar specimen is inoculated on half of blood agar plate.After 24-48 hours of incubation swarming growth is seen on other half of agar.

The specimen is inoculated in three tubes of Robertson's cooked meat broth. First two tubes are heated at 80^0C for 15 minutes and 5 minutes respectively and third is kept without heating. These tubes are incubated at 37^0C and then subcultured on blood agar on its half side for 4 days.*Cl.tetani* are resistant to polymyxin B so by adding into medium polymyxin B it is used as selective medium to confirm the organism.To detect toxicity antitoxin is spread on other half of horse blood agar plate.On the half of medium without antitoxin colonies show haemolysis and no lysis on other half of plate.

Prophylaxis- Tetanus is preventable disease. It is carried out by active immunization.

16.5 Clostridium botulinum

Cl.Botulinum causes botulism, a severe form of food poisoning.The bacillus is widely distributed in vegetables, hay, silage, animal manure and sea mud.

Morphology

Gram positive bacilli about 5µ×1µ, noncapsulated, motile by peritrichate flagella, oval, subterminal and bulging spores.

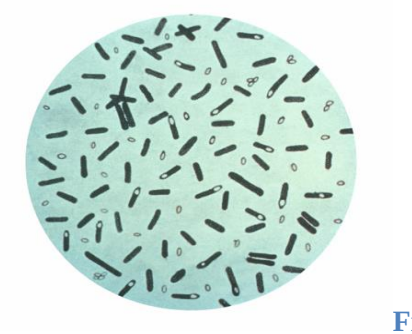


Figure 6.4 Cl. botulinum

Cultural characteristics

It is strict anaerobe. Optimum temperature 35^0C. Grow well on ordinary media.Colonies are large, irregular, semitransparent with fimbriate border.Spores can be produced by growing them in alkaline glucose gelatin media at 20-25^0C.

Resistance

Spores are highly resistant to boiling and at 120^0C for upto 20 minutes.

Toxin

Produces powerful exotoxin that is responsible for pathogenicity.This toxin appears in the medium only on death and autolysis of the cell.

Pathogenicity

Cl.botulinum produces botulism which is of three types-food borne, wound and infant botulism.

Laboratory diagnosis

Demonstration of bacillus or the toxin in food or faeces.

Control

Control can be achieved by proper canning and preservation of food.

Review Questions-

1. What are clostridia?
2. What are metachromatic granules:
3. What are different types of spores present in clostridia?
4. Classify clostridia.
5. Differentiate between Cl.welchii and Cl. tetani.
6. Laboratory diagnosis of gas gangrene.
7. Laboratory diagnosis of tetanus.

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17- **Antibiotic sensitivity test**

Objective- Students shall understand significance of of doing antibiotic sensitivity test and its different methods.

17.1 Introduction

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.

Antibiotic sensitivity is carried out by two methods-

- Diffusion method
- Dilution method

1.1 Diffusion method

In this method drug is allowed to diffuse through a solid medium so that a gradient is established, the concentration being highest near the site of application of drug and decreases with distance. The test bacterium is seeded on the medium and its sensitivity is determined from the inhibition of its growth.

Several methods are used for the application of drug. It may be added to holes cut in medium or to hollow cylinders placed on it. Most commonly employed method is to use filter paper discs, impregnated with antibiotics.

- **Disc diffusion method-**

This method uses filter paper discs six mm in diameter charged with appropriate concentration of the drugs. The discs should be stored dry in the cold.

Technique- A suitable dilution of broth culture or a broth suspension of test bacterium is flooded on the surface of a solid medium. There should be uniform spreading and if there is excess broth it should be pipetted off. Keep the plate in an incubator at 37^0C for 30 minutes. Then antibiotic discs (4-5 per 10 cm plate) are applied with sterile forcep. Then incubate it. After

incubation determine the sensitivity by measuring zone of inhibiton of growth around the disc.

Observation-

- Growth will be inhibited around antibiotic disc to which bacterium is sensitive, i.e. there is clear halo around disc.
- Growth will be there around antibiotic disc to which bacterium is resistant.
- The results are reported as ‘sensitive’, ‘moderately sensitive’ and ‘resistant’ to different drugs.
- Antibiotic disc are applied between the standard and test inocula so that zones of inhibition form around each disc are composed of standard and test bacteria. Comparison of zones of inhibition between the standard and test bacteria indicates the sensitivity of test bacteria**.**

Figure 17.1

Note-

- Antibiotics for sensitivity should be chosen with discrimination. Those antibiotics which are clinically relevant should be used. E.g.

chloramphenicol should not be used for urinary tract infections but nitrofurantoin should be used.

- AST should be done with known or presumed pathogen. It is generally done after the pathogenic bacteria are isolated from clinical samples.

1.2 **Dilution method-**

In this serial dilution of drug are prepared and inoculated with the test bacterium. This method is laborious for routine use. It is generally employed when the therapeutic dose is to be regulated accurately e.g. in the treatment of endocarditis. Also used for slow growing organisms such as tubercle bacilli and when the small degree of resistance is to be determined.

It is of two types- Agar and tube dilution**.**

- **Tube dilution method-** Serial dilutions of the drug in broth are taken in tubes and a standardized suspension of the test bacterium is inoculated. After overnight incubation the 'minimum inhibitory concentration' [MIC] is read by noting the lowest concentration of the drug that inhibits the growth.
- **Agar dilution method-** This is more convenient when several strains are to be tested at the same time. Here serial dilution of the drug is prepared in agar and poured into plates. The advantage is that many strains are inoculated on each plate conataining an antibiotic dilution.

Review questions:-

1. Why it is necessary to perform antibiotic sensitivity test?
2. Describe disc diffusion method.
3. Describe dilution method.
4. Differentiate between diffusion and dilution method.

18- Virology

Objectives:

- Students shall be able to explain general propertieso of viruses & their classification.
- Student will have elementary knowledge of important viral infections like HIV, Influenza, DengueChikungunya, Measles, and Hepatitis& Poliomyelitis.

Definition of viruses: Viruses are obligate intracellular parasites that do not have a cellular organization & are totally dependent on their host cell for survival & multiplication.

18.1 General properties of viruses

- Viruses form the boundary between the living & nonliving. Viruses are living chemicals.
- They do not have a cellular organization.
- They contain only one type of nucleic acid either DNA or RNA, never both.
- They are obligate intracellular parasites.
- They do not have any enzymes that are necessary for protein & nucleic acid synthesis and are dependent for replication on the host cells.
- They multiply by a complex process & not by binary fission.
- They are not affected by antibiotics.
- Viral illnesses can be non-serious like common cold to highly fatal diseases like rabies or yellow fever.
- It is difficult to make vaccines against viruses because they are intracellular.
- It is difficult to make drugs against viruses because theirantigenic structure keeps on changing.

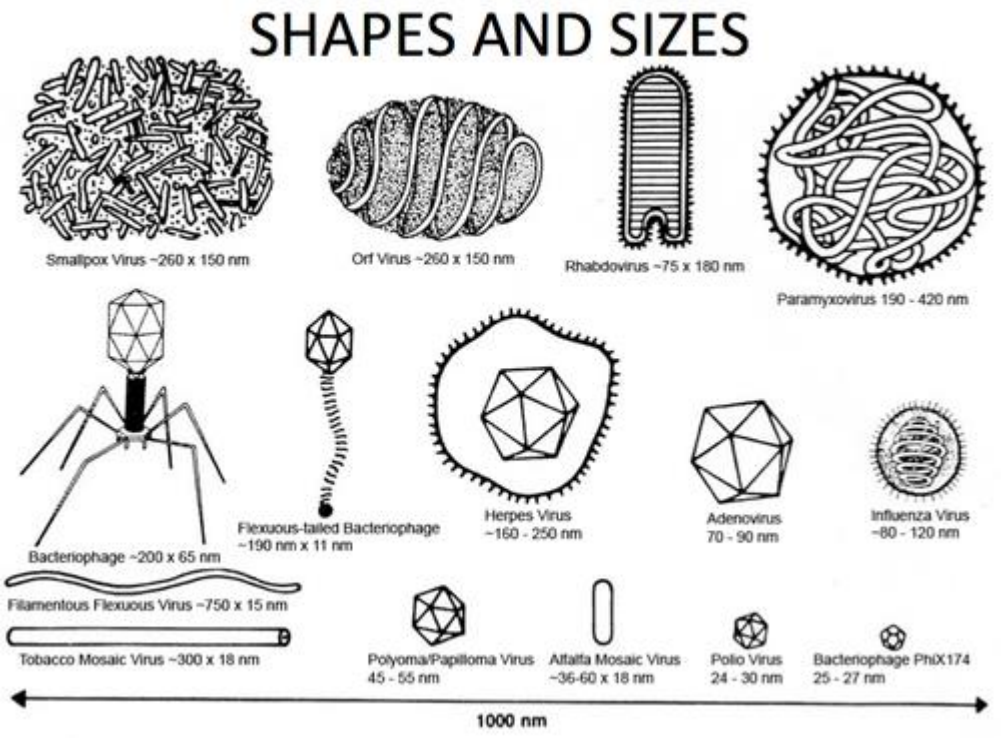


Fig. 18.1 Size & shape of different Viruses

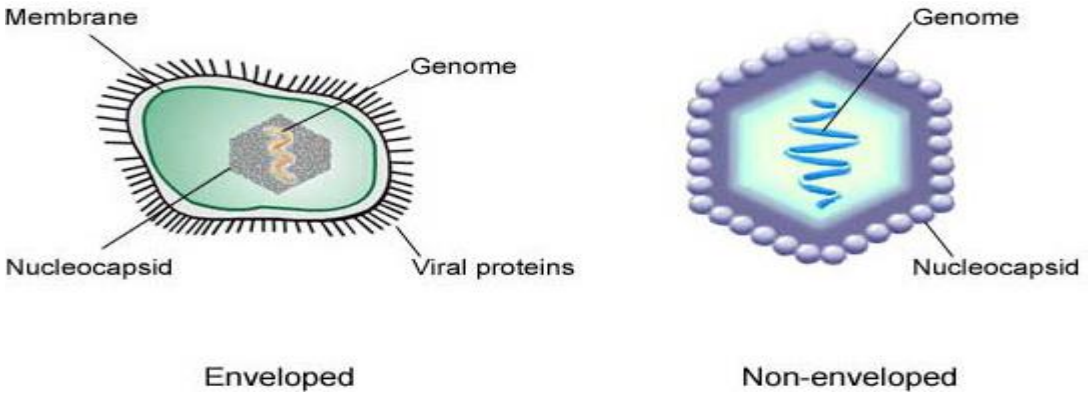
Differences between viruses & bacteria

Viruses	**Bacteria**
1. Much smaller in size – measured in nm.	1. Larger- measured in µ.
2. Contain only one type of nucleic acid either DNA or RNA.	2. Contain both DNA & RNA.
3. Do not have a cellular organization & ribosomes.	3. Possess cellular organization & ribosomes.
4. Inert in extra cellular environment.	4. Active in extracellular environment
5. Replicate only inside the living cells.	5. Replicate outside the living cells
6. Lack the enzymes necessary for synthesis of protein & nucleic acid.	6. Possess enzymes necessary for synthesis of protein & nucleic acid.

7. Obligate intracellular parasites not affected by antibiotics.	7. Not obligate intracellular parasites and affected by antibiotics.
8. Filterable through bacteriological filters because of their small size.	8. Not filterable through bacteriological filters because of their large size.

18.2 Morphology of viruses

- **Size-** They are too small to be seen under the light microscope. Viruses have different sizes.The largest is poxvirus (300nm) & the smallest viruses is the foot and mouth disease virus (20 nm).
- **Shape-** Viruses may be of following shapes
  - Brick- shaped , e.g. Poxvirus
  - Bullet – shaped, e.g. Rabies virus
  - Rod –shaped, e.g. Tobacco mosaic virus
  - Irregular & pleomorphic in some viruses
- **Structure of Viruses-** Viruses may be enveloped or nonenveloped (naked). The envelope or outer covering of viruses is derived from the host cell membrane. The envelope is lipoprotein in nature. Protein subunits may be seen as projecting spikes on the surface of the envelope. These structures are called *peplomers.* The structure of the virus includes nucleocapsid & Envelope



Enveloped Non-enveloped

Fig. 18.2 Structure of virus

Capsid –The nucleic acid core is surrounded by a protein coat known as Capsid. The capsid is composed of a large number of capsomeres which forms its morphological units.

Nucleocapsid - The protein nucleic acid complex-the capsid, together with the enclosed nucleic acid is known as the Nucleocapsid.

Envelope- Is the outer covering of the viruses.

18.3 Classification of viruses-

1. **On the basis of the disease they caused**
   - Dermotropic- Those viruses producing skin lesion e.g. smallpox, chickenpox, measles.
   - Neurotropic- Those viruses affecting nervous system e.g. poliomyelitis, rabies.
   - Pneumotropic- Those viruses affecting the respiratory tract e.g. influenza, common cold
   - Viserotropic- Those viruses affecting the visceral organs e.g. yellow fever, hepatitis.
2. **On the basis of type of nucleic acid they have**
   - DNAviruses- Those containing DNA genome.
   - RNA viruses- Those containing RNA genome.

❖ **DNA Viruses-**

- Poxviridae family

E.g. Molluscum contagious causing benign skin lesion.

- Herpesviridae family-

E.g. Herpes simplex type I & II

- Varicella zoster virus- chicken pox, Herpes zoster
- Cytomegalovirus- congenital infections

- Epstein Barr virus- Infectious mononucleosis
- Adenoviridae Family-
  Gastroenteritis & respiratory tract infection
- Papovaviridae Family

E.g. Papillomavirus causing cervical cancer

- Hepadnaviridae

E.g. Hepatitis B

❖ **RNA Viruses**-

- Picornaviridae Family : e.g Polio virus
- Orthomyxoviridae family.: e.g. Influenza virus
- Paramyxoviridae family : e.g. Mumps virus
- Togaviridae Family ; e.g. Chikungunya virus
- Flaviviridae family: e.g. Dengue, Yellow fever, Hepatitis C virus
- Bunyaviridae Family : e.g. Haemorrhagic fever
- Arenaviridae family : e.g. Arenavirus causing Choriomeningitis
- Rhabdoviridae Family : e.g. Rabies virus
- Retroviridae Family : HIV I/II viruses causing AIDS

Viral Enzymes-

Viruses produce different types of enzymes. These include-

1. Neuraminidase
2. RNA polymerase
3. Reverse transcriptase
4. Enzymes of cellular origin

Resistance & Inactivation of viruses-

With few exceptions, viruses are heat sensitive.They are inactivated within seconds at 56˚C.minutes at 37˚C.They are stable at low temperature. For long term storage they are kept frozen at -70˚C. For prolonged storage, we use **lyophilisation**

or freeze drying. Some viruses do not stand freeze drying (e.g. Polio viruses).

Viruses are inactivated by heat, sunlight, UV rays &ionizing radiations.Active antiviral disinfectants are hydrogen peroxide, potassium permanganate, hypochlorites, organic iodine compound & chlorine.Some viruses (hepatitis & polio) are relatively resistant to chlorination.Formaldehyde & Beta propiolactone are actively virucidal.

Cultivation of Viruses-

The methods commonly used are

- Animal Inoculation
- Embryonated eggs
- Tissue culture

18.4 Influenza Viruses

Influenza viruses have three serotype: Type A, Type B & Type C.

Antigenic classification- A human virus contains two main subtypes

- A1 **(H1N1)**
- A2 (H2N2)

Morphology –

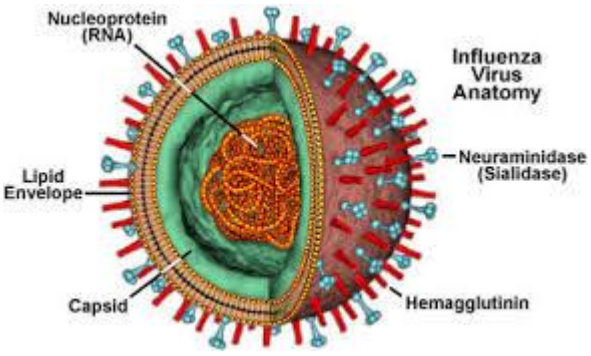


Figure 18.3

It is RNA virus typically spherical, 80 – 120 nm in diameter. It is enveloped virus. Attached to the lipid layer of the envelope are two types of glycoprotein peplomers or spikes, the haemagglutinin (HA) and the neuraminidase (NA). Haemaggutinin spikes are triangular in cross section while neuraminidase spikes are mushroom shaped.

Antigenic Variation- A unique feature of influenza viruses is its ability to undergo antigenic variation due to frequent changes in the antigenicity of HA & NA. The variation is highest in influenza virus type A.Influenza virus enters the body by respiratory tract.

H1N1 INFLUENZA (Swine influenza)

It is a highly contagious respiratory disease in pigs caused by the H1N1 swine influenza A virus. Human cases of H1N1 influenza have been reported worldwide. H1N1 influenza tends to cause high morbidity but low mortality rates if diagnosedearly & treated promptly.

Manifestations of H1N1 influenza are similar to those of seasonal influenza, with patients presenting with symptoms of acute respiratory distress, including fever, cough, sore throat and body pain.Outbreaks of swine influenza are common in pigs throughout the year. When humans have become infected, it is a result of close contact with infected pigs.

Laboratory Diagnosis-

Demonstration of virus Antigens- Viral antigen in clinical specimen can be detected by immunofluorescence. Smears of nasopharyngeal secretion & nasal swab or centrifuged deposit of throatgargling are prepared on slides & treated with

fluorescent tagged influenza antiserum & examined under the fluorescent microscope. The cell containing viral antigens will be found to fluorescent.

Serology – The routinely employed serological tests for the diagnosis of influenza include-

- Complement Fixation Test (CFT).
- Haemagglutination Inhibition Test (HAI test).

18.5 Dengue

Dengue virus has four serotypes: Dengue-1, Dengue-2, Dengue-3, Dengue -4.

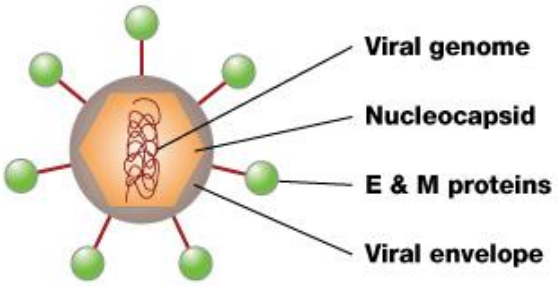


Figure 18.4

Clinical Features –

- Classical dengue fever (break-bone fever)
- Dengue in more serious form with haemorrhagic manifestation

Laboratory diagnosis- Demonstration of IgM antibody in serum provides early diagnosis. IgM antibody appears within 2 to 5 days of the onset of illness & persists for 1 to 3 months. ELISA is used for detection of IgM antibody. A strip immunochromatographic test for IgM is also available for rapid diagnosis, which is used in routine pathology laboratory.

18.6 chikungunya virus

The virus is transmitted by mosquito Aedes aegypti. The disease is characterised by fever, crippling joint pains. Chikungunya is the native word for the disease in which the patient lies 'doubled up' due to severe joint pains.

Laboratory Diagnosis-

In routine laboratory Rapid card test is used for detection of IgM antibodies.

18.7 Measles Virus

Incubation period varies from 10-12 days. It is characterized by high fever, cough & conjunctivitis. Koplik's spot can be seen on the buccal mucosa & are pathognomic of measles. With the decline of acute symptoms in 1-2 days, widespread maculopapular rash appears first on the neck & then spread to the rest of the body.

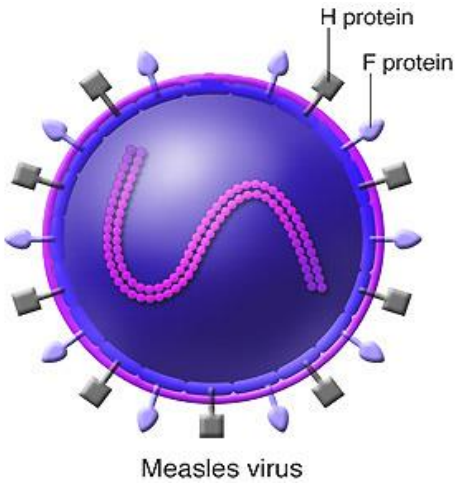


Figure 18.5

Laboratory Diagnosis-

Most cases are diagnosed clinically.

Serology-

Measles specific IgM antibody in the patient serum can be detected by ELISA.

18.8Rabies virus

Rabies virus belongs to Rhabdovirus family it is bullet shaped enveloped virus.

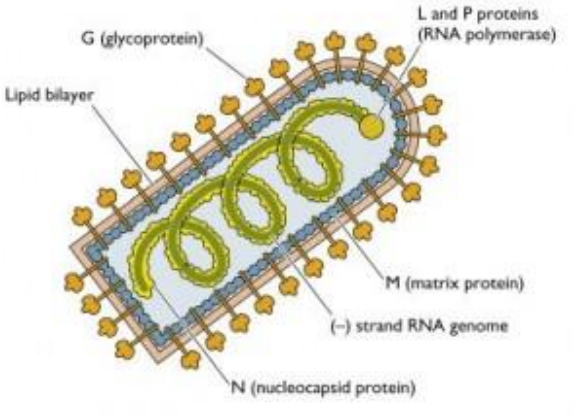


Figure 18.6

Clinical Features of Rabies or Hydrophobia:

- Incubation period is short when wound is on face & hands & long when on legs.
- It is shorter in children than adults.
- Incubations period is related with period required by virus to reach thebrain.

Four stages are seen in human:

1. Prodromal phage
2. Acute neurological phase
3. Coma
4. Death.

Laboratory Diagnosis of Rabies-

Specimens-

- Antemortem – corneal smear. Skin biopsy from nape of neck, saliva CSF
- Postmortem- impression smears of brain

Processing of Specimen

- **Ag Detection by :**
  - Immunofluorescence.`
  - Nucleic Acid Detection
    - PCR
    - Dot blot hybridization
    - Probes.
- **Ab Detection:**
  - In serum &C.S.F by ELISA
- **Virus Isolation**
  - Animal inoculation
  - Cell culture

Postmortem Diagnosis

- Demonstration of Negri bodies- Brain smears from brain are stained by Seller's technique show intracytoplasmic ,3- 27 µ,oval, eosinophillic inclusion with basophilic granules
- Immunofluorescence.

Prophylaxis for rabies virus infection includes following measures.

- **Local treatment**
  - Prompt cleaning with soap water
  - Prompt cauterization
  - Disinfection with cetavlon or iodine
  - In severe wound- infiltration of antirabies serum
  - Tetanus toxoid
  - Antibiotics
  - Wound suturing should be postponed.
- **Active prophylaxis**- by vaccine
  - Serum vaccine

- Beta propiolactone (BPL) vaccine
- Infant brain vaccine

3. **Passive prophylaxis –** Antirabies serum is given in high risk exposures.

18.9 Hepatitis Viruses

Many viruses causes hepatitis of these the following six are commonly described"Hepatitis Viruses"

Hepatitis A, B, C, D, E G Except type B all other viruses are RNA viruses

Type of virus⇨ Criterian of virus⇩	**A**	**B**	**C**
Virus	HAV, 27 nm RNA	HBV,47 nm DNA,	HCV,30-60 nm
Modes of infection	Fecal-oral	Precutaneous, Vertical,Sexual	Precutaneous
Incubation Period(days)	15 – 45	30 – 180	15 - 160
Onset	Acute	Insidious	Insidious
Illness	Mild	Occasionally severe	Moderate
Carrier state	Nil	Common	Present
Oncogenicity	Nil	Present specially after neonatal infection	Present

Prevalence	Worldwide	Worldwide	Probably Worldwide
Specific Prophylaxis	Ig and vaccine	Ig and vaccine	Nil

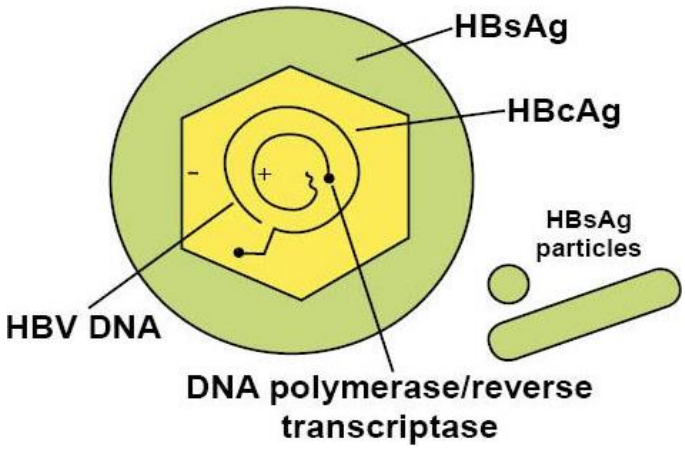


Fig. 18.7 **Structure of Hepatitis B Virus**

- **HBsAg** – It is the first specific marker to appear in blood after infection, becomes detectable about a mouth after exposure to infection. A peak level is seen in preicteric phase and disappears with recovery of clinical disease, but persists for many years in carriers.
- **Anti-HBS** – Antibodies of HBsAg appear within week after the disappearance of HBsAg and persist for years.It is protective antibody.
- **HBcAg** – It is not detectable in serum, but can be demonstrated in liver by Immunofluorescence test.
- **Anti-HBcAb**
- **HBeAg:** Sera containing HBeAg are considered to be highly infectious.

Laboratory Diagnosis: Type A

Diagnosis is usually by:

- Detection of antibody: Demonstration of IgM antibody in serum indicates current or recent infection, while the IgG antibody denotes recent or remote infection & immunity.ELISA kits for detection of IgM &IgG antibodies are available.
- Demonstration of virus in faeces by immunoelectron microscopy
- Biochemical tests: Liver function tests S.Bilirubin, SGPT, and SGOT etc.

18.10 Polioviruses

Morphological Characteristics

- Spherical particle-27nm in diameter
- Nonenveloped RNA virus.
- Viruses are classified into 3 serotypes -1, 2, 3.
- Clinical Features
  - Inapparent infection
  - Minor Illness
  - Major Illness
  - Paralytic Poliomyelitis

Laboratory diagnosis

Specimens

Blood, CSF, Throat swab, Faeces

Virus can be isolated from blood during the stage of viraemia, from throat swab in early stage of disease & from faces in 80% of patient during the first week of infection.

Prevention

Poliovirus infection can be prevented by vaccination with:

- Oral- Sabin vaccine (O.P.V.)

❖ Injectable-Salk vaccine.

18.11Human Immunodeficiency Virus

Introduction: AIDS means Acquired Human immunodeficiency virus (HIV).In this there is a deficiency in the immune system of the individual due to which patient becomes very susceptible to infections with relatively less virulent microorganisms & can die because of these infections.This deficiency is acquired & not present since birth so it is named acquired immunodeficiency.These individuals also become more susceptible to lymphoid & other malignancies. **Luc Montagnier** in 1983 isolated a retrovirusfrom a West African patient.

Structure of virus

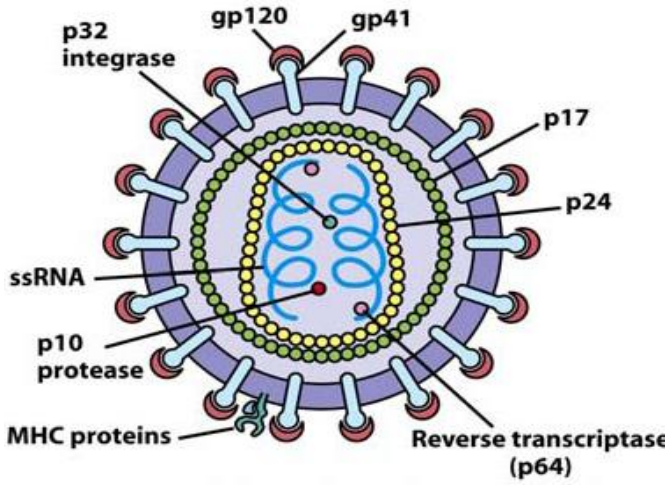


Figure 18.8

- Retroviruses are those viruses which have a RNA dependent DNA polymerase
- HIV is an enveloped virus, 90 to 120 nm is diameter.
- It has nucleoprotein core containing single stranded RNA genome & proteins

- In association with viral RNA is the reverse transcriptase enzyme. When the virus infect cell, the viral RNA is transcribed by the enzyme, first into single stranded DNA & then into doubled stranded DNA(provirus),which is integrated into the host cell chromosome. The provirus can remain latent for long periods but sometimes in response to viral promoters the provirus initiates viral replication by directing synthesis of viral RNA & other components.
- The virus core is surrounded by a protein shell.
- During viral replication when the naked virus buds out through the host cell surface membrane it acquires a lipoprotein envelope which consists of lipid derived from host cell membrane & glycoprotein which are virus coded. The major virus coded envelope proteins are the projecting spikes on the surface & the anchoring transmembrane pedicles.The spikes constitute the major surface component of the virus which binds to the CD4 receptors on susceptible host cells.

Major antigens of HIV

- Envelope antigens
  a. Spike antigen – gp -120 (principal envelope antigen)
  b. Transmembrane pedicle protein gp41.
- Shell antigen.
  a. Necleocapsid protein p18.
- Core antigen
  a. Principal Core antigen-p24.
  b. Other core antigens-p15p55.
- Polymerase antigens-p31, p51, p64.

The principal core antigen is p24 which can be detected in serum during the early stage of HIV infection before antibodies appear.The spike glycoprotein gp120 is the major envelope antigen and antibodies to gp120 are first to appear after infection.

- **Types of HIV Virus**
  - **HIV-1**
  - **HIV-2**

Stages of HIV infection

- Acute HIV infection
- Asymptomatic infection
- Persistent generalised lymphadenopathy
- AIDS-related Complex (ARC)
- AIDS

Symptoms

- Respiratory – Dry cough, breathlessness, Fever, Pneumonia and tuberculosis.
- Gastrointestinal – oral thrush, herpetic stomatitis, gingivitis, Kaposi's sarcoma, diarrhoea, abdominal pain, distention.
- Neurological – toxoplasmosis and cryptococcosis
- Malignancies – Kaposi's sarcoma, Hodgkin's, Non Hodgkin's lymphoma

World Health Organization Clinical Case Definition of AIDS Proposed at Workshop on AIDS (1985)

Adults- The existence of at least two of the following major signs associated with at least one minor sign, in absence of known causes of immunosuppression such as cancer or severe malnutrition or other recognized etiologies.

❖ **Major signs –**

a) Weight loss > 10 % of the body weight.
b) Chronic diarrhea > 1 month.
c) Prolonged fever > 1 month (intermittent or constant).
d) Generalized pruritic dermatitis.

❖ **Minor signs –**

a) Persistent cough > 1 month.
b) Chronic progressive, disseminated herpes simplex.
c) Infection.
d) Recurrent herpes zoster.
e) Oropharyngeal candidiasis.

Children – Pediatric AIDS is suspected in an infant or child presenting with at least two major signs associated with at least two minor signs in the absence of known causes of immunosuppression.

❖ **Major signs –**

- Weight loss or abnormally slow growth.
- Chronic diarrhea > 1 month.
- Prolonged fever > 1 month.

❖ **Minor signs –**

- Repeated common infections (otitis, pharyngitis, etc.)
- Persistent cough.
- Generalized dermatitis.
- Oropharyngeal candidiasis.
- Generalized lymphadenopathy.

❖ **Common modes of transmission of HIV**

➢ Sexual intercourse

- Anal

- Vaginal
- Oral

- Blood and blood products
  - Blood transfusion, factorVIII etc.
  - Tissue and organ donation
  - Semen, Cornea, bone marrow, kidney.
- Injections and injuries
  - Needles shared by drug addicts
  - Infections with unsterile syringes and needles
  - Needle stick and other injuries in health staff
  - Surgical Wounds
- Mother –Baby
  - Transplacental,
  - At birth,
  - Afterbirth

LAB DIAGNOSIS

Lab procedures for the diagnosis of HIV tests include tests for immunodeficiency as well as specific test for HIV

❖ **Non specific Tests**

The following tests are usually done

- TLC- It shows leucopenia.
- Total lymphocyte count <2000/cumm.
- Absolute T4 cell count <200/cumm.
- T4:T8 ratio reversed.
- Platelet count-Thrombocytopenia.
- Raised IgG &IgA levels.
- Low cell mediated immunity as shown by skin tests.

Specific Tests

Laboratory Tests for Detection of Specific Antibodies in HIV Infection

Screening (E/R/S) Tests

- ELISA
- Rapid Tests
  - Dot blot assay.
  - Particle agglutination (latex, gelatin).
  - HIV spot and comb tests.
- Simple Tests
  - These are based on ELISA principle.
- Supplemental Tests.
  - Western blot test.
  - Indirect immunofluorescence test.
  - Radio Immuno Precipitation assay.

Antibody detection – Demonstration of antibodies is the simplest and most commonly employed technique for diagnosis. There are two types of serological tests- **screening and supplemental.**

1. Screening: Screening is done for all donors of blood, blood products, semen, cells, tissues and organs.

ELISA test: It is highly sensitive and specific test. ELISA test is an extremely good screening test and most laboratories use a commercial ELISA kit that contains both HIV-1 and HIV-2.

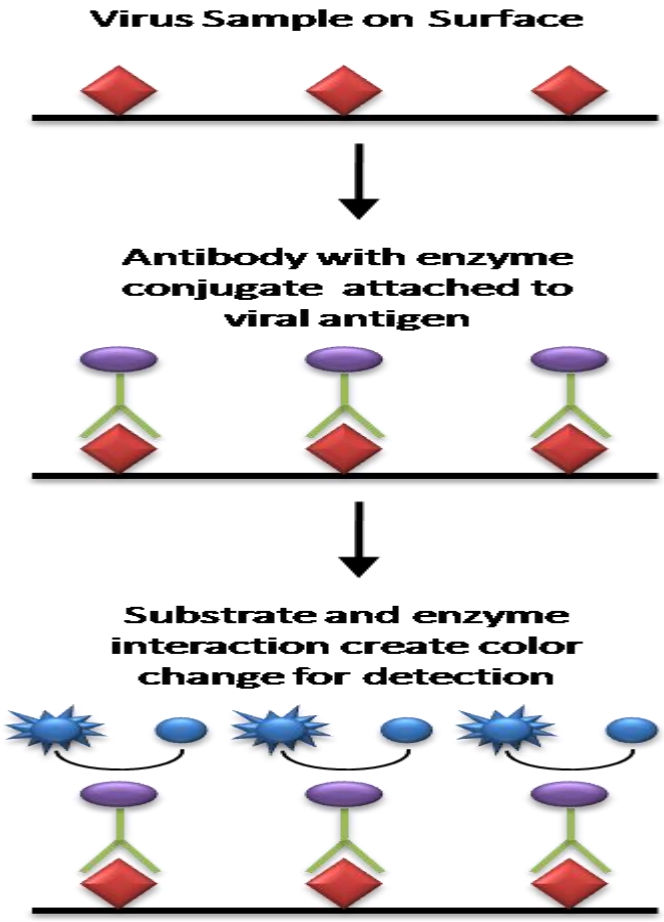


Fig. 18.9 solid phase ELISA test principle

Rapid Tests: These tests take less than 30 minutes and do not require expensive equipments. The rapid tests include dot-blot assay, particle agglutination, HIV spot and comb tests.

2. Supplemental Tests-

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. In a positive serum, bands will be seen with multiple proteins.

Antibodies to p24 (gag gene, core protein), p 31 (pol gene, reverse transcriptase) and gp 41, gp 120 or gp 160 (env gene, envelop 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, gp41, and gp120/160.

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.

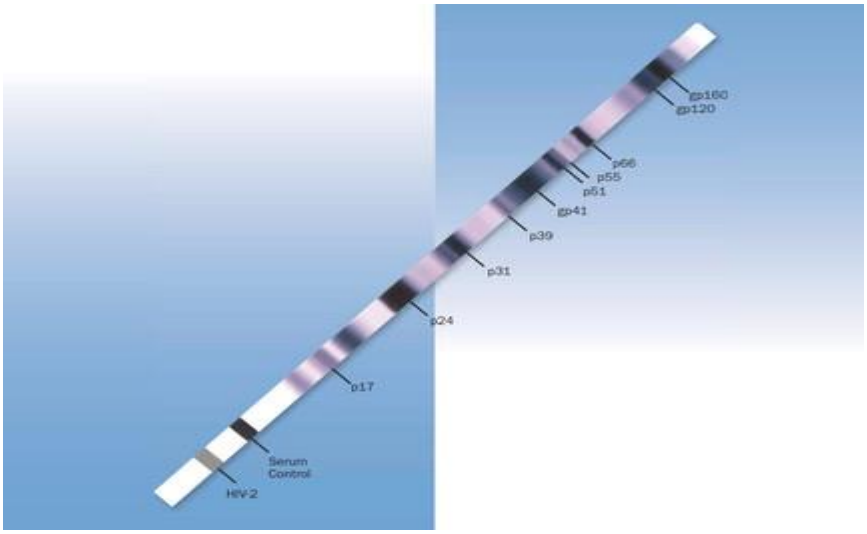


Fig. 18.10 Results of Western blot test

Indirect immunofluorescence test

Specific Tests for HIV Infections-

- **Antigen Detection -** The p24 antigen capture assay (ELISA) using anti-p24 antibody as the solid phase can be used for detection of this antigen.
- **Virus Isolation –** For diagnosis, virus is not routinely isolated. 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.
- **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.

Laboratory Monitoring of HIV Infection

The tests used for monitoring the course of HIV infection include:

- CD4+ T cell count
- Measurement of HIV RNA
- Measurement of B-2-microglobulin and neoprotein.

The most important among these is CD4+ T cell count which reflects the immunological competence of the patient. The count below 500 per mm3 is an indication of disease progression and the need for antiviral therapy.

Prevention:

The following preventive measures are recommended.

- Sexual contact**:** The use of condoms can prevent transmission of the virus.
- Sharing needles**:** contaminated syringes or needles should not be shared. All blood and blood products are to be screened for HIV. This also applies to donation of cornea, semen, marrow, kidney and other organs.

- Isolation of AIDS patient and initiation of treatment.
- Control of infection**:** Screening of individuals within risk groups helps to identify the HIV infected persons.

Prophylaxis

1. **Antiretroviral therapy (ART)**

- Specific treatment with antiretroviral drugs is the mainstay in the management of HIV infection.
- Apart from specific antiretroviral therapy, other measures in the treatment of AIDS include :
    - Treatment and prophylaxis of opportunistic infections and tumours.
    - General management
    - Immunorestorative measures.

Post exposure 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.

Zidovudine 300mg BD and Lamivudine150mg BD

Review Questions

- Classify viruses.
- What are properties of viruses?
- Differentiate between virus and bacteria.
- Differentiate between Hepatitis A, B and C.
- Describe morphology of-Dengue, H1N1, HIV, Rabies virus.
- Lab diagnosis of HIV infection.
- Prophylaxis of HIV/ AIDS.

19- Immunity

Objective: Student should get an idea about defence mechanism of body. Also they should know what are vaccines and their importance.

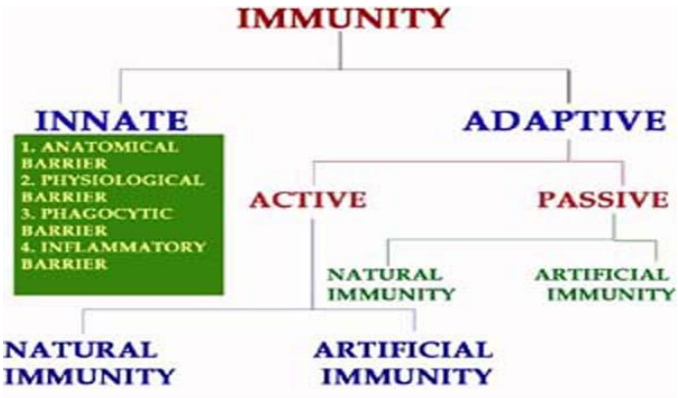
19.1 Introduction

The human system is under constant threat from a variety of infectious organisms like bacteria, fungi, etc, parasites tumour cells, etc.Also nonliving substances like organic or inorganic molecules, pollutants, etc can cause threat. The human body has specific defences against this by which it can keep itself free from harmful effects of these agents.

This host defence mechanism is explained as **Immunity.** Immunity is defined as the resistance exhibited by the host towards injury caused by microorganisms and their products.

19.2 Classification

Immunity can be classified as



19.3 Innate Immunity

Innative immunity is also known as **Native** immunity. It is the resistance to the infection which an individual possesses by virtue of its genetic and constitutional makeup i.e. It is an inherited resistance to infection. It doesn't depend on prior contact with microorganisms or immunization.

19.3.1Classification

- **Nonspecific** – It is thedegree of resistance to infection in general.

- **Specific**- Indicates resistance to a particular pathogen.

Both are studied considered at three different levels

- **Species**-It is the total or relative resistance to a pathogen, e.g. All human beings are totally resistant to plant pathogens and to many animal pathogens such as canine distemper. This type of immunity can be considered as a person obtains as a birthright.
- **Racial-** Within species different variants are there known as Races. They show differences in their pattern of resistance to infection or disease, called racial immunity, e.g. Jews are more resistant to tuberculosis and Negroes are more resistant to yellow fever. Indians are more resistant to GIT infections than those from developed countries.

  Negroes in USA are more susptible to TB than whites.
- **Individua**l- The differences in the immunity shown by individuals in a race is known as individual immunity, e.g. Homozygous twins show similar degree of resistance to TB than heterozygous twins. Individual innate immunity is influences by no. of factors like age, sex, nutrition, stress etc.

19.5 Acquired Imuunity

It is the resistance gained (acquired) by the host during the life. It differs from innate immunity which is inborn.

19.5.1 Classification

1) **Active**- Natural
   Artificial

2) **Passive**- Natural
   Artificial

19.5.2 Comparision of Active and Passive immunity:

Sr No.	Active Immunity	Passive Immunity
1	Resistance developed by an individual as a result of antigenic stimulus.	Resistance that is gained passively i.e. in ready made form.
2	Immune system of an individual must be actively functioning leading to formation of antibody.	No active role of individual's immune system as readymade antibodies is transferred.
3	There is latent phase during which immune system set in to function.	No latent period, protection becomes effective and immediate.
4	There is negative phase during which level of measurable immunity is actually lower than before antigenic stimulis.	No negative phase.
5	Once developed it long lasting.	Immunity is transient, short lasting-for few days or weeks.
6	There is secondary response- if an individual is actively immunized against an antigen and he experiences the same antigen subsequently then immune response occurs more quickly and abundantly. Immunological memory is present	No secondary response, Infact passive immunity decreases with repetition. Immunological memory is absent.
7	It is more effective and gives better protection.	Less effective and immunity produced is inferior to that of active.
8	Not applicable to immunodeficient host.	Applicable in immunodeficient host.
9	Active Natural-Results from either clinical or subclinical infection, e.g. immunity against polio measles, chickenpox etc. Immunity following bacterial infection is less	Passive Natural-Resistance passively transferred in natural course of events, e.g. from mother to baby through placenta and milk. It is of

	permanent than following viral infection.	short duration (few months).
10	Active Artificial- Resistance induced by vaccines e.g. DPT,BCG,etc	Passive Artificial- Resistance passively transferred to a recipient by administration of antibodies e.g. hyperimmune sera of human origin-human ATS,ADS,etc.

19.5.3 Vaccines

A vaccine is a biological preparation that improves immunity to a particular disease. A vaccine typically contains an agent that resembles a disease-causing microorganism, and is often made from weakened or killed forms of the microbe, its toxins or one of its surface proteins. The agent stimulates the body's immune system to recognize the agent as foreign, destroy it, and "remember" it, so that the immune system can more easily recognize and destroy any of these microorganisms that it later encounters.

Vaccines can be prophylactic (example: to prevent the effects of a future infection by any natural or "wild" pathogen), or therapeutic (e.g., vaccines against cancer are also being investigated)

Different types of vaccines

Vaccines are dead or inactivated organisms or purified products derived from them.

There are several types of vaccines in use.These represent different strategies used to try to reduce risk of illness, while retaining the ability to induce a beneficial immune response.

Killed

Some vaccines contain killed, but previously virulent, micro-organisms that have been destroyed with chemicals, heat, radioactivity, or

antibiotics.Examples cholera, bubonic plague, polio, hepatitis A, and rabies.

Attenuated

Some vaccines contain live, attenuated microorganisms. Many of these are active viruses that have been cultivated under conditions that disable their virulentproperties, or that use closely related but less dangerous organisms to produce a broad immune response. Although most attenuated vaccines are viral, some are bacterial in nature. Examples include the viral diseases yellow fever, measles, rubella, and mumps, and the bacterial disease typhoid.The live Mycobacterium tuberculosis vaccine developed by Calmette and Guérin is not made of a contagious strain, but contains a virulently modified strain called "BCG" used to elicit an immune response to the vaccine. The live attenuated vaccine-containing strain Yersinia pestis EV is used for plague immunization. Attenuated vaccines have some advantages and disadvantages. They typically provoke more durable immunological responses and are the preferred type for healthy adults. But they may not be safe for use in immunocompromised individuals, and may rarely mutate to a virulent form and cause disease.

Toxoid

Toxoid vaccines are made from inactivated toxic compounds that cause illness rather than the micro-organism. Examples of toxoid-based vaccines include tetanus and diphtheria. Toxoid vaccines are known for their efficacy. Not all toxoids are for micro-organisms.

Subunit

Protein subunit – rather than introducing an inactivated or attenuated micro-organism to an immune system (which would constitute a "whole-agent" vaccine), a fragment of it can create an immune response. Examples include the subunit vaccine against Hepatitis B virus that is composed of only the surface proteins of the virus (previously extracted from the blood

serum of chronically infected patients, but now produced by recombination of the viral genes into yeast), the virus-like particle (VLP) vaccine against human papilloma virus (HPV) that is composed of the viral major capsid protein, and the hemagglutinin and neuraminidasesubunits of the influenza virus. Subunit vaccine is being used for plague immunization.

Conjugate

Conjugate – certain bacteria have polysaccharide outer coats that are poorly immunogenic. By linking these outer coats to proteins (e.g., toxins), the immune system can be led to recognize the polysaccharide as if it were a protein antigen. This approach is used in the *Haemophilus influenzae* type B vaccine.

Difference between Live and Killed Vaccine

No.	Live	Killed
1	Immunity following live vaccine is similar to following natural infection.	Less immunogenic. Atleast two doses are required to develop immunity.
2	Lasts for several years but booster doses are required	Protection lasts only for a short period. Repeated doses are required.
3	Given orally-Polio(Sabin) Vaccine or parenterally-Measles	Given orally (TAB) but not effective. Can be given parentally.

19.5.4 Immune response

The specific reactivity induced in a host by an antigenic stimulus is known as immune response. Immune response is of two types-Primary and Secondary

Differences between **Primary and Secondary immune response:**

No	Primary	Secondary
1	The antibody response to an initial antigenic stimulus.	The antibody response to subsequent antigenic stimuli following initial stimulus.
2	It is slow, sluggish, and short lived with long lag phase and low titre of antibodies.	It is prompt, powerful and prolonged with short or negligible lag phase and high titre of antibodies.
3	Antibodies are IgM	Antibodies are IgG.

19.5 Antigen

Antigen is defined as any substance which when introduced in body is capable of stimulating immune system and reacting with the product specifically and in an observable manner.

The term involves two criteria:

i. Induction of an immune response(Immunogenicity)
ii. Specific reaction with antibody or sensitized cells(Immunological reactivity)

Based upon the ability of antigen to carry out these two functions they are classified in to

- Complete antigen-Able to induce antibody formation and produce a specific and observable reaction with antibody.
- Hapten- Can not induce antibody formation but can react specifically with antibody. It is thought to be partial antigen.
- Proantigen- Low molecular substances which do not induce antibody formation but cause delayed hypersensitivity when applied on skin.

The smallest unit of antigenicity is called **Antgenic determinant** (**Epitope**). Antigen possesses a number of determinant groups or combining sites.They are responsible for antigenic specificity of a molecule.Antigenicity is affected by number of factors like foreignness,

chemical structure, size, susptibility to tissue enzymes, etc. E.g. only antigens which are foreign to an individual can induce response; large molecules are highly antigenic, etc.

19.6 Antibody-Immunoglobulins

Immunoglobulins or antibodies are glycoproteins found in serum or tissue fluids of all mammals. They are highly specific to to specific antigens. Immunoglobulins are structural and chemical concept while antibody is biological and functional concept. Immunoglobulins constitute 20-25% of total serum proteins. Antibodies are produced by plasma cells which in turn are formed by B-lymphocytes. The plasma cell produces about 2000 molecules of antibodies per second (2000/second).

Structure of immunoglobulins-Antibody

Immunoglobulins are glycoproteins. Each molecule consists of four polypeptide chains-two heavy or H-chains and two light or L-chains. The L-chain has molecular weight of approx. 25000 and H-chain has 50000.

The L-chain is attached to heavy chain by disulphide bond. The two heavy chains are joined together by 1-5 s-s bonds depending on class of immunoglobulins.

It gives Y shape to antibody structure. The region holding the arm and stem of antibody is called Hinge.

The L-chains are similar in all classes of immunoglobulins. They occure in two forms either kappa or lambda. A molecule of Ig will have either kappa or lambda but never both.The kappa and lambda chains occure in the ratio of 2:1.

The H-chains are structurally and antigenically distinct for each class of Ig. And it is designated by Greek letter. It is as follows:

Immunoglobulin class	Designation of H-chain

IgG	Gamma
IgA	Alpha
IgM	Mu
IgD	Delta
IgE	Epsilon

Each chain of antibody consists of two distinct regions – Variable region and Constant region. Variable region constitutes the antigen binding site (**paratope).** This part of antibody recognizes and binds to the specific antigen, forming antigen-antibody complex. Combining site of antigen is known as **epitope.**

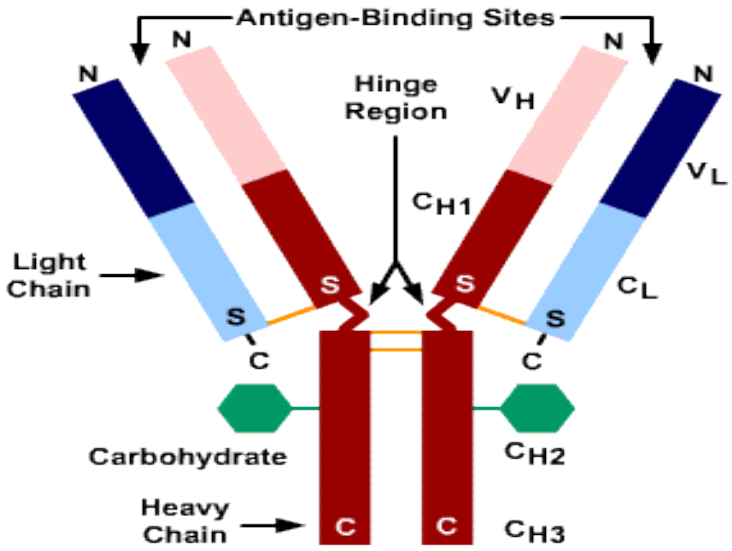


Figure 19.1

Different types of immunoglobulins are:

- **IgG-** Major serum immunoglobulins. Constitutes 80% of the total. It is the late antibody. Normal level-8-16mg/ml

  It can cross the placenta and provides natural immunity to foetus.It enhances phagocytic activity. It is considered as general purpose antibody protective against those infectious agents which are active in blood and tissues.

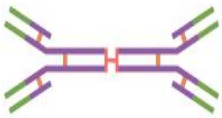
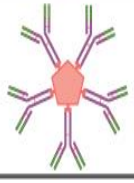
- **IgA**- 2nd most abundant immunoglobulin. Constitutes 10-13% of total. Normal level-0.6-4.2mg/ml

  Major immunoglobulins present in Colostrum, Saliva and Tears.

  It's concentration in secretions and on mucus surfaces forming an "antibody paste" it plays an important role in local immunity against respiratory and intestinal pathogens.
- **IgM**- 5-8% of total. Normal level- 0.5-2mg/ml

  It is a heavy molecule so called ' The millionaire molecule'. It is the first one to reach at the site of infection. Demonstration of IgM in the serum indicates recent infection.
- **IgD**- Resembles IgG in structure. Normal level-3mg/100ml

  Present on the surface of the B-cell which are designed to differentiate in to antibody producing plasma cells.
- **IgE**- Acts as mediater of allergic reactions.

Name	Properties	Structure
IgA	Found in mucous, saliva, tears, and breast milk. Protects against pathogens.	
IgD	Part of the B cell receptor. Activates basophils and mast cells.	
IgE	Protects against parasitic worms. Responsible for allergic reactions.	
IgG	Secreted by plasma cells in the blood. Able to cross the placenta into the fetus.	
IgM	May be attached to the surface of a B cell or secreted into the blood. Responsible for early stages of immunity.	

19.7 Monoclonal antibodies (mAb or moAb)

A Monoclonal antibody, by contrast, represents antibody from a single antibody producing B cell and therefore only binds with one unique epitope. Each individual antibody in a polyclonal mixture is technically a monoclonal antibody; however, this term generally refers to a process by which the actual B-cell is isolated and fused to an immortal hybridoma cell line so that large quantities of identical antibody can be generated.

Applications

Diagnostic tests

Once monoclonal antibodies for a given substance have been produced, they can be used to detect the presence of this substance. The Western blot test and immuno dot blot tests detect the protein on a membrane. They

are also very useful in immunohistochemistry, which detect antigen in fixed tissue sections and immunofluorescence test, which detect the substance in a frozen tissue section or in live cells.

Cancer treatment

One possible treatment for cancer involves monoclonal antibodies that bind only to cancer cell-specific antigens and induce an immunological response against the target cancer cell. Such mAb could also be modified for delivery of a toxin, radioisotope, cytokine or other active conjugate; it is also possible to design bispecific antibodies that can bind with their Fab regions both to target antigen and to a conjugate or effector cell. In fact, every intact antibody can bind to cell receptors or other proteins with its Fc region.

19.8 Polyclonal antibodies (pAbs)

A Polyclonal Antibody represents a collection of antibodies from different B cells that recognize multiple epitopes on the same antigen. Each of these individual antibodies recognizes a unique epitope that is located on that antigen.

Application areas suitable for use of polyclonal antibodies are as follows:

a) Sandwich ELISA for tumor markers or other antigens can be designed with polyclonal antibodies as the coating (trapping) antibody, followed by addition of standard antigen/sample and then addition of relevant MAb conjugated to HRPO.

b) Polyclonal antibodies are useful in histopathological analysis using immunoperoxides staining technique.

c) In some cases of affinity purification of antigens, polyclonals have advantages over MAbs.

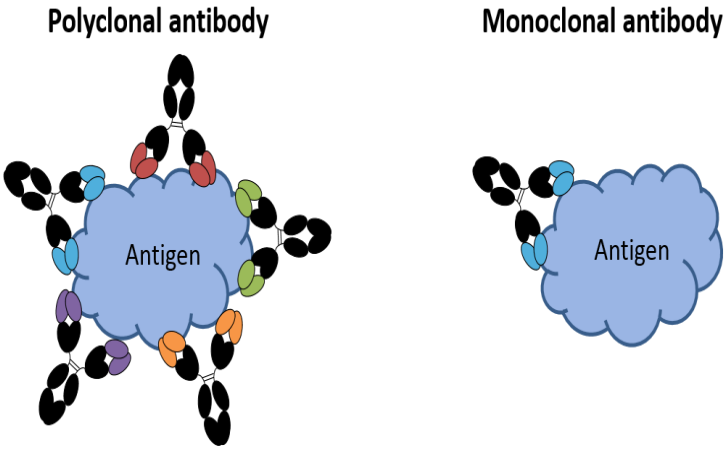


Figure 9.2

Comparision of monoclonal and polyclonal antibodies:

No.	Polyclonal antibodies	Monoclonal antibodies
1	Inexpensive to produce	Expensive to produce
2	Technology required is low.	High technology is required
3	Skills required are low	Training is required for the technology use
4	Time scale is short	Time scale is long for hybridomas
5	Produces large amounts of nonspecific antibodies	Can produce large amounts of specific antibodies but may be too specific
6	Recognises multiple epitopes on any one antigen	Recognises only one epitope on an antigen
7	Can be batch to batch variability	Once hybridoma is made it is constant and renewable source and all batches will be identical

Exercise:

1. Define immunity and classify it.
2. What are the barriers of innate immunity?

3. Describe in short “vaccines”.
4. What is primary and secondary immune response?
5. Define- Antigen, antibody and hapten.
6. Describe structure of an antibody.
7. What are monoclonal and polyclonal antibodies?
8. Types of immunoglobulins.
9. Difference between active and passive immunity.

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20 - Serology

Objective: Students should know various techniques involving antigen antibody reactions, their principles and significance.

20.1 Introduction

Serology is the study of serum, is the science that deals with antigen-antibody reactions or immunologic reactions of the body using serum. Immunology includes antigen antibody reactions but it is not confined to serum sample only.

In practice, the term usually refers to the diagnostic identification of antibodies in the serum. Such antibodies are typically formed in response to an infection against a given microorganism, against foreign proteinsfor example, toa mismatched blood transfusion, or to one's own proteins like in case of autoimmune disease.

There are several serology techniques that can be used depending on the antibodies being studied.

These tests include: ELISA, agglutination, precipitation, complement-fixation, and immunofluorescence.

Some serological tests are not limited to blood serum, but can also be performed on other body fluids such as semen and saliva, which have roughly similar properties to serum.

20.2 Advantages

Antigen antibody reactions serve several purposes:

1. In the body they form antibody mediated immunity in infectious diseases.
2. Helpful in cases of tissue injury in hypersensitivity reactions and autoimmune diseases.
3. In laboratory they help in the diagnosis of infections.

4. In epidemiological survey.
5. Identification of infectious and noninfectious agents like enzymes.

Thus these reactions can be used for detection and quantitation of either antigen or antibody.Antigen antibody reactions *in vitro* are known as serological reactions.

20.3 Characteristics of antigen antibody reactions

Antigen antibody reactions show following characteristics:

1. The reaction is specific; an antigen combines only with its homologous antibody and vice versa. Sometimes cross reactions can take place due to antigenic similarity or relatedness.
2. Entire molecule reacts and not fragments.
3. There is no denaturation of antigen or antibody during reaction.
4. The combination occurs at the surface. So, it is the surface antigen that are immunologically relevant.
5. The combination is firm but reversible. Firmness of union is influenced by the affinity and avidity of reaction.
6. Both antigen and antibody participates in the formation of agglutinate or precipitate.
7. Antigen and antibody can combine in varying proportions. Both antigens and antibodies are multivalent.

20.4 Stages of antigen- antibody reactions

Antigen- antibody reactions occur at two stages:

Primary stage- It is initial interaction between antigen and antibody without any visible effects.

Secondary stage-Interaction leads to visible effects in the form of agglutination, precipitation, complement fixation, etc. Depending on type of reaction antibodies are designated as agglutinin, precipitin etc and antigens as agglutinogen, precipitinogen, etc.An antigen

gives rise to only one antibody which can cause different types of reactions depending on nature of antigen and the condition of reaction.

20.5 Various serological reactions

Serological tests to detect antibodies against infecting microorganisms provide useful means of indirect diagnosis. These tests are of special significance for those organisms which cannot be isolated and cultured in laboratory for e.g. viral infections, syphilis, etc.

Different serological reactions are as follows:

20.5.1 Precipitation

Principle

When a soluble antigen (precipitinogen) combines with its antibody (precipitin) in the presence of electrolyte (NaCl) at a suitable temperature and pH, the antigen-antibody complex forms an insoluble precipitate.

It is carried out as qualitative or quantitative test. It is very sensitive for detecting antigen. It is relatively less sensitive in detecting antibody.

Applications-

- It is helpful in forensic cases in identification of blood and seminal stains.
- Used in testing for food adulteration.

Different precipitation tests are:

Ring test- A precipitate forms at junction of two liquids.

- C-reactive protein

- Ascoli' test

Immunodiffusion (precipitation in gel)-

The reaction is visible in the form of band, it is stable, it can be stained for preservation.

Number of different antigens can be observed in reacting mixture.

There are different types of immunodiffusion modifications available.

Single diffusion in two dimensions (Radial immunodiffusion)

In this antibody incorporated in agar gel poured on slide, wells are cut on it and antigen is added into the wells. Antigen diffuses radially from the well and form ring shape band of precipitation concentrically around the well. The diameter of the halo gives estimation of concentration of antigen. This is used in i) Estimation of Immunoglobulin classes

ii) Screening sera for antibodies to influenza virus

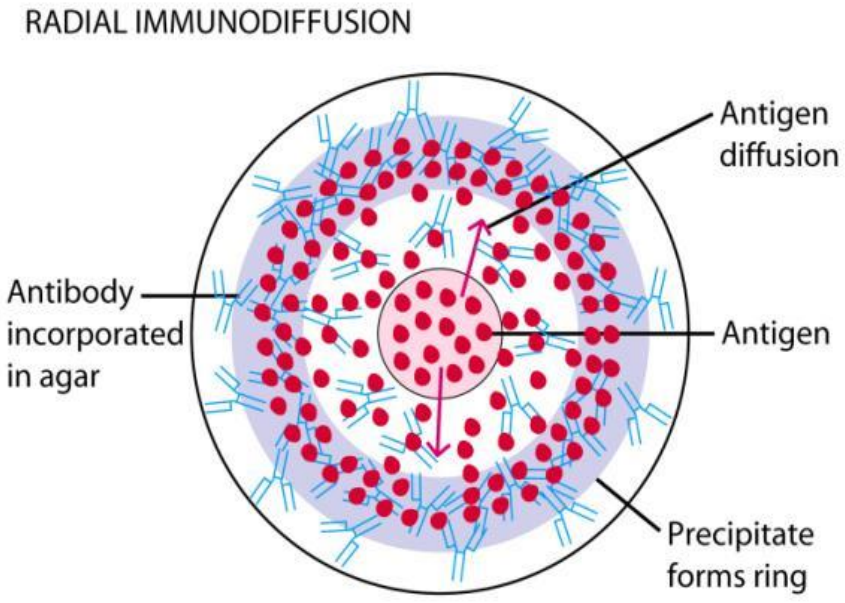

Figure20.2

Double diffusion in two dimensions-

Here agar gel is poured on a slide and wells are cut with template. Antiserum is placed in a central well and different antigens in surrounding wells.If two adjacent antigens are identical, the line of precipitate formed by them will fuse. If not related the line will cross and partial identity is

indicated by spur formation.This is used to compare different antigens and antisera directly.It indicates identity, cross reactions and non-identity.

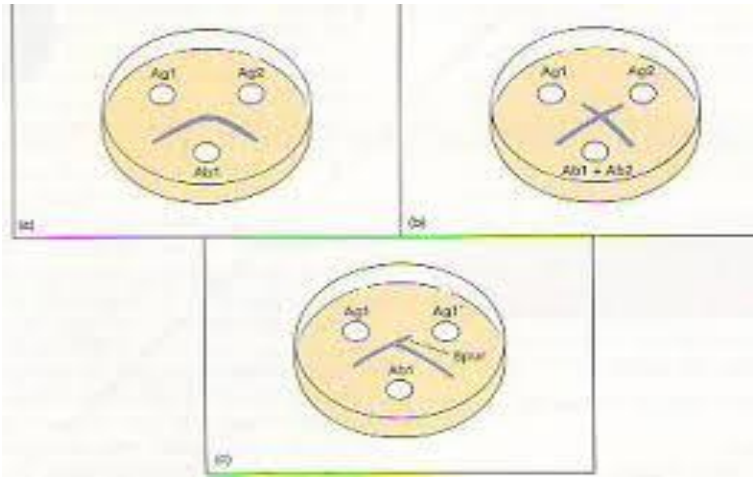

Fig. 20.3 Double immunodiffusion

Immunoelectrophoresis-

In this first agar electrophoresis of the antigen is carried out. Rectangular troughs are cut in the agar and are filled with the antiserum. Lines of precipitate will form with separated components of the antigens.Useful in testing normal and abnormal serum proteins.

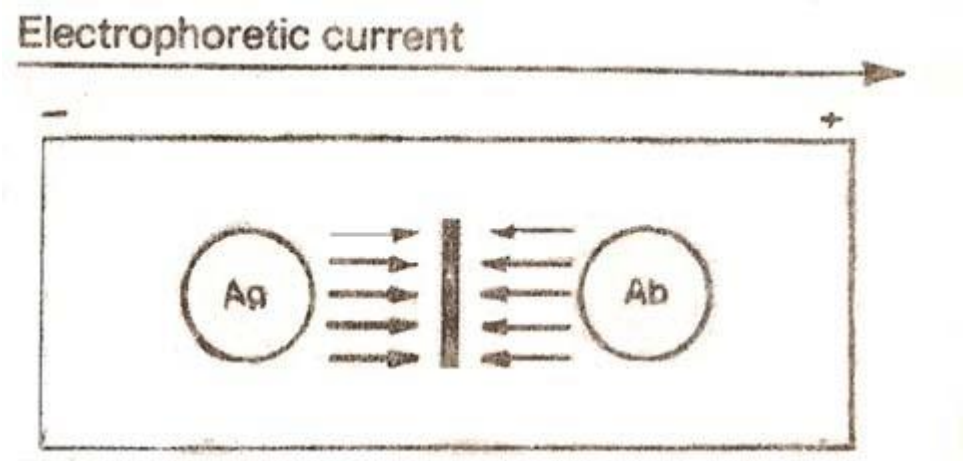

Figure 20.4

20.5.2 Flocculation

Principle

When a soluble antigen combines with its antibody the precipitate which is formed remains suspended as floccules, reaction is known as flocculation. It is used to detect syphilis.

i) Slide test-VDRL test for syphilis

ii) Tube test- Kahn test for syphilis

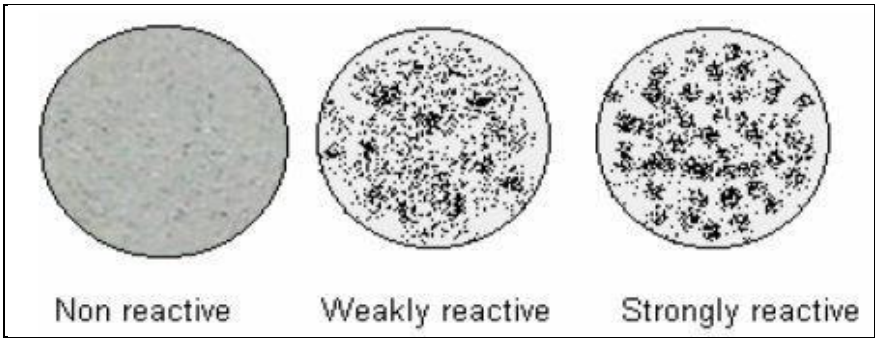

Figure 20.5

20.5.3 Agglutination

Principle-

When a particulate antigen (Agglutinogen) is mixed with its antibody (Agglutinin) in presence of electrolytes at a suitable temperature and pH, the particles are clumped or agglutinated.Agglutination is more sensitive for the detection of antibodies than precipitation.

Applications:

Slide agglutination – A drop of antiserum and particulate antigen is mixed on a slide or a tile. Agglutination is indicated by clumping together of particles and clearing of drop. It is used in –

- Identification of bacterial isolate from clinical specimens
- Blood grouping and Cross matching

Tube agglutination- A standard quantative method for measuring antibodies. A fixed volume of a particulate antigen is added to anequal volume of serial dilutions of an antiserum in test tube, and agglutination titre is estimated. It is used in-

- Widal test for Typhoid fever
- Test for Brucello
- Cold agglutinin test for primary atypical pneumonia

i. The antihuman globulin (AHG) test

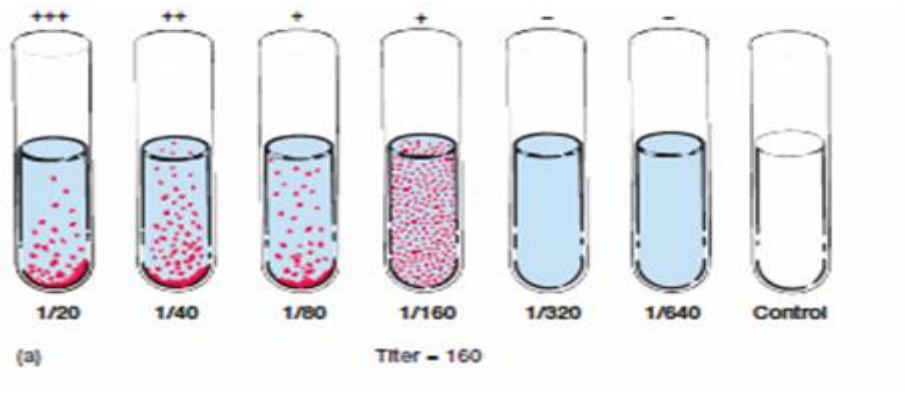

Figure 20.6

Passive agglutination test- By attaching soluble antigen to the surface carrier particles precipitation reaction can be converted in to agglutination reaction. Such reactions are more sensitive for detection of antibodies. The carrier particles commonly used are red cells, Latex particles or bentonite. Latex agglutination tests are used to detect Hepatitis B, ASO, RA factor, etc

20.5.4 Complement fixation test

Principle

It is the ability of antigen-antibody complexes to fix the complement used in CFT. Complement takes part in many immunological reactions and is absorbed during combination of antigen and antibody. In presence of corresponding antibodies,complement lyses red cells,kills and lyses bacteria, immobilizes motile organisms,etc.The antigen may be soluble or particulate.

Applications –

i) Wassermann test for syphilis.

20.5.5 Neutralisation

Principle

The toxin produced by the pathogen is used as reagent in vitro which is neutralized by the antibody produced by the patient which is present in his serum.

Applications- i) ASO test to detect Streptococcus pyogenes infection.

20.5.6 Enzyme-linked immunosorbent assay (ELISA)

Principle-

Antigens from the sample are attached to a surface. Then a further specific antibody is applied over the surface so it can bind to the antigen. This antibody is linked to an enzyme, and in the final step a substance Containing the enzyme's substrate is added. The subsequent reaction produces a detectable signal, most commonly a color change in the substrate.

Types of ELISA- There are three types of ELISA.

Indirect ELISA

Antibody can be detected or quantitated with an indirect ELISA. Serum or some other sample containing primary antibody (Ab_1) is added to an Antigen coated microtitre well and allowed to react with the bound antigen. After any free Ab_1 is washed away, the presence of Antibody bound to the antigen is detected by adding enzyme conjugated secondary antibody (Ab_2) which binds to the Ab_1. Any free Ab_2 then is washed away and a substrate for the enzyme is added. The coloured reaction product that forms is measured by specialized spectrophotometric plate reader.

Indirect ELISA is used to detect the presence of serum antibodies against Human immunodeficiency Virus, the causative agent of AIDS.

Sandwich ELISA

Antigen can be detected or quantitated by a sandwich ELISA. In this technique the antibody is immobilized on a microtitre well. A sample containing antigen is added and allowed to react with the bound antibody. After the well is washed, a second enzyme linked antibody specific for a different epitope on the antigen is added and allowed to react with the bound antigen. After any free second antibody is removed by washing, substrate is added and the coloured reaction product is measured.

Competitive ELISA

Another variation for quantitating antigen is competitive ELISA. In this technique antibody is first incubated in solution with a sample containing antigen. The antigen-antibody mixture is then added to an antigen coated microtitre well. The more antigen present in the sample, the less free antibody will be available to bind the antigen coated well. Addition of an enzyme conjugated secondary antibody (Ab_2) specific for the isotype of the primary antibody can be used to quantitate the amount of primary antibody bound to the well as in an indirect ELISA. In competitive assay, however, the higher the concentration of the antigen in the original sample, the lower the absorbance.

Advantages of ELISA

- It is simple and versatile technique.
- It is as sensitive as RIA.
- It is as reliable and accurate as RIA.
- It is less expensive and safe as here enzyme labeled reagents is used.

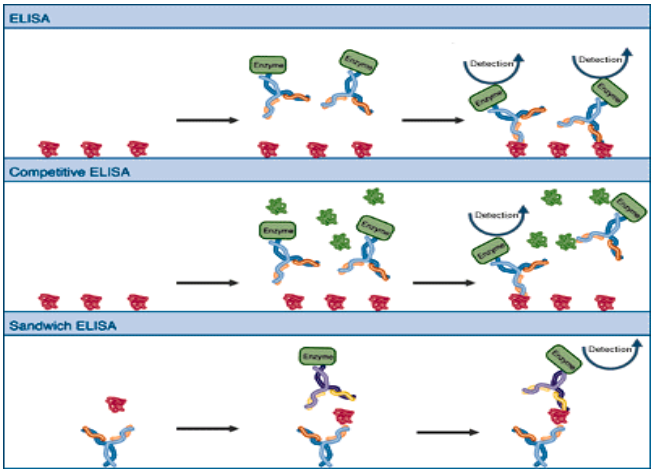

Figure 20.7

Few examples of enzyme and substrates as follows

Enzymes	**Substrate**
Alkaline phosphatase	p-nitrophenyl phosphate
Horse radish peroxidase	o-phenylenediamine
β-D-galactosidase	o-nitrophenyl galactopyranoside
Penicillinase	Penicillin V

Applications-

1. ELISA has been used as a diagnostic tool in medicine and plant pathology, as well as a quality-control check in various industries.
2. Since they are designed to rapidly handle a large numbers of samples in parallel, they are a very popular choice for the evaluation of various research and diagnostic targets.
3. IgG and IgE antibodies against *A.fumigatus* can be detected.
4. Sandwich ELISA can be used for detection of HBs Ag.

20.6 Types of hypersensitivity reactions

20.6.1 Introduction

Normally the immune system plays an important role in protecting the body frommicroorganisms and other foreign substances. If the activity of the immune system is excessive or overreactive, a **hypersensitivityreaction** develops. The reactions may be damaging, uncomfortable or occasionally fatal. It requires presensitised state of host. The initial contact sensitizes the immune system and subsequent contact causes manifestations of hypersensitivity.

20.6.2 Definition

Hypersensitivity refers to undesirable reactions produced by the normal immune system including allergies and autoimmunity.**Or**Hypersensitivity is a state of altered reactivity in which the body reacts with an exaggregated immune response to a foreign protein.

20.6.3 Types of hypersensitivity reactions

Depending on the time require for a sensitized host to develop clinical reactions on reexposure to antigen hypersensitivity reactions are divided intotwo groups-

- **Immediate**
- Anaphylaxis
- Atopy
- Antibody mediated cell damage
- Arthus phenomenon
- **Delayed**
- Infection type
- Contact dermatitis type

Review Questions:

1. Define serology.
2. Name different antigen-antibody reactions.
3. Principle of precipitation/agglutination reaction.
4. Principle of RIA/ELISA.
5. What are advantages of ELISA over RIA?
6. What is hypersensitivity?
7. Classify hypersensitivity reactions.
8. What is immunofluorescence? What are its types?
9. Describe precipitation in gel.

******

21 Collection, Transportation and processing of

Specimen

Objectives: After reading this chapter student will understand importance of proper collection, specimen containers& preservation of specimens. Student will know about use of transport media & processing of different specimens in short.

21.1 Introduction & general considerations

The goal of laboratory is to provide accurate, clinically pertinent results in a timely manner. Accuracy is required in the following aspects of specimen collection:

1. Type of specimen.
2. Collection time.
3. Method of dispatch to the laboratory.
4. Storage & aseptic handling.
5. Information about the patient's condition is required sometimes.

General considerations:

1. Specimen should contain only those organisms from the site where it was collected.
2. Specimen should be collected under strict aseptic conditions.
3. It is necessary to avoid contaminating discharges with skin commensals.
4. Specimens should be collected in dry sterile, leak proof containers free from all traces of disinfectant.
5. Each specimen must be clearly labeled with (a) Patient's name (b) Date (c) Time and (d) OPD/IPD
6. Each specimen should be accompanied by a request form which gives – (a) Patient's above mentioned data (b) Investigations required and (c) Clinical note giving details of (i) The patient's

illness (ii) Suspected diagnosis and (iii) Any antimicrobial treatment (that may have started).

7. Following specimens should be labeled as HIGH RISK and must be handled with extra care.

High – Risk Specimens -

Specimen	Likely to contain
Sputum	M. tuberculosis
Feces	V.cholerae or S. typhi
Fluid from ulcers	Anthrax bacilli or treponemes
Blood	Hepatitis virus, AIDS virus

21.2 Storage of specimen & Transport media

Refrigeration at 2 - 8˚C can help to preserve cells and reduce the multiplication of commensals in un-preserved specimens. Specimens for the isolation of S. pneumoniae, Neisseria species and Haemophilus, should never be stored in the refrigerator since these organisms are killed at low temperature.

Transport media :

Transport medium inhibits self destruction and multiplication of micro organism. Delay during the transport of specimens from the point of collection affects the viability of delicate organisms such as Neisseria, Haemophilus, Shigella and anaerobes. Various media are used for transporting different types of specimens and the following three are of major significance.

Stuart transport medium: is used for CSF and cervical specimens. This medium preserves both Neisseria species as well as Trichomonas vaginalis

Cary - Blair and Amies transport media: are used for transporting faecal material or rectal swabs, and are effective in preserving Salmonella, Shigella and Vibrio. During transport, the medium is mixed with equal parts of stool.

21.3 Specimen Rejection Criteria- They are

- Unlabeled or mislabeled specimen.
- Use of improper transport media.
- Use of improper swab.
- Very long transporting time.
- Improper transportation temperature.
- Improper storage temperature.
- Improper specimen collection site, Leakage of specimen.

21.4 Collection and processing of Urine

Requirements

1. Dry, sterile wide mouth leak proof bottle.
2. First morning midstream urine sample (MSU)

Instructions

- Male patient should wash the genital organ with clean water.
- Female patient should cleanse the area around the urethral opening with clean water and after drying the area, midstream urine should be collected with the labia held apart. For women, do not collect urine during menstrual period.
- For urine chemistry tests, 24hours urine collection is done.
- Catheterization carries a risk of introducing microorganisms into the bladder but it is sometimes unavoidable.

Collection of sample from infants

- A non-invasive method of stimulating urine flow in a baby is by tapping just above the pubis with two fingers.
- Alternatively for infants the urine is collected in a plastic bag with an adhesive mouth. The bag is fixed around the infant's genitalia.

Urethritis and Prostatitis –

For the investigation of these conditions, the initial flow of urine, rather than a midstream specimen should be examined.

Suprapubic Aspiration - When it is not possible to obtain MSU sometimes this method is done.

Collection Procedure –

1. Ask patient to collect about 20 ml of midstream urine after voiding first portion of the urine.
2. Label the container.
3. For transportation of specimen a thermos flask can be used. Urine transport tubes containing boric acid, glycerol and sodium formate have been shown to preserve bacteria without refrigeration for about 24 hours.
4. Since bacteria grow rapidly in urine, it is necessary to test it immediately after collection. In the case of delay in the tests, urine should be preserved at 2 - 8˚C in the refrigerator.

Note –

If renal tuberculosis is suspected, collect first urine passed (entire specimen) on three successive mornings of laboratory investigations.

Additional Information –

1. E. coli is the commonest cause of urinary infections. Women are more frequently infected.
2. Proteus, Klebsiella and Pseudomonas infections follow after catheterization and gynecological surgery.

3. The presence of bacteria in urine is called **Bacteriuria** and it is usually accompanied by pus cells in urine **(pyuria**).
4. **Urethritis** (infection of the anterior urinary tract) is mainly caused by Chlamydiae, Streptococci, Staphylococci and N.gonorrhoeae.
5. M. tuberculosis is often suspected where there is pyuria but routine culture is sterile.
6. S. typhi and S. paratyphi can be found in the urine of patients with enteric fever from the third week of infection.
7. The urinary tract is normally sterile except urethra. Few commensals may be present in urethra. Yeasts may be found in female urethra. Female patient's urine may become contaminated with vaginal organismswhich is indicated by the presence of many epithelial cells with mixed bacterial flora.
8. The urine is markedly acidic with E. coli infections and it is markedly alkaline with Proteus infections.
9. Patients with pus cells in the urine but no significant growth of bacteria on routine culture- Causes are
    - Prior administration of antibiotics.
    - Patient may have infection with an organism that does not grow on media used e.g. Gonococcal infection, Urinary tuberculosis, anaerobic bacteria causing UTI.

21.5 Examination of urogenital specimens –

1. Swabs collected for isolation of gonococci should be transported at room temperature to the laboratory in modified Stuart's or Amie's charcoal transport media. Specimen should be kept at room temperature.
2. In the laboratory specimen can be stored at 2 - 8˚C for 24 hours.

Figure 21.1Culture swab with transport media

21.6 Examination of Feces (stool specimen)

1. Give the patient a clean, dry and disinfectant free, wide mouth bottle.
2. In the case of transportation, transfer a portion of the specimen to a cotton swab and insert it in Cary Blair transport medium. If cholera is suspected transfer 1 ml of the specimen in 10 ml of sterile alkaline peptone water.
3. The container for stool is a 25 ml screw capped, wide mouthed plastic bottle with a spoon projecting from the underside of the cap. Collect 1 – 2 ml of stool sample with spoon and insert it in the bottle. Take care not to soil the rim or outside of the bottle. Apply the cap tightly. Do not collect several spoonfuls. If delay is unavoidable, add approximately
4. 6 ml of buffered glycerol saline transport medium in the bottle.
5. Stool for direct wet-mount examination, must be sent to pathology laboratory without any added preservative.

Container for Collection of Stool

Figure 21.2

21.7 Examination of Sputum

Sputum – Is a mixture of bronchial secretion and inflammatory exudates coughed up into mouth and expectorated. Staff collecting specimens should be instructed in how to obtain and recognize the correct material. Do not accept saliva for examination.

Specimen Collection –

1. Give the patient a dry, clean, wide mouth, leak proof sterile container.
2. Request the patient to cough deeply to get a sputum specimen.
3. Usually early morning sample is collected at home after washing mouth in the open, well ventilated, isolated area. It is never collected in the toilet.
4. Ask the patient to deliver the specimen to the laboratory as quickly as possible, preferably within two hours.

Fig 21.3 Container for sputum

Precautions –

Adequate safety precautions should be taken to prevent spread of infectious organisms. The organisms such as Pneumococci and H. influenzae require immediate culturing. Hence, if pneumonia or bronchopneumonia is suspected, the specimen should be inoculated as early as possible.

21.8 Examination of Throat, Mouth and Nasal Specimens

Specimen Collection

1. Swabs should be collected by a medical officer or by an experienced technician.
2. In routine laboratory cotton wool swabs are prepared for collection of specimens.
3. Commercially available albumin coated or charcoal coated swabs can be used.
4. After collecting specimen from tonsil, pharynx or any other inflamed area, it should be replaced in its tube with care not to soil the rim.
5. If it cannot be delivered to laboratory within about one hour, it should be placed in refrigerator until delivery.
6. Laboratory investigations should be performed within two hours of the swab collection.

Note

- The throat contains a wide variety of commensals which make differentiation of other pathogens difficult.
- If diphtheria is suspected, look for pleomorphic rods. Commensal diphtheroids show little variation in shape and size. Do Albert staining.
- Culturing the specimen: (a) Inoculate blood agar (b) If diphtheria is suspected inoculates on Tellurite Blood agar, (c) Inoculate on Sabouraud agar if thrush is suspected (Candida infection).
- Preferably two blood agar plates are inoculated; one is kept in CO_2 jar and other anaerobically.Perform biochemical tests to confirm the results.

Nasal swabs

1. These are more often taken to detect healthy carriers than to diagnose infection,

2. Deep nasal swabs being taken for S. pyogenes and diphtheria bacillus and swabs from the skin of the anterior nares for S. aureus.
3. Nasal carriers are a more dangerous source of infection for others than are throat carriers of the same organism, for they disseminate much larger numbers of organisms into the environment than the latter.

Oral swabs-Stomatitis- Acute infections of the mouth are commonest in neonates and debilitated elderly persons. They can be caused by Vincent's organism, Candida albicans, and Herpes simplex, Coxsackie A and various other viruses.

Sinusitis

The paranasal sinuses are normally sterile, but in the course of a nasal infection, a nasopharyngeal bacterium such as H. influenzae or Pneumococcus may invade a sinus, most commonly the maxillary or frontal sinus, which then becomes filled with pus.

Pus aspirated from the sinus, or a saline 'wash-out', should be examined in a Gram film and by culture on aerobic and anaerobic blood agar plates.

Nasopharyngeal swabs:

It may be used for the detection of potential pathogens carried in the nasopharynx of healthy persons, e.g. meningococcus, which generally cannot be recovered from parts of the throat bathed with saliva from the mouth.

21.9 Examination of Pus from Wounds, Abscesses, Burns and Sinuses

Specimen Collection

1. Do not apply antiseptic before taking the specimen.

2. Collect specimens from wounds (ulcerations) of different part of the body by touching the infected area with a sterile swab. The swab should be placed immediately in a sterile test tube.
3. Syringe in which sample is collected, can be directly send to laboratory.
4. Put in a transport medium if delay is expected.
5. Two swabs are generally collected. One is used for direct microscopic examination and the other is used for culture.
6. If the infection is suspected to be due to an anaerobe, aspirate the draining pus into a sterile syringe and immediately put it into a thioglycolate broth.
7. Specimens collected during operation or curetting from infected sinuses should be homogenized with little nutrient broth under aseptic conditions. It is treated as exudate.

21.10 Examination of Ear Discharge

Specimen Collection –

1. A specimen of ear discharge should be collected by a medical officer.
2. Collect a specimen on a sterile dry cotton swab.
3. If fungal infection is suspected, mix a small amount of the discharge with a drop of 10 g/dl potassium hydroxide and cover it with a cover slip.
4. For transportation use Amies transport medium.

Figure 21.4Amies transport medium

21.10 Examination of Eye specimens:

Specimen Collection:

1. Eye specimens should be collected by a doctor.
2. Collect specimens of the discharge by using a dry sterile cotton swab. The specimens must be cultured as soon as possible since the natural secretions of the eye contain antibacterial enzymes.

Note

- The principle difficulty in laboratory diagnosis is that of obtaining an adequate specimen in which the viability of the delicate pathogens is necessary.
- It is best to make smears and seed culture plates beside the patient immediately after collecting material from the eye because the volume of exudates obtained is small & dry cotton wool swab absorbs most of the specimen.
- It should be collected without contamination from the skin and margin of the eyelid.
- If it is necessary to dispatch a specimen to the laboratory, it should be taken on an albumen coated swab which is placed at once in Stuart's transport medium.

- For examination for viruses, a swab from the conjunctiva should be submitted in a virus transport medium & dispatched to virology laboratory.

21.11 Examination of Blood –

Specimen Collection –Blood (and bone marrow) require immediate culturing after collection before clotting takes place.

1. Collect about 5 - 10 ml of blood by using sterile syringe and needle, for pediatric and adult patient respectively.
2. Insert the needle through the rubber liner of the bottle cap and dispense 5 ml of blood into Tryptone soya diphasic medium in the thioglycollate broth medium or Castaneda medium. The blood should not be allowed to clot in the culture media.
3. Brain heart infusion broth with cooked meat particles is also used for inoculation.

21.12 Examination of CSF

Specimen Collection – It must be collected by doctor.

It is collected by lumbar puncture under all aseptic precautions.

Note

Inflammation of the meninges (the covering membrane of brain and spinal cord) is called **meningitis**. It is a serious disease and requires urgent treatment. Hence, the Gram-stained smear must be examined immediately for pus cells and bacteria.

21.13 Examination of other body fluids

- Sample should be collected by doctor.
- Samples of pleural, pericardial and peritoneal fluid (Ascitic fluid), synovial fluid are collected by tapping.

- They are examined generally as CSF. If the fluid is relatively clear and its volume sufficient, it should first be centrifuged to deposit the cell and bacteria and the supernatant discarded into disinfectant.
- As nutritionally exacting pathogens, such as pneumococcus, haemophilus and gonococcus may be present, an extra plate of chocolate agar should be seeded for incubation in air plus 5 – 10% CO_2.
- Deposits from joint and pleural fluid not yielding other bacteria should be examined for tubercle bacilli by Ziehl – Neelsen film & culture on Lowenstein – Jensen medium.
- Ascitic fluid from peritoneal dialysis patient :
- Many patients with renal failure are now treated by the procedure of peritoneal dialysis, which exposes them to the risk of bacteria being introduced into the peritoneum and causing a serious peritonitis.

Review questions

1. Write about collection of urine sample in microbiology laboratory.
2. Write about collection, transport & processing of stool sample.
3. What instructions are given to the patient while collecting sputum sample? How it is processed in microbiology laboratory?
4. Write about processing of pus/CSF/ear swabs/eye sample.
5. Write about blood culture procedure.

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22-Parasitology

Objective- Students shall get an idea about various parasites which are commonly observed in different samples and their

22.1 Introduction-

Parasites of Medical Importance

In diagnosing parasitic infections and participating in the control of parasitic diseases, laboratory personnel need to understand the relevant features of parasites of medical importance, particularly those that are found in their area.

Parasitism:-A parasite is an organism that is entirely dependent on another organism, referred to as its host, for all or part of its life cycle and metabolic requirements. The degree of dependence of a parasite on its host varies, parasitism is therefore a relationship in which a parasite benefits and the host provides the benefit.

Types of Hosts:-

1. Definitive Hosts:-This is the host in which sexual reproduction of parasites takes place or in which the most highly developed form of a parasite occurs. When the most mature form is not obvious the definitive host is the mammalian host.
2. Intermediate Hosts: -This is the host which alternates with the definitive host & in which the larval or asexual stages of a parasite are found some parasites required to intermediate host in which to complete their life cycle.
3. Reservoir Hosts:-This is an Animal host serving as a source from which other animal can become infected, epidemiologically reservoir host are important in the control of parasitic diseases.

22.2 Practical Classification of Parasites of Medical

Importance-

1. **Protozoa**: -Single celled organisms multiply in human host.
   a. **Amoebae**: Amoebae consist of a shapeless mass of moving cytoplasm which is divided into granular endoplasm and clear ectoplasm. They move by pushing out the ectoplasm to form pseudopodia (false feet) into which the endoplasm then flows. Amoebae reproduce asexually by simply dividing into two (binary fission) Example:-
      - Entomoeba histolytica

   b. **Flagellates**: - Flagellates possess at some stage of their life cycle one or more long hair like flagella for locomotion. They reproduce by binary fission. Example: -
      - Giardia Lamblia
      - Trichomonas Vaginalis
      - Trypanosoma Species
      - Leishmania Species

   c. **Ciliates**: - Ciliates trophozoites are covered with short hairs (cilia) by which they move. They reproduce asexually by binary fission and sexually by conjugation. They form cysts by which they are transmitting diseases. Example: -
      - Balantidium coli

   d. **Coccidia**: Coccidia are intracellular parasites that reproduces asexually by a process called schizogony & sexually by sporogony. Example: -Blood & tissue coccidia
      - Plasmodium Species
      - Toxoplasma gondii
      - Intestinal coccidia

   e. **MicroSporidia**:-MicroSporidia are obligate intracellular spore

Farmimg. Microorganism normally pathogenic in fish & insects but in humans they have been reported as pathogens in those infected with HIV.Example: -

- Encephalitozoon Species

2. Helminths: -Multicellular worms, do not normally multiply in human host.

   a. **Trematodes (Flukes)** : -Flukes are unsegmented mostly flat leaf like worms (schistosomes) without a body cavity. They vary in size from 1 mm to 70 mm in length. They have oral and ventral suckers (attachments organs) in humans only the adults are found. Examples: -

   - Schistosoma species,
   - Fasciola hepatica

   b. **Cestodes (Tapeworm)**: -The body of a Tape worm is segmented and tape like. It consists of a head (Scolex) & many proglottids (Segments). There is no body cavity. The scolex has suckers that attached the tapeworm to its host. Proglottids are from behind the head mature proglottid contains fully developed reproductive organs. Proglottids that contain eggs are called gravid segments. In most species the eggs are released when a gravid segment become detached and ruptures. Examples: -

   - Taenia species,
   - Hymenolepis nana.

   c. **Nematodes (Round Worms):** - Nematodes are cylindrical worms. They have a body cavity & a cuticle (skin) which may be smooth, spine or ridged. The adult of some species are very long measuring 1 metre or more. Sexes are separate with male worms being smaller than the females. Females are either viviparous (produce larvae) or oviparaous (lay eggs). For most nematodes humans are the only or most significant hosts.Examples: -

Intestinal Nematodes

a) Ascaris lumbricoides (large round worm)
b) Enteribius vermicularis (thread worm)
c) Trichuris Trichiura (whip worm)
d) Strongyloides stercoralis (pin worm)
e) Ancylostoma duodenale (hook Worm)
f) Necator Americans (hook Worm)
g) Filarial & Other Tissue Nematodes
h) wuchereria bancrofti
i) Drancunculus Medinensis (Guinea Worm)

22.3 Factors Contributing Transmission of Parasitic Infection: -

1. Inadequate Sanitation and unhygienic living condition leading to faecal contamination of the environment.
2. Lack of Health education.
3. Insufficient water and contaminated water supplies.
4. Failure to control vectors due to ineffective interventions, insecticides resistance, lack of resources and control measures.
5. Poverty malnutrition and for some parasites increase susceptibility due to co- existing HIV infection.
6. Failure of drugs to treat parasitic infection effectively.
7. Climatic Factors.
8. Population Migration causing poor health, loss of natural immunity, overcrowded conditions, etc.

22.4 Commonly found parasites in stool sample:

Protozoa

1. **Entamoeba histolytica**

Distribution: - It is endemic in many parts of tropical and subtropical Africa, Asia, Mexico, and South America, China.

Transmission:-It is transmitted by the faecal oral route with infective cysts being ingested in food water or from hands contaminated with faeces.

Definitive Host:-Human

Intermediate host:-No

Diseases:-Amoebic dysentery, Amoebic Liver abscess.

Lab Diagnosis:-The Lab diagnosis of amoebic dysentery is by finding E-histolytica trophozoites in a fresh dysenteric faecal specimen or rectal scrape specimen must be examined without delay.

Trophozoite of E histolytica:-

- Average size is about 25 x 20 micro meter
- Shows active amoeboid movement (directional) in fresh warm specimen.
- In dysenteric specimen, the amoebae contain ingested red cells.
- This diagnostic of E histolytica

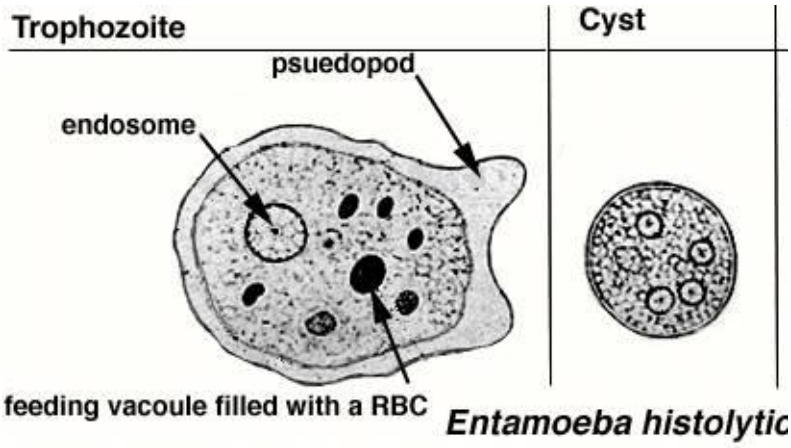


Figure 22.1 Trophozoite and cyst of E. Histolytica

2. Giardia Lamblia

Distribution:- It has a Worldwide distribution and is particularly common in the tropics and sub tropics, in areas where water supplies & the environment become faecally contaminated. Young children are more frequently infected than adult.

Transmission: lambia is transmitted by the faecal Oral route.

Definitive host:-Human

Intermediate host: No

Diseases:-Giardiasis

Lab diagnosis: Fecal specimen containing G.lamblia may have an offensive odour & are pale coloured fatty and float in water. Finding G.lamblia trophozoites in fresh diarrhoeic specimen particularly in mucus they are difficult to detect because they attach themselves to the wall of the intestine so several specimens collected at different times may need to be examined.

Trophozoites of G.lamblia:-

- Small pear shaped flagellate with a rapid tumbling and spinning motility, often likened to a falling leaf.
- Measures 12.15 x 5-9 micro Metre.
- Has a large concave sucking disc on the ventral surface which attached to the intestine
- It has four pairs of flagella two axonemes & two nuclei which stains well.
- A single or two curved median bodies are present.

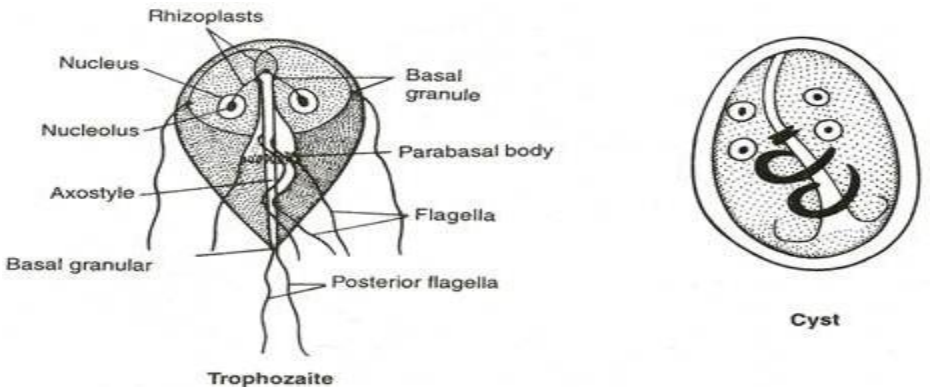


Figure 22.2 Giardia lamblia

Helminths:

A. Nematodes – (Round worms)

1. Ascaris Lumbricoides :-

Distribution:-A. lumbricoides has a world wide distribution. It is particularly common in the tropics and sub tropics in places where environmental sanitation is inadequate & untreated human faecus are used as fertilizer.

Transmission:-A. lumbricoides is spread by faecal pollution of the environment. A person becomes infected by ingesting infective eggs in contaminated food or from hands that have become faecally contaminated.

Definitive host: In the human the natural worms live free in the intestine. Fertilized female worms produce many eggs per day.The eggs can remain viable in soil & dust for several years.The worms can live 1-2 years in their host.

Intermediate host – No

Diseases: - Ascariasis.

Lab diagnosis: - Finding A. Lumbricoides eggs in feaces. Concentration techniques are rarely required. A. Lumbricoides worms expelled through the anus or mouth. Freshly expelled Ascaris worms are pinkish in colour

with an appearance similar to earthworms, they measure 12-35 cm in length and taper at both the ends.

Eggs of A. lumbricoides:-

Usually fertilized eggs are found in feaces but occassionaly infertile eggs are produced by unfertilized female worms.

Fertlized Eggs:-

- Yellow, Brown, Oval or round measueing 50-70 Micro metre long by 30-50 Micro Metre wide.
- Shell is often covered by an uneven albuminous coat.
- Contains a central granular mass which is the unsegmented fertlilized ovum.

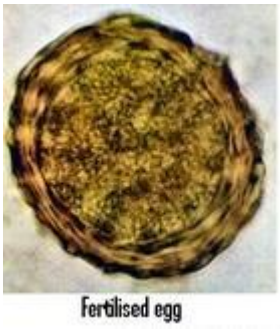


Fig 22.3.1

Infertile Eggs:-

- It is darker in colour & has a thinner wall & more granular, albuminous coat.
- More elongated than a fertilized eggs measuring about 90-45 micro meter.
- Contains a central mass of large granules.

Fig 22.3.2

2. **Trichuris trichiura :- (Whipworm)**

Distribution: - T. trichiura is found worldwide but is more common in moist warm climates in areas where faecal contamination of the environment occurs.

Transmission: - Infection is by ingesting infective eggs in contaminated food or from contaminated fingers. Childre are more often infected than adults, due to playing on faecally contaminated ground.

Definitive host: - Human

Intermediate host: - No

Diseases: - Trichuriasis

Lab Diagnosis: - The lab diagnosis of T.trichiura infection is by finding its eggs in faeces. Concentration techniques are rarely required to detect significant infection.

Eggs of T.trichiura:-

- It is yellow, Brown, & measure about 50 x 25 Micro metre
- Has a characteristic barrel shape with a colouless protruding mucoid plug at each end contains a central granular mass which is unsegmented ovum.

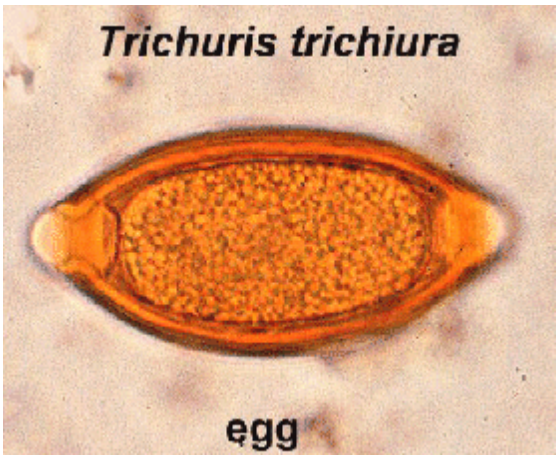


Fig.22.4

3. Hookworms :-

Necator Americans and Ankylostoma duodenale

Distribution: - Hookworms are widespread in the tropics and sub tropics N Americans is commonly found in the far East, South Asia, pacific Island, Tropical Africa, and Central & South America. Ancylostoma duodenale is found in the middle East in the countries around the Mediterranean, North China & North India.

Transmission: - Hookworm infection is spread by faecal pollution of the soil. Infection occurs when infective filariform larvae penetrate the skin, especially when a person is walking barefooted on the infected ground. Ancylostoma duodenale can also be transmitted by ingesting infective larvae.

Definitive host:-Human

Intermediate host:-No

Diseases: - Hookworm infection & Hookworm anaemia

Lab Diagnosis:-By direct examination of hookworm eggs in feaces and it is adequate to detect the eggs or by concentration method. Hookworm infection is usually accompanied by a blood eosinophilia.

Eggs of Hookworm:--

In faecal specimen less than 12 hours old, a hookworm egg has the following appearance.

- It is colourless with a thin shell which appears microscopically as a black line around the ovum.
- Oval in shape, measuring about 65 x 40 micro meters.
- Contains an ovum which appears segmented .

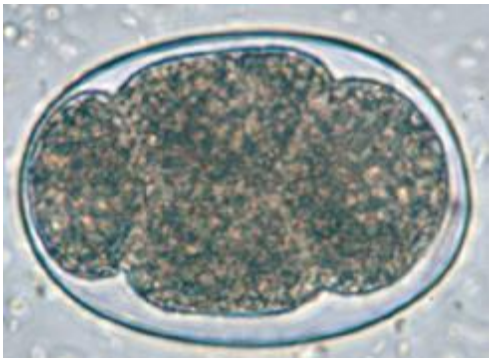


Fig 22.5 Hook worm egg

4. **Strongyloides Stercorallis** :- (Dwarf Thread worm)

Distribution: - Strongyloides Stercorallis has a world wide distribution. It is endemic in many tropical and sub tropical countries including those of Africa, Asia and South America.

Transmission: - By infective flariform larvae penetrating the skin and by auto infection (self infection).

Definitive host: - Human

Intermediate host: - No

Diseases: - Strongyloidiasis

Lab Diagnosis: - By Finding Strongyloides Stercorallis larvae in fresh faeces. The larvae can also be found in duodenal aspirates. In disseminated infections larvae can be found in most body fluids.

Rhabditiform Larvae of S Stercorallis

- It is actively motile.
- It is large measuring 200-250 x 16 Micro Metre & Unsheathed.
- Shows typical rhabditiform large bulbed oesophagus.
- It can be distinguished from hookworm larvae by its shorter buccal cavity.

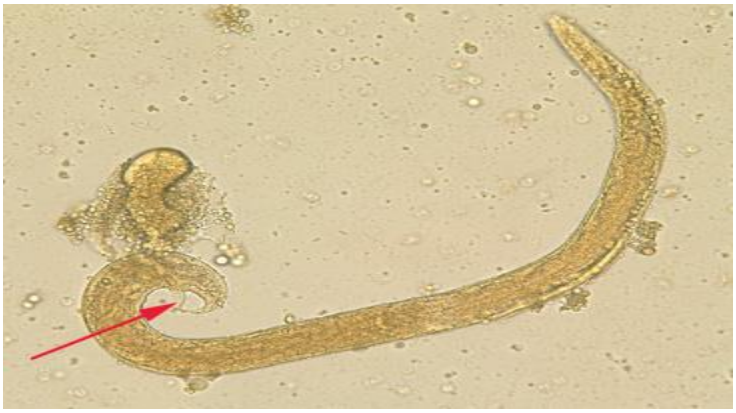


Fig. 22.6 S.Stercoralis

5. **Cestodes :- (Tapeworm)**

a. Taenia saginata :-

Distribuition: - Taenia saginata has a worldwide distribution in countries where cattle are raised & beef is eaten. High infections rates are found particularly in the highlands of Ethiopia. In Egypt & Morocco the camel is the main source of human infection.

Transmission: - Mass Travel, the Migration of workers, and an inadequate disposal of sewage have contributed to increases in the prevalence rates of infection with Taenia Saginata. It is transmitted by eating raw or undercooked beef or other animal meat which contains infective cysticercus larvae.

Definitive host: - Human

Intermediate host: - Cattle

Diseases: - Taenia saginata taeniasis

Lab Diagnosis:-

Identifiying gravid segments recovered from clothing or passed in faeces usually passes singly.

Detecting eggs in faeces, morphologically the eggs of Taenia Saginata & T. solium are indistinguishable.

Gravid Segment of Taenia saginata:-

- Appears white and opague & measures about 20 mm long by 6mm wide when freshly passed. It is therefore longer than a T. Solium gravid segment.
- Uterus has a central stem ehich has more than 13 main side branches on each side. (T. solium has fewer than 13).The main side branches are sub divided into smaller branches.

Examination of feaces for Taenia eggs:-

A concentration techniques & the examination of several specimen may be necessary to detect Taenia eggs in faeces.

Eggs of T. saginata or T. solium:-

- It is round to oval measuring 33-40 micrometer in diameters.
- Embryo is surrounded by a thick, brown, radially striated wall.
- Hooklets are present in the embryo careful focusing is necessary to see the three pairs of hooklets.
- Sometimes a clear membrance can be seen surrounding the egg but usually this losts when the gravid segment disintergrates.

b. Taenia solium**:-**

Distribution: - T. solium is not as widely distributed as T. saginata. It occurs mainly in Southern Africa, China, India,.

Transmission: - T- Solium transmitted by eating raw or insufficiently cooked pork which contains infective cysticercus larvae.

Definitive host: - Human

Intermediate host: - Pigs

Diseases: - T. solium taeniasis, Cystcercosis, Neuro Cystcercosis.

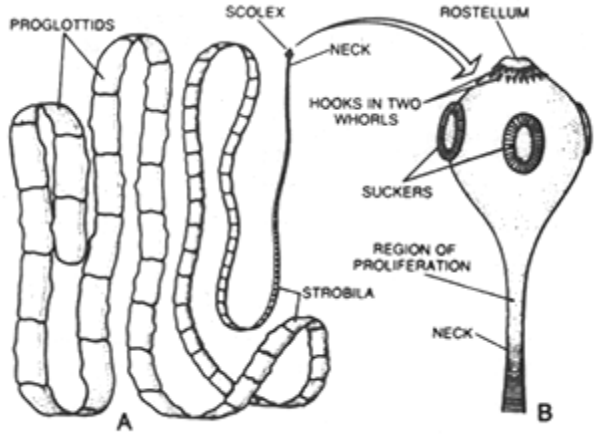
Lab Diagnosis:-

- Identifying gravid segment passed in faeces.
- Detecting eggs in faeces, morphologically eggs of T. Solium & T. Saginata are similar in appearance.

- Cysticercoids: - This is usually diagnosed serologically, when calcified; the cyst can be detected by x-ray. Cyst in the brain can also be detected by CT scan, CSF examination.

Gravid Segment of T. solium:-

- Appears grey blue & translucent and measures about 13 mm long by 8mm wide when freshly passed. Shorter than T. saginata.
- Uterus has a central stem which has upto 13 main side branches on each side which is subdivided into smaller branches.



Taenia solium : A. whole; B. its scolex

Fig.23.7 T.solium

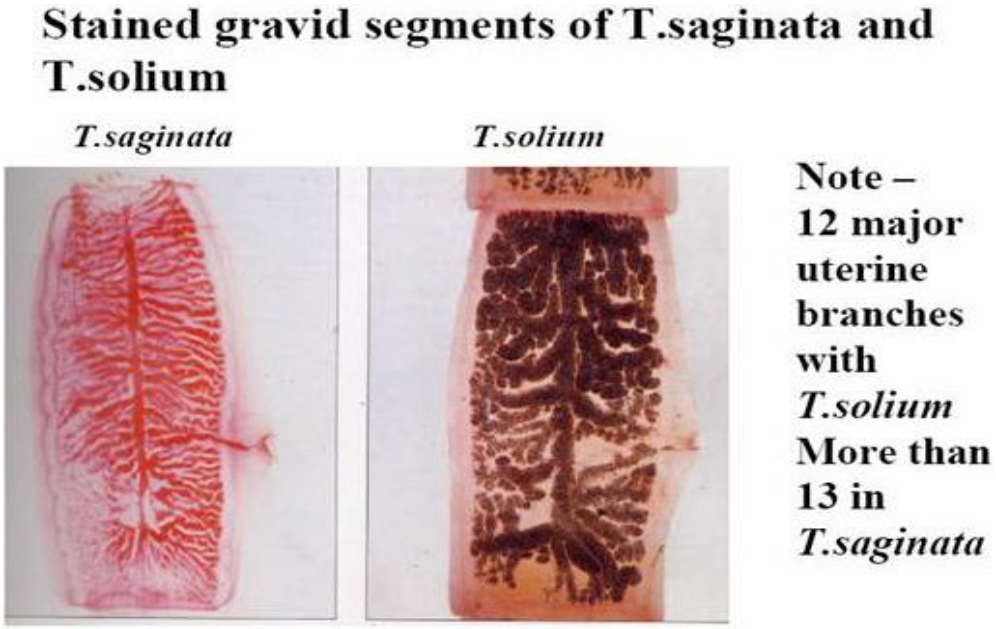


Fig 22.8

Review Questions

1. What is parasitism?
2. Define definitive and intermediate host.
3. Classify parasites.
4. Differentiate between fertilized and unfertilized egg of Ascaris lumbricoides.
5. Differentiate between T. solium and T.saginata.
6. Describe morphology of trophozoite and cyst of E histolytica and Giardia lamblia.

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Glossary

1. **Anaerobi**c – Does not required oxygen.
2. **Inclusions** – Present within.
3. **Invasion** – Entered inside.
4. **Unaided** – Not supported.
5. **Extraneous** – Something from outside.
6. **Susceptibility** – can be acted upon by.
7. **Pathogenicity**- Damage done within the body by organism.
8. **Fastidious** – Power to act fast.
9. **Commensals** – normally present
10. **Affinity** – attraction for
11. **Slender**- thin and long
12. **Ferment**- decay
13. **Intradermally** – within the layers of skin
14. **Aerobes**- Requires oxygen
15. **Syndrome**- group of diseases
16. **Lymhangitis** – inflammation of lymph vessels
17. **Cellulitis**- Inflammation of skin and subcutaneous tissue
18. **Isolated**- kept separate
19. **Virulent**- infectious
20. **Emulsifable**- can be melted
21. **Ig**- immunoglobulin (antibodies)
22. **Attenuated**- made less active
23. **Epidemiology**- study, survey, analysis and planning to control a condition
24. **Viable -** Living
25. **Aseptic** – far from infections

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