STUDENT PARTICULARS

Name	.....
Semester	.....
Class Roll No.	.....
Batch (Regular/Suppl)	.....
University Enrollment No.	.....

Certified to be the bonafide record of work done by

_____

_____ of class MBBS 2nd Professional during the year 2013 to 2014 at

Hamdard Institute of Medical Sciences and Research (HIMSR)
&
Associated Hakeem Abdul Hameed Centenary (HAHC) Hospital.

Professor In-Charge

Submitted for the practical examination held in Dec/July at

Hamdard Institute of Medical Sciences and Research (HIMSR)
&
Associated Hakeem Abdul Hameed Centenary (HAHC) Hospital.

New Delhi - 110062

Examiner

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Safe laboratory conduct and practice

In a microbiology laboratory students will be dealing with live microorganisms along with toxic and hazardous chemicals. In order to protect themselves from accidental laboratory infection or accidents, strict observance of the following safe laboratory conduct is essential.

1. The students should enter the laboratory wearing a clean white apron with full sleeves and buttons up.
2. Students with long hairs should tie up their hairs or keep under the apron.
3. Eating and drinking is strictly prohibited inside the laboratory
4. No bag, books, copies except the practical record book and writing/drawing stationeries should be carried in the laboratory.
5. Fingernails should be kept short and trim.
6. Habit of biting fingernails, pen , pencil etc should be avoided
7. Familiarize yourself with the laboratory set up viz. source of light, gas, water, wash basin and disinfectant jar.
8. Working table and microscope should be thoroughly cleaned with separate tissue paper or cloth before and after use.
9. No reagent, chemicals, stain etc should be wasted unnecessarily.
10. Any accidental spillage of reagent, specimen material and breakage of glassware should be reported immediately to the faculty or laboratory staff present.
11. Labeled diagram of the day's observation should be drawn in the area specified in the chapter.
12. On completion of day's work, one should get the record signed by the concerned teacher. Only then the attendance will be marked.
13. One should check if all the equipments, reagents, chemicals etc are kept at their appropriate places before leaving the working table.
14. The students should make sure that the table is clean and no slide is left on the microscope or on the table. The electric switch, water tap and gas tap should be switched off/closed.
15. **Every student should wash their hand with disinfectant/soap and water before leaving the practical laboratory**.

Exercise Number 1: Microscopy and Micrometry

Learning objectives:

On completion of the exercise the students should be able to

- **a)** Name the major parts of the microscope.
- **b)** Determine the diameter of a field of view.
- **c)** Prepare a wet mount.

Introduction :

Microorganisms are too small to be seen with the naked eye so a microscope must be used to visualize these organisms. While a microscope is not difficult to use it does require some practice to develop the skills necessary to use the microscope to its maximum capabilities. Bacteria and other cellular microorganisms are measured in micrometers (μm) or 1 x 10^{-6} meters. The microscopes used in an basic microbiology laboratory is a compound light or bright-field microscope.

Parts of a Microscope:

1) Ocular /Eyepiece: The ocular, or eyepiece contains low power lenses. It fits into the top of the body tube and gives a secondary magnification of the image formed by the objective lenses. The eyepieces of most microscopes have a magnification of 10. The total magnification is calculated by multiplying the objective and ocular magnifications. If the microscope is tilted too much, the eyepiece may fall out and break.

2) Head/tube: It is the structure from which the oculars protrudes out. The binocular tube allows adjustment of the two oculars for the particular spacing of your eyes. The binocular tube can be rotated on the stand allowing the microscope to be used with the stage directly in front or with the focusing knobs in front of the user.

3) Revolving Nosepiece: The nosepiece, located at the lower end of the tube, holds the objectives. it can be turned gently to bring in place the objectives. There is a faint click when an objective is in proper alignment.

4) Objective Lenses: These are mounted on the revolving nosepiece. Each lens is marked with its Magnification/Numerical Aperture and Focal Distance. Most standard microscopes have 4X, 10X, 40X, and 100X oil immersion lenses. The first three are used with only air between the lens and the slide, the highest power lens (100X) is used with a drop of immersion oil between the lens and the slide. That is the light passing through the slide (material being viewed) passes through only oil, not air, as it is transmitted to the lens system. Although the oil immersion lens is designed to work in oil, the oil must be removed after use and before you put the microscope away at the end of the period. All the other lenses do not operate with oil on them. If oil gets on the air lenses it will ruin their mounts and surfaces.

5) Stage: This is the platform on which slide is placed to be examined. The slide is placed in the center of the stage over the aperture through which light enters from underneath. Stage clips, or a mechanical stage, are used to hold the slide in place. The specimen can be centered with the "mechanical stage adjustment knob."

6) Mechanical Stage: Resting on top of the stage is the mechanical stage. This contains a spring clip that will hold the slide in place. To the right of the mechanical stage are two control knobs that allow you to move the slide left and right and backwards and forwards. This enables the user to look at all areas of the specimen.

7) Condenser Lens: Below the stage is the condenser lens. This focuses light onto the object and is not involved in the magnification. The focusing adjustment is a rack and pinion movement to permit vertical movement of the condenser. Clear images are obtained only when the condenser lens is in proper focus: when the cone of rays illuminating the object is equal to that observed by the objective lens. A blurry image may be due to out of focus condenser.

8) Iris diaphragm: The iris diaphragm is located just above the light source on the bottom side of the stage, bellow the condenser. Using the lever attached to it you can increase or decrease the amount of light reaching the specimen.

9) Light Source: Most modern microscopes contain a built in base illuminator with a facility to vary the intensity of light by varying the voltage to the lamp (light intensity) as well as an iris diaphragm in the condenser. For optimal results, the proper amount of illumination should be obtained: fields that are too bright or too dim will not allow to see the details of the Preparation.

10) Coarse and Fine Adjustment Wheels: These are used to raise and lower the body tube or stage, depending upon the manufacture of the microscope. The coarse adjustment is used to first bring the object into approximate focus starting first with the stage as close as possible to the objective lens without touching. Then move the coarse adjustment so that the stage moves away from the lens until the object is in relative focus. If this is always done in this way, there is no possibility that the lens gets jammed into the slide, damaging both. After the object is in relative focus, it can then be brought into sharp, critical focus with the fine adjustment knob.
11) Arm: At the back of the stage is the arm of the microscope that supports the head of the microscope. It is connected to the base and is a good place for user to grab hold of the microscope when he need to carry it or lift it out of its storage cabinet.

Focusing:

Place the slide in the center of the stage, and start with low power. Looking from the side bring the slide and objective very close together using the coarse adjustment. Then looking through the microscope focus by turning the coarse adjustment knob so that the distance between the slide and objective increases until you see the object in question, then finish the focus with the fine adjustment (the smaller knob). If you don't see anything, adjust the light; too much light will wash out the image. Most scopes are par-focal. This means that when you want to change view of the specimen from low power to high power, all that is needed after revolving the nosepiece to high power, is to adjust the focus with only the fine focus knob. You should never focus with the coarse adjustment while using high power, as this leads to broken slides. When changing from low to high power you will notice that more light is also required.

Adjustment of light:

Remember that the condenser controls the quality of light which reaches the slide while the diaphragm controls the quantity of light reaching the slide. If it is too low refraction causes distortion of the object being examined. To control the amount of light reaching the slide adjust only the diaphragm. When the diaphragm is on the largest opening,, the greatest resolution of detail is obtained while when the diaphragm opening is small, resolution is reduced but contrast is high. Resolution is the ability to see two separate points instead of them combining into one fuzzy point.

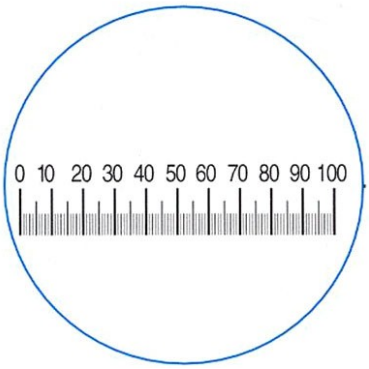
Safety and the Compound Microscope:

The compound microscope is a delicate instrument that needs to be used safely. Some tips to keep in mind include the following:

1) Always keep the microscope upright when handling it. Use two hands to carry the microscope—one under the base and one on the arm. Place the microscope near the centre of the desk or table where it will be used.
2) Be careful when handling glass slides—they may shatter if dropped.
3) When you are observing a specimen through the microscope, keep both eyes open to avoid straining your eyes.
4) Always store the microscope with the lower-power objective lens in place and the stage lowered. This will prevent the objective lens from being accidentally scratched by the slide when you begin using the microscope.
5) Only use the coarse-adjustment knob with low-power objective lenses. Use the fine-adjustment knob at higher powers.
6) Use the microscope in a dry area. Your hands should also be dry when using a microscope.
7) Remember to unplug the microscope from the electrical outlet by grasping and pulling the plug, not by pulling on the power cord.
8) Coil the power cord neatly around the arm of the microscope when returning the microscope to its storage area.
9) The 100X objective should be cleaned with alcohol before and after used. Xylene can be used as an alternative for alcohol.
10) The stage should be cleaned after use and should be kept dry and free from oil.

Micrometry

Micrometry is the measurement of microorganisms. Since microorganisms can be seen only under a microscope, a suitable scale for their measurements should be somewhere in the microscope itself. For this an ocular micrometer serves as a scale or rule. Ocular micrometer is simply a disc of glass upon which are etched lines. By determining how many divisions of ocular micrometer superimpose a known distance on the stage micrometer, it is easy to find out the exact value of one division of ocular micrometer in the microscope field. Once calibrated, the ocular micrometer can be used to measure the size of various microbes in terms of length, breadth, diameter.



Procedure

There are usually etched 100 equally spaced divisions, marked 0 to 100 upon an ocular micrometer. When placed in the ocular (eye piece), the ruled lines superimpose certain distance markers on the microscope field. However, the scale on ocular micrometer does not have any standard value. We can find out the value of one division of this unknown scale by calibrating it with a known scale. Thus actual value of one division of ocular micrometer is found by using another known scale, the stage micrometer. Stage micrometer is simply a microscope glass slide having in its centre a known (one millimeter) distance etched into 100 equally spaced divisions. This 1 mm (1000 micro.m) distance is encircled and mounted by a cover glass. Thus each division of stage micrometer equals to 0.01 mm or 10 micro meter. The distance of each division of stage micrometer becomes correspondingly enlarged under high power and oil immersion objectives of the microscope. Ocular micrometer is, therefore, calibrated under different objective lens systems of the microscope.

Demonstrations

The teacher should Demonstrate the following things during the class:

a) The different parts of a microscope.
b) Method of focusing a slide under the microscope
c) Method of adjusting of illumination according to requirement.
d) Method of cleaning and safety of a microscope.
e) Micrometer and its use.

Students Exercise:

1. The total magnification obtained while using the 100X objective lens is.....
2. How do you adjust the condenser and iris diaphragm when observing under oil immersion lens?

   .....

   .....

   .....

3. Glass lenses are present at following positions in a microscope:

   a) .....

   b) .....

   c) .....

4. what are the possible faults that may cause inability to focus a smear under high or oil immersion objective?

   .....

   .....

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   .....

5. What is the use of a Micrometer ?

   .....

   .....

Exercise Number 2: Bacterial Morphology & Staining Techniques

Learning objectives:

On completion of the exercise the students should be able to:

- **a)** Note the bacterial growth on culture media and understand different colony characteristics.
- **b)** Understand the basic principle of the common staining methods.
- **c)** Perform the different staining methods and observe the stained slides under the microscope.

Bacterial Morphology

Introduction:

Bacteria are very small unicellular microorganisms ubiquitous in nature. They are micrometers (1μm = 10^{-6} m) in size. They have cell walls composed of peptidoglycan and reproduce by binary fission. Bacteria vary in their morphological features. Bacteria grow on solid media as colonies.

A colony is defined as a visible mass of microorganisms all originating from a single mother cell, therefore a colony constitutes a clone of bacteria all genetically alike.

I n the identification of bacteria and fungi much weight is placed on how the organism grows in or on media. Although one might not necessarily see the importance of colonial morphology at first, it really can be important when identifying the bacterium. Features of the colonies may help to pinpoint the identity of the bacterium.

Different species of bacteria can produce very different colonies.The most common morpholologies are:

1) **Coccus (pleural: Cocci):** Spherical bacteria; may occur in pairs (diplococci), in groups of four (tetracocci), in grape-like clusters (*Staphylococci)*, in chains (*Streptococci*) or in cubical arrangements of eight or more (sarcinae).For example: *Staphylococcus aureus, Streptococcus pyogenes.*

2) **Bacillus (pleural: Bacilli):** Rod-shaped bacteria; generally occur singly, but may occasionally be found in pairs (diplo-bacilli) or chains (streptobacilli). Example: *Bacillus cereus, Clostridium tetani*

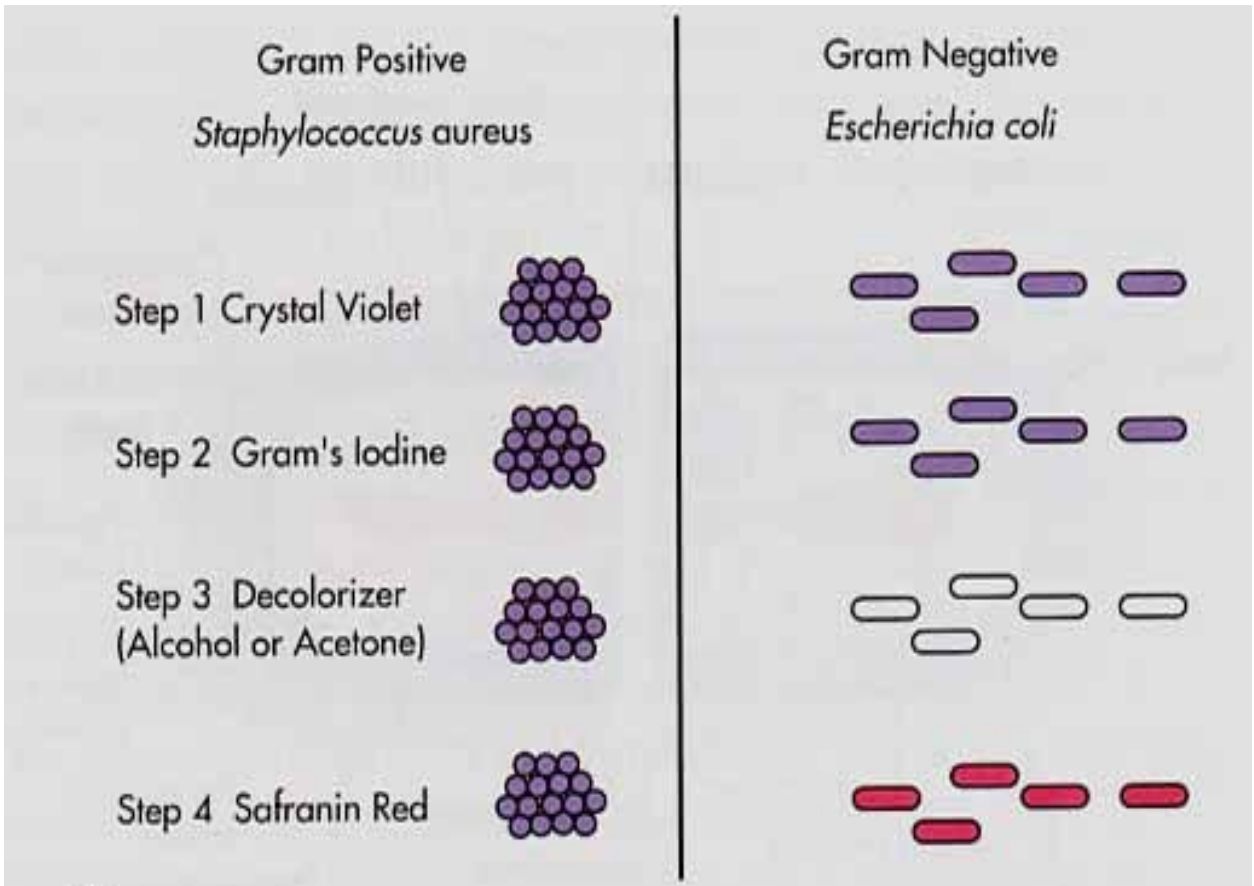
3) **Coccobacilli:** These are short and stout bacilli which appears as cocci under low power of a microscope. Example: *Hemoplilus*

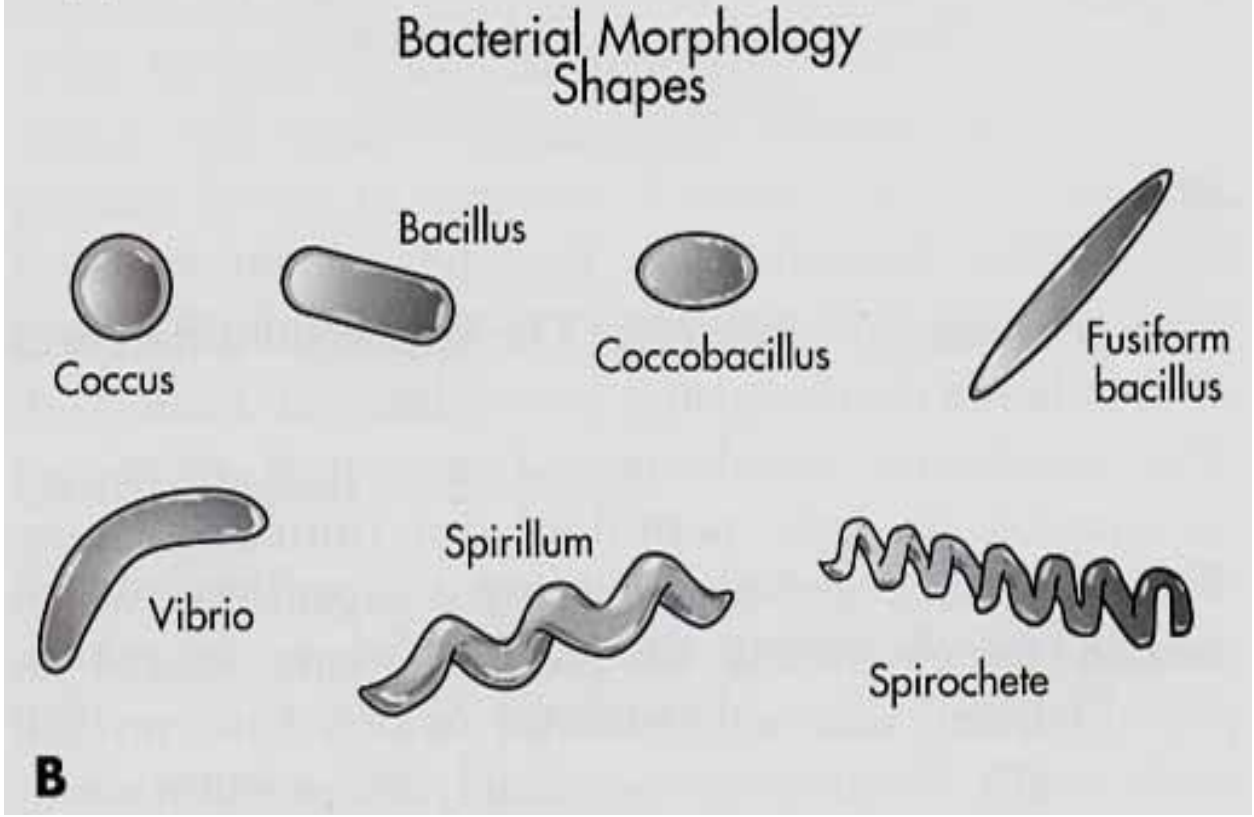
4) **Vibrio:** These are bacilli with a slightly curved structure. Example: Vibrio cholrae.

5) **Spirillum(pleural:Spirilla):**Spiral-shaped bacteria. Example: *Spirillum, Spirochete* species.

6) **Filamentous:** Bacilli that occur in long chains or threads.

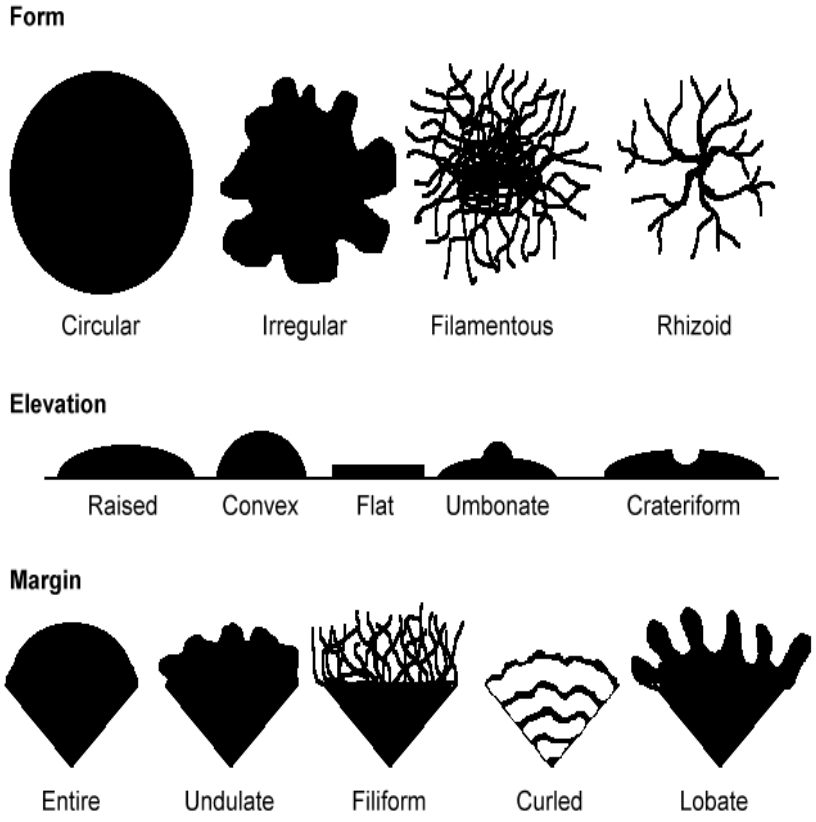
7) **Fusiform:** Bacilli with tapered ends.


Gram Positive
Staphylococcus aureus
Gram Negative
Escherichia coli
Step 1 Crystal Violet
Step 2 Gram's Iodine
Step 3 Decolorizer
(Alcohol or Acetone)
Step 4 Safranin Red
A


Bacterial Morphology
Shapes
Bacillus
Coccus
Coccobacillus
Fusiform
bacillus
Vibrio
Spirillum
Spirochete
B

Different Colony morphologies:

Colony characteristics	Observations
Size	Very small, Small, Medium, large, very large
Form	Punctiform, circular, Filamentous,irregular,rhizoid, spindle
Elevation	Flat, raised, convex, pulvinate, umbonate
Margin	Entire, undulate, lobate, erose, filamentous, curled
Color	White, grey, yellow, black, orange,pink, red, etc
Haemolysis	Haemolysis α , β or γ
Pigment production	Color of the pigment production e.g red,yellow,Green etc
Odor	Fruity, freshly cut apple, fishy, fecal or putrid, bleach, pungent
Consistency	Buttery, viscid, Brittle, mucoid
Surface	Smooth, Glistening, Rough, dull
Opacity	Transparent, Opaque, Translucent



Haemolysis: Certain bacteria produce enzymes called hemolysin that cleaves RBC. When these organisms are grown on Bloor agar, they produce visible change on the medium depending on the type of hemolysis they perform.

Type of hemolysis	Description
Alpha	This refers to the **partial lysis** of red blood cells and hemoglobin. This results in a greenish-grey discoloration of the blood around the colonies. Example : *Streptococcus pneumoniae*
Beta	**Complete lysis** of red blood cells and hemoglobin. This results in complete clearing of the blood around the colonies. Example: *Staphylococcus aureus*
Gamma	**No hemolysis**, results in no change in the medium. Example : Few strains of *Enterococcus*

Odor : Certain organism when grown on artificial culture medium, produces a typical smell that can be used as a presumptive method of identification. Few typical odors are:

Odor:	Probable organism
Fruity	Pseudomonas species
Fishy	Proteus species
Freshly cut apple	Alcaligenes faecalis
Fecal or Putrid	Clostridium species

Bacterial Staining Techniques

Introduction: Bacteria are microscopic organisms and are not seen with unaided eye. They can be seen even in unstained preparations such as a wet mount or hanging drop preparation but the morphology is not clear. Bacteria are colorless and when suspended in saline they don't offer any contrast. Besides, bacterial motility makes it difficult to observe the morphology clearly. Hence, bacteria have to be stained to observe them. The dyes often used are toxic chemicals that kill the bacteria. The process of smearing, fixing and drying often kill the bacteria. This process fixes the bacteria to the slide and their position on slide remains unaltered.

Types of Dyes

a) Acidic dyes : these have an affinity for positively-charged components in the cell because their auxochrome group is negatively charged (anionic). Example: Picric Acid
b) Basic dyes : These have an affinity for negatively-charged components in the cell because their auxochrome group is positively-charged (cationic). Example: Methylene Blue

Different staining methods

a) **Simple Staining:** A simple stain employs a single stain that is used primarily to examine shape and arrangement of cells. Basic stains such as methylene blue, crystal violet, or carbol fuchsin, are often used for this type of staining.

b) **Negative staining:** Negative staining is a technique by which bacterial cells are not stained, but are made visible against dark background.
Acidic dyes like eosin and Nigrosin are employed for this method. Though, this staining technique is not very popular, it has an advantage over the direct or positive staining methods for the study of morphology of cells. This is because of the fact that the cells do not receive vigorous physical or chemical treatments.

Procedure:

1. Place a drop of India ink on a slide.
2. Sterilize the loop. Aseptically add a loopful of the *Klebsiella pneumoniae* colony to the ink drop and mix evenly.
3. Place a cover slip on the drop. Press down firmly on the cover slip
4. Examine in the microscope at 100X before the preparations dry out.

c) **Differential Stains**: Staining procedure which differentiates or distinguishes between types of bacteria is termed as differential staining technique. Differential staining procedure utilizes more than one stain, and imparts distinctive colour only to certain types of bacteria. The basic principle underlying this differentiation is due to the different chemical and physical properties of cell and as a result, they react differently with the staining reagents.

1) Gram Stain: Discovered by the Danish scientist and physician **Hans Christian Joachim Gram** in 1884, Gram stain is one of the most important and widely used differential stains.

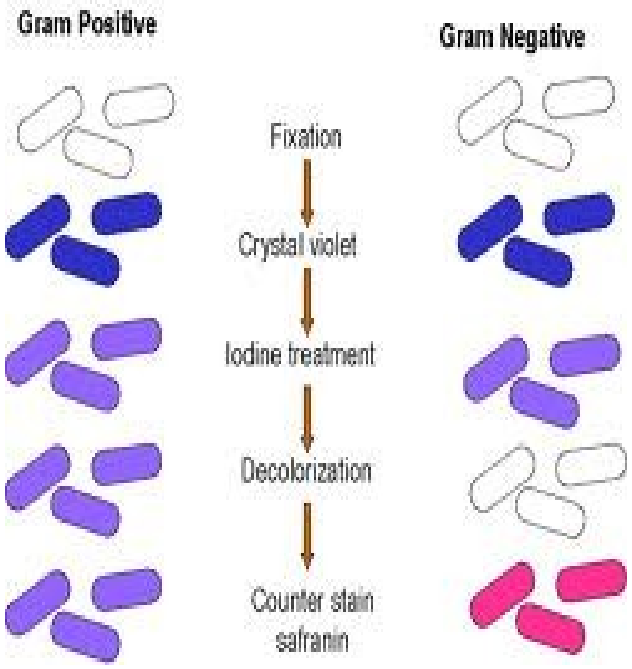
Principle:

Gram staining differentiates bacteria into two groups: gram-positives and gram-negatives. The procedure is based on the ability of microorganisms to retain color of the stains used during the gram stain reaction. A heat fixed smear is first treated with the primary stain- **Crystal violet(CV)**. CV dissociates in aqueous solutions into CV+ and Cl – ions. These two ions then penetrate through the cell wall and cell membrane of both Gram-positive and Gram-negative cells. The CV+ ions later interacts with negatively charged bacterial components and stains the bacterial cells purple. Further Iodine is added which acts as a **mordant** and as a trapping agent. A mordant is a substance that increases the affinity of the cell wall for a stain by binding to the primary stain, thus forming an insoluble complex which gets trapped in the cell wall. In the Gram stain reaction, the crystal violet and iodine form an insoluble complex (CV-I) which serves to turn the smear a dark purple color. At this stage, all cells will turn purple. Then a decolorizing agent (either 95% ethanol or acetone) is applied. Gram-negative bacteria are decolorized by the alcohol, losing the color of the primary stain, purple. Alcohol or acetone dissolves the lipid outer membrane of Gram negative bacteria, thus leaving the peptidoglycan layer exposed and increases the porosity of the cell wall. The CV-I complex is then washed away from the thin peptidoglycan layer, leaving Gram negative bacteria colorless. On the other hand, the peptidoglycan layer in gram positive cell wall is much thicker and the alcohol is unable to dissolve it or form pores in it completely. It just has a dehydrating effect on the cell walls of Gram positive bacteria which causes the pores of the cell wall to shrink. Hence the gram-positive bacteria are not decolorized by alcohol and will remain as purple.

After decolorization step, a counter stain is used to impart a pink color to the decolorized gram-negative organisms.

Procedure:

a) Add the primary stain Crystal Violet (CV) to a heat-fixed smear and keep for 1 min.
b) Wash the slide after 1 minute with distilled water
c) Add Gram's Iodine for 1 minute.
d) Wash the slide.
e) Decolorization: add 95% ethyl alcohol for 10-15 seconds or acetone for 3-5 seconds.
f) Counter stain with Safranine for 45 sec to 1 minute
g) Dry the slide by keeping it in a slant position or by putting it on a hot plate.
h) Observe under microscope under 100 X objective



Result:

a. **Gram positive bacteria**: Stain dark purple due to retaining the primary dye called Crystal Violet in the cell wall. Example: *Staphylococcus aureus*

b. **Gram negative bacteria**: Stain red or pink due to retaining the counter staining dye called Safranine. Example: *Escherichia coli*

2. Acid Fast Stains: Acid fast staining is another widely used differential staining procedure in bacteriology. This stain was developed by Paul Ehrlich in 1882, during his work on etiology of tuber culosis (5). Some bacteria resist decolourization by both acid and alcohol and hence they are referred as acid-fast organisms. Acid alcohol is very intensive decolourizer. This staining technique divides

bacteria into two groups (i) acid-fast and (ii) non acid-fast. This procedure is extensively used in the diagnosis of tuberculosis and leprosy.

Acid-fastness property in certain *Mycobacteria* and some species of *Nocardia* is correlated with their high lipid content. Due to high lipid content of cell wall, in some cases 60% (w/w), acid-fast cells have relatively low permeability to dye and hence it is difficult to stain them. For the staining of these bacteria, penetration of primary dye is facilitated with the use of 5% aqueous phenol which acts as a chemical intensifier. In addition, heat is also applied which acts as a physical intensifier. Once these cells are stained, it is difficult to decolourize.

Ziehl-Neelsen method: Ziehl and Neelsen independently proposed acid fast stain, in 1882-1883 is commonly used today. The staining reagents are much more stable than those described by Ehrlich.

Procedure: The procedure for staining is as follows.

1. Prepare a smear and fix it by gentle heat.
2. Flood the smear with carbol fuchsin and heat the slide from below till steam rise for 5 minutes. Do not boil and ensure that stain does not dry out.
3. Allow the' slide to cool for 5 minutes to prevent the breakage of slide in the subsequent prevent step.
4. Wash well with water.
5. Decolourize the smear till red colour no longer comes out in 20% sulphuric acid.
6. Wash with water.
7. Counter-stain with 1% aqueous solution of malachite green or Loeffler's methylene blue for 15-20 seconds.
8. Wash, blot dry and examine under oil-immersion objective.

3. ALBERT STAIN: Albert's staining technique is a type of special staining technique since it is used to demonstrate a special structure in bacteria. It is chiefly used to demonstrate metachromatic granules found in *Corynebacterium diphtheriae*. The storage granules in this bacterium is called metachromatic granules because it exhibits the property of metachromasia, wherein the granules appear in a colour other than the colour used for staining. When stained with polychrome methylene blue, the granules appear violet while the rest of the bacillus appears blue. The granules are made up of polymetaphosphates and are known by various other names such as volutin bodies, Babe-Ernst granules or polar bodies. The bacterium produces the granules in abundance when grown on nutrient rich medium such as Loeffler's serum slope.When stained with Albert's stain, the bacillus stains green whereas the granules stain bluish black. There are two reagents that are used in the staining process:

a) Albert's A :Toluidine blue, malachite green, glacial acetic acid, and ethyl alcohol.

b) Albert's B: Iodine and Potassium iodide in water.

Requirements: Smear on glass slide, staining rack, Albert's A solution , Albert's B solution, blotting paper, immersion oil, microscope

Procedure:

a) Make a smear and place the slide on the staining rack with smear facing upwards.

b) Cover the smear with Albert's A solution and allow it to act for 7 minutes.

c) Pour off the stain and wash the slide in Albert's B solution (NOT tap water).

d) Place the slide back on the rack and cover the smear with Albert's B solution and let it act for two minutes.

e) Pour off the stain and wash the slide in tap water.

f) Dry the slide using blotting paper, place a drop of immersion oil on the smear and observe under oil immersion objective.

Observation: Green coloured rod arranged at angles to each other resembling English letter 'L', 'V' or Chinese letter pattern along with bluish black metachromatic granules at the poles.

4. Endospore staining: Bacterial **endospores** are metabolically inactive, highly resistant structures produced by some bacteria as a defensive strategy against unfavorable environmental conditions. The bacteria can remain in this suspended state until conditions become favorable and they can germinate and return to their **vegetative** state.

Procedure:

a) Add **malachite green**(**primary stain)**, which stains both vegetative cells and endospores.

b) **Heat** is applied to help the primary stain penetrate the endospore.

c) The cells are then **decolorized** with **water**, which removes the malachite green from the vegetative cell but not the endospore.

d) **Safranin** is then applied to **counter stain** any cells which have been decolorized.

Observation: vegetative cells will be pink, and endospores will be dark green**.**

Demonstrations:

The teacher should demonstrate the following things during the class:

a) Different morphological forms and arrangements of bacteria through a microscope or/and with the help of electronic visual aids
b) Different colony morphologies on solid medium and growth characteristics on liquid culture medium.
c) Common changes produced by bacterial growth onto the culture mediums.
d) Material required and procedure of common staining methods

Students Exercise:

1. Record gross colonial characteristics for two types of the colonies grown on agar plates in the given table.

No.	Colony Characteristics	Observation			
		Colony 1	Colony 2	Colony 3	Colony 4
1	Size				
2	Form				
3	Elevation				
4	Margin				
5	Color				
6	Haemolysis				
7	Pigment production				
8	Odor				
Name of media					
Possible Organism					

2. The cocci which mostly occur in chains or pairs are
   A. Streptococci
   B. Diplococci
   C. Tetracocci
   D. None of these
3. Bacteria having no flagella are unable to
   A. Move
   B. Reproduce
   C. stick to tissue surfaces
   D. grow in nutrient agar
4. The arrangement, in which flagella are distributed all round the bacterial cell, is known as
   A. Lophotrichous
   B Peritrichous
   C. Amphitrichous
   D. Monotrichous

5. The common word for bacteria which are helically curved rods is

A. Cooci

B. Bacillus

C. Pleomorphic

D. Spirilla

6. The bacteria deficient in cell wall is

A. Treponema

B. Mycoplasma

C. Staphylococcus

D. Klebsiella

7. Which of the following has Chinese letter arrangement?

A. *Bacillus anthracis*

B. *Mycobacterium tuberculosis*

C. *Clostridium tetani*

D. *Corynebacterium diphtheria*

8. Which of the following may be most likely to be missing from a gram-positive bacterium?

A. Penicillin binding protein

B. Peptidoglycan

C. Lipopolysaccharide

D. Phospholipid bilayer membrane

9. Perform gram stain and report your finding. Draw a neat labeled diagram of your observation.

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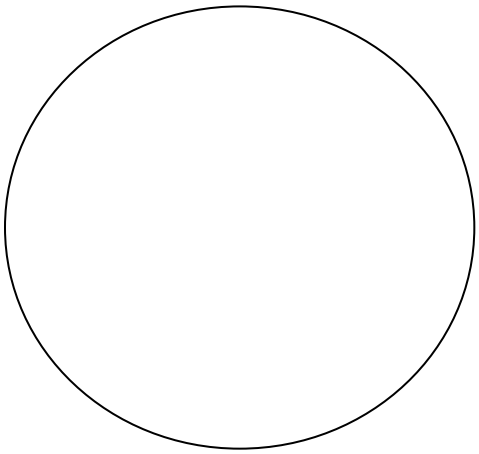
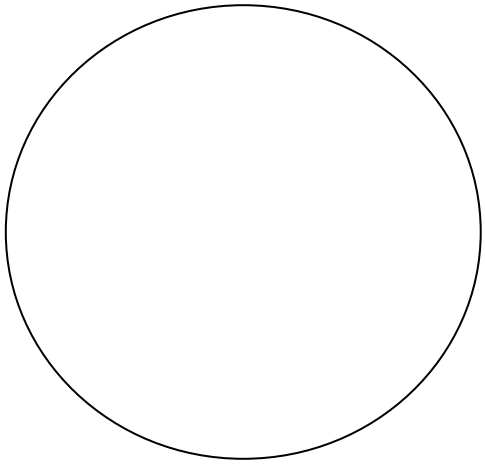


10. Perform a ZN stain and report your finding. Draw a neat labeled diagram.



11. How are staining techniques classified?

12.Which are the alternative primary and secondary stains used in Gram stain?

13.What are the conditions when Gram positive bacteria can appear Gram negative?

14 .Why are Mycobacteria acid fast?

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15.Which are the various dilutions of sulfuric acid used?

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Exercise Number 03: Culture media

Learning objectives:
On completion of the exercise the students should be able to:

- **a)** Identification and use of different culture media.
- **b)** Methods of sterilization of various media.

Introduction :

For diagnosing a bacterial infection bacteria have to be grown (cultured) in the laboratory on artificial culture media. Isolating a bacterium from sites in body normally known to be sterile is an indication of its role in the disease process. Culturing bacteria is also the initial step in studying its morphology and its identification. Bacteria have to be cultured in order to obtain antigens from developing serological assays or vaccines. Certain genetic studies and manipulations of the cells also need that bacteria be cultured in vitro. Culturing bacteria also provide a reliable way estimating their numbers (viable count). Culturing on solid media is another convenient way of separating bacteria in mixtures.

An artificial culture medium must provide all the nutritional components that a bacterium gets in its natural habitat. Most often, a culture medium contains water, a source of carbon & energy, source of nitrogen, trace elements and some growth factors. Besides these, optimum pH, oxygen tension and osmolarity too have to be taken into consideration.

Classification of culture media:
Classification based on consistency:

Liquid media (Broths): Are available for use in test-tubes, bottles or flasks. In liquid medium, bacteria grow uniformly producing general turbidity. Certain aerobic bacteria and those containing fimbriae (Vibrio & Bacillus) are known to grow as a thin film called 'surface pellicle' on the surface of undisturbed broth. *Bacillus anthracis* is known to produce stalactite growth on ghee containing broth. Sometimes the initial turbidity may be followed by clearing due to autolysis, which is seen in penumococci. Long chains of Streptococci when grown in liquid media tend to entangle and settle to the bottom forming granular deposits but with a clear medium. Culturing bacteria in liquid media has some drawbacks. Properties of bacteria are not visible in liquid media and presence of more than one type of bacteria can not be detected. Liquid media tend to be used when a large number of bacteria have to be grown.

Solid media: Any liquid medium can be rendered by the addition of certain solidifying agents. Agar agar (simply called agar) is the most commonly used solidifying agent. For preparing agar in Petri plates, 2% agar (by weight) is added to the broth and autoclaved, when the medium is at ~50^{o}C, it is poured on to sterile Petri plates and allowed to set. For preparing agar containing media in test-tubes, the culture medium is mixed with 2% agar and heated with stirring to melt. This ensures that all the tubes get equal amounts of agar. These tubes can then be sterilized by autoclaving.
Besides agar, egg yolk and serum too can be used to solidify culture media. While serum and egg yolk are normally liquid, they can be rendered solid by coagulation using heat. Serum containing medium such as Loeffler's serum slope and egg containing media such as Lowenstein Jensen medium and Dorset egg medium are solidified as well as disinfected by a process of inspissation.

Semi-solid media: Reducing the amount of agar to 0.2-0.5% renders a medium semi-solid. Such media are fairly soft and are useful in demonstrating bacterial motility and separating motile from non-motile strains (U-tube and Cragie's tube). Certain transport media such as Stuart's and Amies media are semi-solid in

consistency. Hugh & Leifson's oxidation fermentation test medium as well as mannitol motility medium are also semi-solid.

Biphasic media: Sometimes, a culture system comprises of both liquid and solid medium in the same bottle. This is known as biphasic medium (Castaneda system for blood culture). The inoculum is added to the liquid medium and when subcultures are to be made, the bottle is simply tilted to allow the liquid to flow over the solid medium. This obviates the need for frequent opening of the culture bottle to subculture.

Classification based on nutritional component:
Simple media: Simple media such as peptone water, nutrient agar can support most non-fastidious bacteria.

Complex media: Complex media such as blood agar have ingredients whose exact components are difficult to estimate.

Synthetic media: Synthetic or defined media such as Davis & Mingioli medium are specially prepared media for research purposes where the composition of every component is well known.

Classification based on functional use or application:

Basal media are basically simple media that supports most non-fastidious bacteria. Peptone water, nutrient broth and nutrient agar considered basal medium

Enriched media: Addition of extra nutrients in the form of blood, serum, egg yolk etc, to basal medium makes them **enriched media**. Enriched media are used to grow nutritionally exacting (fastidious) bacteria. Blood agar, chocolate agar, Loeffler's serum slope etc are few of the enriched media.

Blood agar is preparing by adding 5-10% (by volume) to a basal medium such as nutrient agar or other blood agar bases. Since blood can not be sterilized, it has to be collected aseptically from the animal. Animals have to be bled and the blood is collected in sterile containers with anticoagulant or glass beads.

Chocolate agar is also known as heated blood agar or lysed blood agar. The procedure is similar to that of blood agar preparation except that the blood is added while the molten blood agar base is still hot. This lyses the blood cells and releases their contents into the medium. This process turns the medium brown, hence the name. This medium is especially useful in growing Hemophilus and Neisseria.

Serum for medium can be obtained from animal blood but must be filtered through membrane or Seitz filter before use.

Selective and enrichment media: These are designed to inhibit unwanted commensal or contaminating bacteria and help to recover pathogen from a mixture of bacteria. While selective media are agar based, enrichment media are liquid in consistency. Both these media serve the same purpose. Any agar media can be made selective by addition of certain inhibitory agents that don't affect the pathogen. Various approaches to make a medium selective include addition of antibiotics, dyes, chemicals, alteration of pH or a combination of these. For example, Lowenstein Jensen Medium used to recover *M.tuberculosis* is made selective by incorporating Malachite green. Wilson & Blair's Agar for recovering *S.typhi* is rendered selective by the addition of dye Brilliant green. Selective media such as TCBS Agar and Monsur's Tellurite Taurocholate Gelatin Agar used for isolating *V. cholerae* from fecal specimens have elevated pH (8.5-5.6), which inhibits most other bacteria.

Enrichment media are liquid media that also serves to inhibit commensals in the clinical specimen. Selenite F broth, tetrathionate broth and alkaline peptone water are used to recover pathogens from fecal specimens.

Differential media: Certain media are designed in such a way that different bacteria can be recognized on the basis of their colony colour. Various approaches include incorporation of dyes, metabolic substrates etc, so that those bacteria that utilize them appear as differently coloured colonies. Such media are called **differential media or indicator media**. This approach is used in MacConkey's agar, CLED agar, TCBS agar, XLD agar etc. MacConkey's agar is the most commonly used media to culture and identify gram negative bacilli (especially enterobacteriaceae members). It contains bile salts (selective agent), lactose (sugar), peptone and neutral red (pH indicator), agar and water. Those bacteria that can ferment lactose produce pink coloured colonies where non-lactose fermenting colonies produce colourless colonies.

Transport media: Clinical specimens must be transported to the laboratory immediately after collection to prevent overgrowth of contaminating organisms or commensals. This can be achieved by using **transport media**. Such media prevent drying (desiccation) of specimen, maintain the pathogen to commensal ratio and inhibit overgrowth of unwanted bacteria. Some of these media (Stuart's & Amie's) are semi-solid in consistency. Addition of charcoal serves to neutralize inhibitory factors. Cary Blair medium and Venkatraman Ramakrishnan medium are used to transport feces from suspected cholera patients. Sach's buffered glycerol saline is used to transport feces from patients suspected to be suffering from bacillary dysentery. Pike's medium is used to transport streptococci from throat specimens.

Anaerobic culture media: Anaerobic bacteria need special media for growth because they need low oxygen content, reduced oxidation –reduction potential and extra nutrients. Media for anaerobes may have to be supplemented with nutrients like hemin and vitamin K. Such media may also have to be reduced by physical or chemical means. Boiling the medium serves to expel any dissolved oxygen. Addition of 1% glucose, 0.1% thioglycollate, 0.1% ascorbic acid, 0.05% cysteine or red hot iron filings can render a medium reduced. Robertson cooked meat that is commonly used to grow Clostridium spps medium contain a 2.5 cm column of bullock heart meat and 15 ml of nutrient broth. Before use the medium must be boiled in water bath to expel any dissolved oxygen and then sealed with sterile liquid paraffin. Thioglycollate broth contains sodium thioglycollate, glucose, cystine, yeast extract and casein hydrolysate. Methylene blue or resazurin is an oxidation-reduction potential indicator that is incorporated in the medium. Under reduced condition, methylene blue is colourless.

Demonstrations

The teacher should demonstrate the following things during the class:

a) Different types of culture media.
b) Specific growth patterns of different bacteria on culture media.

Students Exercise:

1. Name 2 enriched media.

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2. Which media are not auto-clavable.

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3. Give example of following media with the specific selective agent.

Selective agent	Name of selective agent	Media
Antibiotic		
Dye		
Salt		
Chemicals		
Ph		

4. Name two Transport media.

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5. What id the percentage of blood used in blood agar.

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6. What is the percentage of agar used for making solid and semisolid agar.

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Exercise Number 4:

Collection, storage and transport of specimens and laboratory diagnosis of infectious diseases

Learning objectives:
On completion of the exercise the students should be able to:

- **a)** Know the various specimens to be collected in different infections .
- **b)** Know the methods of collection and transport of various specimens safely to laboratory.
- **c)** Understand the laboratory diagnosis of infectious diseases.

Introduction:

COLLECTION OF MICROBIOLOGICAL SPECIMENS

The value and reliability of microbiological reports are directly affected by the quality of the specimen received by the laboratory and the length of time between its collection and processing.

- The amount and type of specimen required, container to use, and need for any preservative or transport medium.
- Best time to collect a specimen.
- Aseptic and safe methods of collection to avoid contamination and accidental infection.
- Labelling of the specimen container.
- Conditions in which specimens need to be kept prior to and during their transport to the laboratory.
- Arrangements for processing specimens that are urgent and those collected outside of normal working hours, e.g. blood cultures collected by medical staff.

Type of specimen
The correct type of specimen to collect will depend on the pathogens to be isolated, e.g. a cervical not a vaginal swab is required for the most successful isolation of *N. gonorrhoeae* from a woman. Sputum not saliva is essential for the isolation of respiratory pathogens.

Time of collection
Specimens such as urine and sputum are best collected soon after a patient wakes when organisms have had the opportunity to multiply over several hours. Blood for culture is usually best collected when a patient's temperature begins to rise. The time of collection for most other specimens will depend on the condition of the patient, and the times agreed between the medical, nursing, and laboratory staff for the delivery of specimens to the laboratory.
Important: Every effort must be made to collect specimens for microbiological investigation *before* antimicrobial treatment is started and to process specimens as soon after collection as possible.

Collection techniques

The following apply to the collection of most microbiological specimens:

- Use a collection technique that will ensure aspecimen contains only those organisms from the site where it was collected. A strictly sterile (aseptic) procedure is essential when collecting from sites that are normally sterile, e.g. the collection of blood, cerebrospinal fluid, or effusions.
- Avoid contaminating discharges or ulcer material with skin commensals. The swabs used to collect the specimens must be sterile and the absorbent cotton-wool from which the swabs are made must be free from antibacterial substances.
- Collect specimens in sterile, easy to open, leakproof, dry containers, free from all traces of disinfectant. Containers must be clean but need not be sterile for the collection of faeces and sputum.
- Patients should be instructed in the aseptic collection of specimens and asked to avoid contaminating the outside of containers.
- When contamination occurs, wipe the outside of the container with a tissue or cloth soaked in disinfectant before sending the specimen to the laboratory.
- Report any abnormal features, such as cloudiness in a specimen which should appear clear, abnormal coloration, or the presence of pus, blood, mucus, or parasites.
- The appearance of urine, pus, vaginal discharge, faeces, effusions, and cerebrospinal fluid should
- be described routinely.

Labelling specimens

- Each specimen must be clearly labelled with the date and time of collection, and the patient's name, number, ward or health centre.
- Each specimen must be accompanied by a correctly completed request form

Transport of microbiological specimens collected in a hospital

Specimens should reach the laboratory as soon as possible or a suitable preservative or transport medium must be used. Refrigeration at 4–10 °C can help to preserve cells and reduce the multiplication of commensals in unpreserved specimens. Specimens for the isolation of *Haemophilus*, *S. pneumoniae*, or *Neisseria* species, however, must never be refrigerated because cold kills these pathogens.

Preservatives and transport media for microbiological specimens

In general, specimens for microbiological investigations should be delivered to the laboratory without delay and processed as soon as possible. This will help to avoid the overgrowth of commensals.

When a delay in delivery is unavoidable, a suitable chemical preservative or transport culture medium must be used. This will help to prevent organisms from dying due to enzyme action, change of pH, or lack of essential nutrients.

A transport medium is needed to preserve anaerobes.

Amies transport medium is widely used and effective in ensuring the survival of pathogens in specimens collected on swabs, especially delicate organisms such as *Neisseria gonorrhoeae*. Amies medium is a modification of Stuart's transport medium .

Boric acid which may be added to urine.

Cary-Blair medium is used as a transport medium for faeces .

The laboratory investigation of microbial diseases involves:

Examining specimens to detect, isolate, and identify pathogens or their products using:

a) Microscopy
b) Culture techniques

c) Biochemical methods
d) Immunological (antigen) tests
e) Testing serum for antibodies produced in response to infection, i.e. serological response.

A . MICROSCOPY: Microorganisms are examined microscopically for their motility, morphology, and staining reactions in 10x ,40x and 100x .

B . CULTURE TECHNIQUES: The culture of pathogens enables colonies of pure growth to be isolated for identification and, when required, for antimicrobial susceptibility testing.

C . BIOCHEMICAL METHODS

Following culture, biochemical tests are often required to identify pathogens including the use of substrates and sugars to identify pathogens by their enzymatic and fermentation reactions.

Examples

- Catalase test to differentiate staphylococci which produce the enzyme catalase from streptococci which are noncatalase producing.
- Oxidase test to help identify *Vibrio*, *Neisseria*, *Pasteurella* and *Pseudomonas* species, all of which produce oxidase enzymes.
- Coagulase test to help identify *Staphylococcus aureus* which produces the enzyme coagulase (coagulates plasma).
- Sugar Fermentation tests to differentiate enterobacteria, e.g. use of glucose and lactose in Kligler iron agar medium to assist in the identification of *Shigella* and *Salmonella* like organisms.
- Indole test to detect those organisms that are able to break down tryptophan with the release of indole. It is mainly used to differentiate *Escherichia coli* from other
- Urease test to assist in the identification of organisms such as *Proteus* species which produce the enzyme urease.

D . IMMUNODIAGNOSTIC TESTS

Antigen tests often enable an early diagnosis or presumptive diagnosis of an infectious disease to be made. They involve the use of specific antibody (antisera or labelled antibody):

- To identify a pathogen that has been isolated by culture, e.g. identification of *Salmonella* serovars, *Shigella* species, and *Vibrio cholerae* by direct slide agglutination.

- To identify pathogens in specimens using direct immunofluorescence, e.g. identification of respiratory viruses, rabies virus, cytomegalovirus, *Pneumocystis jiroveci*, and *Chlamydia*.

- To identify antigens of microbial origin that can be found in serum or plasma, cerebrospinal fluid, urine, specimen extracts and washings, or fluid cultures. Highly specific monoclonal antibody reagents are often used by agglutination techniques (direct, latex, coagglutination), enzyme immunoassays (EIA), or more recently developed immunochromatographic (IC) tests and dipstick dot immunoassays. Because many of these antigen tests are rapid, simple to perform, and have good stability, they are becoming increasingly used at labs . Adequate controls must be used.

PRINCIPLES OF ANTIGEN TESTS

- Direct slide agglutination
- Latex agglutination
- Coagglutination (COAG)
- Direct immunofluorescence
- Enzyme immunoassays (EIA)* to detect antigen
- Dipstick comb immunoassays to detect antigen

Testing serum for antibodies (serological tests)

In laboratories, serological testing in which antigen is used to detect and measure antibody in a person's serum is used mainly:

- To help diagnose a microbial disease when the pathogen or microbial antigen is not present in routine specimens or if present is not easily isolated and identified by other available techniques, e.g. dengue, brucellosis, rickettsial infections, syphilis, leptospirosis.
- To test individuals and screen donor blood for antibody to HIV-1 and HIV-2.
- To measure antibody levels to determine the prevalence of infectious disease in a community and immune status of individuals.
- To screen for rises in anti-streptolysin O, e.g. in the investigation of rheumatic fever, acute glomerulonephritis, and other complications of Group A streptococcal infection.
- To screen pregnant women for infections such as syphilis and HIV infection.

Serological techniques used in laboratories

These techniques include agglutination tests, flocculation tests, enzyme immunoassays (membrane based EIA), immunochromatographic strip, cassette and card tests, and dipstick comb immunoassays.

PRINCIPLES OF ANTIBODY TESTS

Agglutination techniques

- Latex agglutination tests
- Indirect (passive) haemagglutination (IHA)
- Flocculation tests
- Enzyme immunoassays (EIA) to detect antibody
- Immunochromatographic (IC) strips, cassettes and cards to detect antibody
- Dipstick comb immunoassays to detect antibody

E . NUCLEIC ACID TESTS TO DIAGNOSE MICROBIAL INFECTIONS

Recent advances in nucleic acid probe technologies and gene amplification techniques (e.g. polymerase chain reaction, PCR), have resulted in the development of a new generation of rapid, highly sensitive and specific tests to identify pathogens in clinical specimens and cultures, often at an earlier stage than by other tests.

DEMONSTRATION:

The teacher should Demonstrate the following things during the class:

1) Containers for collection of various clinical specimens.
2) Stained smears under microscope.
3) Colony morphology on various culture media.
4) Biochemical tests of various microorganisms.
5) Immunodiagnostic tests.

Students Exercise:

1. Why transport media are used ? Name few transport media .

2. What important precautions should be taken in laboratory while collecting samples ?

3. Enumerate important tests done for microbial isolation and identifications .

Exercise Number 5: Virology

Introduction

The choice of materials and methods for laboratory confirmation of viral infection depends on the stage of illness. The following methods are commonly employed.

A. *Microscopy:* Cytopathic effect and inclusion bodies
B. *Culture and isolation:* Laboratory animals, chick embryo, - tissue culture, cell culture.
C. *Serology:* HI, CFT, EIA, Western blot test.
D. *Detection of viral proteins and genetic material:* DNA probes, PCR.

Specimen

Specimen should be collected early in the acute phase of the disease before the virus ceases to shed.

Collection and transport of specimens

Ideally all specimens for detection of virus should be processed by the laboratory immediately because many viruses are labile and the samples are also susceptible to bacterial and fungal overgrowth. Specimen should be placed in ice and transported in special media (Stuart's viral transport media) containing proteins.

Blood for serological test is transported to laboratory in sterile test tubes. Serum is separated as soon as possible. Blood for vial culture is to be transported in a sterile viral containing anticoagulant. Blood should be refrigerated at 4^0C until processing and can be stored for months at – 20^0C or below. Virus specific IgM should be tested before freezing since IgM may form insoluble aggregates upon thawing.

OBJECT: Study the various spots demonstrated to you which are used for diagnosis of various viral infections.

1. **Inclusion bodies**

   These are virus specific intracellular masses produced during replication of virus in host cells and can be seen under the light microscope. Inclusion bodies can be demonstrated by histopathological examination and are much larger than the virus particle usually 10-40 μm in size. These may be intra – nuclear or intra – cytoplasmic.

   Negri bodies: These are intra cytoplasmic eosinophilic inclusions present in nerve cells in brain tissue and are diagnostic or rabies virus infection. Microscopic examination of impression smear of brain tissue stained by Seller's stain which has the advantage that fixation and staining are done simultaneously. Negri bodies appear as round or oval, pink coloured structure 5–27 μm in diameter with basophilic granules within the cytoplasm of neurons

   Molluscum bodies: These inclusions are diagnostic of Molluscumcontagiosum virus infection. It is a pox group of virus. This disease is seen in children and is characterized by pink or pearly white wart like nodules on the skin. This virus cannot be grown in eggs, tissue culture or animals.

Inclusion bodies (Molluscum bodies) are large and 20-30 μm in size. On microscopic examination of stained smear inclusions are seen as eosinophilic mass which occupy whole of the cytoplasm and displaces the nucleus to the margin.

2. **Chorioallantoic membrane of chick embryo:**

For inoculation on chorioallantoic membrane 10-12 days old embryonatedhens eggs are taken, as at this age it is well developed. Viruses grown on it are

Variola Major

Variola Minor

Vaccinia

Cow pox:

Herpes simplex

Pocks or Vaccinia Virus:

Pocks ar4e seen as white circular plaques of epithelial hyperplasia which are visible to naked eye after 48 hours of incubation and these are much larger than the pocks of Variola major and are 4-5 mm in diameter after 72hrs. The pocks formed are not uniform in size and are yellow in colour due to central necrosis whereas the pocks formed by Variola major are uniform in size white in colour and are more commonly seen near the blood vessels.

3. **Growth of cell culture**

Continuous cell lines are most commonly used in virology laboratory for isolation of viruses. Continuous cell lines such as HeLa, Hep-2, KB, Vero, McCoy and BHK-21 are commonly used. These cells are grown in chemically define3d media which contain balanced salt solution containing essential amino acids, fatty acids, vitamins and carbohydrates. To this medium 10% foetal calf serum is added and medium is buffered with bicarbonate to give a pH 7.2-7.2 and phenol red is added as a pH indicator. Penicillin and streptomycin are added to prevent bacterial contamination.

Cytopathic effect

Picornaviruses: Many viruses produce morphological changes in the cultured cells in which they grow and these are called cytopathic effects. Picornaviruses lead to nuclear pyknosis, cells become refractile, their margins become crenated and there is degeneration of cells. There is complete or partial detachment of infected cells from glass.

4. **Viral Haemagglutination**

A large number of viruses agglutinate RBC from different species. These viruses have haemagglutinin spikes on the capsid or envelope which agglutinate RBCs. The viruses which agglutinate RBCs are:

S.No.	Name	RBCs
1.	Influenza Viruses	Fowl, human, guinea pig. Elution at 37^0C
2.	Parainfluenza& Mumps viruses	Fowl, human, guinea pig. Elution at 37^0C
3.	Rubella virus	One day old chick RBC, pigeon RBC at + 4^0C
4.	Adenoviruses	Monkey and rat

5.	Measles	Monkey at 37^0C
6.	Enteroviruses and ECHO viruses	Human RBC at + 4^0C and 37^0C
7.	Pox Viruses	Fowl RBC
8.	Rabies Virus	Goose RBC at + 4^0C
9.	Variola, Dengue, J. encephalitis, West Nile virus also agglutinate RBCs	

Viral haemagglutination of Influenza virus

The haemagglutinins are glycoprotein in nature. These are rod shaped spikes present on the envelope, have affinity for receptors (glycoprotein) present on RBCs. The viral envelope also carries, other spikes which are mushroom – shaped and are for the enzyme neuraminidase which are also called as receptor destroying enzyme (RDE). It is also produced by some bacteria like Vibrio cholera. RDE enzyme also acts on the receptors of haemgglutination present on RBC and destroy them, which leads to reversal of haemagglutination and release of virus called elution. The eluted virus can agglutinate fresh RBCs but the eluted RBC cannot agglutinate fresh viral suspension.

Test proper: The test is performed in micro or macro titre plates. This test is done mainly to titrate the viral suspension (viral antigens). Viral suspension is diluted by serial doubling dilution starting from 1:2; 1:4; 1:8, 1:16, 1:32……..1:512

Then to it is added equal volume of 1% RBC suspension. The plate is incubated for one hour. The highest dilution that produces haemagglutination gives the haemagglutination titre. Each test include control positive and RBC control (No Haemagglutination).

No agglutination - The red cells which are not agglutinated settle at the bottom in the form of a button

Haemagglutination -The agglutinated red cells are seen spread like a sheet.

Elution -It occurs only in the myxoviruses that possess neuraminidase. Haemagglutination is stable with other viruses.

Uses: Viral HA is used to –
Detect and assay the influenza viruses.
Titrate influenza virus vaccines.

5. Haemagglutination Inhibition Test (HI)

As haemagglutination is specifically inhibited by the antibody to the virus, haemagglutination inhibition is a convenient test for the detection of anti – viral antibodies. Here patient's serum is diluted by serial doubling dilutions and a fixed amount of viral antigen is added. Then to it RBCs are added. The last well showing no haemagglutination is the end titre.

6. **ELISA Test for HBsAg**(Refer to Exercise Number 6)
7. **ELISA Test for HIV1 and HIV2 (**Refer to Exercise Number 6)

Students Exercise:

1. How will you collect and transport samples for viral diagnosis.

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2. What are inclusion bodies? Name a few of them.

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3. Enumerate 5 cell culture medium used for viral cultivation.

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Exercise Number 6: Sterilization & methods of waste disposal.

Learning objectives:
On completion of the exercise the students should be able to**:**

- **a)** To understand the terms sterilization, disinfection and antisepsis.
- **b)** To know the physical and chemical methods of sterilization.
- **c)** To understand the modes of action of physical agents of sterilization and their applications.
- **d)** To know the concentration, contact time and uses of commonly used disinfectants.
- **e)** To understand the necessity and methods of proper management of Bio-medical waste.

Introduction:

Sterilization: The process by which an article, surface or medium is freed of all living micro-organisms either in the vegetative or spore state.

Disinfection: Destruction or removal of all pathogenic organisms.

Antisepsis: Prevention of infection, usually by inhibiting the growth of bacteria in wounds or tissues.

1. <u>Physical methods of sterilization:</u>

Sunlight: Important source of spontaneous sterilization under natural conditions. Action is due to ultra-violet rays and heat.

Drying: As moisture is essential for growth of bacteria, drying has a deleterious effect on many bacteria.

Dry heat: Heating is the most reliable source of sterilization. Killing effect of dry heat is due to protein denaturation, oxidative damage and the toxic effect of elevated levels of electrolytes.

a) **Flaming:** Inoculating loops or wires, tips of forceps etc. can be sterlised by holding in Bunsen flame till red hot.

b) **Incineration:** An excellent method for safely destroying materials such as contaminated cloth, pathological materials etc.

c) **Hot air oven:** A widely used method of sterlising glassware, swabs, dusting powder, oils etc. Holding period is one hour at 160^{O} C.

Moist heat: The lethal effect of moist heat is due to denaturation and coagulation of proteins. The advantage of steam lies in the latent heat liberated on condensation.

a) **Pasteurisation:** Involves moist heating below 100^{O} C. For pasteurization of milk milk is heated at 63^{O} C for 30 minutes (holder method) or at 72^{O} C for 15-20 seconds (flash method) followed by quickly cooling to 13^{O} C or lower.
Intermittent heating below 100^{O} C is called **inspissation**.

b) **Boiling:** Involves moist heating at 100□C. The material to be treated needs to be immersed in water and boiled for 10-30 minutes.
Intermittent heating at 100□C is called **tyndallisation**.

c) **Steam under pressure:** This principle is used in **autoclave**. Water boils when its vapour pressure equals the atmospheric pressure. Increasing the pressure also increases the boiling temperature. Sterilization by steam under pressure is carried out between 108□C and 147□C. (working temperature of a common laboratory autoclave is 121^0C at 15 lbs pressure for 15 minutes)

Filtration: Helps to remove bacteria from heat labile liquids. Commonly used filters are candle filters, asbestos filters, sintered glass filters and membrane filters.

Radiation: Non-ionizing and ionizing radiations can be used for sterilization. Infra red and ultraviolet rays are non-ionizing, gamma rays and high energy electrons are ionizing rays.

2. Chemicals

Alcohols: These act by denaturing bacterial proteins. Used mainly as skin antiseptics at concentration of 60-90%.

Aldehydes: These are bactericidal, sporicidal and virucidal. Formaldehyde is used for preserving specimens,sterlising instruments and fumigation. It is irritant and toxic. Glutaraldehyde is used to sterilize instruments such as bronchoscopes, cystoscoptes etc.

Dyes: Aniline and acridine dyes are used as skin antiseptics. Both are bacteriostatic at high dilutions.

Halogens: Iodine as an aqueous and alcoholic solution is used as a skin disinfectant. Chlorine is used as a disinfectant in water supplies and food industries.

Phenols: The lethal effect of phenols is due to cell lysis. Phenol (carbolic acid) is a powerful microbicidal substance.

Surface active agents: Act on cell membranes, causing them to lose semi-permeability and denaturing cell proteins.

Metallic salts: Salts of silver, copper and mercury are used as disinfectants. These are protein coagulants when used in appropriate concentrations.

Gases: Ethylene oxide, formaldehyde and beta-propiolactone are commonly used gaseous disinfectants. All are bacteriocidal, sporiidal and virucidal. Ethylene oxide is used for sterlising equipments and instruments; formaldehyde is used for fumigation; and beta-propiolactone is used for sterilizing biological products.

Bio-Medical Waste (BMW): Any solid and/or liquid waste including its container and any intermediate product, which is generated during the diagnosis, treatment and / or immunization of human beings or animals.

For category wise list of all biomedical wastes and their disposal methods refer Annexure I.

Demonstrations: The teacher should Demonstrate the following things during the class:

a) Demonstrate the working of autoclave and the use of controls.

b) Familiarize the students with working of hot air oven, inspissator, filters etc.

c) Familiarize the students with the working of common disinfectants used in the hospital.

d) Familiarize the students with segregation of bio-medical waste and its disposal.

Students Exercise:

1. Enumerate different methods of sterilization by dry heat. Give their applications.

2. Enumerate different methods of sterilization by moist heat. Give their applications.

3. Draw a labeled diagram illustrating working of an autoclave.

4. Mention the working concentration, contact time and uses of following disinfectants:

    a. Sodium hypochlorite.....
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    b. Lysol.....
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    c. Glutaraldehyde.....
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5. What do you understand by segregation of biomedical waste? What are different color-coded bags?

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6. How is the waste segregated in color-coded bags treated and disposed?

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Exercise number 7: Antibiotic Sensitivity Tests

Objective: Observe various methods for determining antibiotic sensitivity.

Introduction

Antibiotic susceptibility testing has become a very essential step for the proper treatment of infectious diseases. It is used to guide the clinicians in selecting the best antimicrobial agent and, to accumulate epidemiological information on the resistance of microorganisms of public health important. Antimicrobial susceptibility test measures the ability of an antibiotic or other antimicrobial agent to inhibit bacterial growth in vitr4o. This ability may be estimated by either the dilution method or the diffusion method.

A. DISC DIFFUSION METHOD

Here culture plate is taken and a lawn culture of organism is done and 6mm filter paper discs impregnated with antibiotics are put on the culture plate. Antibiotic diffuses out around the disc and zone of growth inhibition around the discs is seen. There are two methods for disc diffusion.

1. Stoke's disc diffusion test.
2. Kirby – Bauer dis diffusion test.

Stokes Method: (Same plate comparative disc diffusion test)

Here nutrient agar or Mueller Hinton agar plate is taken. The control strain and the test strains are inoculated on the same plate on each side of the same disc, thus controlling the effect of every single disc used. The culture plate is divided into three parts (Fig. 12.1) in the central part test strain is inoculated. The standard strain is inoculated in upper and lower part. Control strain is sensitive to all the antibiotics (Plate 5; Fig. 1). Three controls strains are used.

Control Strains

1. Escherichia coli ATCC 25922 - For coliform organisms
2. Staphylococcus aureus ATCC 25923 - For Staphylococcus and Enterococcus
3. Pseudomonas aeruginosa ATCC 27853 - Against pseudomonas

Results

Here radial with of the zone, outside the disc is measured i.e. the inhibition zones are measured from the edge of the disc to the edge of the zone and the following interpretation is made. The test organism is-

Susceptible: Zone size equal to, wider than or not more than 3mm smaller than the control.
Intermediate: Zone size greater than 3mm but smaller than the control by more than 3mm.
Resistant: Zone size 3mm or less.

Kirby – Bauer Disc Diffusion Method

Here Muller – Hinton agar plates are used. The inoculum is prepared by touching 3-5 colonies and inoculated into peptone water which is incubated at 35^0C for 4-6 hours. The Mueller Hinton plate is inoculated with the help of sterile cotton swab dipped into the inoculum. Allow the plate to dry

before antibiotic discs are applied. On a plate of 9cm diameter seven discs are usually applied (Fig. 12.2). The plates are then incubated at 35^0C for 16-18 hours. The zone of inhibition of growth around each disc is measured (diameter including the disc) and compared with the standard chart for interpretation as sensitive, inter – mediate and resistant (Plate 5; Fig. 2).

Standard strain's used

Escherichia coli (ATCC 25922) - For coliform organism

Pseudomonas aeruginosa (ATCC 27853) - For Pseudomonas

Staphylococcus aureus (ATCC 25923) - For Staphylococcus and Enterococcus.

Results: The results are given as resistant, intermediate and susceptible to the antibiotic by measuring the zone of inhibition diameter and comparing the zone size with standard zone size chart

Difficulties in Reading of results

1. Proteus vulgaris and Proteus mirabilis may swarm into the area of inhibition around some antibiotics but the zone of inhibition is usually clearly seen and the **thin layer of swarming growth should be ignored.**
2. When penicillinase producing strains of staphylococcus are tested against penicillin or other Beta Lactam drugs they sometimes fail to form enough penicillinase enzyme. There is zone of inhibition around the disc with a heaped up margin. The colonies at the edge are large and there is not gradual fading of growth as seen in control strain **Regardless of size of the zone of inhibition they should be regarded as resistant.**
3. Polymyxin diffuses poorly in agar and the zones are small.
4. Methicillin resistant Staphylococcus aureus (MRSA) may appear fully sensitive and 5% NaCl is added to overcome this problem or plates are incubated at 30^0C.

3. Epsilometer or E-Test:

It is a recently introduced test and is a modification of the agar diffusion sensitivity test. Here a strip absorbed with a known gradient of antibiotic concentration is placed on the plate inoculated with the test organism. The antibiotic diffuses into the medium and zone of inhibition appears around the strip which is elliptical shaped. The minimum inhibitory concentration (MIC) is recorded as the lowest concentration of the gradient which inhibits the growth of the organism (Plate 5; Fig. 3)

B. DILUTION METHODS

These are used for determination of Minimum inhibitory concentration (MIC).

1. **Broth dilution Method:** This is done in a liquid medium which is inoculated with the pathogen (Fig. 12.4). Doubling dilutions of the antibiotic are added to a series of the test tubes containing inoculated liquid media. After incubation at 37^0C growth in each tube is seen. The concentration of the antibiotic in the last tube that shows no visible growth (turbidity) is taken as MIC. Subculture from this tube and the tubes with subsequent lower dilution (showing no turbidity) is done on nutrient agar to

determine the minimum bactericidal concentration (MBC). ***The lowest concentration of drug that fails to produce growth on subculture is the MBC of the drug for that particular strain of the organism. MIC inhibits the bacterial growth but MBC kills the bacteria.***

2. **Agar dilution Method:** Serial dilutions of the drug are prepared and added to the agar in the plates. This is a more convenient method as large number of strains can be tested on each plate containing particular concentration of the antibiotic dilution. Each plate is divided into small squares and strains to be tested are inoculated into each square. The MIC is the lowest concentration of drug in the plate at which growth appears on the plate.

Uses of MIC determination

1. To regulate the therapeutic dose of treatment as in bacterial endocarditis.
2. To test the antimicrobial sensitivity of slow growing bacteria such a Mycobacterium tuberculosis.
3. When some bacteria show small degree of resistance and there is increase in the MIC value, for example certain strains of Salmonella typhi is showing some degree of resistance to ciprofloxacin and here higher dosage of drug for prolonged duration may be required for treatment of enteric fever.

Students Exercise:

1. Diameter of filter paper discs impregnated with antibiotics is .....
2. The two methods for disc diffusion technique for AST are.....

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3. How E test differs from conventional disc diffusion test?

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4. Why Dilution method is considered superior than disc diffusion method for AST?

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Exercise Number 8:
Serological tests for diagnosis of microbial infections.

Learning objectives:

On completion of the exercise the students should be able to:

a) Enumerate the different serological reaction.

b) Identify the common serological test done for diagnosis of microbial infection.

c) Interpret the results of the common serological test.

Introduction:

Serology and Serological reactions:

Serology is the study of plasma serum and other bodily fluids. Antibodies are serum proteins(gamma-globulins) that are typically formed in response to an infection (against a given microorganism), against other foreign proteins, or to one's own proteins (in instances of autoimmune disease). In practice, the term **serological reactions** usually refers in vitro antigen-antibody reactions, used for diagnostic identification and quantitation of antibodies or antigens in the serum.

Table no 6.1: Types Serological reactions

	Serological reactions	Examples	Usages
a)	Precipitation reaction	VDRL/RPR	Nonspecific identification of Syphilis.
b)	Agglutination reaction	WIDAL CRP ASO RA	Diagnosis of typhoid
c)	Complement fixation test	HCFT(Histoplasma Complement fixation test)	Diagnosis of Histoplasmosis
d)	Neutralization test	Dengue-PRNT (plaque reduction neutralization test)	Diagnosis of Dengue by Identification and quantification of circulating levels of anti-dengue virus neutralizing antibody
e)	Immunofluorescence	Direct Immunofluorescence Indirect Immunofluorescence	Identification of many bacterial and viral infections like *Coxiella burnetii*, Rickettsia or Colorado tick virus etc.
f)	Enzymeimmunoassay(EIA)	ELISA (Enzyme Linked Immunosorbent Assay)	Diagnosis of Diseases like HIV, HBsAg and many more
g)	Chemiluminescence Immunoassay (CLIA)	HCV-CLIA	Diagnosis of HCV
h)	Immunoelectroblots	Western blot	Confirmatory test for HIV infection and other diseases
i)	Immunochromatographic test	Tridot ICT Malaria	Diagnosis of HIV Diagnosis of Malaria

Important serological tests:

a) Rapid Plasma Reagin (RPR) Test:

Principle:

RPR is a non specific card test for the serologic detection of syphilis. In this method, cardiolipin antigen combines nonspecific antibodies(reagin) serum from syphilitic persons, and occasionally in serums of persons with other acute or chronic conditions. RPR is a Flocculation reaction.

Flocculation /precipitation reaction: in this type of reaction a soluble antigen combines with its corresponding antibody at appropriate temperature and pH. The byproduct in precipitation reaction is chalky precipitate that settles down at the bottom of reaction mixture. In Flocculation reaction the precipitate is cottony and fluffy and floats on the surface.

The antigenic suspension used in this reaction contains cardiolipin antigen, choline chloride (to eliminate the need of heating the serum to deactivate complement), EDTA (to enhance the stability of the suspension), and finely divided charcoal particles as a visualizing agent.

The RPR test measures IgM and IgG antibodies to lipoidal material released from damaged host cells as well as to lipoprotein-like material, and possibly cardiolipin released from the treponemes. The antilipoidal antibodies are antibodies that are produced not only as a consequence of syphilis and other treponemal diseases, but also in response to nontreponemal diseases of an acute and chronic nature in which tissue damage occurs. If antibodies are present, they combine with the lipid particles of the antigen, causing them to flocculate. The charcoal particles coagglutinate with the antibodies and show up as black clumps against the white card.

The term "reagin" means that this test does not look for antibodies against the actual bacterium, but rather for antibodies against substances released by cells when they are damaged by *T. pallidum*.

Material Required:

1) RPR antigen suspension.
2) Positive control.
3) Negative control.
4) Plastic-coated RPR cards.
5) Micro pipette- adjusted at 50µl.
6) Plastic applicator stick.
7) Mechanical rotator.
8) Disposable gloves, discard jar.

Procedure:

1) Place 50 µl of serum or plasma onto a circle of the RPR test card, using micro pipette. Add positive and negative control to the adjacent circles.
2) Add 1 free-falling drop of antigen suspension to each of the circles containing serum or plasma, positive and negative control.
3) Place the card on the mechanical rotator. Rotate the card for 8 minutes at 100 ∀ 2 rpm.
4) Remove the card from the rotator; briefly rotate and tilt the card by hand
5) (three or four to-and-fro motions) to aid in differentiating nonreactive from minimally reactive results.
6) Read the test reactions under a high-intensity incandescent lamp. Read the test without magnification.

Report the results :

Reading	Report
Characteristic clumping ranging from marked and intense to slight but definite (minimally to moderately)	reactive Reactive (R)
Slight roughness or no clumping	Nonreactive (N)

Note: Only two reports with the RPR card test are possible: reactive, no matter how much clumping, or nonreactive.

Sources of error:

1) If the temperatures of the sera, reagents, or testing area are less than 23°C, test reactivity decreases; if temperatures are greater than 29°C (85°F), test reactivity increases.
2) If the speed of the mechanical rotator is too fast or too slow, improper antigen-antibody interaction will cause unpredictable test results.
3) If the time of rotation is too long test reactivity may be increased, or if too short test reactivity may be decreased.
4) If the antigen is outdated or not adequately tested for standard reactivity, the results may be inaccurate.
5) If the serum is unevenly spread in the circle, the antigen and antibody may not mix properly.
6) If hemolyzed, contaminated, or improperly collected serum or plasma specimens are tested, the reaction may be masked.
7) Biological false-positive (BFP) reactionsBiological false-positive (BFP) reactions occur occasionally with cardiolipin antigens.

Biological false-positive reactions is defined as a positive test result with cardiolopin antigen but negative result with a specific treponemal test in absence of past or present treponemal infection and not due to any technical fault.

Acute BFP lasts for few weeks or months and is associated with acute infection, injuries or inflammatory conditions.
Chronic BFP presists longer than 6 months. It is seen in SLE and other collagen diseases. Leprosy, malaria, relapsing fever, infectious mononucleosis, hepatitis and tropical eosinophilia are examples where BFP is seen.

VDRL(Venereal Disease Research Laboratory) is the original nonspecific test for Syphilis. RPR is a modification of VDRL test. VDRL antigen suspension lacked choline chloride and hence the serum needed to be heated to destroy complement which can interfare in the reaction. Other modification includes addition on EDTA and charcoal to the antigenic suspension.

b) WIDAL Test

Principle:

It is a serological test for the diagnosis of typhoid and paratyphoid fever. It is a type of agglutination reaction.A Patients suffering from enteric fever would possess antibodies in their sera which can react and agglutinate dilutions of killed, coloured Salmonella antigens.

Agglutination reaction: in this type of reaction an insoluble antigen combines with its corresponding antibody at appropriate temperature and pH to form visible clumps that settles down at the bottom of reaction mixture.

Four antigen suspensions are used. S.typhi O antigen (O), S.typhi H antigen (H), S. paratyphi A antigen (AH) and S. paratyphi B antigen (BH).O antigens for S.paratyphi A and S.paratyphi B are not taken as they cross-react with S.typhi O antigen. WIDAL can be performed by either Slide method or Tube method

Slide Test

Material Required:

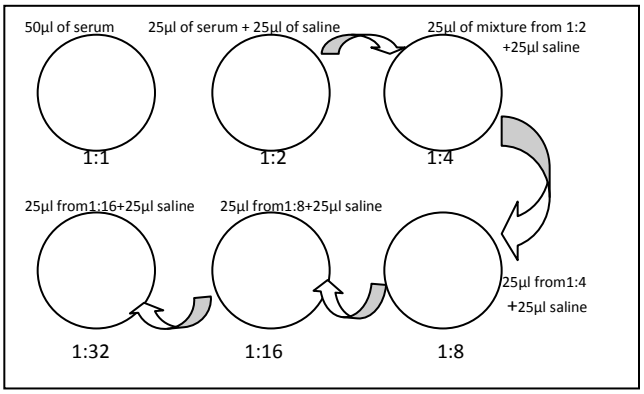
a) Antigen suspensions-O, H, AH, BH.
b) Polyspecific positive cotrol
c) Glass Slides with 6 reaction circles.
d) Mixing sticks.

Procedure:

1) Place one drop of positive control on one reaction circles of the slide.
2) Pipette one drop of Isotonic saline on the next reaction circle. (negative Control)
3) Pipette one drop of the patient serum to be tested onto the remaining four reaction circles.
4) Add one drop of Widal TEST antigen suspension 'H' to the first two reaction circles. (PC & NC)
5) Add one drop each of 'O', 'H', 'AH' and 'BH' antigens to the remaining four reaction circles.
6) Mix contents of each circle uniformly over the entire circle with separate mixing sticks.
7) Rock the slide, gently back and forth and observe for agglutination macroscopically within one minute.

Semi-Quantitative method:

1) Mark each of the circle on the test slide as 1:1, 1:2, 1:4, 1:8, 1:16, 1:32
2) Put 50µl of patient serum to the first circle (1:1)
3) Put 25µl of normal saline on the rest of the circles.
4) Put 25µl of patient serum on the second circle(1:2) and mix well with the pipette.
5) Transfer 25µl from the current circle to the next. Repeat the process till the last circle is reached.



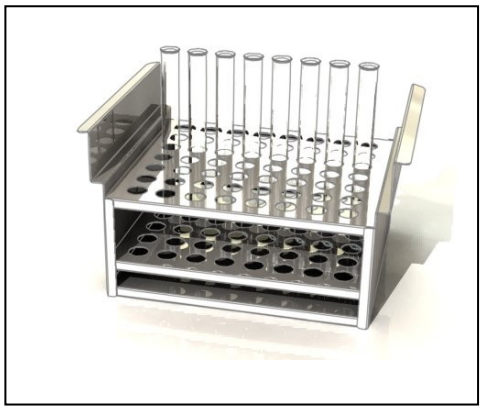
6) Take 25µl from the last circle and discard.
7) Add to each reaction circle, a drop of the antigen which showed an agglutination with the test sample in the screening method.
8) Rotate slide back and forth for 2 minutes and read result under an indirect oblique light source.

Interpretation of result:

Agglutination indicates a antibody titre of ≥1: 20 in the specimen. The lack of agglutination indicates a antibody titre of ≤ 1:20 in the sample.

DILUTION	ANTIBODY TITRE
No agglutination	≤ 1:20
1:1	≥1: 20
1:2	≥1: 40
1:4	≥1: 80
1:8	≥1: 160 and so on...

Standard tube test method



1) Take 4 sets of 8 test tubes and label them 1 to 8 for O, H, AH and BH antibody detection. Use 1 set of round bottom (Felix) tub for O, and conical bottom (Dreyer's) tube for H antigens.
2) Pipette into the tube No.1 of all sets 1.9 ml of isotonic saline.
3) To each of the remaining tubes (2 to 8) add 1.0 ml of isotonic saline.
4) To the tube No.1 tube in each row add 0.1 ml of the serum sample to be tested and mix well.
5) Transfer 1.0 ml of the diluted serum from tube no.1 to tube no.2 and mix well.
6) Transfer 1.0 ml of the diluted sample from tube no.2 to tube no.3 and mix well. Continue this serial dilution till tube no.7 in each set.
7) Discard 1.0 ml of the diluted serum from tube No.7 of each set.
8) Tube No.8 in all the sets, serves as a saline control.
   Now the dilution of the serum sample achieved in each set is as follows:

Tube No	1	2	3	4	5	6	7	8
Dilutions	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	control

9) To all the tubes (1 to 8) of each set add one drop of the respective WIDAL TEST antigen suspension (O, H, AH and BH) from the reagent vials and mix well
10) Cover the tubes and incubate at 37^0C overnight (approximately 18 hours) preferably in a water bath
11) observe for agglutination.

Interpretation of result:

Agglutination indicates a antibody titre of ≥1: 20 in the specimen. The lack of agglutination indicates a antibody titre of ≤ 1:20 in the sample.

TUBE	ANTIBODY TITRE
No agglutination	≤ 1:20
Only in 1	≥1: 20
Upto tube 2	≥1: 40
Upto tube 3	≥1: 80
Upto tube 4	≥1: 160
Upto tube 5	≥1: 320
Upto tube 6	≥1: 640
Upto tube 7	≥1: 1280

c) CRP(C Reactive Protien) Test.

Principle:

CRP is a acute phase protein (Abnormal β-globulin) secreted by hepatocytes. CRP level increases in the sera of patients in the acute stages of a number of inflammatory conditions such as most bacterial and some viral infections; acute rheumatoid fever with or without carditis; rheumatoid arthritis and most other collagen diseases; and in a number of other conditions characterised by inflammation. CRP is considered to be a sensitive indicator of inflammation. Changes in the serum level of CRP with time from the same patient can be used as an index of recovery. The use of the CRP test to measure the effectiveness of therapy is of great clinical significance.

The Rapid CRP Test is based on the latex-agglutination method . it's an immunological reaction between CRP as an antigen and the corresponding antibody coated on the surface of biologically inert latex particles.

Material required:

1) CRP Latex Reagent
2) CRP Positive Control:
3) CRP Negative Control
4) Glass test slide(with 6 circles).
5) Serological pipettes.
6) Test tubes 12x75 mm.
7) Distilled water
8) physiological saline 0.9%

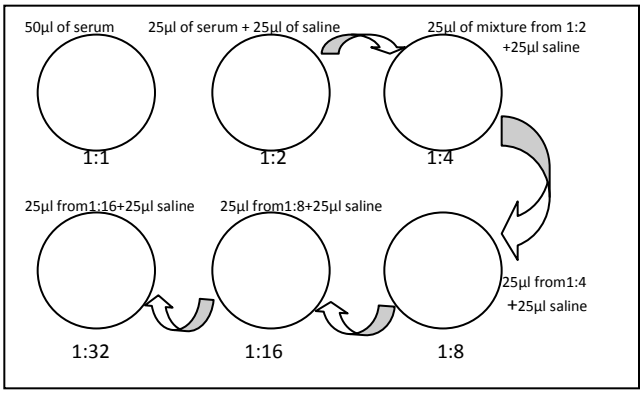
Procedure:

Method I (Qualitative):

1) Bring all reagents, controls and serum samples to room temperature.
2) Put one drop of CRP latex reagent to the test circle on the glass test slide. Using the disposable pipettes, add one drop (50µl) of the undiluted patient serum onto the same circle and mix both together with the end of the pipette.
3) Positive and negative controls should be run simultaneously in the same way as in Step 2.
4) Rotate slide back and forth for 2 minutes and read result under an indirect oblique light source.

Method II (Semi-Quantitative method):

1) Mark each of the circle on the test slide as 1:1, 1:2, 1:4, 1:8, 1:16, 1:32
2) Put 50µl of patient serum to the first circle (1:1)
3) Put 25µl of normal saline on the rest of the circles.
4) Put 25µl of patient serum on the second circle(1:2) and mix well with the pipette.
5) Transfer 25µl of the mixture from second circle to the third circle(1:4) and mix well.
6) Again transfer 25µl from the current circle to the next. Repeat the process till the last circle is reached.
7) Take 25µl from the last circle and discard.
8) Add one drop of CRP latex reagent to all the test circles and mix well.
9) Rotate slide back and forth for 2 minutes and read result under an indirect oblique light source.



Interpretation:

Positive reaction is indicated by agglutination. Since negative results may be caused by antigen excess, the test should be repeated using a diluted serum sample in case prozone effect is suspected. For the SemiQuantitative Method, multiplication of the dilution factor with 6 mg/L will yield the approximate level of CRP in the serum sample.

DILUTION	CONCENTRATION
1:1	6 mg/L
1:2	12 mg/L
1:4	24 mg/L
1:8	48 mg/L
1:16	64 mg/L
1:32	128 mg/L

Precautions

1) The strength of the agglutination reaction is not indicative of the CRP concentration. Weak reactions may occur with slightly elevated or markedly elevated concentrations.
2) A prozone phenomena (antigen excess) may cause false negative results.
3) Reaction times longer than specified may produce apparent false reactions due to a drying effect.
4) Strongly lipemic or contaminated sera can cause false positive reactions.
5) Only serum should be used in this test.

d) ASO(Anti-Streptolysin O) Test.

Principle:

The group A beta-hemolytic streptococci produces various toxins that can act as antigens. One of these exotoxins is streptoysin-'O', an oxygen labile hemolysin. Antistreptolysin-O test detect serum antibodies to streptolysin 'O'. The detection of antistreptolysin-O may be the single best test for documenting antecedent streptococcal infections. Over 80% of patients with acute rheumatic fever and 95% of patients with acute glomerulonephritis have elevated titers of ASO.

ASO Test method is a rapid latex agglutination test for the qualitative and semiquantitative determination of antistreptolysin-O in serum. In the presence of ASO in the serum, the latex suspension agglutinates due to the antigen-antibody reaction.

Material required:

1) ASO latex reagent
2) ASO Positive control
3) ASO Negative control
4) Stirrers
5) Glass slide
6) Physiological saline 0.9%

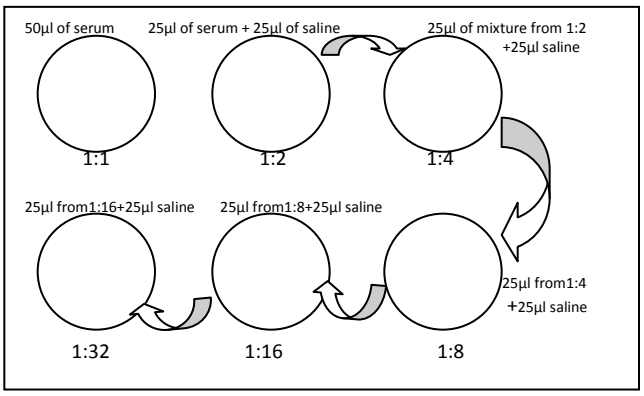
Procedure:

Method I (Qualitative):

1) Bring all reagents and samples to Room Temperature prior to testing.
2) Label circles on the slide provided with appropriate sample identification.
3) Dispense one drop (50µl) of sample, positive and negative control to the appropriately identified circle on the test slide.
4) Put one free falling drop into each circle, being used on the test slide.
5) Using separate stirrers mix the contents of each circle completely together over the entire surface area of the circle.
6) Rock the card by a to and fro motion for up to 2 minutes.
7) Observe the for any sign of agglutination.

Method II (Semi-Quantitative method):

9) Mark each of the circle on the test slide as 1:1, 1:2, 1:4, 1:8, 1:16, 1:32
10) Put 50µl of patient serum to the first circle (1:1)
11) Put 25µl of normal saline on the rest of the circles.
12) Put 25µl of patient serum on the second circle(1:2) and mix well with the pipette.
13) Transfer 25µl from the current circle to the next. Repeat the process till the last circle is reached.
14) Take 25µl from the last circle and discard.
15) Add one drop of ASO latex reagent to all the test circles and mix well.



16) Rotate slide back and forth for 2 minutes and read result under an indirect oblique light source.

Interpretation of result:

Agglutination indicates an ASO content of more than 200 lU/ml in the specimen. The lack of agglutination indicates an ASO level lower than 200 lU/ml in the sample.

DILUTION	CONCENTRATION
1:1	200 lU/ml
1:2	400 lU/ml
1:4	800 lU/ml
1:8	1600 lU/ml and so on...

Precautions:

1) It is advisable to compare the results of 2 separate samples taken in 2 week intervals.
2) The early use of pencillin, as well as other antibiotics, will prevent the ASO titre from rising.
3) Compare the agglutination with that of positive and negative control for a correct interpretation.
4) A prozone phenomena (antigen excess) may cause false negatives.
5) Reaction times longer than specified may produce apparent false reactions due to a drying effect.
6) The average ASO titre for a healthy individual is less than 200 IU/ml. Titres above the upper limits may be indicative of a streptococcal infection, but only a two dilution rise in titer between acute and convalescent stage specimens should be considered significant.
7) Following acute streptococcal infection, the ASO titer will usually rise after one week, increasing to a maximum level within 3 to 5 weeks and usually returning to the pre-infection levels in approximately 6 to 12 months.

e) RF(rheumatoid factor) Test.

Principle:

Serum from patients with rheumatoid arthritis usually contains auto-antibodies to the Fc portion of human IgG. these autoantibodies are termed "rheumatoid factors" because of their association with rheumatoid arthritis. Rheumatoid factors belong predominantly to the IgM class of immunoglobulins. However, rheumatoid factors have been associated with each human IgG subclass and with IgA and IgE. Elevated levels of rheumatoid factor(s) are not present in other joint diseases such as osteoarthritis, ankylosing spondylitis, gout, rheumatic fever, suppurative arthritis, psoriatic arthritis, and Reiter's syndrome. Because of this high degree of specificity, the detection of rheumatoid factor(s) is particularly useful as an indicator of rheumatoid arthritis. Circulating RF levels have been shown to parallel clinical condition. Rheumatoid arthritis patients with active disease tend to have higher levels of RF than those whose disease is under control. Thus, RF tests can aid the physician in the detection, diagnosis, prognosis, and therapeutic monitoring of rheumatoid arthritis.

Material required:

1) RF latex reagent
2) RF Positive control
3) RF Negative control
4) Stirrers
5) Glass slide
6) Physiological saline 0.9%

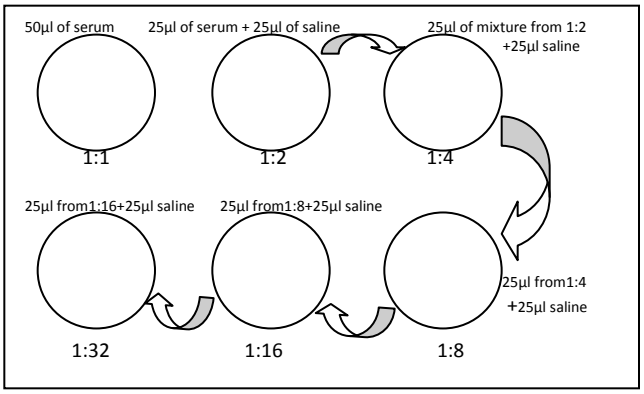
Procedure:

Method I (Qualitative):

1) Bring reagents and specimens to room temperature before use.
2) Place one drop (50 µl) of the RF Positive Control, RF negative Control and test serum on the separate circles on the reaction slide.
3) Add one drop of RF latex Reagent to each test field. Use Stir Stick to spread reaction mixture over entire test field.
4) Rotate slide for 3 minutes and read immediately under direct light.

Method II (Semi-Quantitative method):

1) Mark each of the circle on the test slide as 1:1, 1:2, 1:4, 1:8, 1:16, 1:32
2) Put 50µl of patient serum to the first circle (1:1)
3) Put 25µl of normal saline on the rest of the circles.
4) Put 25µl of patient serum on the second circle(1:2) and mix well with the pipette.
5) Transfer 25µl from the current circle to the next. Repeat the process till the last circle is reached.
6) Take 25µl from the last circle and discard.
7) Add one drop of ASO latex reagent to all the test circles and mix well.



8) Rotate slide back and forth for 2 minutes and read result under an indirect oblique light source.

Interpretation of result:

Agglutination (positive reaction) indicates the level of rheumatoid factor(s) in the undiluted test sample is approximately 15 IU/ml or greater in the specimen. the lack of agglutination (negative reaction) indicates the level of rheumatoid factor(s) is within normal range

DILUTION	CONCENTRATION
1:1	15 lU/ml
1:2	30 lU/ml
1:4	60 lU/m
1:8	120 lU/ml and so on...

Precautions:

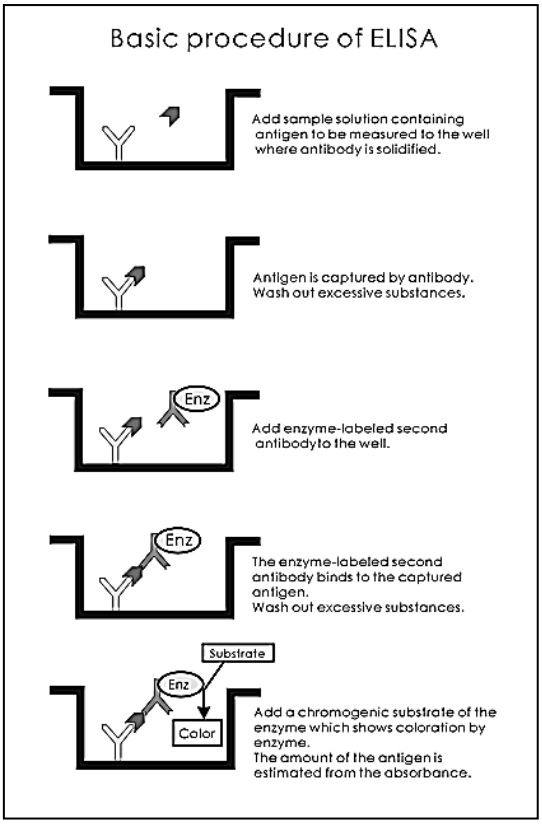
1) Although the RF latex Reagent is sensitive and specific, a diagnosis of rheumatoid arthritis should not be made on the basis of a positive test result without the support of patient history and other clinical evidence.
2) A negative test result cannot completely rule out rheumatoid arthritis.
3) Incubation of the test for longer than the recommended time may cause false positive reactions.
4) Healthy persons may have rheumatoid factor(s).These individuals usually have low titers.
5) The incidence of positive reactions in increases with age and is similar in females and males.

f) ELISA (Enzyme Linked Immunosorbent Assay)

The purpose of an ELISA is to determine the presence and quantity of a particular protein a sample. There are two main variations on this method: to determine how much antibody is in a sample, or to determine how much antigen is in sample.

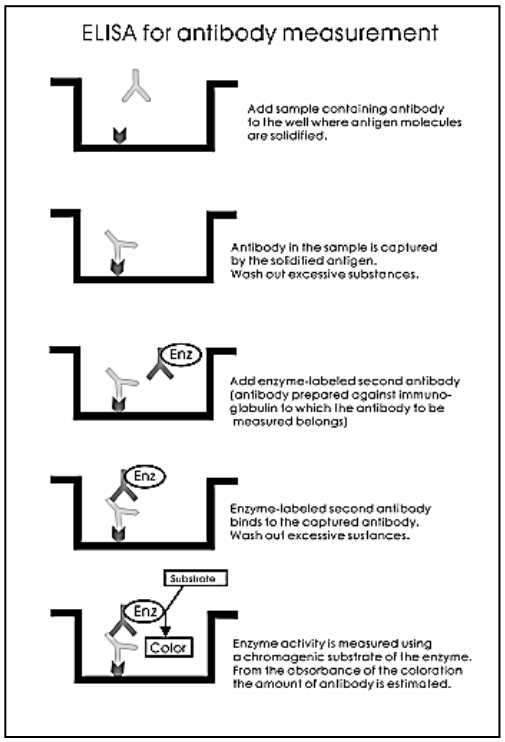
Principle for antigen detection(**Direct ELISA**):

1) samples are added to the antibody-coated wells, and incubated for 1-1.5 hours so as to the antigen molecules are captured by "capture antibody".
2) wells are thenwashed to remove excessive unbound materials.
3) The second antibody which recognizes another epitope in antigen is added. This second antibody has been labeled with an enzyme such as horseradish peroxidase (HRP).
4) The enzyme-labeled second antibody will bind to the antigen which is bound to the capture antibody on the bottom area of wells.
5) Enzyme activity is measured by adding a chromogenic substrate of this enzyme. In the case of HRP, tetramethylbenzidine (TMB) is often used.
6) After incubation for some period, the chromogenic substrate is changed to a colored product.
7) The reaction is stopped by adding a reaction stopper, e.g. diluted sulfuric acid, and absorbance is measured using a plate reader.



Principle for Antibody detection(**Indirect ELISA**)

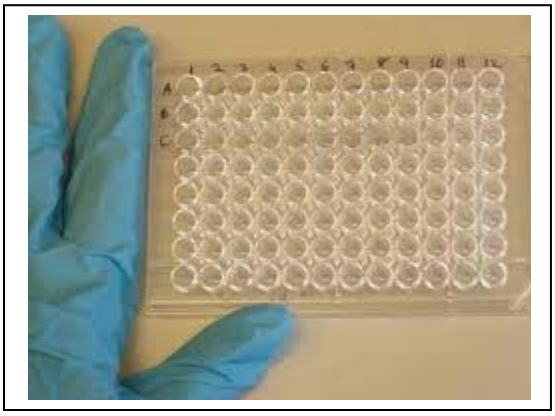
1) In this system, antigen molecule, specific to the antibody to be measured, is adsorbed on the bottom of wells, and samples containing antibody are added to the wells.
2) For estimation of the captured antibody, the second antibody directed against Fc fragment of human immunoglobulin, labeled with enzyme is added and washed out after the binding reaction.
3) The rest of the process is similar to antigen detection method.



Detection of Hepatitis B surface Antigen

Material required:

1) Antibody coated micro-titre well plate
2) Positive control
3) Negative control
4) Test sample
5) Specimen diluent
6) Conjugate solution
7) Substrate solution
8) Stopping Solution
9) Washing buffer
10) Distilled water
11) ELISA absorbance reader
12) Incubator
13) Micro pipette



Procedure:

1) Add 75 µL of positive control, negative control and sample in micro titre wells and then add 25 µL of Specimen diluent to each well
2) Incubate the plate for 1 hour at 37° C
3) Wash the wells 5 times using washing buffer. Pat the wells dry.
4) Add 100 µL of Working Conjugate Solution.
5) Incubate the plate for 1 hour at 37° C
6) Wash the wells 5 times using washing buffer. Pat the wells dry.
7) Add 100 µL of Working substrate Solution.
8) Incubate for 30 minutes in the dark at room temperature.
9) Add 100 µL of Stopping Solution.
10) Put the plate in ELISA reader for absorbance reading.

Precautions:

1) To minimize the risk of spread of infection, all blood and human materials used in ELISA should
2) be treated as potentially infectious.
3) Standard precautions include hand hygiene, wearing of gloves and use of other appropriate protective clothing and equipment as well as safe disposal of waste.
4) Some reagents may be harmful by inhalation, if swallowed, or in contact with eyes and skin.
5) Some reagents contain preservatives such as sodium azide which may react with lead and copper plumbing to form explosive metallic azides. Exercise caution during disposal.
6) Never pipette by mouth - always use safety pipetting devices for all pipetting.
7) Do not eat, drink, smoke or apply cosmetics in the laboratory.
8) Wash hands thoroughly with soap and water after work is completed.

g) Immuno-chromatographic test

Principle:

Immuno-chromatographic tests are membrane-based immunoassays that allow visual detection of an antigen/antibody in liquid specimens. In clinical assays, specimens such as urine, whole blood, serum or plasma, saliva and other body fluids may be employed.

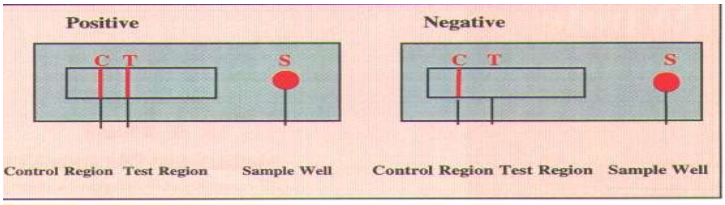
The tests system comprise of a base membrane such as nitrocellulose. A detector reagent (antigen/antibody–indicator complex) specific to the analyte (Antigen or antibody, which ever to be detected) is impregnated at one end of the membrane. A capture reagent is coated on the membrane at the test region. When the specimen is added to the sample pad, it rapidly flows through the conjugate pad. antigen/antibody if present in the specimen, binds to the detector reagent. As the specimen passes over the test band to which the capture reagent is coated, the analyte-detector reagent complex is immobilized. A colored band proportional to the amount of analyte present in the sample, develops. The excess unbound detector reagent moves further up the membrane and is immobilized at the control band.

Material Required: a) Serum/whole blood b) Test kit c) pipette d) buffer(optional)

Procedure:

1) Take out the test kit from its packing. The kit should be brought to room temperature before use.
2) Put 1-2 drop of patient serum onto the sample window made on the kit
3) Wait for few minutes to read the result. Maximum observation time is difrrent for different test kits.

Result: Appearance of a coloured band at the area marked as "C"(control) indicates that the kit is working. In a positive reaction an additional band appears at the area marked as "T"(test). Absence of band at T region indicates negative reaction



Demonstrations

The teacher should Demonstrate the following things during the class:

a) Demonstrate the materials, kits and equipments required for various serological tests
b) Explain and demonstrate the procedure different Serological tests.
c) Explain and demonstrate the method of interpretation and reporting of each serological test.
d) Explain all the safety precautions to be taken during serological test.

Students Exercise:

1. Explain Agglutination reaction.

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2. What are the reasons of Biological false positive reactions associated with RPR test?

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3. Name the antigen suspensions used in WIDAL.

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4. What is CRP? What is its significance?

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5. ASO test is based on.....reaction.

6. The full form of ELISA is.....

7. What is the major difference between antigen detection and Antibody detection by ELISA?

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Exercise Number 9: Staphylococcus

Learning Objectives:
On completion of the exercise the students should be able to do:

- **a)** Understand the morphology and culture characteristics of *Staphylococcus aureus*.
- **b)** Know the other medically important *Staphylococc*i and their identification.

Introduction:

Taxonomically, the genus ***Staphylococcus*** is in the Bacterial family *Micrococcaceae*

The *Staphylococci* are gram (+) cocci which appear to be arranged in clusters. These organisms are salt tolerant and catalase positive.Three species are important in humans as part of the normal flora and as pathogens.

Staphylococcus aureus

Causes boils, carbuncles, impetigo, abcesses, postsurgical wounds, pneumonia and osteomyelitis.There are several toxin associated pathologies associated with this organism including:toxic epidermal necrosis (scalded skin syndrome),toxic shock syndrome,food poisoning.Many strains of *Staphylococcus aureus* are resistant to antibiotics. Methicillin resistant *Staphylococcus aureus* (MRSA) is particularly threatening.Staphylococcus aureus is mannitol (+), coagulase (+), often beta-hemolytic, sensitive to novobiocin.,Phosphatase (+) and liquefies gelatin.

Staphylococcus epidermidis

Causes bacteremia, intravenous catheter infections, CSF shunt infections, endocarditis, urinary tract infections.Staphylococcus epidermidis is mannitol (-), coagulase (-) , novobiocin sensitive.

Staphylococcus saprophyticus

Second most common cause of cystitis, after *E. coli*, in young women.Staphylococcus saprophyticus is mannitol (+ or -), coagulase (-) and novobiocin resistant.

Lab diagnosis of Staphylococci:

Morphology: Gram positive cocci in clusters.

Colony appearance: Medium to large circular raised colonies.Most colonies are pigmented yellow to cream and opaque producing beta hemolysis on blood agar.On Mac Conkey colonies are small and pink due to lactose fermentation.

Biochemical identification:

Coagulase test: Staphylococcus aureus is known to produce coagulase, which can clot plama into gel in tube or agglutinate cocci in slide. This test is useful in differentiating S.aureus from other coagulase-negative staphylococci. Most strains of S.aureus produce two types of coagulase, free coagulase and bound coagulase. While free coagulase is an enzyme that is secreted extracellularly, bound coagulase is a cell wall associated protein. Free coagulase is detected in tube coagulase test and bound coagulase is detected in slide coagulase test. Slide coagulase test may be used to screen isolates of S.aureus and tube coagulase may be used for confirmation. While there are seven antigenic types of free coagulase, only one antigenic type of bound coagulase exists. Free coagulase is heat labile while bound coagulase is heat stable.

Slide coagulase test:

Principle:The bound coagulase is also known as clumping factor. It cross-links the α and β chain of fibrinogen in plasma to form fibrin clot that deposits on the cell wall. As a result, individual coccus stick to each other and clumping is observed.

Procedure: Dense suspensions of Staphylococci from culture are made on two ends of clean glass slide. One should be labeled as "test" and the other as "control". The control suspension serves to rule out false positivity due to auto-agglutination. The test suspension is treated with a drop of citrated plasma and mixed well. Agglutination or clumping of cocci within 5-10 seconds is taken as positive. Some strains of S.aureus may not produce bound coagulase, and such strains must be identified by tube coagulase test

Tube coagulase test:

Principle: The free coagulase secreted by S.aureus reacts with coagulase reacting factor (CRF) in plasma to form a complex, which is thrombin. This converts fibrinogen to fibrin resulting in clotting of plasma. Procedure: Three test tubes are taken and labeled "test", "negative control" and "positive control". Each tube is filled with 0.5 ml of 1 in 10 diluted rabbit plasma. To the tube labeled test, 0.1 ml of overnight broth culture of test bacteria is added. To the tube labeled positive control, 0.1 ml of overnight broth culture of known S.aureus is added and to the tube labeled negative control, 0.1 ml of sterile broth is added. All the tubes are incubated at 37°C and observed up to four hours. Positive result is indicated by gelling of the plasma, which remains in place even after inverting the tube. If the test remains negative until four hours at 37°C, the tube is kept at room temperature for overnight incubation.

Application: Coagulase test is used to identify and differentiate S.aureus from coagulase negative staphylococci. While slide coagulase test is useful in screening, tube coagulase test is useful in confirmation of coagulase test. Not all S.aureus strains produce coagulase; such rare strains are identified by thermonuclease test. Some coagulase negative staphylococci such as S.lugdensis and S.schleiferi are known to give positive slide coagulase test while S.hyicus and S.intermedius are known to give positive tube coagulase test.

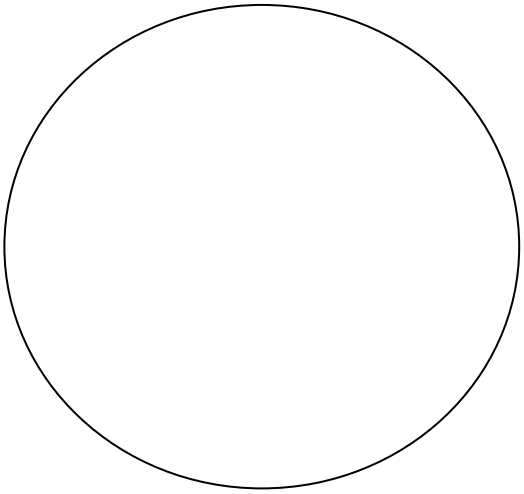
Demonstrations

The teacher should Demonstrate the following things during the class:

1) Colony morphology of Staphylococcus aureus .
2) Gram staining of S aureus
3) Slide and tube Coagulase test.

Students Exercise:

1. Study the colony morphology and gram stained smear. Perform slide Coagulase test with the given isolate. Record your findings with labeled diagram.



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2. The bacteria which is novobiocin resistant is
   A. *Staphylococcus aureus*
   B. *S epidermidis*
   C. *S saprophyticus*
   D. None of these
3. Coagulase-reacting factor is necessary for
   A. slide coagulase test
   B. tube coagulase test
   C. precipitation test
   D. none of these
4. Staphylococcal food poisoning usually manifests itself following ingestion of contaminated food after
   A. 2-6 hours
   B. 8-12 hours
   C. 12-18 hours
   D. 18-36 hours

5. Which of the following can be used to detect clumping factor?

A. tube coagulase test

B. slide coagulase test

C. precipitation test

D. none of these

6. How do you differentiate between Staphylococci and Streptococci?

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7. Name two Coagulase negative Staphylococci.

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Exercise Number 10: Identification of Streptococci

Learning objectives:
On completion of the exercise the students should be able to:

- **a)** Understand the morphology and culture characteristics of different Streptococci.
- **b)** Know the different diseases caused by Streptococci and their laboratory diagnosis.

Introduction: Streptococci are responsible for more infectious disease processes than any other type of bacteria. Therefore, differentiation and identification of streptococci is an important step in diagnosis.

There are many different species of streptococci, which makes them more difficult to identify. The type of hemolysis on blood agar is the most important test in the identification of the different groups of streptococci. The three groups of streptococci are:

1. beta-hemolytic streptococci

2. alpha-hemolytic streptococci

3. non-hemolytic (gamma) streptococci

Beta-hemolytic streptococci produce colonies on blood agar that are surrounded by a relatively clear zone of hemolysis in which the red blood cells in the agar are completely lysed. Many serious infections such as pharyngitis, scarlet fever, impetigo, rheumatic fever, and glomerulonephritis are caused by the beta-, is often the cause of bacterial meningitis in newborns, and can also cause childbirth sepsis. (This is due to the fact hemolytic species Streptococcus pyogenes. Another beta-hemolytic streptococcus species, Streptococcus agalactiae that S. agalactiae is present in the vaginal normal flora of up to 25% of all women.)

Alpha-hemolytic streptococci produce colonies on blood agar that are surrounded by a greenish zone of hemolysis, due to the incomplete breakdown of the hemoglobin in the red blood cells. Streptococcus pneumonia is an example of a pathogenic alpha-hemolytic streptococcus. S. pneumoniae causes pneumonia, ear infections (otitis media), and meningitis. Other alpha-hemolytic streptococci are primarily normal flora, such as Streptococcus salivarius and Streptococcus mutans, found in the mouth. Collectively, these non-pathogenic streptococci are called "viridans" streptococci.

Gamma or non-hemolytic streptococci do not produce any hemolysis on blood agar. Enterococcus faecalis is an example of a non-hemolytic streptococcus that is normally found in the intestinal tract, and is therefore included in a group of streptococci called the "enterococci". These enterococci can migrate to other areas of the body to cause conditions such as urinary tract infections or peritonitis.

Sl no	***S. Pyogenes***	***S.pnemoniae***	***Enterococcus***
1	Beta-hemolytic Strep	Alpha-hemolytic Strep	Gamma-hemolytic Strep
2	bacitracin sensitivity	optochin sensitivity	bile esculin
3	hippurate hydrolysis	Bile solubility	Growth in 6.5% salt

Lancefield antigenic group typing.

Beta-hemolytic streptococci and enterococci possess chemicals called CH (carbohydrate) antigens. The presence and type of CH antigen can be demonstrated by extraction of the antigen from the cell, and reacting it with antibodies specific to each antigen. Lancefield found thirteen different antigenic groups, A-O. Of these, Groups A, B, and D are most commonly implicated in human infections. Groups C, F, and G are also occasionally cultured from patients.

Group	Major Species
A	S. pyogenes
B	S. agalactiae
D	E. faecalis*
	S. faecium*
	S. durans*
	S. avium*

Identification of Beta-hemolytic Streptococci:

Bacitracin sensitivity

S. pyogenes is sensitive to the antibiotic bacitracin, whereas other beta-hemolytic strep are not.

When a paper disk impregnated with bacitracin is placed on a blood agar plate upon which S. pyogenes is growing, there will be a zone of inhibition around the bacitracin disk where the S. pyogenes cannot grow. This is a positive test for S. pyogenes.

Hippurate Hydrolysis

To confirm the I.D. of S. agalactiae, the sodium hippurate hydrolysis test is often used. Sodium hippurate broth is inoculated with the organism and incubated overnight. The tube is then centrifuged and the supernatant fluid removed. A reagent called ferric chloride is added to the supernate and observed for the development of a heavy precipitate, which is a positive test for S. agalactiae. (If little or no precipitate results, the test is negative, and the organism is not S. agalactiae.)

Identification of Alpha-hemolytic Streptococci:

Optochin sensitivity

The optochin sensitivity test is similar to the bacitracin sensitivity test, except that the disk used is impregnated with the chemical optochin. The presence of a zone of inhibition around the optochin disk is a presumptive identification of S. pneumoniae.

In summary: optochin sensitive = S. pneumoniae

optochin resistant = possible viridans streptococci

Identification of the non-hemolytic (gamma) streptococcus group

Bile esculin hydrolysis

BE media can be made into agar plates or slants. The surface is then inoculated with the suspected organism and incubated for 24-48 hours. If blackening of the media occurs, the test is positive for bile esculin hydrolysis, and the organism can be identified as part of the group of streptococci called the enterococci.However, some streptococci that are BE + are not enterococci species. Therefore, another test must be done to differentiate these strep species from the true enterococci. The test used for this purpose is the 6.5% NaC1 tolerance test.

Growth in media with 6.5% Salt

The salt can be incorporated into an agar plate or a tube of broth. The media is then inoculated with the strep, incubated for 24-48 hours, and checked for growth. If growth occurs, the organism is an Enterococcus.

Demonstrations:
The teacher should demonstrate the following things during the class:

a) Demonstrate the morphology of *Streptococci* in Gram stained smears.
b) Demonstrate the growth of *Streptococci* in Blood agar, with different species showing different types of hemolysis.
c) Demonstrate key identification reactions for *S. pyogenes* and *S. pneumoniae*.

Students Exercise:

1. How is S. pyogenes differentiated from other hemolytic streptococci?

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2. How do you differentiate between Staphylococci and Streptococci ?

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3. What is Quellungs test?

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4. How do you identify Enterococci?

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5. What is CAMP test?

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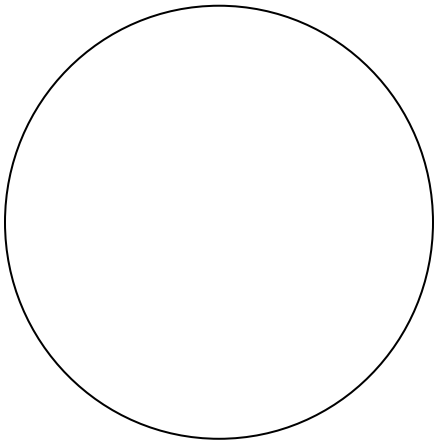
6. Draw a neat labeled diagram of the slide shown to you.



Exercise Number 11 : Gram Negative Diplococci

Learning objectives:
On completion of the exercise the students should be able to

- **a)** Understand the morphology and culture characteristics of Neisseria
- **b)** Know the important diseases caused by Neisseriae.
- **c)** Know the biochemical tests to identify Neisseria.
- **d)** Know the difference between different species of Neisseria .

Introduction: The members of the genera Neisseria are Gram negative cocci, aerobic, non sporulating, arranged in pairs , non motile. Important pathogenic species are Neisseria menigitidis, which causes meningococcal meningitis and also septicaemia; and Neisseria gonorrhoeae- which causes gonorrhoea, pelvic Inflammatory Disease (PID) in women and neonatal gonorrheal ophthalmia in newborns.

Laboratory diagnosis: Usually based on microscopy and culture, antigen detection can also be done.

Microscopy: These are Gram negative cocci, usually appearing in pairs, with adjacent sides flat (N. meningitides) or concave (N. gonorroheae). Demonstration of diplococci in the clinical specimen is very specific of Neisserial infection, however the sensitivity is less. N. gonorroheae usually occur intracellularly in polymorphs.

Culture: Can be grown in media enriched with blood or serum. Selective media such as modified Thayer Martin medium may be required for specimens from probable contaminated sites, e.g. genital swabs. The species identification can be done based on biochemical reactions.

Neisseria menigitidis: Oxidase positive, produces acid by oxidising glucose and maltose.

Neisseria gonorrhoeae: Oxidase positive, produces acid by oxidising glucose only.

Antigen detection and **molecular diagnosis** can also be used, but are not very popular for diagnosing Neisserial infections.

Prophylaxis:

Immunoprophylaxis is available for sero-groups A, C, Y and W-135. Chemoprophylaxis is given with rifampicin, ciprofloxacin or cefiriaxone.

Demonstrations
The teacher should demonstrate the following things during the class:

a) Gram stained smears of Gram negative diplococci.
b) Culture characteristics of Neisseriae.
c) Display biochemical reactions of meningococcus and gonococcus.

Students Exercise:

1. Which specimen is collected for laboratory diagnosis of gonorrhoea?

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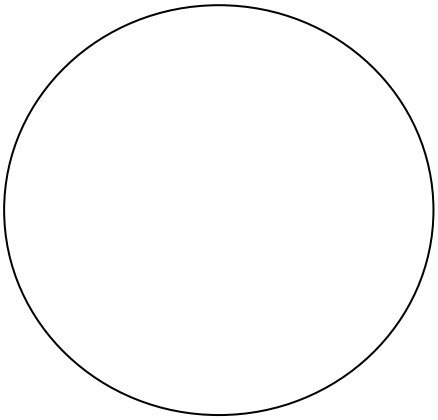
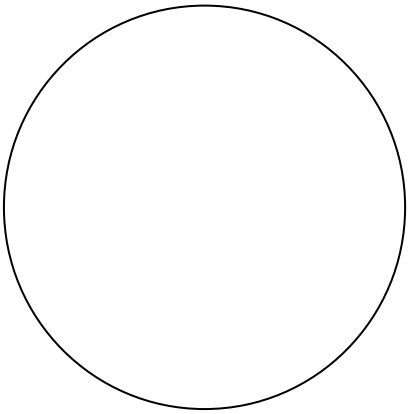
2. Draw diagrams of Gram stained smears of gonococcus and meningococcus seen by you.





3. Describe the important morphological features of gonococcus and meningococcus

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4. Mention the prophylaxis against meningococcal infections.

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Exercise Number 12:

Corynebacterium diphtheriae and other Gram positive non-sporing bacilli

Learning objectives:

On completion of the exercise the students should be able to:

- **a)** Understand the morphology and culture characteristics of *Corynebacterium diphtheriae*.
- **b)** Know the other non-diphtheria Corynebacteria and their medical importance.
- **c)** Know important anti-diphtheria vaccines.
- **d)** Understand the role of laboratory animals in reference to diphtheria.

Introduction:

Corynebacteria are Gram positive, non acid fast, non-sporing, non-motile rods. *Corynebacterium diphtheriae*, the causative agent of diphtheria- is the most important species in this genus.

Diphtheria: It is the infection caused by *Corynebacterium diphtheriae,* the site of infection may be faucial, laryngeal, nasal, otitic, conjunctival, genital or cutaneous. Faucial diphtheria is the commonest. Locally, a tough, leathery pseudomembrane is formed which is constituted of fibrinous exudates, local disintegrating cells and bacteria ; the systemic effects are due to a potent exo-toxin. Common complications are asphyxia- due to mechanical obstruction by pseudomembrane, circulatory failure, post diphtheritic paralysis and sepsis.

C. diphtheriae: These are slender Gram positive bacilli, with a tendency to clubbing at both ends. Granules composed of polymetaphosphate are seen in the cells. They are also called *volutin* or *Babes-Ernst* granules. In smears the bacteria are arranged at angles to each other, called as *Chinese letter* or *Cuneiform* arrangement. This is due to incomplete separation of daughter cells after binary fission.

Laboratory diagnosis:It includes isolation and identification of the bacillus and demonstration of its toxicity.

Swabs from the lesions are collected and subjected to microbiological procedures.

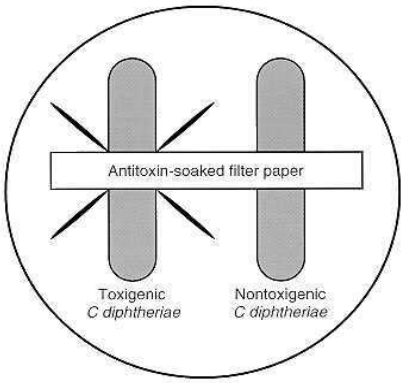
Microscopy: The smears are stained with Gram's stain to demonstrate Gram positive bacilli and specific arrangement. Meta-chromatic granules are demonstrated using Albert's stain.

Isolation: The organism is a facultative anaerobe. Culture media employed for growth are Loeffler's serum slope and tellurite blood agar. On Loeffler's serum slope the colonies are obtained with-in 6-8 hours. The tellurite blood agar acts as a selective and indicator medium, as it inhibits the growth of most of throat commensals and the colonies obtained are black in colour due to reduction of tellurite to metallic tellurium.

Toxigenicity testing: The toxigenicity of *C diphtheriae* strains is determined by a variety of *in vitro* and *in vivo* tests.

Several sensitive *in vivo* tests for diphtheria toxin have been described (e.g., guinea pig challenge test, rabbit skin test).

The most common *in vitro* assay for toxigenicity is the Elek immunodiffusion test. This test is based on the double diffusion of diphtheria toxin and antitoxin in an agar medium. A sterile, antitoxin-saturated filter paper strip is embedded in the culture medium, and C diphtheriae isolates are streak-inoculated at a 90° angle to the filter paper. The production of diphtheria toxin can be detected within 18 to 48 hours by the formation of a toxin-antitoxin precipitin band in the agar.



Many eukaryotic cell lines (e.g., African green monkey kidney, Chinese hamster ovary) are sensitive to diphtheria toxin, enabling *in vitro* tissue culture tests to be used for detection of toxin.

Prophylaxis:

Active Immunisation: Because of the high degree of susceptibility of children, artificial immunization at an early age is universally advocated. Toxoid is given in 3 doses (1 month apart) for primary immunization at an age of 3 - 4 months. A booster injection should be given about a year later, and it is advisable to administer a further booster injection at school entry. Usually, immunization is done with a trivalent vaccine containing diphtheria toxoid, pertussis vaccine, and tetanus toxoid (DPT vaccine).

Passive Immunisation: Anti Diphtheric Serum (ADS) is administered as an emergency measure when susceptibles are exposed to infection. As this is horse serum, precaution against hypersensitivity should be observed.

Demonstrations:

The teacher should Demonstrate the following things during the class:

1. Demonstrate the morphology of *Corynebacterium diphtheriae* in Gram stained and Albert's stained smears.
2. Demonstrate the growth of *C. diphtheriae* in Loeffler's serum slope and Potassium-tellurite agar.
3. Display the vials of DPT and DT vaccines and ADS.

Practical and Exercise:

- Perform Gram stain on the given fixed smear and observe bacilli of *C. diphtheriae.*
- Perform Albert's stain on the given fixed smear and observe granules of *C. diphtheriae.*

Students Exercise:

1. Which specimen is collected for laboratory diagnosis of diphtheria?

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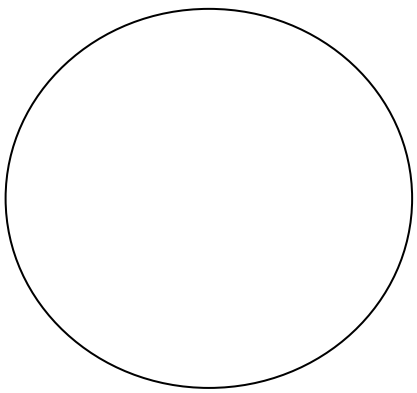
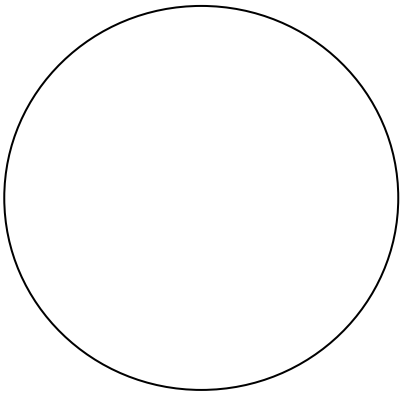
2. Draw diagrams of Gram stained and Albert's stained smears of *C. diphtheria* seen by you.





3. Describe the important morphological features of *C. diphtheriae.*

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4. Mention the importance of Loeffler's serum slope and Potassium-tellurite agar in diagnosis of diphtheria.

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5. What are the in-vivo tests of toxigenicity of *C. diphtheriae*?

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6. What are the in-vitro tests of toxigenicity of *C. diphtheriae*?

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7. Mention the immunization against diphtheria.

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Exercise Number 13: Mycobacteria

Learning objectives:

on completion of the exercise the students should be able to

- **a)** Enumerate the important pathogenic mycobacteria
- **b)** Perform staining procedures for mycobacteria
- **c)** Identify methods culture and identification of mycobacterium

Table no 11.1: classification of pathogenic mycobacteria

	Group	Organism	Disease caused
a)	*M. tuberculosis* complex	*M. tuberculosis* *M. bovis* *M. africanum* *M. microti*	Tuberculosis
b)	Leprosy bacillus	*M. leprae*	Leprosy
c)	Nontuberculous mycobacteria	Photochromogens *M.kansasii*, *M. simiae* *M. marinum* Scotochromogens *M. scrofulaceum*, *M. szulgai* Non-chromogens *M.avium* complex (MAC),*M. ulcerans*, *M. xenopi*, *M. malmoense M. genavense etc* Rapid growers *M.chelonae*, *M.fortuitum, M.smegmatis* *M. flavescens etc*	pulmonary disease resembling tuberculosis, lymphadenitis, skin disease, or disseminated disease.

Morphology and staining:

M. *tuberculosis* and Nontuberculous mycobacteria and non-sporing, non-capsulated straight or slightly curved slender rods, measuring 1–4 µm X 0.2–0.6 µm. Although they does not Gram stain well due to its waxy surface, the organism has a Gram positive cell wall. They are best demonstrated using the Ziehl-Neelsen staining technique or a fluorescence technique.

M. *leprae* is a non-motile, non-sporing, straight or slightly curved rod measuring 5–8 X 0.2–0.5 µm. Bacteria with pointed or enlarged ends are sometimes seen. The organisms can be found singly, in clusters, and in large groups within macrophages. Organisms in large groups are called "**Globi**".

M. *leprae* is acid fast when stained by the Ziehl-Neelsen technique. But it is not as acid fast as the tubercle bacillus and therefore a weaker acid solution, i.e. 1% acid alcohol is used to decolorize smears. M. leprae is also less heat resistant than tubercle organisms and therefore smears must be gently heat-fixed for just a fewseconds.

Concentration method and smear preparation for detection of AFB in sputum

Chances of detecting AFB in sputum smears are significantly increased when sputum is treated with 5% sodium hypochlorite (NaOCl), followed by centrifugation. Since NaOCl kills M. tuberculosis, the NaOCl concentration technique is also safer for laboratory staff.

Sodium hypochlorite centrifugation technique to concentrate AFB

1) Transfer 1–2 ml of sputum (particularly any yellow caseous material) to a screw-cap Universal bottle or other container of 15–20 ml capacity.

Caution: Open specimen containers with care and at arms length to avoid inhaling infectious aerosols. When available, handle the specimen inside a safety cabinet.

2) Add an equal volume of 5% NaOCl solution and mix well.
3) Leave at room temperature for 10–15 minutes, shaking at intervals to break down the mucus.
4) Add about 8 ml of distilled water. Mix well.
5) Centrifuge at 3000 g for 15 minutes or at 250–1000 g for 20 minutes.
6) discard the supernatant fluid. Mix the sediment.
7) Transfer a drop of the well-mixed sediment to a clean scratch-free glass slide. Spread the sediment to make a thin preparation and allow to air-dry.
8) Heat-fix the smear and stain it using the Ziehl-Neelsen technique, as described in exercise number-2.

Reporting of sputum smears

When any definite red bacilli are seen, report the smear as 'AFB positive', and give an indication of the number of bacteria present as follows:

More than 10 AFB/field ---------- report +++
1–10 AFB/field ---------- report ++
10–100 AFB/100 fields ---------- report+
1–9 AFB/100 fields ---------- report "Scanty" and write exact number
When no AFB are seen after examining 100 fields: Report the smear as 'No AFB seen'

Note: Up to three specimens may need to be examined to detect M. tuberculosis in sputum. One specimen should be collected as an early morning sputum

Smear preparation for *M. leprae* **(slit skin smear)**

1) Take a sterile scalpel blade.
2) Wearing protective rubber gloves, cleanse the area from where the smear is to be taken, using a cotton wool swab moistened with 70% v/vethanol (alcohol). Allow the area to dry.
3) Pinch the skin tightly between the thumb and index finger until it becomes pale due to loss of blood.
   Important: The area must be kept bloodless while the smear is collected because a smear which contains red cells will be difficult to examine and report.
4) Using the sterile blade, make a small cut through the skin surface, about 5 mm long and deep enough into the dermis (2–3 mm) where the bacteria will be found. Continue to hold the skin tightly.
5) Using a dry piece of cotton wool, blot away any blood which appears at the site of the cut.
6) Turn the scalpel blade until it is at a right angle to the cut. Using the blunt edge of the blade, scrape firmly two or three times along the edges and bottom of the cut to collect a sample of tissue juice and cells.
7) Transfer the sample to a slide. Make a small circular smear, covering evenly an area measuring 5–7 mm in diameter.
8) When the smear has dried, gently heat-ix it over the flame for a few seconds.

Staining for *M. leprae*: Stain the smear by ZN staining technique as for tubercle bacilli, but using **1% acid alcohol/5% H_2SO_4** as decoloriser.

Reporting *M. leprae* smear

Report the smear as 'Positive' if *M. leprae* bacteria are seen or 'Negative' if no bacteria are seen after examining the entire smear or at least 100 high power microscope fields.

Culture of Mycobacterium:

Solid mediums:

Egg-based – 1) Lowenstein–Jensen medium (recommended by the International Union against Tuberculosis, IUAT)
2) Petragnini medium
3) Dorset medium

Agar based- Middlebrook 7H10 Agar, Middlebrook 7H11 Agar

Blood based – Tarshis medium

Serum based – Loeffler medium

Potato based – Pawlowsky medium

Liquid mediums:-

1) Dubos' medium
2) Middlebrook 7H9 Broth
3) Proskauer and Beck's medium
4) Sula's medium
5) Sauton's medium

Cultural characteristics on LJ medium:

The tubercle bacillus is an obligate aerobe and grows at temperatures from 30-41^{0}C, optimally at 35-37^{0}C. Lowenstein-Jensen media with glycerol or sodium pyruvate is generally used for isolation. The specimen is incubated on slopes of L-J medium at 37^{0}C. Growth is slow so that colonies only appear after 2-3 weeks. The slopes should be incubated for a total of 6-8 weeks before discarding. M. tuberculosis colonies on L-J media are **rough, buff to yellowish in colour, and tough when picked off.** The addition of glycerol to L-J medium improves the growth of *M. tuberculosis*, but not that of *M. bovis*.

Most NTMs show similar growth characteristics as of *M. tuberculosis*. Rapid growers take much lesser time and colonies can seen within 7 days.

Rapid Culture technique: *Mycobacterium* can be cultured using rapid culture tequniques like BacT/ALERT (BioMerieux, Durham) system. It is a fully automated and non radiometric system that utilizes a bottle containing a colorimetric sensor embedded in its bottom. Carbon dioxide produced by microbial metabolism causes reduction in pH of medium and changes the sensor color from dark green to yellow. The color change is continuously monitored and promptly reported by the instrument.

This type of rapid growth systems have shorter turn out time (4-21 days) compared to that of the conventional culture method (3- 8 weeks).

Biochemical identification of Mycobacterium:

M. tuberculosis can be differentiated from NTMs by following biochemical tests:

1) Niacin accumulation test.
2) Nitrate reduction.
3) Pyrazinamidase test
4) Urease test.
5) Arylsulfatase test
6) Catalase test
7) Iron uptake
8) Tellurite reduction
9) Tween 80 hydrolysis
10) Growth on LJ containing p-nitrobenzoic acid (PNB)

Biochemical tests	*M. tuberculosis* clx.	NTM
Niacin accumulation	+	-
Nitrate reduction	+	-
Pyrazinamidase activity	+	+/-
Urease activity	+	-/+
Arylsulfatase test	-	-/+
Catalase test	-	+/-
Iron uptake	-	+
Tellurite reduction	-	+
Tween 80 hydrolysis	-	+

Molecular diagnosis:

Molecular methods are genetic procedures that make use of genetic materials (DNA or RNA) to detect specific proteins or genes of the test organism using specific probes or short stranded oligonucleotides (primers) complementary to the test DNA strand.
The high degree of DNA polymorphism, repetitive DNA sequences and presence of insertion sequences (IS) characteristic of MTB strains have been used as basis for the study of strain-strain relatedness/diversity of MTB.
Primer sequences of specific regions in the MTB genome have been done and cloned for use in detection, identification and typing from clinical samples and cultures.

Insertion sequence 6110 polymerase chain reaction based diagnostic method:

The IS6110 PCR technique is useful in the rapid detection of MTB complex strains in clinical specimens from naturally sterile anatomical sites with minimal bacillary load, often undetectable by the conventional methods. E.g. CSF, pleural effusion, joint and marrow taps, gastric washings. The method is cost-effective and simple to perform. It is specific, sensitive, reproducible and able to generate results within hours.
The target DNA is PCR amplified using IS6110 primers sequences. The amplified product is electrophoresed using 2% agarose and observed by UV illumination for DNA band of 123 base pair.

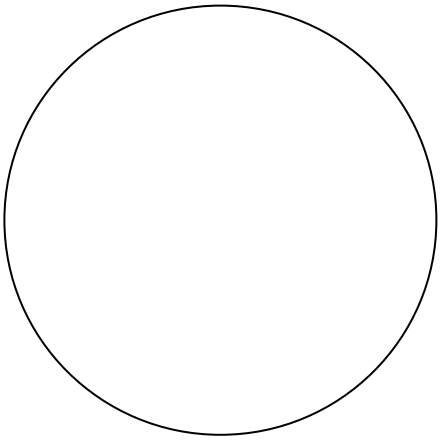
Demonstrations

The teacher should Demonstrate the following things during the class:

a) Explain and demonstrate the different steps involved in preparation of sputum smear.
b) Explain and demonstrate the different steps of ZN staining.
c) Demonstrate AFB under microscope and explain the method of reporting.
d) Demonstrate the methods of *Mycobacterium* culture.
e) Demonstrate the colony characteristics of *Mycobacterium*.

Students Exercise:

1. Draw and label the microscopic view of the stained sputum smear.



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2. What precautions should be taken during preparation of a sputum smear?

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3. Write the various medium used for culture of mycobacterium.

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4. Write the minimum incubation time & colony morphology of *M. tuberculosis* on LJ medium.

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5. Write the difference in ZN staining procedure done for staining of *M. tuberculosis* and *M. leprae*.

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6. what are the benefits of concentration technique for smear preparation for detection of AFB in sputum

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Exercise Number 14: Spore bearing aerobic and anaerobic bacilli.

Learning objectives:
On completion of the exercise the students should be able to**:**

a) Know about the diseases caused by and diagnosis of organisms Bacillus and Clostridium.
b) Observe the position of endospores in different Clostridium sp.

Introduction:

Bacillus: In 1872, Ferdinand Cohn, a contemporary of Robert Koch, recognized and named the bacterium ***Bacillus subtilis.*** The organism is **Gram-positive**, capable of **growth in the presence of oxygen**, and forms a unique type of resting cell called an **endospore**. The organism represented what was to become a large and diverse **genus** of bacteria named ***Bacillus***, in the Family ***Bacillaceae.***

Most members are saprophytic organisms prevalent in soil, water and air and on vegetation Some are human pathogens.**Bacillus anthrax – principal pathogen**, a major agent of bioterrorism and biologic warfare, causes anthrax. B. anthracis has poly-D-glutamic acid capsule.The spores are located in the center of nonmotile bacilli. Spores are Resistant to environmental changes withstand dry heat and certain chemical disinfectants for moderate periods Persist for years in dry earth Animal products contaminated with anthrax spores e.g. hides, bristles, hair, wool, bone can be sterilized only by autoclaving.

Lab Diagnosis of Bacillus anthracis:
Specimens: Include fluid aspirated from cutaneous lesions and when indicated, sputum, cerebrospinal fluid, and blood for culture.

Microscopy

B. anthracis is a large, 5–8 _ 1.5 µm, Gram positive (or Gram variable) non-motile bacillus, often appearing joined end to end in chains.*In smears from specimens*: Bacilli are capsulated. The capsular material often appears irregular and fragmented. When stained using Loeffler's polychrome (McFadyean) methylene blue , the bacilli stain blue and the capsular material stains purple-pink as shown in colour .*In smears from aerobic cultures*: Bacilli are non-capsulated but contain oval spores (same diameter as the bacilli), giving the organisms a beaded appearance.

Culture

B. anthracis grows aerobically and anaerobically(facultative anaerobe). The temperature range for growth is 12–45 ºC with an optimum of 35–37 ºC. Spore formation is best between the range 25–30 ºC.
Blood agar: B. anthracis produces large 2–5 mm in diameter, grey-white, irregular colonies with wavy edges. The colonies are nonhaemolytic or only slightly haemolytic. Saprophytic *Bacillus* species are markedly haemolytic.
Broth cultures: They are not usually turbid,but they often show a thick skin (pellicle) and a sediment.
Gelatin stab culture: Occasionally this is used to assist in the identification of *B. anthracis*. The organism slowly liquefies the gelatin along and out from the line of inoculation. The treelike pattern formed by the liquefaction lines is characteristic of *B. anthracis*, but the reaction is slow and in practice anthrax bacilli are usually identified microscopically by their morphological appearance.

Bacillus cereus

It is a large, 1 x 3-4 μm, Gram-positive, rod-shaped, endospore forming, facultative aerobic bacterium . It was first successfully isolated in 1969 from a case of fatal pneumonia in a male patient and was cultured from the blood and pleural fluid . B. cereus is mesophilic, growing optimally at temperatures between 20°C and 40°C, and is capable of adapting to a wide range of environmental conditions. It is distributed widely in nature and is commonly found in the soil as a saprophytic organism. B. cereus can spread easily to many types of foods such as plants, eggs, meat, and dairy products, and is known for causing 25 % of food-borne intoxications due to its secretion of emetic toxins and enterotoxins . Food poisoning occurs when food is left without refrigeration for several hours before it is served. Remaining spores of contaminated food from heat treatment grow well after cooling and are the source of food poisoning.

Mannitol egg-yolk phenol-red polymyxin agar (MYPA) is recommended as a selective medium for the isolation of *B. cereus* from faeces, vomit, or food. After overnight incubation at 35–37 ºC, large 3–7 mm flat, dry grey-white colonies surrounded by an area of white precipitate are produced.

Clostridium:

Clostridia are, spore-forming, Gram-positive, anaerobes (although some species are microaerophilic). Most Clostridium species decompose proteins or form toxins and some do both. Their natural habitat is the soil or intestinal tract as saprophytes. They are known to produce a variety of toxins, some of which are fatal. *Clostridium tetani* is the etiological agent of tetanus,*Clostridium botulinum* is the etiological agent of botulism, and *Clostridium perfringens* is one of the etiological agent of gas gangrene.

Morphology: Large anaerobic gram positive motile rods.The spore is usually wider than the rods. Spores are placed centrally, terminally, or subterminally according to the genus.

Culture: Anaerobic culture conditions are established by one of the following:

1. Agar plates or culture tubes in anaerobic jar.

2. Fluid media contain either**:**

a. Fresh animal tissue (chopped meat)

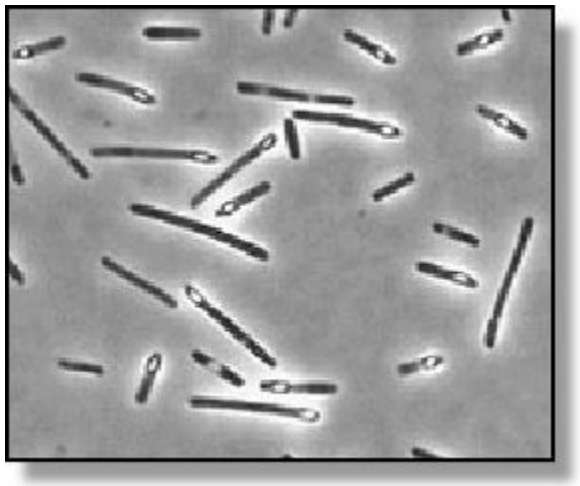
b. Reducing agent

eg. Thioglycollate broth, Robertsons cooked media.

Special tests for the Identification of various species of Clostridium

Cl.perfringens

Morphology: *C. perfringens* is a non-motile Gram positive thick brick-shaped rod as shown in colour Plate 34. Spores are rarely seen in smears from infected tissue. Some strains are capsulate in tissue. Pus cells, if seen, usually appear damaged due to toxin action. Often other bacteria are also present.

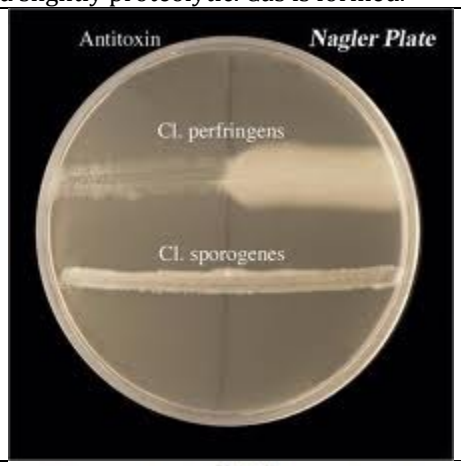


Colony Morphology*:C. perfringens* is a facultative anaerobe which can be cultured, both anaerobically and microaerophilically. Optimum temperature range is 37–45 ºC.

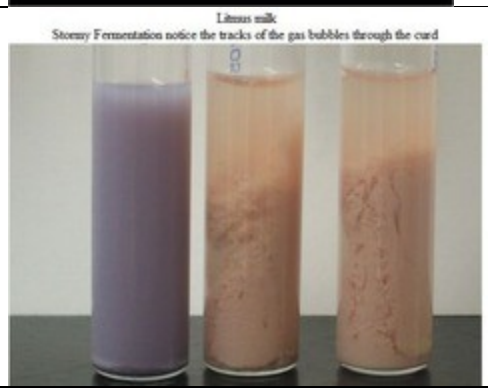
Blood agar: Large *beta*-haemolytic colonies are produced (most food-poisoning strains are non-haemolytic). Some strains produce a double zone of haemolysis as shown in colour

Robertson's cooked meat medium (RCMM): In this medium *C. perfringens* is saccharolytic and slightly proteolytic. Gas is formed.

Naegler reaction: *C. perfringens* produces an opacity in medium containing lecithin due to lecithinase C activity (*alpha* toxin). This opacity can be inhibited by applying specific antitoxic serum to the medium which will inactivate the lecithinase. The technique is referred to as the Nagler reaction.

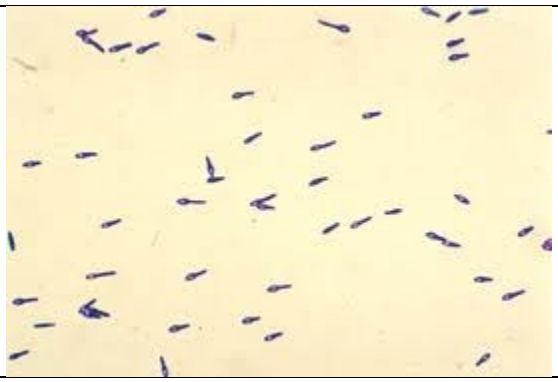


Stormy milk fermentation:



Morphology

C. botulinum is a Gram positive, motile, pleomorphic rod with oval sub-terminal spores. It is rarely isolated from specimens.



Culture and biochemical reactions

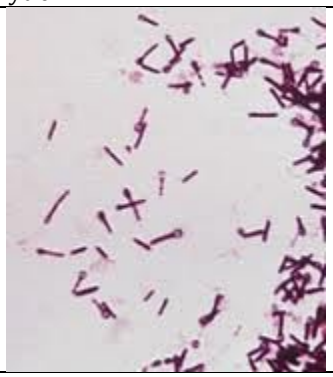
C. botulinum is a strict anaerobe. Grows best at 30–35 °C

Robertson's cooked meat medium(RCMM): Inoculate the emulsified specimen (in 0.1% peptone water) in several containers ofRCMM. Heat half of them at 80 ºC for 30 minutes (spores remain). Incubate the heat treated and untreated inoculated RCMM at 35 ºC for 3–5 days. Types A, B and F blacken and digest cooked meat medium (proteolytic
reaction) and produce hydrogen sulphide gas (types C, D, and E do not).

Blood agar subculture from RCMM (anaerobic culture): *C. botulinum* produces large semi-transparent colonies with a wavy outline. Most strains are *beta*-haemolytic.

Morphology

C. tetani is a Gram positive, non-capsulate rod that forms spores. Most strains are motile (type 6 is nonmotile).It can sometimes be seen in Gram smears of exudate from wounds, appearing as a long thin Gram positive (weakly staining) rod with a rounded unstained spore at one end.



Culture and biochemicals:

C. tetani is a strict anaerobe with a temperature range of 14–43 °C (37 °C optimum).***Blood agar:***When isolated (only very occasionally),*C. tetani* produces a fine film of feathery growth. Use a hand lens to examine the plate.On fresh blood agar, *C. tetani* is haemolytic (*alpha* first followed by *beta* haemolysis).

Robertson's cooked meat medium: (RCMM): *C. tetani* is slowly proteolytic. If clostridial growth occurs (check a Gram smear),divide the culture and heat one half at 80 °C for 30 minutes and cool. Subculture both the unheated and heated cultures on fresh blood agar and incubate anaerobically.

Antitoxin test

If growth occurs, subculture on a blood agar antitoxin plate (half the plate covered with antitoxin). Incubate the plate anaerobically. The haemolysis produced by *C. tetani* is inhibited by the antitoxin.

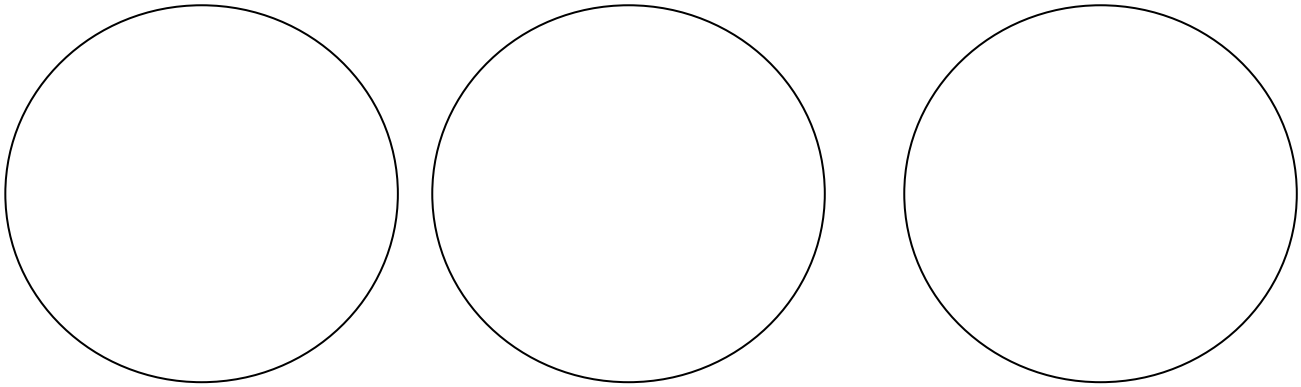
Demonstrations

The teacher should Demonstrate the following things during the class:

a) Slides of anthrax and different species of Clostridium to be demonstrated.

Students Exercise:

1. Draw a neat labeled diagram of the slides you observed in class.



2. Which of the following types of *Clostridium perfringens* produces alpha toxin most abundantly?

   A. Type A

   B. Type B

   C. Type C

   D. Type D

3. Types of exotoxin, most commonly associated with botulism in man, is/are?

   A. Type A

   B. Type B

   C. Type E

   D. All of these

4. The most toxic exotoxin is

   A. tetanus toxin

   B. diphtheria toxin

   C. botulinum toxin

   D. cholera toxin

5. Stormy clot reaction is useful in identification of

   A. *C tetani*

   B. *C botulinum*

   C. *C perfringens*

   D. *C difficile*

6. McFadyean's reaction is employed for the presumptive diagnosis of

A. Anthrax

B. Tetanus

C. Typhoid

D. all of these

7. The bacteria, used to examine the efficacy of autoclave, is

A. *B polymyxa*

B. *B brevis*

C. *Bacillus stearothermophilus*

D. *B megaterium*

8. Medusa head appearance of the colonies may be due to

A. *Bacillus anthracis*

B. *Proteus mirabilis*

C. *Clostridium tetani*

D. *Pseudomonas aeruginosa*

Exercise Number 15: *Escherichia coli* and other lactose fermenting Gram Negative Bacilli.

Learning objectives:
On completion of the exercise the students should be able to:

a) Understand the morphology and culture characteristics of members of *Enterobacteriaceae*.
b) Know the medical importance and identification of enteric bacilli especially *Escherichia coli.*

Introduction: The **Enterobacteriaceae** is a large family of Gram-negative bacteria that includes, along with many harmless symbionts, many of the more familiar pathogens, such as *Salmonella*, *Escherichia coli*, *Yersinia pestis*, *Klebsiella* and *Shigella*. Other disease-causing bacteria in this family include *Proteus*, *Enterobacter*, *Serratia*, and *Citrobacter*. Members of the Enterobacteriaceae are rod-shaped, and are typically 1-5 μm in length. They are Gram negative, facultative anaerobes, fermenting sugars to produce lactic acid and various other end products. Most also reduce nitrate to nitrite, although exceptions exist (e.g. *Photorhabdus*). Unlike most similar bacteria, enterobacteria generally lack cytochrome C oxidase, although there are exceptions (e.g. *Plesiomonas shigelloides*). Most have many flagella used to move about, but a few genera are nonmotile. They are not spore-forming. Usually they are catalase positive.

I. Escherichia coli

Escherichia coli is the most important member of the genus *Escherichia*. It is associated with gastroenteritis, UTI, meningitis and sepsis. By and large it is the most common bacterium causing UTI, both in community and hospital setting. Although in most circumstances it is a commensal in gut, but may be associated with gastroenteritis as in case if traveler's diarrhea, food poisoning and paediatric diarrhea.

Diagnosis is achieved by identification of the organism in the specimen from site of infection.

It readily grows on routine culture media. Further identification by the standard set of biochemical reaction.

Laboratory indications:

- Indole+
- MR+
- VP –
- Citrate–
- Urease–
- +/+ TSI (with/without gas)
- Motile

II. Klebsiella Spp

The most clinically important species of this genus is *Klebsiella pneumoniae*. This large, non-motile bacterium produces large sticky colonies when plated on nutrient media. Klebsiella's pathogenicity can be attributed to its production of a heat-stable enterotoxin. *K. pneumoniae* infections are common in hospitals where they cause pneumonia (characterized by emission of bloody sputum) and urinary tract infections in catheterized patients. In fact, *K. pneumoniae* is second only to *E. coli* as a urinary

tract pathogen. *Klebsiella* infections are encountered far more often now than in the past. This is probably due to the bacterium's antibiotic resistance properties. *Klebsiella* species may contain **resistance plasmids (R-plasmids)** which confer resistance to such antibiotics as ampicillin and carbenicillin. To make matters worse, the R-plasmids can be transferred to other enteric bacteria not necessarily of the same species.

Laboratory indications:

- Indole –
- MR –
- VP +
- Citrate+
- Urease+
- +/+ TSI (with gas)
- Non-motile

III. Enterobacter

Enterobacter includes eleven species of highly motile bacteria. The *Enterobacter* species is biochemically similar to Klebsiella, but unlike *Klebsiella Enterobacter* is ornithine positive. Although this bacterium is part of the normal flora of the human intestinal tract, several species cause opportunistic infections of the urinary tract as well as other parts of the body. *E. aerogenes* and *E. cloacae* are two such pathogens that do not cause diarrhea, but that are sometimes associated with urinary tract and respiratory tract infections.

Laboratory indications:

- Lysine + (except *E. cloacae*)
- Citrate +
- Indol -
- +/+ TSI (with gas)
- Motile
- Ornithine +

IV. Serratia

Members of the *Serratia* genus were once known as harmless organisms that produced a characteristic red pigment. Today, *Serratia marcescens* is considered a harmful human pathogen which has been known to cause urinary tract infections, wound infections, and pneumonia. *Serratia* bacteria also have many antibiotic resistance properties which may become important if the incidence of *Serratia* infections dramatically increases. *Serratia* can be distinguished from other genera belonging to Enterobacteriaceae by its production of three special enzymes: DNase, lipase, and gelatinase.

Laboratory indications:

- Lysine +
- Citrate +
- Indol -
- +/+ TSI (No gas)
- DNase +

Demonstrations:

The teacher should demonstrate the following things during the class:

a) Demonstrate the morphology and motility of *E. coli* in Gram stained smears.
b) Demonstrate the growth of members of *Enterobacteriaceae* in Blood agar and MacConkey agar.
c) Display the biochemical reactions of *E. coli*, *Klebsiella* etc.

Students Exercise:

1.The reaction(s) that is/are usually positive in Escherichia coli, is/ are

A. Glucose fermentation

B. Indole reaction

C. Methyl red reaction

D. All of these

2. Prodigiosin (red pigment) is produced by members of genus

A. Hafnia

B. Enterobacter

C. Serratia

D. Cirobacter

3.Enterobacter can be differentiated from Klebsiella by

A. ornithine positivity

B. motility

C. both (a) and (b)

D. none of these

4. Traveller's diarrhea is caused by

A. enteropathogenic Escherichia coli (EPEC)

B. entero invasive E. coli (EIEC)

C. entero invasive E. coli (ETEC)

D. entero haemorrhagic E. coli (EHEC)

5. Name two H_2S producing bacteria.

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6.Name two non motile Enterobacteriaceae.

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Exercise Number 16: Non lactose fermenters: Salmonella and Shigella

Learning objectives:
On completion of the exercise the students should be able to

- **a)** Understand the morphology and culture characteristics of Salmonella and Shigella.
- **b)** Appreciate the motility of Salmonella and Shigella.
- **c)** Know the important biochemical test to identify Salmonella and Shigella.
- **d)** Know the difference between Salmonella and Shigella.
- **e)** Know the serotypes of Salmonella and Shigella.

I. Salmonella

Introduction

The genera Salmonella comprises of gram negative rods which are non capsulated non sporing and motile bacteria .They are aerobe and facultative anaerobes and grow rapidly on ordinary media . Member of genus Salmonella are pathogenic to man .Typhoid fever is caused by Salmonella Typhi and paratyphoid fever is caused by Salmonella Paratyphi A and B. The term enteric fever includes both typhoid and paratyphoid fever . Other important infections caused by this genus are food poisoning and septicemia .

Important pathogenic species

a) S. typhi
b) S.paratyphi
c) S.typhimurium

Laboratory Diagnosis:

Sample : Depending on the site of infection specimen can be blood ,stool or urine .

Morphology : Gram negative rods 1-3µm x 0.5 µm and motile .

Cultural Characteristics

On Nutrient agar : smooth ,convex ,circular , 2-3 mm in diameter and are translucent.

On MacConkey Agar : Salmonella produces pale colonies due to non fermentation of lactose .

On Wilson and Blairs Bismuth sulphite medium – It produces black colonies due to production of H_2S.

Biochemical Characters : S. Typhi produce only acid and no gas from glucose . Do not ferment lactose sucrose and maltose .It ferments mannitol , do not utilize citrate ,MR positive,VP negative , Indole negative and citrate positive except S.typhi which is anaerogenic and citrate negative .

The isolated organism is confirmed by serotyping .

Widal test - Detection of circulating antibodies aganist the causative agents .

Most of the strains of S.Typhi have become resistant to multiple antibiotics . Multi drug resistant typhoid fever has become endemic in India .At present later fluoroquinolones and third generation ciprofloxacin are given . So antibiotic sensitivity should be done.

II. Shigella

It is a causative organism of bacillary dysentery . Dysentry is a clinical condition of multiple etiology ,characterized by the frequent passage of blood stained and mucopurulent stools .

Important pathogenic species

1. Shigella dysentriae
2. Shigella flexneri
3. Shigella boydii
4. Shigella soneii

Morphology : Shigella are short, gram negative rods 0.5x 1-3 mic m in size.They are non motile ,nonsporing and non capsulated .

Cultural Characterstics : They are aerobes and facultative anaerobes .They grow on ordinary media but less readily than other enterobacteriacae .

Blood Agar : After overnight incubation colonies are small , about 2mm

in diameter circular , convex , smooth and translucent .

MacConkey Agar: Colonies are colourless due to the absence of lactose fermentation .

Deoxycholate citrate Agar : Colonies are pale and tiny .

Biochemical Reactions

They are nitrate positive and MR positive . Citrate negative ,H2S negative Catalse is produced except Sh. dysentriae type 1. Glucose is fermented with out gas except some strains .

Demonstrations

The teacher should demonstrate the following things during the class:

a) Colony characteristics of Salmonella and Shigella.
b) Gram stained slides and motility of Salmonella and Shigella.
c) Common biochemical tests used for identification of Salmonella and Shigella.
a) Sero-typing of Salmonella .

Students Exercise:

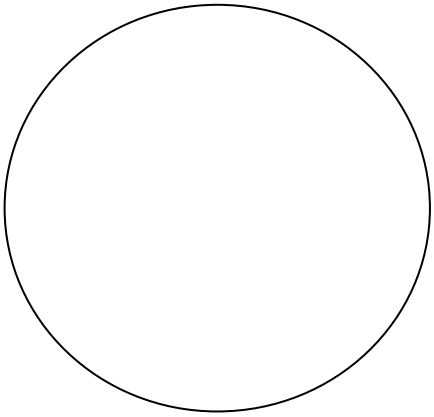
1. Write the culture characteristics of Salmonella.

	characters	Blood Agar	McConkey Agar	Nutrient Agar
1	Size			
2	Shape			
3	Surface			
4	Elevation			
5	Edge			
6	Opacity			
7	Consistency			
8	Colour			
9	Any change in medium			

2. Write the culture characteristics of Shigella.

	characters	Blood Agar	McConkey Agar	Nutrient Agar
1	Size			
2	Shape			
3	Surface			
4	Elevation			
5	Edge			
6	Opacity			
7	Consistency			
8	Colour			
9	Any change in medium			

3. Draw the findings of Gram stain done by you.



Observation

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3. Mention the motility characteristics of Salmonella and Shigella.

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4. Mention the biochemical reactions of of Salmonella and Shigella.

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5. Mention the principle of Widal test.

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Exercise Number 17:*Vibrio cholerae* and other Vibrio like organisms

Learning objectives:
On completion of the exercise the students should be able to**:**

- **a)** To understand the morphology and culture characteristics of *Vibrio cholerae*.
- **b)** To appreciate the darting motility of *Vibrio cholerae*.
- **c)** To know important bio-chemical tests to identify *Vibrio cholerae*.
- **d)** To know the differences between classical and El Tor Vibrios.
- **e)** To know the other non-cholera Vibrios and their medical importance.

Introduction:

Vibrios are Gram negative, rigid, curved rods that are actively motile by means of a polar flagellum. *Vibrio cholerae*, the causative agent of cholera is the most important species in this genus.

Cholera is a potentially epidemic and life-threatening secretory diarrhoea characterized by numerous, voluminous watery stools, often accompanied by vomiting, and resulting in hypovolemic shock and acidosis. It is caused by certain members of the species *Vibrio cholerae* which can also cause mild or inapparent infections. The diarrhea is mediated by entero-toxin and endo-toxin produced by the organism.

Other pathogenic members of the genus are non-agglutinable (NAG) vibrios and *V. parahemolyticus.* NAG vibrios produce mild cholera like syndrome and *V. parahemolyticus* causes marine food poisoning.

Laboratory diagnosis: includes microscopy to demonstrate darting motility and curved rods; culture on special media; biochemical and agglutination reactions.

Sample: Watery rice water stool

Microscopy: Demonstration of characteristic "darting" motility of the organism in fresh or enriched stool specimen is done in hanging drop preparation; and its inhibition by appropriate antisera confirms the diagnosis. Gram stain of diarrheac stool may reveal curved Gram negative rods.

Culture: A number of special media have been employed for the cultivation of vibrios. Th ey are classified as follows:

Transport media:

a) Venkataraman-Ramakrishnan medium at pH 8.6-8.8.
b) Cary Blair medium at pH 8.4
c) Autoclaved sea water

Enrichment media

a) Alkaline peptone water at pH 8.6
b) Monsur's taurocholate tellurite peptone water at pH 9.2

Plating media

a) Alkaline bile salt agar (BSA): The colonies are round, about 1-2 mm-diameter, moist translucent with a bluish tinge in transmitted light.
b) Monsur's gelatin Tauro cholate trypticase tellurite agar (GTTA) medium: Cholera vibrios produce small, translucent colonies with a greyish-black center.
c) TCBS medium: This is the mostly widely used medium; it contains thiosulphate, citrate, bile salts and sucrose. Cholera vibrios produce flat, 2–3-mm-diameter, yellow-nucleated colonies.

Biochemical Reactions:

Sl no	Biochemical Tests	Expected Reactions
1	Oxidase	Positive
2	Nitrate	Positive
3	Indole	Positive
4	Triple sugar iron agar	A/A, no gas, no H_2S
5	Glucose (acid production)	Positive
6	Glucose (gas production)	Negative
7	Sucrose (acid production)	Positive
8	Lysine	Positive
9	Arginine	Negative
10	Ornithine	Positive
11	Cholera red reaction	Positive
12	String test	Positive

Diagnosis can be confirmed, as well as serotyping done by agglutination with specific sera.

Prophylaxis: An ideal cholera vaccine is yet to be found. Two types of oral vaccines are available- killed oral whole cell vaccines with and without inclusion of the B subunit of cholera toxin; and live oral vaccines with classical, El Tor and O-139 strains.

Demonstrations:
The teacher should Demonstrate the following things during the class:

a) Demonstrate the darting motility in hanging drop preparation and morphology of *Vibrio cholerae* in Gram stained smears.
b) Demonstrate the growth of *V. cholerae* in blood agar and TCBS agar.
c) Display the important biochemical reactions of *V. cholerae*, viz- oxidase test, indole test, cholera red reaction, string test etc.

Students Exercise:

- Perform Gram stain on the given fixed smear and observe curved bacilli of *V. cholerae*.
- Make a hanging drop preparation and observe darting motility of *V. cholerae.*

1. Which specimen is collected for laboratory diagnosis of cholera?

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2. How will you collect stool specimen for laboratory diagnosis of cholera?

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3. What is meant by darting motility? How will you demonstrate it?

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4. The wet smear examination of rice-water stools shows ABSENCE of following:

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5. How will transport a stool specimen from a suspected case of cholera to the laboratory at a distant place?

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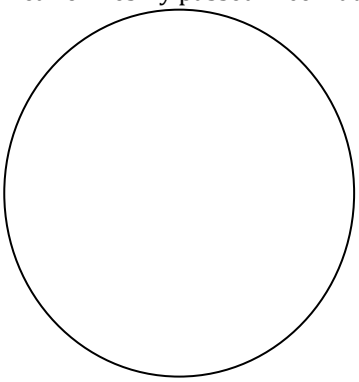
6. Draw a diagram of Gram stained smear of freshly passed 'rice water' stool showing *V. cholerae.*



7. Mention the important culture media used in diagnosis of *V. cholerae.*

8. Describe the important biochemical reactions of *V. cholerae.*

9. How is an isolate of *Vibrio cholerae* confirmed?

10. How will you differentiate between classical and El Tor vibrios?

11. Mention the immunization against cholera.

Exercise Number 18 : Proteus and Pseudomonas

Learning objectives:

On completion of the exercise the students should be able to

- **a)** Enumerate the important pathogenic species of *Proteus* and *Pseudomonas.*
- **b)** Describe the cultural characteristics and biochemical properties of *Proteus* and *Pseudomonas.*

I) *Proteus*

Introduction:

The genus *Proteus* comprises of motile, Gram-negative rods with peritrichous flagella, and are assigned to the family Enterobacteriaceae. *Proteus* species are most commonly found in the human intestinal tract as part of normal human intestinal flora, along with *Escherichia coli* and *Klebsiella* species, of which *E coli* is the predominant resident. *Proteus* is also found in multiple environmental habitats, including long-term care facilities and hospitals.

Important pathogenic species:

1) ***P. mirabilis***: P. mirabilis causes 90% of Proteus infections .It causes serious urinary tract infections(in elderly and young males and often following catheterization or cystoscopy. Infections are also associated with the presence of renal stones), abdominal and wound infections(often a secondary invader of ulcers, pressure sores, burns and damaged tissues), septicemia, occasionally meningitis and lung infections.
2) ***P. vulgaris***: This species is occasionally isolated from urine, pus, and other specimens. Infections usually respond better to antimicrobial therapy than those caused by *P. vulgaris* and other related organisms.

Laboratory Diagnosis:

Sample: Depending on the site of infection, specimen can be urine, pus, sputum, blood or CSF.

Morphology: *P. mirabilis* and *P. vulgaris* are actively motile, noncapsulate, Gram negative pleomorphic rods. The size of individual cells varies from 0.4-0.6µm X 1.2-2.5µm.

Cultural Characteristcs: *Proteus* grows well in most of the media used commonly in laboratory. Optimum growth temperature is 35-40^{0}C.

On **Blood agar**: *Proteus* forms non hemolytic swarming growth on blood agar. The swarming is due to the high motility of the bacteria.

On **MacConkey agar**: *Proteus* produces individual non-lactose fermenting colonies after overnight incubation at 35–37^{0}C. Swarming is prevented on MacConkey agar and XLD agar because these media contain bile salts. Swarming is inhibited on CLED agar because it is electrolyte deficient.

Swarming: it is a rapid (2–10 µm/s) and coordinated translocation of a bacterial population across solid or semi-solid surfaces. Generally motile bacteria find it difficult to move over solid media and hence forms defined colonies on the hard surface. But organism like Proteus shows higher degree on molitlity and forms concentric layers/waves of bacterial growth.

figure: A typical Proteus growth on Blood agar

Biochemical Characters: Lactose non-fermenter , Rapidly hydrolyze urea (within 4 hours), Phenylalanine deaminase (PDA), positive. Beta-galactosidase (ONPG) negative, Indole negative (P. vulgaris is indole positive).

Table:16.1: *Proteus* biochemical properties

Biochemicals	***P. mirabilis***	***P.vulgaris***
Nitrate	+	+
Indole	-	+
MR	+	+
VP	+/-	-
Citrate	+/-	-/+
Urease	+	+
ONPG	-	-
TSI	Red/Yellow, Gas	Red/Yellow, Gas
H_2S	++	++
Lactose	-	-
Glucose	+	+
Sucrose	-/+	+
Arginine	-	-
Ornnithine	+	-
Lysine	-	-
PDA	+	+

+/- mostly positive

-/+ mostly positive

++ strongly positive

Red -alkaline reaction

Yellow -acidic reaction

Antimicrobial susceptibility: Antibiotics with activity against P. mirabilis include ampicillin, cephalosporins and aminoglycosides. Some strains of P. mirabilis are beta-lactamase producing and therefore resistant to ampicillin. Proteus species are resistant to polymyxin and nitrofurantoin.

II) ***Pseudomonas***

Pseudomonas is a genus of Gram-negative aerobic gamma-proteobacteria, belonging to the family Pseudomonadaceae, and contains 191 species. *Pseudomonas* can be found in the intestinal tract, water, soil and sewage and is frequently found in moist environments in hospitals(sinks, cleaning buckets, drains, humidifiers etc). It is able to grow in some eye drops (especially quaternary ammonium compounds), saline and other aqueous solutions.

Important pathogenic species:

1) ***P. aeruginosa:*** Causes skin infections, especially burn sites, wounds, pressure sores, and ulcers (often as secondary invader), Septicemia, urinary tract infections, usually following catheterization or associated with chronic urinary disease. Respiratory infections are especially in patients with cystic fibrosis or conditions that cause immuno-suppression. External ear infections (otitis externa) and eye infections is also common, often secondary to trauma or surgery.

2) ***P. putida*** and ***P. fluorescens*** :These are less commonly isolated opportunistic pseudomonads of low pathogenicity. They are however able to grow at 4^0C and are occasionally found as contaminants of blood stored in blood banks. Severe reactions can occur if such contaminated blood is transfused.

Laboratory Diagnosis:

Sample: Depending on the site of infection, specimen can be urine, pus, sputum, blood, or effusions etc.

Morphology: *Pseudomonas* are motile (one or more polar flagella), rod shaped and aerobic, Gram-negative, non-fermentative bacteria. The typical bacterial size is 0.5 – 1.0 x 1.5– 5.0 µm.

Cultural Characteristcs: *P. aeruginosa* is an obligatory aerobe. It is usually recognized by the pigments it produces including pyocyanin a blue-green pigment, and pyoverdin (fluorescein) a yellow-green fluorescent pigment. A minority of strains are non-pigment producing. Cultures have a distinctive smell due to the production of 2-aminoacetophenone. *P. aeruginosa* grows over a wide temperature range 6–42 ºC with an optimum of 35–37 ºC.

On Blood agar: *P. aeruginosa* produces large, flat, spreading colonies which are often haemolytic and usually (90% of strains) pigment-producing.The pigments diffuse into themediumgiving it a dark greenish-blue colour. Some strains produce small colonies or mucoid colonies. When the culture is left at room temperature, pigment colour becomes more intense.

On MacConkey agar: *P. aeruginosa* produces pale pink coloured (lactose non-fermenting) colonies on MacConkey agar. Compared with blood agar, pigment production is less marked.

Biochemical Characters: P. aeruginosa is oxidase positive and produces acid only from glucose (no gas). These features together with the typical pigments produced by most strains and the distinctive smell of cultures are usually sufficient to identify the organism. Growth at 42^0C differentiates *P. aeruginosa* from the less commonly isolated *P. putida* and *P. fluorescens*.

Table:16.2: *Pseudomonas* biochemical properties

	Indole	MR	VP	Citrate	Urease	TSI	Oxidase	Growth at 4^0C	Growth at 42^0C	Mannitol	Maltose
P. aeruginosa	-	-	-	+	-	Red/red	+	-	+	+	-
P. putida	-	-	-	+	-	Red/red	+	-	+	+	-
P. fluorescens	-	-	-	+	-	Red/red	+	+	_	_	_

Antimicrobial susceptibility

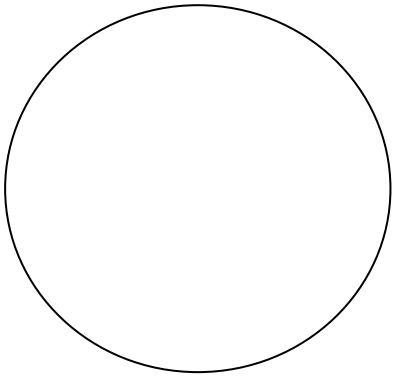
P. aeruginosa is resistant to most of the commonly used antibiotics. Antimicrobials that usually show activity against Pseudomonas include aminoglycosides, polymyxin, and some penicillins and cephalosporins

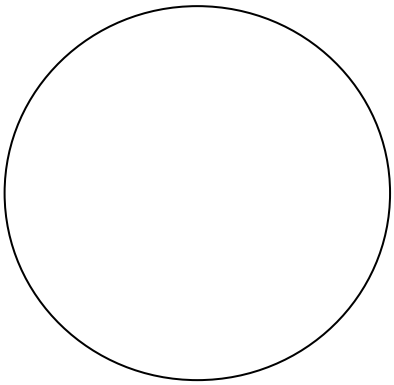
Demonstrations

The teacher should demonstrate the following things during the class:

a) Colony characteristics of Proteus and Pseudomonas. (Swarming and pigment production)
b) Gram stained slides and motility of Proteus and Pseudomonas.
c) Common biochemical tests used for identification of Proteus and Pseudomonas.

Students Exercise:





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1. Write the colony characteristics of:

    a) Pseudomonas on Nutrient agar……

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    b) Proteus on Blood agar……

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2. Write the TSI reactions of Both Pseudomonas and Proteus.

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3. what is the characteristic odor produced by the colonies of Pseudomonas and Proteus?

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4. Why Proteus fails to swarm on CLED agar?

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Exercise Number 19: Spirochetes

Learning objectives:
On completion of the exercise the students should be able to:

a) Enumerate the important genera of spirochaetes
b) Identify serological tests for syphilis.

Introduction:

Spirochaetes are thin-walled, flexible, spiral rods. They are motile through the undulation of axial filaments that lie under the outer sheath. Three genera of Spirochaetes cause human infection:

- ***Treponema.***
- ***Borrelia.***
- ***Leptospira.***

Disease-causing members of these genera including the following:

Sl no	Organism	Disease
1	*Treponema pallidum*	syphilis
2	*Treponema pertenue*	yaws
3	*Treponema carateum*	pinta
4	*Treponema endemicum*	bejel.
5	*Borrelia burgdorferi*	Lyme disease
6	*Borrelia recurrentis*	relapsing fever
7	*Leptospiral species*	leptospirosis

Treponemes and Leptospirae are so thin that they are seen only by dark-field microscopy, silver impregnation or immunofluorescence. When examined by dark-field microscope, spirochaetes have characteristic motility, including apparent rotation around their long axis and a boring corkscrew rotation.

Laboratory diagnosis of *T. pallidum*:

Microscopic morphology: T. pallidum is a thin, delicate, lightly wound spirochaete. T. pallidum cannot be seen in Gram stain smears. The organisms are best seen by dark-field microscopy in serous fluid collected from a primary chancre or secondary skin lesion.

Culture: Pathogenic Treponemes have not yet been reproducibly cultured in the routine laboratory. The organisms, however, are able to survive in some fluids, including donated blood.

Non-specific serological tests: These tests involve the use of nontreponemal antigen. Extracts of normal mammalian tissues (e.g. Cardiolipin) react with antibodies in serum samples from patients with syphilis. These antibodies, which are a mixture of IgG and IgM, are called “reagin” antibodies. Flocculation tests e.g. VDRL (Venereal Disease Research Laboratory) and RPR (Rapid Plasma Reagin) tests, detect the presence of these antibodies.

Specific serological tests: These tests involve the use of treponemal antigens and therefore are more specific than the non-specific tests. In these tests, T. pallidum reacts in immunofluorescence (FTA-ABS) or haemagglutination (TPHA, MHA-TP) assays.

Rapid Plasma Reagin test:

Rapid Plasma Reagin (RPR) refers to a type of test that looks for non-specific antibodies in the blood of the patient that may indicate that the organism (*Treponema pallidum*) that causes syphilis is present. The term "reagin" means that this test does not look for antibodies against the actual bacterium, but rather for antibodies against substances released by cells when they are damaged by *T. pallidum*.

In addition to screening for syphilis, an RPR level (also called a "titer") can be used to track the progress of the disease over time and its response to therapy.

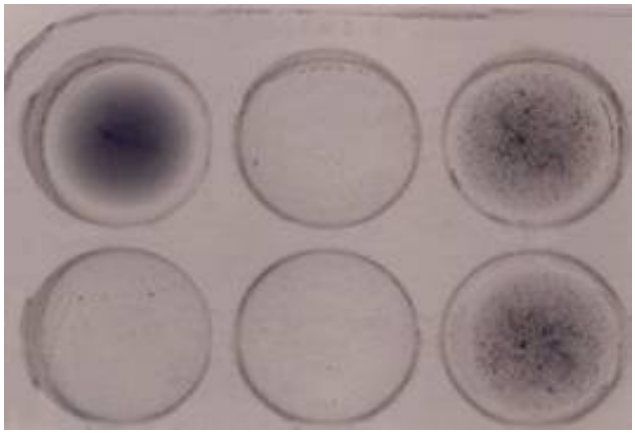
Principle:

RPR Card Test is a non-treponemal test for the serologic detection of syphilis and is recommended when venous blood collection is employed. In this method, carbon particle cardiolipin antigen detects "reagin" a substance present in serum from syphilitic persons, and occasionally in serums of persons with other acute or chronic conditions. Specimens that contain reagin cause flocculation with a coagulation of the carbon particles of the RPR Card antigen, which appear as black clumps against a white background. The coagulation can be read macroscopically. In contrast, non-reactive specimens appear to have uniform light-gray color.

Specimen required:

2ml serum collected in a red top tube without anticoagulant. Separate blood as soon as possible to prevent hemolysis. Store sera at 2-8°C and use within 48 hours. Freeze at -80°C if not tested within 48 hours. Plasma: Collect blood in tubes containing EDTA as anticoagulant. Keep plasma in original collection tube and centrifuge prior to use. If not used immediately, store at 2-8°C and use within 48 hours of collection.

Procedure : Agglutination Test



Result:Reactive/Non reactive

Laboratory diagnosis of *Leptospira:*

Leptospirosis is a disease having widespread manifestations with major brunt on liver and lungs. Diagnosis is achieved by following means:

- Direct examination of blood, body fluids and tissues under dark ground microscope.
- Culture of leptospires from clinical specimens.
- Testing for antibodies against leptospires.
- Molecular approaches.

Laboratory diagnosis of *Borrelia:*

Borrelia causes borreliosis, a zoonotic, vector-borne disease transmitted primarily by ticks and some by lice, depending on the species. Laboratory diagnosis mainly relies on serological tests and immune-fluorescence tests.

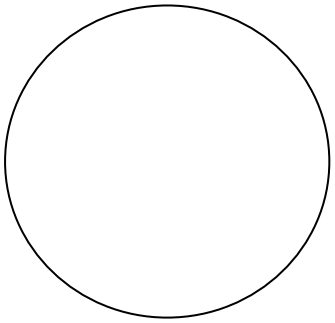
Demonstrations

The teacher should demonstrate the following things during the class:

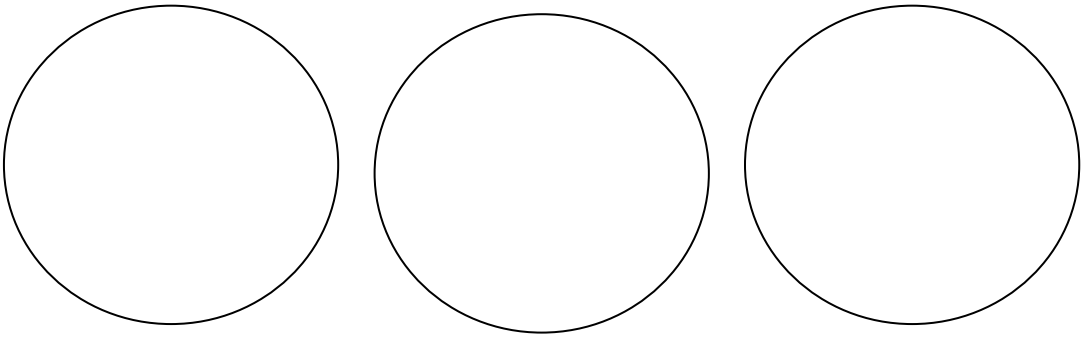
a) Serological tests for syphilis
b) Slides/photos of Treponema, Borrelia and Leptospira.
c) Antibody test kit for leptospirosis.

Students Exercise:

1. Record the readings of RPR test given to you. Draw a labelled diagram.



2. Draw a labeled diagram of the slides shown to you.



3. Which two stages of syphilis are most infectious?

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4. What disease state(s) other than syphilis can cause a true positive RPR and FTA-ABS and why?

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5. What are specific problems when performing laboratory testing to diagnose congenital syphilis?

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6. What test is used to diagnose Neurosyphilis?

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7. Interpret the following results:

(i.e., biological false positive, primary syphilis, secondary syphilis, etc.)

Observation	Interpretation
a. RPR: reactive FTA-ABS: reactive	
b. RPR: nonreactive FTA-ABS: reactive	
c. RPR: nonreactive FTA-ABS: nonreactive in the past (assuming immunocompetent)	
d. RPR: reactive FTA-ABS: nonreactive	
e. EIA screen: reactive RPR: reactive RPR titer: 1:32	

Exercise Number 20: Actinomycetes and Nocardia

Learning objectives:
On completion of the exercise the students should be able to do:

- **a)** Enumerate the pathogenic species of Actinomyces and Nocardia.
- **b)** Identification of Actinomyces and Nocardia slides

Introduction:

Actinomycetes

Actinomycosis is a chronic suppurative and granulomatous disease of the cervico-facial, thoracic or abdominal areas.The most common cause of actinomycosis is the organism *Actinomyces israelii* which infects both man and animals. In man, *A. israelii* is an endogenous organism that can be isolated from the mouths of healthy people. Frequently, the infected patient has a tooth abscess or a tooth extraction and the endogenous organism becomes established in the traumatized tissue and causes a suppurative infection. These abscesses are not confined to the jaw and may also be found in the thoracic area and abdomen.

Laboratory diagnosis:

The patient usually presents with a pus-draining lesion, so the pus will be the clinical material sent to the laboratory. This diagnosis can be made on the hospital floor. When the vial of pus is rotated, the yellow sulfur granules, characteristic of this organism, can be seen with the naked eye. These granules can also be seen by running sterile water over the gauze used to cover the lesion. The water washes away the purulent material leaving the golden granules on the gauze.

This organism, which occurs worldwide, can be seen histologically as "sulfur granules" surrounded by polymorphonuclear cells (PMN) forming the purulent tissue reaction. The organism is a gram positive rod that frequently branches.

The laboratory must specifically be instructed to culture for this anaerobic organism. These lesions must be surgically drained prior to antibiotic therapy and the drug of choice is large doses of penicillin.

Nocardia

Nocardiosis primarily presents as a pulmonary disease or brain abscess in the U.S. In Latin America, it is more frequently seen as the cause of a subcutaneous infection, with or without draining abscesses. It can even present as a lesion in the chest wall that drains onto the surface of the body similar to actinomycosis. Brain abscesses are frequent secondary lesions.

The most common species of *Nocardia* that cause disease in human beings are *N. brasiliensis* and *N. asteroides*. These are soil organisms which can also be found endogenously in the sputum of apparently healthy people. *N. asteroides* is usually the etiologic agent of pulmonary nocardiosis while *N. brasiliensis* is frequently the cause of sub-cutaneous lesions.

Laboratory diagnosis:

The material sent to the lab, depending on the presentation of the disease, is sputum, pus, or biopsy material. These organisms rarely form granules.

The *Nocardia* are aerobic and grow readily on most bacteriologic and TB media. These are gram-positive rods and stain partially acid-fast (i.e., the acid-fast staining is not uniform) . There are no serological tests.

Demonstrations

The teacher should Demonstrate the following things during the class:

a) Gram stained slides of actinomyces granules and Nocardia.
b) ZN stained slides of Nocardia.

Students Exercise:

1. What is the most common type of actinomycosis?
   A. Thoracic
   B. abdominal
   C. Cervicofacial
   D. Genital

2. Nocardiosis is caused by which of the following?
   A. Actinomyces nocardia
   B. Nocardia asteroides
   C. Actinomyces asteroides
   D. Nocardia actinomyces

3. Why is Nocardia acid fast?

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4. What are the differences between bacteria ,fungi and actinomycetes.

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Exercise Number 21: Medically important fungi.

Learning objectives:
On completion of the exercise the students should be able to:

- **a)** Know the important fungi which are pathogenic to humans, and the infections caused by them.
- **b)** Know the specimens needed to be collected for diagnosis of fungal infections.
- **c)** Know the methods of diagnosing fungal infections.

Introduction:

The term "mycology" is derived from Greek word "mykes" meaning mushroom. Therefore mycology is the study of fungi. The ability of fungi to invade plant and animal tissue was observed in early 19th century but the first documented animal infection by any fungus was made by Bassi, who in 1835 studied the muscardine disease of silkworm and proved the that the infection was caused by a fungus Beauveria bassiana.

In 1910 Raymond Sabouraud published his book Les Teignes, which was a comprehensive study of dermatophytic fungi. He is also regarded as father of medical mycology.

<u>Classification of fungi:</u>

Based on Sexual reproduction:

1. Zygomycetes: which produce through production of zygospores
2. Ascomycetes: which produce endogenous spores called ascospores in cells called asci.
3. Basidiomycetes: which produce exogenous spores called basidiospores in cells called basidia.
4. Deuteromycetes (Fungi imperfecti): fungi that are not known to produce any sexual spores (ascospores or basidiospores). This is a heterogeneous group of fungi where no sexual reproduction has yet been demonstrated.

Based on Morphology:

1. Moulds (Molds): Filamentous fungi Eg: *Aspergillus* sps, *Trichophyton rubrum*
2. Yeasts**:** Single celled cells that buds Eg: *Cryptococcus neoformans, Saccharomyces cerviciae*
3. Yeast like**:** Similar to yeasts but produce pseudohyphae Eg: *Candida albicans*
4. Dimorphic: Fungi existing in two different morphological forms at two different environmental conditions. They exist as yeasts in tissue and in vitro at 37 0C and as moulds in their natural habitat and in vitro at room temperature. Eg: *Histoplasma capsulatum, Blastomyces dermatidis, Paracoccidiodes brasiliensis, Coccidioides immitis* .

Some 200 "human pathogens" have been recognized from among an estimated 1.5 million species of fungi.

Fungal Diseases (Mycoses):

Mycoses can be conveniently studied as:

1. **Superficial mycoses**
   I. Superficial phaeohyphomycosis

II. Tinea versicolor
III. Black piedra
IV. White piedra

2. **Cutaneous mycoses**
   I. Dermatophytosis
   II. Dermatomycosis
3. **Subcutaneous mycoses**
   I. Chromoblastomycosis
   II. Rhinosporidiasis
   III. Mycetoma
   IV. Sporotrichosis
   V. Subcutaneous phaeohyphomycosis
   VI. Lobomycosis
4. **Systemic (deep) mycoses**
   I. Blastomycosis
   II. Histoplasmosis
   III. Coccidioidomycosis
   IV. Paracoccidioidomycosis
5. **Opportunistic mycoses**
   I. Candidiasis
   II. Cryptococcosis
   III. Aspergillosis
6. **Other mycoses**
   I. Otomycosis
   II. Occulomycosis
7. **Fungal allergies**
8. **Mycetism and mycotoxicosis**

Laboratory diagnosis of mycoses:

Specimen collection:

specimen collection depends on the site affected. Different specimens include hair, skin scrapings, nail clippings, sputum, blood, CSF, urine, corneal scraping, discharge or pus from lesions and biopsy.

- All specimens must be transported to the laboratory without any delay to prevent bacterial overgrowth. In case of delay specimens except skin specimen, blood and CSF may be refrigerated for a short period.

- Infected hairs may be plucked using forceps. Those hairs that fluoresce under Wood's lamp may be selectively plucked. Hairs may be collected in sterilized paper envelopes.

- Surface of the skin must be disinfected with spirit before specimen collection. The advancing edge of the lesion is scraped with the help of a blunt forceps and collected in sterilized paper envelopes.

- Discoloured or hyperkeratotic areas of nail may be scraped or diseased nail clipping may be collected in sterilized paper envelopes.

- Specimens from mucus membranes (oral) must be collected by gentle scraping and transported to laboratory in sterile tube containing saline. Swabs may be collected from vagina.

- Corneal scrapings may be collected using a fine needle and inoculated at bedside.

- Pus may be collected by aspiration; use of cotton swabs may give false positive microscopic results.
- Clean catch urine may be collected in a sterile wide-mouthed container.
- Biopsy specimens must be transported in saline. In certain cases, pus or exudates must be looked for presence of granules.

Microscopy:

Microscopy is used to observe clinical specimens for the presence of fungal elements or to identify the fungus following culture. In the latter case, lactophenol cotton blue is stain of choice, which stains the fungal elements blue. Direct examination of clinical specimens could be stained or unstained.

- **Wet mount**: *Candida* may be observed in urine wet mounts
- 10-20% KOH mount: Several specimens are subjected to KOH mount for direct examination. The material is mixed with 20% KOH on a slide and a cover slip is placed. The slide is then gently heated by passing through the flame 2-3 times. The slide is observed on cooling. KOH serves to digest the protein debris and clears keratinised tissue and increases the visibility. Addition of Dimethyl sulphoxide (DMSO) permits rapid clearing in the absence of heat.
- **Calcofluor white:** This is a fluorescent dye, which binds selectively to chitin of the fungal cell wall. The specimen then can be observed under fluorescent microscope.
- **India Ink:** Capsules of Cryptococcus neoformans can be demonstrated by this negative staining technique.
- **Periodic Acid-Schiff (PAS) stain:** On staining by this stain, fungal elements appear bright magenta coloured while the background stains green. It is useful in staining tissue specimens
- **Giemsa's stain:** It is particularly useful in the detection of Histoplamsa capsulatum in the bone marrow smears.
- **Haematoxylin and Eosin (H&E) stain:** Useful for staining tissue sections.
- **Gomori's methenamine silver nitrate (GMS) stain:** Outlines of the fungi are black, internal parts stain pinkblack while the background stains light green. Candida and Aspergillus may be missed in H&E stained **sections,** therefore GMS stained sections are essential for tissue pathology.
- **Gridley's stain: It** stains hyphae and yeasts dark blue-pink, tissues deep blue and background yellow.
- **Meyer mucicarmine stain:** Capsules of C. neoformans and inner walls of Rhinosporidium seeberi's sporangium are stained pink.
- **Gram stain:** Candida is best demonstrated in clinical specimen by Gram stain.
- **Masson-Fontana:** stain is helpful in staining phaeoid (dematiaceous) fungi in tissue.
- **Immunofluorescence:** Monoclonal antibody labelled with fluorescent dyes can be used to detect several fungi in the clinical specimens.

Culture:

One of the most common media used to culture fungi in laboratory is Sabouraud's Dextrose Agar (SDA). It consists of peptone, dextrose and agar. High concentration of sugar and a low pH (4.5-5.5) prevents growth of most bacteria and makes it selective for fungi. Emmon's modification of SDA contains 2% dextrose and has pH of 6.8.

Other basal media to grow fungi include Potato Dextrose Agar, Malt Extract Agar etc. Most fungi are able to grow at room temperature while few pathogenic fungi (e.g, Cryptococcus, dimorphic fungi) can grow at 37^{o}C. Saprophytic fungi grow much quickly than pathogenic fungi (e.g, dermatophytes). In such situations the saprophytic fungi can be inhibited by the addition of cycloheximide (actidione) to the SDA. Addition of antibiotics such as Chloramphenicol, Gentamicin or Streptomycin to SDA serves to inhibit bacterial multiplication. An example of SDA with cycloheximide and Chloramphenicol is Mycosel agar.

Other specialized media used for different fungi include:

• Brain Heart Infusion Agar general isolation of fungi and conversion of dimorphic fungi.

• Inhibitory Mould Agar, an isolation medium with Chloramphenicol to suppress most bacteria.

• Caffeic Acid Agar and Birdseed Agar for isolation of Cryptococcus neoformans.

• Corn Meal Agar: Enhances production of chlamydospores in Candida albicans and formation of conidia in fungi.

• Trichophyton Agars: Used for selective identification of Trichophyton species.

• Dermatophyte Test Medium: Used for recovery of dermatophytes from clinical specimens.

• Sabhi Medium: Isolation of Histoplasma capsulatum.

• 'CHROM agar Candida' is useful in identification of Candida species.

Conversion of mould to yeast phase must be demonstrated in vitro for identification of dimorphic fungi. Since some fungi grow slowly cultures should not be discarded for 4-6 weeks. Fungi are identified on the basis of colony morphology (including pigmentation) and microscopic observation by tease-mount preparation or slide culture technique**.**

Serology:

Detection of anti-fungal antibody is helpful in diagnosis of sub-cutaneous and systemic mycoses, prognosis and response to anti-fungal drugs. Different serologic techniques that are used include agglutination, immunodiffusion, counter-immunoelectrophoresis, complement fixation test, immunofluorescence, RIA and ELISA.

Antigen detection:

It is particularly useful in the diagnosis of cryptococcal meningitis from CSF specimens. The test is performed by Latex Agglutination or immunodiffusion tests. It is also helpful in the detection of Aspergillus and Candida antigens in systemic infections.

Skin tests:

Delayed hypersensitivity reactions to fungal antigens can be demonstrated by skin tests. A positive skin does not necessarily indicate an active infection; it only indicates sensitization of the individual. Hence, its value is in epidemiological studies than diagnosis. These tests may be performed in Histoplasmosis, Candidiasis, Sporotrichosis, Coccidioidomycosis, Blastomycosis, Paracoccidiodomycosis and dermatophytosis.

Molecular techniques:

Newer techniques such as DNA hybridization, PCR are useful in diagnosis of mycoses in a shorter period as well as detect those fungi that are difficult or dangerous to cultivate in vitro.

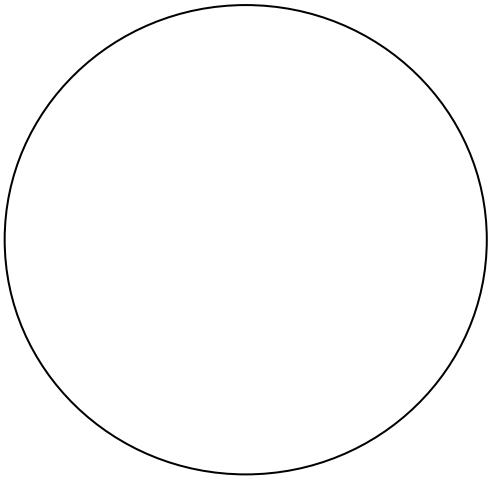
Demonstrations:

The teacher should demonstrate the following things during the class:

a) Methods of collection of specimens for diagnosing fungal infections.
b) Growth media for fungi.
c) Morphological features of medically important fungi.

Student Exercise:

1. Draw the diagram of the fungi shown to you.



2. Match the common name of the disease with the clinical name of the disease.

1. Darling's disease	A. paracoccidioidomycosis
2. Valley fever	B. oral candidiasis
3. Chicago disease	C. aspergillosis
4. South American or Brazilian Blastomycosis	D. blastomycosis
5. Mycotic Mycetoma	E. pseudoallescheriasis
6. Farmer's lung disease	F. histoplasmosis
7. Thrush	G. coccidioidomycosis
8. Balanitis	H. candidiasis of the penis

3. The two growth forms of fungi include:

   A. Yeast and hyphae
   B. Yeast and mould
   C. Blastoconidia and mould
   D. Conidia and hyphae

4. What is the atmospheric requirement for fungi to grow?

   A. Anaerobic
   B. Ambient air
   C. 5-10% CO2
   D. >10% CO2

5. In the Lactophenol aniline blue stain, what reagent stains the chitin in the fungal cell wall?

   A. Aniline blue
   B. Phenol
   C. Lactic acid
   D. Glycerol

6. The purpose for using a KOH preparation when doing a direct examination for fungi is to:

   A. Dissolve the keratin to unmask the fungus elements
   B. Stain the hyphae and conidia of the fungi
   C. Reveal capsules that may be found around yeast cells
   D. Kill any bacteria that may be present in the specimen

Exercise Number 22:Rickettsia, Chlamydia and Mycoplasma

Learning objectives:
On completion of the exercise the students should be able to

- **a)** Understand the morphology of the infectious agents Rickettsia ,Chlamydia , Mycoplasma and Viruses
- **b)** Know the culture media and growth characteristics of these infectious agents.
- **c)** Enumerate the important pathogenic species and infections /diseases caused by them .

Rickettsiae

Introduction:

Rickettsiae are obligate intracellular parasites. They are very short rods that are barely visible in the light microscope.Structurally, their cell wall resembles that of gram-negative rods, but they stain poorly with the standard Gram stain.

Rickettsiae are obligate intracellular parasites. Therefore, rickettsiae must be grown in cell culture, embryonated eggs, or experimental animals.

Rickettsiae are maintained in nature in certain arthropods such as ticks, lice, fleas, and mites and, with one exception, are transmitted to humans by the bite of the arthropod. The rickettsiae circulate widely in the bloodstream (bacteremia), infecting primarily the endothelium of the blood vessel walls.

Table 20.1: Summary of Selected Rickettsial Diseases.

Disease	Organism	Arthropod Vector	Mammalian Reservoir	Important in the United States
Spotted fevers				
Rocky Mountain spotted fever	R. rickettsii	Ticks	Dogs, rodents	Yes (especially in southeastern states such as North Carolina)
Rickettsial pox	R. akari	Mites	Mice	No
Typhus group				
Epidemic	R. prowazekii	Lice	Humans	No
Endemic	R. typhi	Fleas	Rodents	No
Scrub	R. tsutsugamushi	Mites	Rodents	No

Laboratory Diagnosis

Morphology : In smear from infected tissues appear as pleomorphic coccobacilli .3-.6 x .8 -.2 in size .They are non motile and non capsulated .They are gram negative and stain bluish purple with Giemsa and Casteneda stains and deep red with Machiavello and Giminez stains .

Cultivation :Rickettsia cannot grow in cell free media . They are readily cultivated in the yolk sac of developing chick embryos. They are grown on mouse fibroblast, HeLa, HEP2, Detroit and other continuous cell lines but tissue cultures are not satisfactory for primary isolation.

Of the serologic tests, the indirect immune-fluorescence and ELISA tests are most often used.

The Weil-Felix test is of historic interest but is no longer performed because its specificity and sensitivity are too low. A 4-fold or greater rise in titer between the acute and convalescent serum samples is the most common way the laboratory diagnosis is made. The Weil-Felix test is based on the cross-reaction of an antigen present in many rickettsiae with the O antigen polysaccharide found in *P. vulgaris* OX-2, OX-19, and OX-K.

Chlamydia

Intoduction:

Chlamydia are obligate intracellular bacterial parasites of humans, animals and birds.They differ from viruses as they posses both DNA & RNA , have cell walls and ribosomes, replicate by binary fission. It occurs in two forms the elimentary body and the reticulate body .

Important pathogenic species

C.trachomatis : leading cause of ocular and genital infection .It causes trachoma .:

C. psittaci : Clinical disease varies from mild influenza- like syndrome to fatal pneumonia .

C. pneumonia : It appears to be a common cause of respiratory disease in older children.

Lab Diagnosis

1. Microscopy:
   demonstration of inclusion or alimentary bodies : conjunctival scraping stained with Giemsa stain should be observed under microscope for the inclusion bodies .Immunofluoroscence is more sensitive and specific method of microscopic examination using monoclonal antibodies and can be done forcervical or urethral specimen
2. Culture:
   Isolation of Chlamydia can be done by inoculation into embryonated eggs , experimental animals and tissue cultures .
3. Demonstration of chlamydial antigen : commonly used method is microIF . ELISA is a rapid metod for screening .Molecular methods like DNA probes and amplification techniques is more sensitive and specific .
4. Demonstration of antibodies or hypersensitivity : may be done by group specific CF or type specific micro –IF .

Antibiotic sensitivity : Erythromycin ,tetracycline and azithromycin are most effective drugs .

Mycoplasma

Introduction:

Smallest free living organism known , have no cell wall , but are bounded by three layered cell membrane . Also known as PPLO (pleuropneumonialike organism). Important pathogenic species are *M.pneumoniae* and *M. hominis* .*M . pneumonia* cause mycoplasmal or atypical pneumonia *M. hominis* cause genital infection ,non gonococcal urethritis and pelvic inflammatory disease.

Lab Diagnosis:

1) Isolation of the mycoplasma : Specimen throat swab or respiratory secretion inoculated in mycoplasma medium containing glucose and phenol red . Growth may take 1-3 weeks . Growth is indicated by acid production. They don't produce spore , flagella or fimbria . Some may show gliding motility .They are gram negative but better stained with Giemsa .Colony is biphasic , with a fried egg appearance and best studied after staining by Dienes method.

2) Serological test :

specific test using mycoplasmal antigen**-** IF , HAI , metabolic inhibition

non specific methods : Streptococcus MG and cold agglutination tests

3) PCR

Antibiotic sensitivity: Treatment with erythromycin and tetracycline is usually effective.

Demonstrations:
The teacher should Demonstrate the following things during the class:

a) To show the power point presentation and various slides related to morphology, culture media and culture techniques of Rickettsia , Chlamydia , Mycoplasma.

Students Exercise:

1. Mention the insect vectors for the following rickettsial diseases:
   Scrub typhus -.....
   Rickettsial pox-.....
   Rocky Mountain Spotted Fever-.....

2. Mention the important diseases caused by the following Chlamydiae:
   C. trachomatis: .....
   C. psittaci:.....
   C. pneumoniaie: .....

3. Mention the ways of culturing rickettsiae and chlamydiae.
   .....
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4. Mention the laboratory diagnosis of mycoplasma.

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Exercise Number 23: Introduction to Parasitology and Protozoal Infections

(Including *Isospora* and *Cryptosporidium*)

Learning objectives:
On completion of the exercise the students should be able to

a) To know about different pathogenic parasites.
b) To know the classification of parasites and the important differences between various classes of parasites.
c) To know the laboratory diagnosis of parasitic infections.
d) To know the pathogenic and opportunistic protozoa.
e) To understand the morphology of important stages of protozoal parasites of medical importance.

Introduction:

Parasitology is the study of parasites and host-parasite relationship. The animal parasites belong to three phyla:

- Phylum Protozoa
- Phylum Platyhelminths
- Phylum Nemathelminthes

The study of parasites involves the knowledge of geographical distribution, habitat, morphology, life - cycle, modes of transmission, pathogenesis and clinical manifestations. The human medical parasitology essentially also involves the study of laboratory diagnosis, treatment and prophylactic measures for a given pathogenic parasite.

Protozoa are unicellular organisms consisting of a nucleus and cytoplasm. They are classified based on the locomotory organs into:

- **Rhizopoda:** Pseudopodia are the organs of locomotion, e.g. *Entamoeba histolytica*.
- **Ciliata:** Cilia are the organs of locomotion, e.g. *Balantidium coli*.
- **Sporozoa:** Do not have any organ of locomotion, e.g. *Plasmodium spp*., *Toxoplasma gondii*, *Isospora belli*, *Cryptosporidium spp*.
- **Mastigophora:** Possess one or more flagellae for locomotion, e.g. *Giardia spp*., *Trichomonas spp*., *Leishmania spp*.

Protozoa can also be classified based on their habitation in human body:

- **Intestinal protozoa:** Inhabiting the gastro intestinal tract.
- **Blood protozoa:** Inhabiting blood and blood forming organs.
- **Atrial protozoa:** Inhabiting mouth, vagina and urethera.

The following are medically important intestinal protozoa:

- ***Entamoeba histolytica:*** Causative agent of amoebic dysentery and colitis. It exists in two stages- vegetative form called trophozoite and cystic form. Laboratory diagnosis involves

detection of trophozoites and cysts in saline and iodine mounts of stool specimen, culture, immune-diagnostic tests and molecular tests.

- ***Giardia spp.:*** Most important species is *G. intestinalis*. Causative agent of steatorrhoea and mal-absorption syndrome. It exists in trophozoite and cystic forms. Laboratory diagnosis involves detection of trophozoites and cysts in saline and iodine mounts of stool specimen, culture, immune-diagnostic tests and molecular tests.

- ***Cryptosporidium spp.:*** It is an opportunistic pathogen. Most important species is *C. parvum*. Laboratory diagnosis involves detection of oocysts in smears of stool specimen stained with modified Zeihl-Neelsen stain, fluorescence microscopy, immune-diagnostic tests and molecular tests.

- ***Isospora belli:*** This is also an opportunistic parasite. Laboratory diagnosis involves detection of oocysts in wet mounts of stool specimen and in smears stained with modified Zeihl-Neelsen stain, fluorescence microscopy, immune-diagnostic tests and molecular tests.

Wet mounts: For detection of ova, larvae, trophozoites, cysts and segments of enteric parasites saline and iodine mounts are prepared for observation under microscope.

Saline wet mount: Label a microscope slide with the patient's lab number and with the help of an applicator stick mix a small portion of the specimen with a drop of saline on the slide. Apply cover slip taking care to reduce the chances of forming air bubbles in the mount. Scan under 10X objective; confirm any suspicious structure using higher magnification.

Iodine wet mount: On a labeled microscope slide, make emulsion of stool specimen in a drop of 1% iodine. Apply cover slip. Scan under 10X objective; confirm any suspicious structure using higher magnification.

Demonstrations:

The teacher should Demonstrate the following things during the class:

a) Demonstrate the preparation of saline and iodine mounts.
b) Demonstrate the trophozoites of *E. histolytica* and *G. intestinalis* in saline mounts/ photographs.
c) Demonstrate the cysts of *E. histolytica* and *G. intestinalis* in iodine mounts/ photographs.
d) Familiarize the students with differences between amoebic and bacillary dysentery.
e) Familiarize the students with differences between cysts of *E. histolytica* and other non-pathogenic amoebae e.g. *E. coli*, *Iodamoeba sp.* etc.
f) Demonstrate the oocysts of *Isospora belli* and *Cryptosporidium spp.* in smears stained with modified Zeihl-Neelsen stain.

Students Exercise:

- Prepare saline and iodine wet mounts of the specimen provided to you and observe cysts of *G.intestinalis* and *E.histolytica*.

1. What are the differences between amoebic dysentery and bacillary dysentery?

   .....
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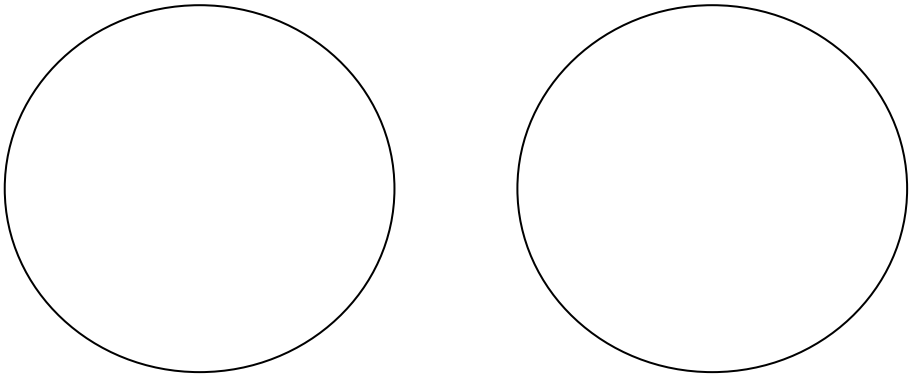
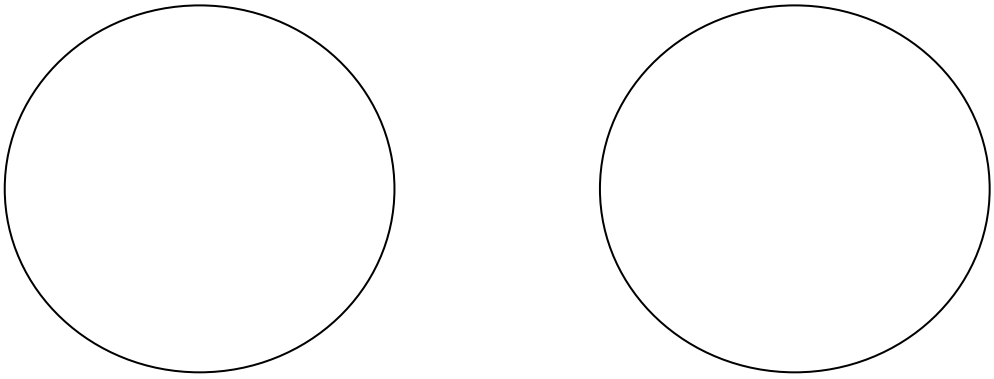
2. Draw the diagrams of following protozoal parasites:

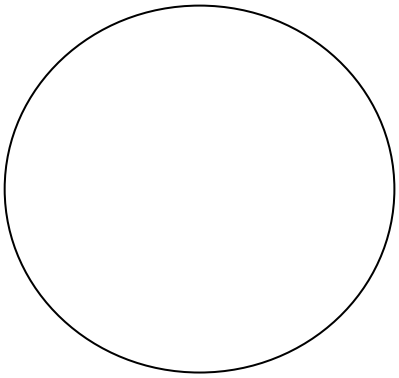
   a) Trophozoite and cyst of *E. histolytica* (saline and iodine wet mounts respectively)



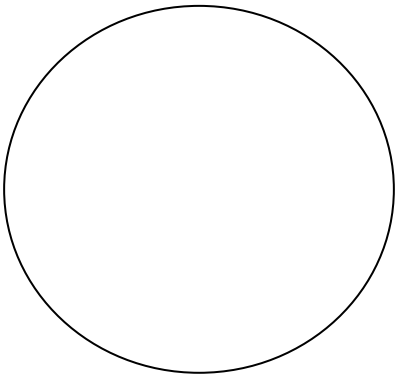
   b) Trophozoite and cyst of *G. intestinalis* (saline and iodine wet mounts respectively)



c) Oocyst of *Isospora belli* (modified ZN stained smear)



d) Oocysts of *Cryptosporidium spp.* (modified ZN stained smear)



Exercise Number 24: Leishmaniasis

Learning objectives:

On completion of the exercise the students should be able to

- **a)** Enumerate the important species of Leishmania found in India.
- **b)** Differentiate the different morphological forms of leishmania
- **c)** Describe the method laboratory diagnosis of Leishmaniasis.

Introduction:

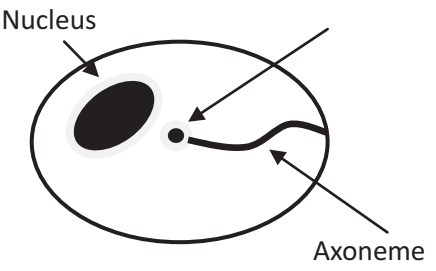
leishmaniasis is caused by any of several species of flagellate protists belonging to the genus *Leishmania* in the order Kinetoplastida. These are parasites of vertebrates, to which they are transmitted by species of *Phlebotomus,* a genus of bloodsucking sand flies. The leishmanial parasites assume two morphological forms one seen in sandfly and other in the vertebrate host. The spectrum of disease depends on the species of *Leishmania*.

Table22.1: Important species of Leishmania

No.	Important species	Disease	Epidemiology
1	*L. donovani* *L. infantum*	Visceral leishmaniasis Post kala azar dermal leishmaniasis (PKDL)	Bangladesh, Brazil, India, Nepal, Sudan and some part of China
2	*L. tropica* *L. major*	Cutaneous leishmaniasis	Afganisthan, Brazil, Iran, peru, Saudi Arabia and Syria
3	*L.braziliensis* *L. panamensis*	Mucocutaneous leishmaniasis	Bolivia, Brazil and Peru

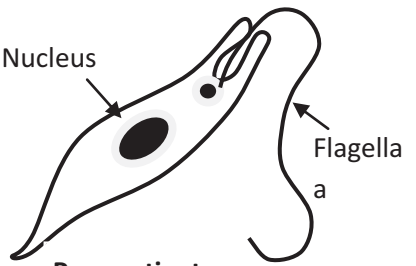
Morphological Forms:

a) **Amastigote:** Amastigote is the aflagellate stage of leishmania. It is ovoid, 2-4 micrometers in size, has one nucleus and one kinetoplast; sometimes a short intracytoplasmic portion of the flagellum can be seen. Amastigotes are found in cells of the reticulo-endothelial system of vertebrate hosts (skin, bone marrow, liver, spleen, and lymph nodes). It is an obligatory intracellular parasite and it may at times be present in the blood stream in large mononuclear cells.



Amastigote

b) **Promastigote**: Promastigote is the flagellate stage and is 10-20 X 7-4 micrometers in size, fusiform in shape, and has one nucleus. The nucleus is central in position. There is a single anterior flagellum which is just as long as its body length. Promastigotes are found in the digestive tract of sandflies which serve as an arthropod vector. Promastigote form can also be grown in artificial culture medium.



Promastigote

Laboratory Diagnosis:

Specific Tests:

a) Microscopy
b) Testing for leishmanial antibodies using antigen known to detect local parasite strains.
c) Culture.

a) **Microscopy:** Amastigotes can be seen by staining of:

–Material aspirated from the spleen, bone marrow or an enlarged lymph node.
–Nasal secretions.
–Peripheral blood smears, monocytes and less commonly in neutrophils (buffy coat preparations).

Sample collection: For peripheral blood smear collect 2-3 ml of venous blood and put in a EDTA container. Aspirates should always be collected by trained and experienced personnel in a secure environment.

Smear preparation:

For buffy coat peraration

1. Centrifuge the EDTA blood in a Eppendorf plastic tubes or glass tubes 6 X 50 mm.
2. Centrifuge for 15 minutes at medium to high speed, to obtain the buffy coat (cream coloured layer of white cells and platelets above the red cells)
3. Using a micro-pipette or Pasteur pipette, remove and discard the plasma above the buffy coat. Transfer the buffy coat to a slide and mix.
4. Place a drop of this on another slide and spread to make a thin smear.
5. Air-dry the smear and fix it with methanol for 2 minutes.

Smear from Aspirates:

1. make at least 2 thinly spread smears of the aspirate by putting a drop of aspirate on each of the clean slides.
2. Dilution with blood should be avoided.
3. Air-dry the smears.
4. Fix the slides for 2-3 minutes by covering each smear with a few drops of absolute methanol (methyl alcohol).

Staining of smears: The fixed smears should be stained by Giemsa or Leishmann's staining technique

Observation: A peripheral blood smear under 100 X will show monocytes containing groups of amastigotes. Occasionally amastigotes can be detected in neutrophils. Small amastigotes may also be seen lying between cells.

A spleenic/bone marrow aspirate will show large mononuclear phagocytic cells containing groups of amastigotes.

b) **Detection of anti-leishmanial antibody:** Antibodies in patient's blood against leishmanial antigen can be detected by serological tests.

1. Direct agglutination test (DAT): this test is useful for screening of Visceral leishmaniasis in endemic areas. The test uses suspension of trypsin-treated fixed promastigotes, stained with Coomasie brilliant blue. The test is performed in V shaped wells in microtitration plates with the serial dilution of patient's serum. The test requires incubation at room temperature for about 18 hours. It is read visually against a white background. The endpoint of the test (titre) is taken as the last well where agglutination is seen. The titre which is taken as indicating a positive test must be decided locally.
2. Detection anti-rK39 antibody: Circulating antibody to rK39 antigen has high sensitivity and specificity for active Visceral Leishmaniasis. Several rapid immunochromatographic dipstick (strip) and cassette format tests have been developed to detect anti-rK39 antibody in patients. The antigen used in the test is a recombinant (r) antigen rK39derived from *L. chagasi*. The patient's serum is applied to the strip and reacts with the colloidal gold dye conjugate. A buffer solution is added. As the antibody-colloidal gold complex migrates up the strip it is captured by a line of rK39 antigen in the test area, producing a pink line. A positive pink control line above the test area indicates satisfactory migration of the reagents.

c) **Culture:** Leishmania spp. can be cultured onto artificial medium. Biopsy specimens from patients of cutaneous leishmaniasis and aspirates from patients with visceral leishmaniasis can be put on NNN(Novy, MacNeal, Nicolle) medium. The medium is taken in MacCartney bottle and incubated at 25^0C. motile promastigote can be seen after incubation of 2-4 weeks. Culture can also be done on Schneider's insect medium.

Nonspecific Tests:

a) **Hematological investigations**: blood test from a patient of Visceral Leishmaniasis may show
- Decreased hemoglobin
- Leukopenia
- Thrombocytopenia.
- Raised ESR.

b) **Albumin/globulin ratio**: albumin- globulin ratio is reversed in Visceral Leishmaniasis.

c) **Formol gel (aldehyde) test**: This is a non-specific screening test which detects marked increases in IgG. Large amounts of polyclonal non-specific immunoglobulin are produced by patients with active Visceral Leishmaniasis.

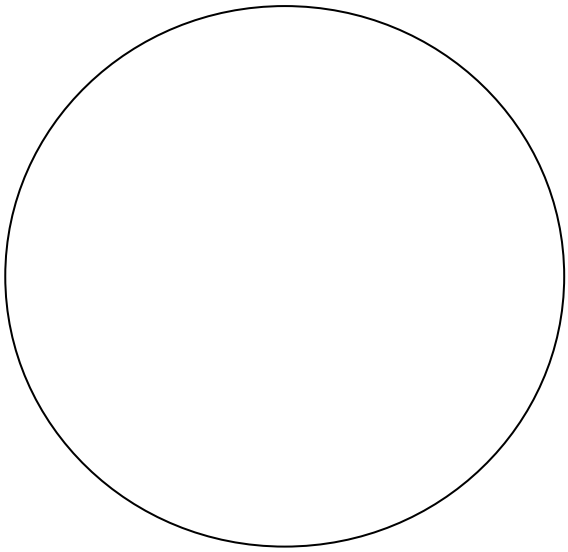
Demonstrations

The teacher should demonstrate the following things during the class:

a) Stained slide of aspirate/peripheral blood smear with amastigotes.
b) Power point presentation depicting the morphological forms of *Leishmania*, culture techniques or any other relevant presentations.

Students Exercise:

1. Draw and label your observation of the given slide as seen under the microscope.



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2. Write the names of important species of Leishmania and the disease caused by them.

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3. Write the two main morphological forms of *Leishmania* and where they can be seen?

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4. What are the serological tests used for detection of Leishmaniasis?

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5. Name the culture media on which Leishmania culture can be done. Which morphological form can be grown on culture?

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Exercise Number 25: Malaria

Learning objectives:

On completion of the exercise the students should be able to do

a) Identification of various stages of malaria parasite in blood film.

Introduction:

Malaria is a serious, sometimes fatal disease resulting from infection with Plasmodium spp. transmitted by the bite of Anopheles mosquitoes.. The clinical diagnosis, where malaria is suspected based on the history, symptoms and clinical findings must always be confi rmed by a laboratory diagnosis. Laboratory diagnosis of malaria involves identification of malaria parasite or its antigens/products in the blood of the patient.

Laboratory Diagnosis of Malaria

Microscopy: Microscopy is gold standard for laboratory confirmation of malaria. A drop of the patient's blood is collected by finger prick, or from a larger venous blood specimen. It is then spread on a glass slide (blood smear), dipped in a reagent that stains the malaria parasites (Giemsa stain), and examined under a microscope at a 1000-fold magnification. Malaria parasites are recognizable by their physical features and by the appearance of the red blood cells that they have infected.

Advantages: Microscopy is an established, relatively simple technique that is familiar to most laboratorians.

Disadvantages: In many developing countries, microscopy is not reliable because the microscopists are insufficiently trained and supervised and are overworked, the microscopes and reagents are of poor quality, and often the supply of electricity is unreliable. Conversely in non-endemic countries, laboratory technicians are often unfamiliar with malaria and may miss the parasites.

Table .1: Morphological features of the different stages of Plasmodia in stained thin blood films

	P. falciparum	***P. vivax***	***P. ovale***	***P. malariae***
Trophozoites	Always present in peripheral blood. Ring-shaped, small to medium size in dimension (2-4 mm) depending on maturation. Young form may lay in marginal position. Polyparasitism and double chromatin dots possible.	Polymorphous in shape from large ring forms younger forms) to ameboid mass occupying the entire red blood cell (mature forms).	Polymorphous in shape from ring forms often showing a central clear vacuole surrounded by cytoplasm (young forms) to large ameboid masses (mature forms). slightly smaller to *P. vivax* in size .	Ring form,small and regular in shape, with no pseudopodes. Older forms may be large, with vacuole Occasionally, equatorial band form present
Schizonts	Solely present in more severe infections. Small and compact, containing 15 to 30 merozoites and a dense dark brown pigmented	Normally present in peripheral blood. Large (= 12-16 mm), round bodies containing 12 to	Normally present in peripheral blood. Large (= 10-12 mm), round bodies containing 4 to 12 merozoites and	Compact, rosetta-like forms with 8-10 merozoites surrounding a central pigmented area

	residual body.	24 merozoites and loose golden brown residual pigmentation	dark pigmentation	
Gametocytes	Present in the second phase of the erythrocytic cycle. Crescent-shaped with coarse rice-like granules and pigment. The female is blue in colour and granules are in central position, while the male form is violet and granules are scattered over the parasite	Round regular bodies with a single voluminous nucleus (dense and red purple in female gametocytes, loose and pink in male forms).	Round regular bodies with a single voluminous nucleus (dense and red purple in female gametocytes, loose and pink in male forms). Their dimensions are usually inferior than in *P. vivax*	Compact large single dense purple nucleus (female form) or loose violet nucleus (male form). Scattered coarse pigment granules are present
Parasitic density	may be very high (average 20-500.000, max 2.000.000)	intermediate level (average 20.0000, max 50.000)	usually moderate (average 9.000, max 30.000)	usually very low (average 6,000, max 20,000)

Table.2: Changes produced by Malarial parasites in blood cells

	P. falciparum	*P. vivax*	*P. ovale*	*P. malariae*
Age	Young and old erythrocyes infected	Young erythrocytes infected	Young erythrocytes infected	Older erythrocytes infected
Dimensions	Normal	Enlarged	Enlarged, sometimes assuming oval shape with *fimbriae* at one pole	Normal
Color	Normal to dark	Normal to pale	Normal	Normal
Granules	Unusual coarse scattered red stippling in mature trophozoites or schizonts (*Maurer's clefts*)	Frequent fine red diffuse stippling in all stages of erythrocytic developmental cycle (*Schuffner's dots*)	Frequent fine red diffuse stippling in all stages of erythrocytic developmental cycle (*Schuffner's dots*, also called *James' dots*)	None
Pigment	Dark brown and usually compact.	Golden brown and usually loose	Brown coarse pigment granules	Brown coarse scattered pigment granules
Leucocytes	The presence of malaria pigment in neutrophils and monocytes is a prognostic marker of severe disease (Hoan Phu et al., 1995)			

Other Laboratory Tests:

In addition to microscopy, other methods have been developed recently for detection of malaria parasites. Parasite antigens and other products can be detected by rapid “dipstick” tests, and parasite DNA can be detected by polymerase chain reaction (PCR). PCR is currently the most accurate test and can identify low levels of infection not detectable by other methods. Serology detects antibodies against malaria parasites. Such antibodies are produced by the immune response of the infected person and can persist in the blood for several months after the infection is over.

Antigen Detection

Antigen detection kits most often use a dipstick or cassette format, and provide results in 2-15 minutes. These "Rapid Diagnostic Tests" (RDTs) offer a useful alternative to microscopy in situations where reliable microscopic diagnosis is not available.

Molecular Diagnosis

Parasite nucleic acids are detected using polymerase chain reaction (PCR). This technique is more accurate than microscopy. However, it is expensive, and requires a specialized laboratory.

Serology

Serology detects antibodies against malaria parasites, using either indirect immunofluorescence (IFA) or enzyme-linked immunosorbent assay (ELISA). Serology does not detect current infection but rather measures past experience.

Indirect Fluorescent Antibody Test

Malarial parasite detection is performed using the indirect fluorescent antibody (IFA) test. Species-specific testing is available for the four human species: *P. falciparum*, *P. vivax, P. malariae,* and *P. ovale.* Cross reactions often occur between *Plasmodium* species and *Babesia* species. When examined with a fluorescence microscope, a positive reaction is when the parasites fluoresce an apple green color.

Drug Resistance Tests

Drug resistance tests are performed in specialized laboratories to assess the susceptibility to anti malarial compounds of parasites collected from a specific patient. Two main laboratory methods are available:

- In vitro tests: The parasites are grown in culture in the presence of increasing concentrations of drugs; the drug concentration that inhibits parasite growth is used as endpoint;
- Molecular characterization: molecular markers assessed by PCR or gene sequencing allow also the prediction, to some degree, of resistance to some drugs; however, the predictive values of these molecular tests are still being evaluated.

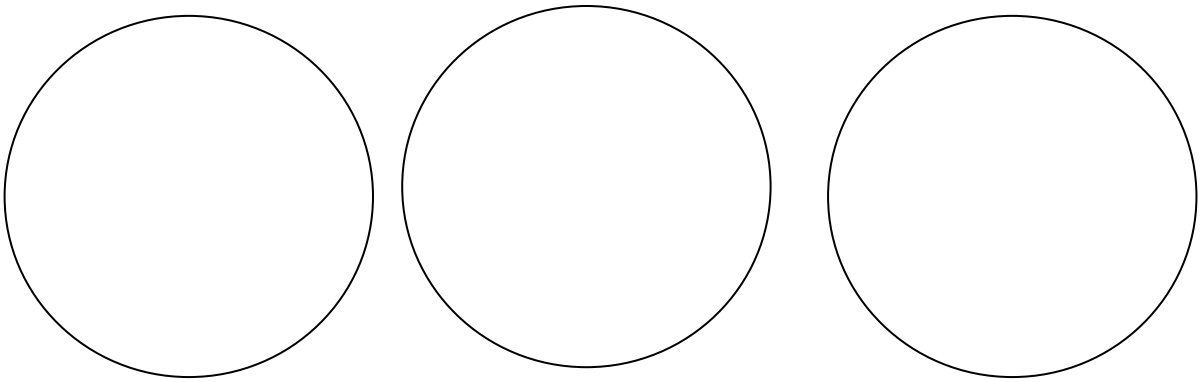
Demonstrations

The teacher should demonstrate the following things during the class:

1. The various stages of Plasmodium vivax and Plasmodium falciparum.
2. Rapid detection test for malarial parasite.

Students Exercise:

1. Observe the given slide and draw the diagram of various stages of malaria parasite.



2. In malaria, the form of plasmodia that is transmitted from mosquito to human is:
    A. Sporozoite
    B. Gametocyte
    C. Merozoite
    D. Hypnozoite

3. Malaria infected cells rupture and release the many progeny of the parasite - what is the name of the stage released from the red cell?

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4. Which species of malaria produces a sausage-shaped gametocyte?

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5. Name the genus and species of the malaria that is sometimes described as benign tertian malaria.

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6. Which species of malaria can infect liver cells and "hide" there, and can cause a "relapse".

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7. Trophozoites, Schizonts and gametocytes of all the malarial parasites are seen in the peripheral blood smear except:

   A. P. falciparum
   B. P. malariae
   C. P.ovale
   D. P.vivax

8. After sporozoite gain entrance to human body it undergoes developmental cycle first in liver than in RBC, only after which fever is seen. This incubation period varies between plasmodium species. The species with longest incubation period is:

   A. P. falciparum
   B. P. malariae
   C. P.ovale
   D. P.vivax

9. Black water fever is a special manifestation of malaria caused by;

   A. P. falciparum
   B. P. malariae
   C. P.ovale
   D. P.vivax

Exercise Number 26 :Toxoplasma gondii

Learning Objectives:
On completion of the exercise the students should be able to

a) Identification of Toxoplasma slides.

Introduction

Toxoplasma gondii, the causative organism of toxoplasmosis, was first observed in 1927 in the gondi, a North African rodent. The first human case of toxoplasmosis was also reported that year. The organism is a coccidian protozoa belonging to the sub-phylum Apicomplexa and has a world wide distribution occurring in all warm-blooded animals.

Cats are the definitive hosts and they become infected by ingesting oocysts or cysts in tissues of paratenic hosts, such as mice, or transplacentally. Man becomes infected either by direct ingestion of oocysts from a cat or by eating raw or undercooked meat. Those who handle raw meat are particularly at risk. Infection can be transmitted transplacentally.

Laboratory Diagnosis

1. Serological Techniques

The detection of toxoplasma specific antibodies is most commonly used in clinical laboratories. Specific IgG antibodies typically persist for life whereas specific IgM antibodies begin to decline after several months. Most laboratories carry out preliminarytests for IgG antibodies and more definitive tests including IgM and IgA are carried out in reference laboratories. The Sabin-Feldman Dye Test is the benchmark for detecting the presence of specific antibodies. It measures the total amount of specific antibody in a serum which is capable of participating in antibody-mediated killing of tachyzoites by complement. This test involves the use of live tachyzoites which are derived from infected mice or rats. Because of the use of live organisms, this test is not recommended in the use of routine laboratories and is thus only employed in reference centers.

2. Isolation Techniques

Culture of parasites in animals is the best overall method but it can take up to six weeks before the result is available and is thus a disadvantage. Tissue culture is more rapid taking three or four days to obtain a result, but is not as sensitive.

3. Antigen Detection

The direct detection of very small amounts of specific nucleic acid has been made possible by the introduction in 1985 of the polymerase chain reaction (PCR). This technique results in the amplification of a specific fragment of DNA from within the parasite genome which is detected by ethidium bromide staining, following gel electrophoresis. PCR is so sensitive it should detect Toxoplasma DNA in one cyst. However this may indicate a latent infection rather than an active infection. However its sensitivity may create problems since it will detect very small amounts of DNA from latent as well as active infections and it does not differentiate between cyst and tachyzoite DNA. Thus samples like blood, CSF, urine and

amniotic fluid should be used as they do not contain the latent stages. PCR shows great promise but as yet is still labor intensive and expensive for routine use in the laboratory.

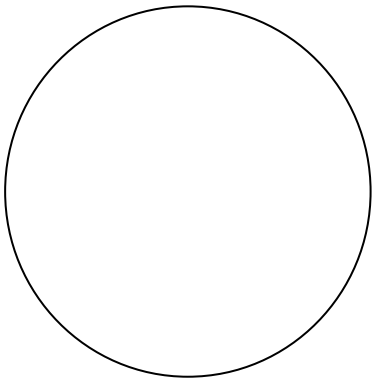
Demonstrations

The teacher should demonstrate the following things during the class:

a) Archived slides/ photos of Toxoplasma tachyzoites.
b) Serological kits for diagnosis of Toxoplasma infections.

Students Exercise:

1. Draw a neat labeled diagram of toxoplasma slide.



2. Who are most at risk of contracting Toxoplasmosis?

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3. What are the manifestations of Congenital toxoplasmosis?

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4. What are the diagnostic stages of Toxoplasmosis gondii?

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Exercise Number 27: Cestode and trematodes.

Learning Objectives:
On completion of the exercise the students should be able to**:**

- **a)** Enumerate the different species of Cestodes causing infection in humans.
- **b)** Identify of various cestode infection by identifying ova or proglottids in stool.
- **c)** List the various disease causing Trematodes
- d) Identify the ova of different trematodes

Introduction:

The cestodes (or tapeworms) form a group of worms, exhibiting two unmistakable morphological features; they all possess flat, ribbon like bodies and lack an alimentary canal. Adult tapeworms usually inhabit the alimentary canal of their hosts (though they occasionally are found in the bile or pancreatic ducts) and attach themselves to the mucosa by means of a scolex. Despite the lack of a digestive system they do absorb food from the hosts intestine; thereby providing the tapeworms a habitat that is associated with high nutritional levels, feeding the tapeworms high growth rate. Larvae on the other hand show a wide range of habitat preferences, being found in almost any organ of both vertebrate and invertebrate hosts. Though most larval species show a preference for a particular site. This lack of an alimentary canal markedly separates tapeworms from nematodes and trematodes.

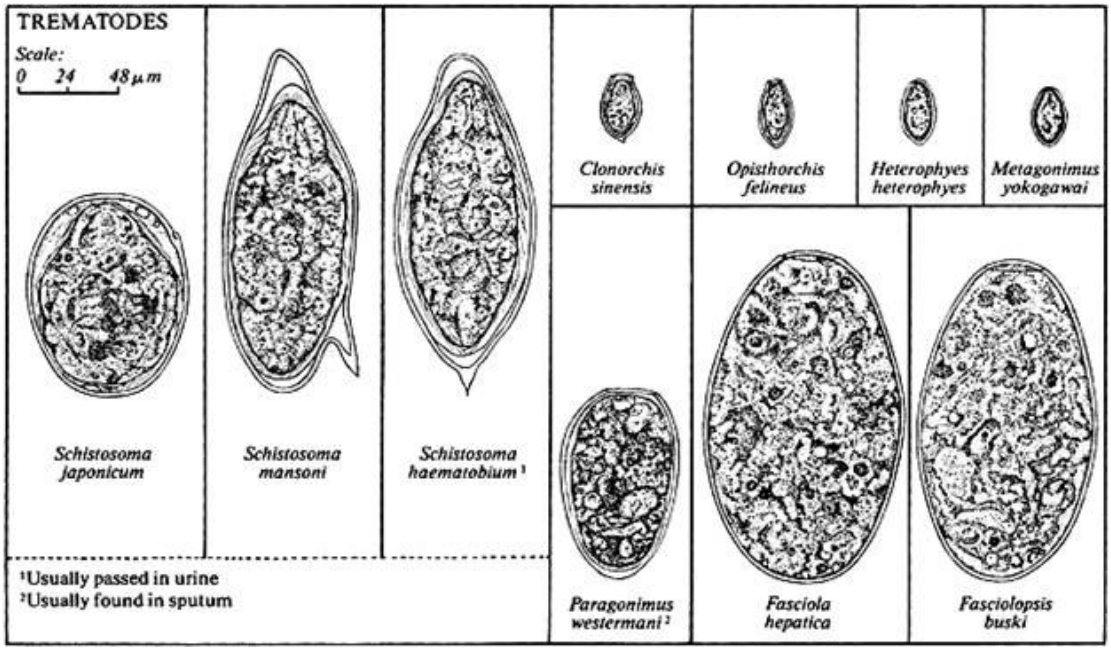
The outer tegument of the body must serve not only as a protective coating but also as a metabolically active layer through which nutritive material can be absorbed, along with secretions and waste material to be transported out of the body. The body consists of a chain of segments or proglottids, which can be immature, mature or gravid; the latter of which contain a fully developed uterus packed with eggs. Therefore, each tapeworm is made up of a 'string of individuals' having a complete set of reproductive organ, progressive degrees of sexual maturity and budding off from a body attached to the host tissue by a head or scolex. Except for Hymenolepis nana, which can develop directly in the same host, the lifecycle of tapeworms involves both an intermediate and definitive host.

Table 25.1: properties of ova of different cestodes

Species	Size	Shape	Color	Stage of Development When Passed	Specific Features and Variations
Cestodes					
Taenia saginata *Taenia solium*	31-43 µm.	Spherical with thick striated shell.	Walnut brown.	Embryonated. 6-hooked oncosphere present inside a thick shell.	Thick, striated shell. Eggs of*T. solium* and *T. saginata* are indistinguishable and species identification should be made from proglottids or scoleces. "*Taenia*" spp. should be reported if only eggs are found.
Hymenolepis nana	47 µm x 37 µm. Range, 40-60 µm x 30-50 µm.	Oval. Shell consists of 2 distinct membranes. On inner membrane are two small "knobs" or poles from which 4 to 8 filaments arise and spread out between the two membranes.	Colorless, almost transparent.	Embryonated. 6-hooked oncosphere inside shell.	Polar filaments.
*Hymenolepis diminuta**	72 □ m. Range, 70-86 □ m x 60-80□ m.	Round or slightly oval. Striated outer membrane and thin inner membrane with slight poles. Space between membranes may appear smooth or faintly granular.	Yellow.	Embryonated. 6-hooked oncosphere inside shell.	Resembles *H. nana* but lacks polar filaments. Poles are rudimentary and often hard to see.

*Dipylidium caninum**	35-40 □ m. Range, 31-50 □ m x 27-48 □ m.	Spherical or oval. 5-15 eggs (or more) are enclosed in a sac or capsule.	Colorless.	Embryonated. 6-hooked oncosphere inside shell.	Eggs are contained in a sac or capsule which ranges in size from 58 □ m to 60 □ m x 170□ m. Occasionally capsules are ruptured and eggs are free.
Diphyllobothrium latum	66 □ m x 44 □ m. Range, 58-76 □ m x 40-51 □ m.	Oval or ellipsoidal with an inconspicuous operculum at one end and a small "knob" at the other end.	Yellow to brown.	Unembryonated. Germinal cell is surrounded by a mass of yolk cells which completely fills inner area of shell. Germinal cell is usually not visible.	Egg resembles hookworm egg but has a thicker shell and an operculum.

The Trematodes (or flukes) are leaf shaped with an outer cover called the tegument which may be smooth or spiny. There are two suckers or attachment organs, an anterior oral sucker and a posterior ventral sucker. The suckers form a characteristic feature of the group, from which the name Trematode is derived from the Greek word for "hole." They can occur in a variety of host environments, with the majority being endoparasites but some are found to be ectoparasitic. Most trematodes are hermaphroditic and most of the body consists of reproductive organs and their associated structures. The digestive system is well developed; they generally feed on intestinal debris, blood, mucus and other tissues, depending on the host environment**.**



Trematodes require an intermediate host in their life cycle with vertebrates being the definitive host. Larval stages may occur in either invertebrate or vertebrate hosts.

There are three groups of trematodes:

1. **Monogenea**, which typically are external parasites of fish with direct life cycles
2. **Aspidogastrea**, these are endoparasites with the entire ventral surface as an adhesive organ
3. **Digenea,** these are endoparasites with simpler adhesive organs and life cycles involving one or more intermediate hosts (indirect life-cycle). This section concentrates on the Digenean trematodes.Most Digenean trematodes inhabit the alimentary canal of vertebrates and many of the associated organs, such as the liver, bile duct, gall bladder, lungs, bladder and ureter. These organs are rich in cavities containing food such as blood, mucus, bile and intestinal debris.

The Digenean trematodes have a complex life cycle, with rare exceptions, always involve a mollusk host. There may be six larval stages – the miracidium, sporocyst, redia, cercaria, mesocercaria (rare) and the metacercaria (the majority have 4 or 5 stages).

Trematode eggs have a smooth hard shell and the majority of them are operculate.

Table 25.2: Differential morphology of diagnostic stages of helmiths.

Species	Size	Shape	Color	Stage of Development When Passed	Specific Features and Variations
Trematoda					
Schistosoma mansoni	140 mm x 66 mm. Range, 114-180 mm x 45-73 mm	Elongated with prominent lateral spine near posterior end. Anterior end tapered and slightly curved.	Yellow or yellow brown.	Embryonated. Contains mature miracidium.	Lateral spine. Found in feces; in rare cases, in urine also. Eggs are discharged at irregular intervals and may not be found in every stool specimen. Are rare in chronic stages of infection.
Schistosoma japonicum	90 mm x 70 mm. Range, 68-100 mm x 45-80 mm.	Oval. Small lateral spine is often seen or may appear as a small hook or "knob" located in a depression in the shell.	Yellow or yellow brown.	Embryonated. Contains mature miracidium.	Found in feces. Often coated with debris and may be overlooked.
Schistosoma haematobium	143 mm x 60 mm. Range, 112-170 mm x 40-70 mm.	Elongated with rounded anterior end and terminal spine at posterior end.	Yellow or yellow brown.	Embryonated. Contains mature miracidium.	Terminal spine. Found in urine, occasionally in feces. Egg often covered with debris.
Schistosoma intercalatum	175 mm x 60 mm. Range, 140-240 mm x 50-85 mm.	Elongated with tapered anterior end and terminal spine. Sometimes "spindle-shaped."	Yellow or yellow brown.	Embryonated. Contains mature miracidium.	Terminal spine long, slender with bent tip. Resembles S. haematobium egg except it is longer, is thinner, and has a longer spine. Found in feces. May have debris adhering to shell.
Schistosoma mekongi	69 mm x 56 mm* Range, 51-73 mm x 39-66 mm.	Spherical. Small lateral spine, not always visible or may appear as a small "knob" in a depression in the shell.	Yellow or yellow brown.	Embryonated. Contains mature miracidium.	Found in feces. Closely resembles S. japonicum egg except it is smaller. May be coated with debris.
Clonorchis sinensis	30 mm x 16 mm. Range, 27-35 mm x 11-20 mm.	Small, ovoidal, or elongated with broad rounded posterior end and a convex operculum resting on "shoulders." A small "knob" may be seen on the posterior end.	Yellow brown.	Embryonated. Contains mature miracidium.	Small size, operculum and "knob" on posterior end. Shell often is covered by adhering debris.
Opisthorchis felineus	30 mm x 12 mm. Range, 26-30 mm x 11-15 mm.	Elongated with operculum on anterior end and pointed terminal "knob" on posterior end.	Yellow brown	Embryonated. Contains mature miracidium.	Lacks prominent shoulders characteristic of Clonorchis and has more tapered end.

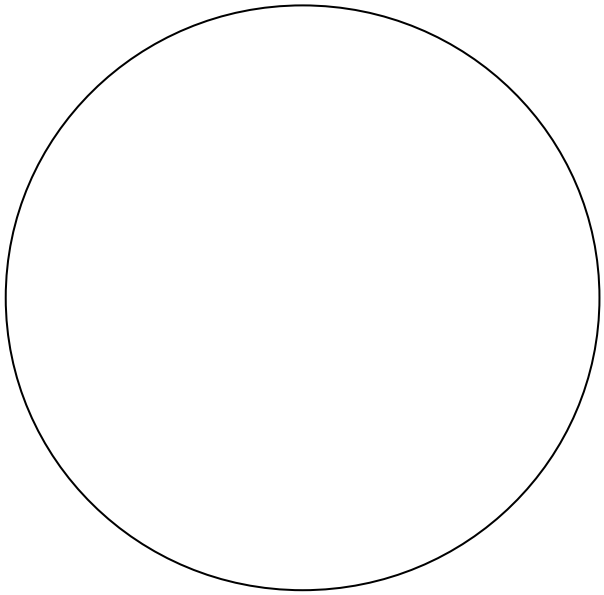
Demonstrations:

The teacher should Demonstrate the following things during the class:

a) Show and explain the specimens of different cestodes and trematodes
b) Explain the methods of identification of ova in stool
c) Show the different ovas in stool under the microscope.

Students Exercise:

1. Draw neat labeled diagram of the ova you as seen under the microscope in the slide provided.



2. Tissue infections caused by Taenia solium is known as:

A. Cysticercosis
B. Taeniasis.

3. Host of Taenia solium is:

A. Pig
B. Human
C. None
D. Both

4. What are the differences between cestodes and trematodes?

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5.Name the definitive and intermediate hosts of the following.

Hymenolepis nana,Taenia solium and Echinococcus granulosus.

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6. What is the specimen of choice to recover Paragonimus westermani?

A. Stool
B. Sputum
C. Urine
D. Tissue biopsy

7. What Cestode has an operculated egg similar to the Trematodes?

A. Diphyllobothrium latum
B. Echinococcus granulosus
C. Paragonimus westermani
D. Taenia species

Exercise Number 28: Intestinal nematodes

Learning objectives:
On completion of the exercise the students should be able to

a) To know about medically important intestinal nematodes.
b) To know about morphological features and clinical manifestations of different intestinal worms.

Introduction:

Nematodes are elongated, cylindrical and unsegmented worms. Following are the medically important intestinal nematodes.

1. Ascaris lumbricoides

Ascaris lumbricoides is the giant roundworm of humans, it is responsible for the disease ascariasis in humans. Ascariasis is prevalent worldwide and more so in tropical and subtropical countries. This is a large round worm which is normally located in the small intestine. The distribution is cosmopolitan and more common in children.

Laboratory diagnosis:

Most diagnoses are made by identifying the appearance of the worm or eggs in feces. Males are 2–4 mm in diameter and 15–31 cm long. The males' posterior end is curved ventrally and has a bluntly pointed tail. Females are 3–6 mm wide and 20–49 cm long. Usually fertilized of eggs *A. lumbricoides* are found in feces. But occasionally unfertilized eggs are also seen.

Fertilized egg:

- Yellow-brown, oval or round, measuring 50–70µm long by 30–50µm wide.
- Shell is often covered by an uneven albuminous coat (mammilated).
- Contains a central granular mass which is the unsegmented fertilized ovum. There is a area of clearing around the poles over the ovum

Unfertilized egg

- It is darker in colour and has a thinner wall and more granular albuminous covering.
- More elongated than a fertilized egg, measuring about 9045µm.
- Contains a central mass of large granules.

Decorticated egg: This term is used to describe an egg that has no albuminous coat. A decorticated egg has a smooth shell and appears pale yellow.

So there is a possibility of finding four different types of Ascaris egg: Fertilized corticated, Fertilized decorticated, unfertilized corticated and Unfertilized decorticated.

2. Trichinella spiralis:

Trichinella spiralis is a nematode parasite, occurring in rats, pigs, bears and humans, and is responsible for the disease trichinosis. It is sometimes referred to as the "pork worm" due to it being found commonly in undercooked pork products.

Laboratory diagnosis:

Muscle biopsy is used for trichinosis detection. Several immunodiagnostic tests are also available.

Males of *T. spiralis* measure between 1.4 mm and 1.6 mm long and are more flat anteriorly than posteriorly. The females of *T. spiralis* are about twice the size of the males and have an anus found terminally

3. Hook Worm:

Necator americanus is a species of hookworm commonly known as the New World hookworm. It lives in the small intestine of hosts such as humans, dogs, and cats.

Ancylostoma duodenale is a species of the worm genus *Ancylostoma*. It is a parasitic nematode worm and commonly known as Old World hookworm. It lives in the small intestine of hosts such as humans, cats and dogs, where it is able to mate and mature.

The two hook worms are mainly differentiated based on morphological features of adult worms.

Necator americanus has two dorsal and two ventral cutting plates around the anterior margin of the buccal capsule. It also has a pair of subdorsal and a pair of subventral teeth located close to the rear. Males are usually 7-9 mm long, whereas females are about 9-11 mm long.

Ancylostoma duodenale is small cylindrical worm, greyish-white in color. It has two ventral plates on the anterior margin of the buccal capsule. Each of them has two large teeth that are fused at their bases. A pair of small teeth can be found in the depths of the buccal capsule. Males are 8 mm to 11 mm long with a copulatory bursa at the posterior end. Females are 10 mm to 13 mm long, with the vulva located at the posterior end.

Laboratory diagnosis: Rests on demonstration of parasite eggs in the stool specimens. The eggs of *Ancylostoma duodenale* and *Necator americanus* cannot be distinguished. Larvae cannot be found in stool specimen unless they are left at ambient temperature for a day or more. through ELISA, and duodenal fumigation..

4. Enterobius vermicularis

The pinworm (genus *Enterobius*), also known as threadworm is a common intestinal parasite. The pinworm has a worldwide distribution, Finger sucking has been shown to increase both incidence and relapse rates, and nail biting has been similarly associated. Because it spreads from host to host through contamination, pinworms are common among people living in close contact, and tends to occur in all people within a household.

Laboratory diagnosis:

The laboratory diagnosis of enterobiasis is achieved by finding *E. vermicularis* eggs in samples collected from perianal skin or recovered from clothing worn at night.Eggs may also be found in faeces and occasionally in urine from females.

5. Strongyloides stercoralis

S. stercoralis is endemic in many tropical and subtropical countries. Infection with S. stercoralis can occur:

By infective filariform larvae penetrating the skin.

By autoinfection (self-infection) with rhabditiform (first stage) larvae developing into infective filariform larvae in the intestine or on perianal skin followed by penetration of the intestinal wall or perianal skin.

Morphology:

Whereas males grow to only about 0.9 mm (0.04 in) in length, females can grow from 2.0 to 2.5 mm

Laboratory Diagnosis: The laboratory diagnosis of S. stercoralis infection isby finding motile S. stercoralis larvae in fresh faeces.

Demonstrations

The teacher should Demonstrate the following things during the class:

1. Demonstrate the ova of important intestinal nematodes.
2. Display the adult worms of Ascaris, Enterobius, hookworms etc.
3. Demonstrate the larvae of Strongyloides.

Students Exercise:

1. What is auto-infection?

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2. What are the two hook worms? How do you differentiate them?

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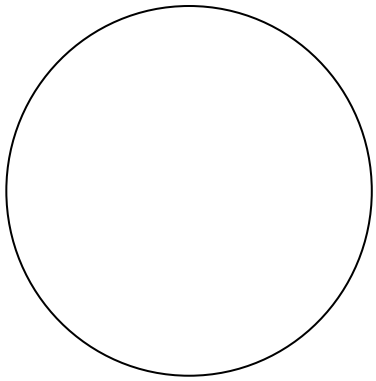
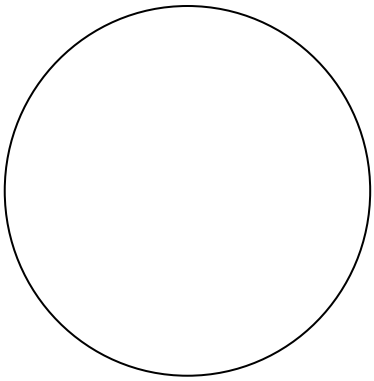
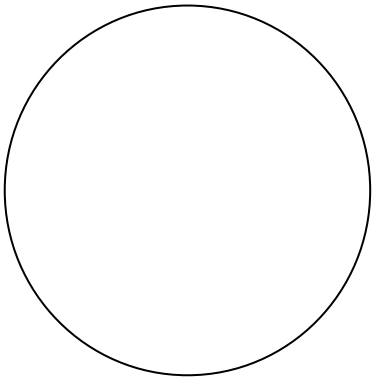
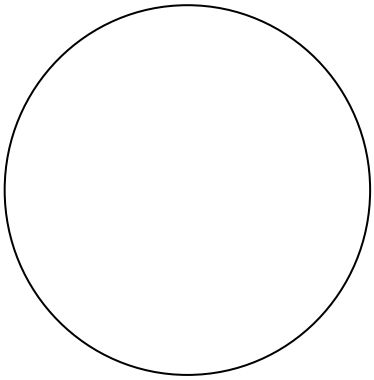
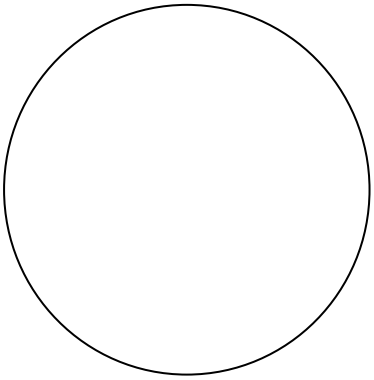
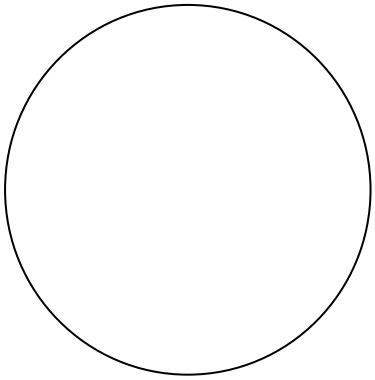
3. Draw well labeled diagrams of the parasite ova demonstrated to you.









Exercise Number 29:Extra Intestinal Nematodes

Learning objectives:
On completion of the exercise the students should be able to

a) To know about medically important extra-intestinal (tissue nematodes).
b) To know about different filarial worms and their clinical manifestations.
c) To know about Guinea worm.

Introduction:

Extra intestinal nematodes include a group of tissue nematodes called filariae and another worm *Dracunculus medinensis* (Guinea worm).

Filarial worms: These are long and slender worms. The female is viviparous, and the larvae called microfilariae are liberated in tissues and find their way to blood vessels (except in case of *Oncocerca volvulus*). The microfilariae of different filarial worms vary in morphology. Microfilariae bancrofti and malayi are found in peripheral blood at night and microfilariae loa during the day, others do not exhibit periodicity.

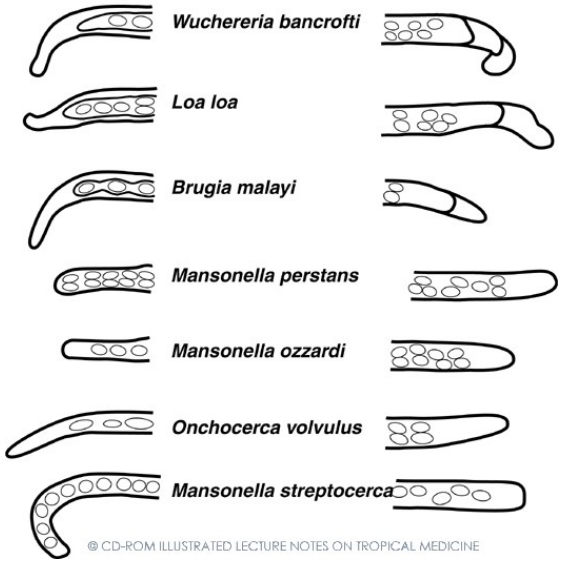
The filarial worms differ in their habitation in human body and clinical manifestations, important ones are:

- ***Wuchereria bancrofti:*** Causes lymphatic filariasis, inhabits lymphatics.
- ***Brugia malayi:*** Causes lymphatic filariasis, inhabits lymphatics.
- ***Oncocerca volvulus:*** Causes skin and eye lesions, inhabits sub-cutaneous tissues.
- ***Loa loa:*** Causes skin and eye lesions, inhabits sub-cutaneous tissues.

Laboratory Diagnosis:

Microscopy: Mainstay of diagnosis is demonstration of microfilariae in blood and other sites. Wet preparations are used to demonstrate actively motile microfilariae. For species identification, peripheral smear stained with Giemsa or any other Romanowsky's stain is used. Examination of tip of tails and cephalic ends is of importance in species identification.

Chart with principle differences between microfilaria.



The microfilaria of *Wuchereria bancrofti* is sheathed, its body is gently curved, and the tail is tapered to a point. The nuclear column (the cells that constitute the body of the microfilaria) is loosely packed, the nuclei can be visualized individually and do not extend to the tip of the tail.

The microfilaria of *Brugia malayi,* like micrifilaria of *Wuchereria bancrofti*, has a sheath. Differently from *Wuchereria*, the microfilariae in this species are more tightly coiled, and the nuclear column is more tightly packed, preventing the visualization of individual cells.

Antigen detection using an immunoassay for circulating filarial antigens constitutes a useful diagnostic approach, because microfilaremia can be low and variable. A rapid-format immunochromatographic test, applicable to *Wuchereria bancrofti* antigens, has been recently evaluated in the field.

Molecular diagnosis using polymerase chain reaction is available for *W. bancrofti* and *B. malayi*.

Identification of adult worms is possible from tissue samples collected during nodulectomies (onchocerciasis), or during subcutaneous biopsies or worm removal from the eye (loiasis).

Antibody detection is of limited value. Substantial antigenic cross reactivity exists between filaria and other helminths, and a positive serologic test does not distinguish between past and current infection.

Guinea worm: *Dracunculus medinensis* is the causative agent of dracunculosis. Man is the definitive host and intermediate host is *Cyclops*. Adult lives in deep viscera or somatic tissues, and at the end of life span migrates to sub-cutaneous tissue forming a blister. The blister bursts when comes in contact with water liberating larvae in water.

Diagnosis is mainly by detection of adult worm when female appears on skin surface; or by obtaining larvae on bathing the blister in water.

Demonstrations:
The teacher should Demonstrate the following things during the class:

1. Demonstrate the morphology of microfilaria bancrofti in a stained peripheral blood smear.
2. Familiarize the student with the differences in morphology of different microfilariae.
3. Display the specimens or photos of Guinea worm and *Cyclops*.

Students Exercise:

1. Which specimen is collected for laboratory diagnosis of lymphatic filariasis?

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2. What are the differences between microfilariae of *W. bancrofti* and *B. malayi*?

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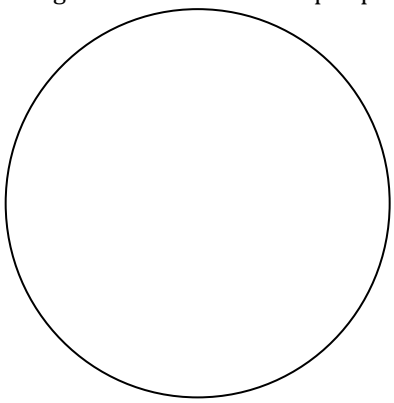
3. Draw a diagram of microfilaria in peripheral blood smear demonstrated to you.



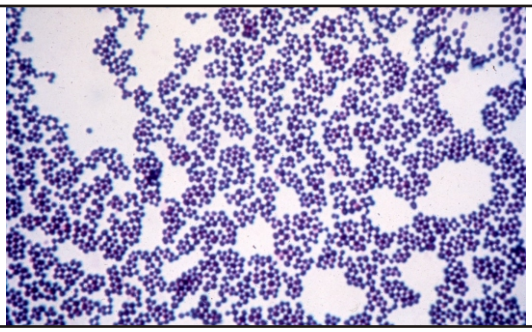
4. Draw diagrams of the tail tips of different microfilaria to exhibit the differences.

Colour Atlas Glossary

Chapter 1	Figure 1	Micrometer
Chapter 2	Figure 2	Gram positive cocci
	Figure 3	Gram negative bacilli
Chapter 3	Figure 4	Peptone water
	Figure 5	Blood agar
	Figure 6	MacConkey Agar
	Figure 7	Muller Hinton Agar
	Figure 8	Lowenstein Jensen Agar
	Figure 9	BacT/Alert Culture Bottle
Chapter 4	Figure 10	Blood vial
	Figure 11	Cotton swab
	Figure 12	Universal container
Chapter 6	Figure 13	Autoclave
	Figure 14	Hot air oven
	Figure 15	Water bath
	Figure 16	Waste disposal coloured buckets
Chapter 8	Figure 17	RPR
	Figure 18	WIDAL
	Figure 19	Malaria Rapid Test Kit
	Figure 20	HIV Rapid Test Kit
	Figure 21	ELISA machine and titre plate
Chapter 9	Figure 22	Staphylococcus aureus Microscopy
	Figure 23	Staphylococcus aureus colony
	Figure 24	Coagulase Test
Chapter 10	Figure 25	Streptococcus pyogenes morphology
	Figure 26	Streptococcus pyogenes colony
	Figure 27	Streptococcus pneumoniae morphology
	Figure 28	Streptococcus pneumoniae colony
	Figure 29	Bacitracin sensitivity test
	Figure 30	Catalase Test
	Figure 31	Bile Esculin agar
Chapter 11	Figure 32	N. gonorroheae microscopy
	Figure 33	N. meningitidis colony
Chapter 12	Figure 34	Albert Staining smear of Corynebacterium diphtheriae
	Figure 35	Elek gel precipitation test
Chapter 13	Figure 36	M. tuberculosis microscopy
	Figure 37	M. tuberculosis colony
Chapter 14	Figure 38	B. anthracis Microscopy
	Figure 39	Robertson's cooked meat medium
Chapter 15	Figure 40	E.coli colony
	Figure 41	Klebsiella colony
	Figure 42	E.coli biochemical reactions

	Figure 43	Serratia colony
Chapter 16	Figure 44	Salmonella colony on Wilson Blair agar
	Figure 45	Salmonella with flagella
	Figure 46	Shigella microscopy
	Figure 47	Shigella colony
Chapter 17	Figure 48	Vibrio cholerae microscopy
	Figure 49	Vibrio cholerae colony on TCBS
Chapter 18	Figure 50	Proteus swarming colony on blood agar
	Figure 51	Pseudomonas colony with pigment production
Chapter 19	Figure 52	Treponema pallidum microscopy silver staining
	Figure 53	Syphilis Chancre on finger
	Figure 54	Leptospira microscopy
	Figure 55	Armadillo
Chapter 20	Figure 56	Actinomycetes Microscopy
	Figure 57	Nocardia colony
Chapter 21	Figure 58	Aspergillus colony
	Figure 59	Aspergillus Microscopy
	Figure 60	Trychophyton Microscopy
	Figure 61	Candida Microscopy
Chapter 22	Figure 62	Scanning electron micrograph of a cultured human cervix cancer cell infected by Chlamydia trachomatis
	Figure 63	Mycoplasma seen under electron Microscope
	Figure 64	Mycolplasma colony-fried egg appearance
Chapter 23	Figure 65	Entamoeba morphology
	Figure 66	Giardia morphology
	Figure 67	Cryptosporidium morphology
	Figure 68	Isospora belli morphology
Chapter 24	Figure 69	Leishmania -LD bodies
	Figure 70	Leishmania Promastigote
Chapter 25	Figure 71	Plasmodium vivax morphology (ring form)
	Figure 72	Plasmodium falciparum morphology (ring form)
	Figure73	Plasmodium vivax schizont
	Figure 74	Plasmodium falciparum gametocyte
Chapter 26	Figure 75	Toxoplasma morphology
Chapter 27	Figure 76	Taenia solium- head
	Figure 77	Taenia solium egg
	Figure 78	Hydatid cyst
Chapter 28	Figure 79	Ascaris ova
	Figure 80	Hookworm ova
	Figure 81	Trichiris ova
	Figure 82	Enterobius vermicularis ova
	Figure 83	Filarial worm in peripheral smear
	Figure 84	Guinea worm

Figure-1



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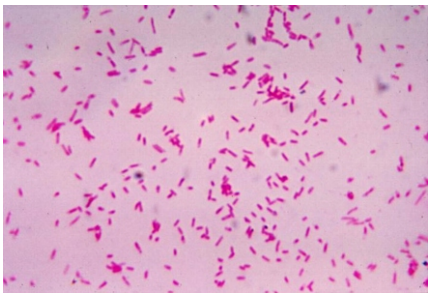

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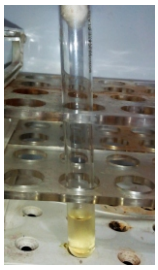

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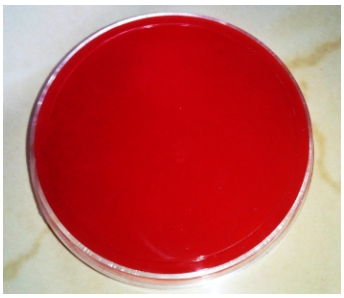

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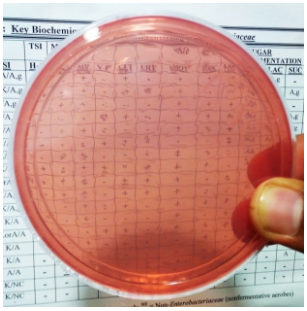

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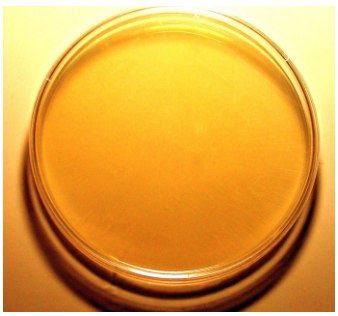

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Figure-8

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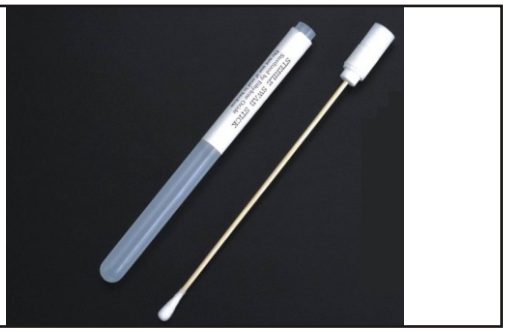
Figure-10



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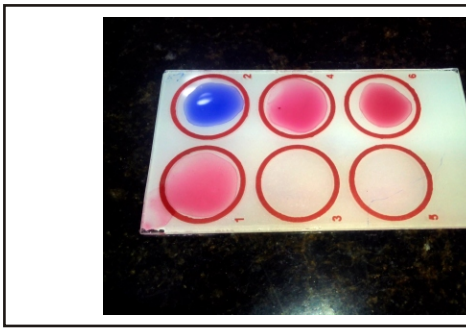

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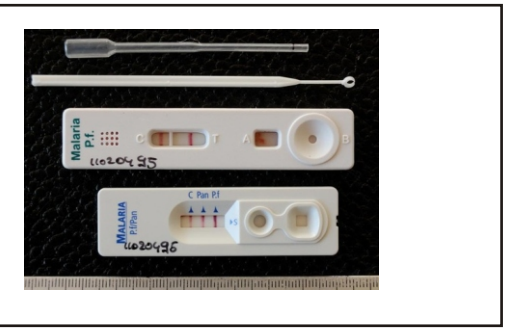

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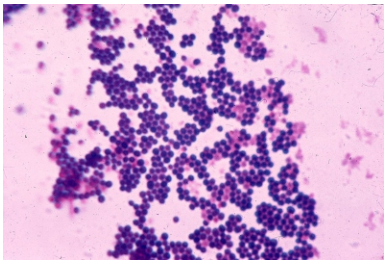
Figure-21



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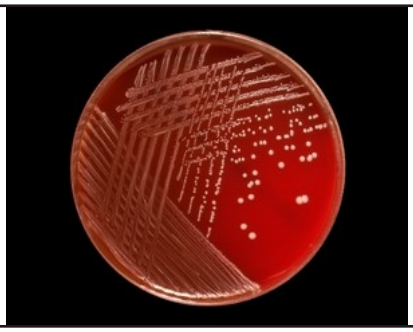

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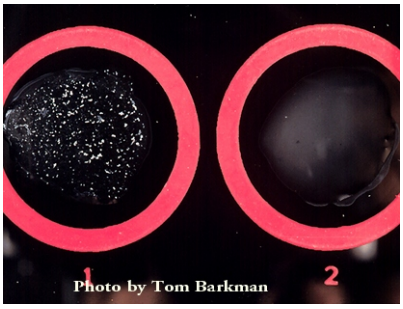

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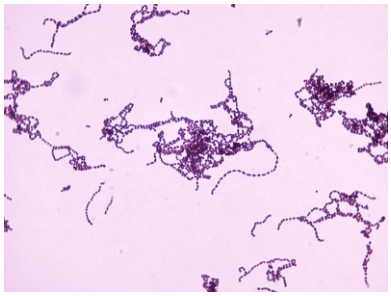

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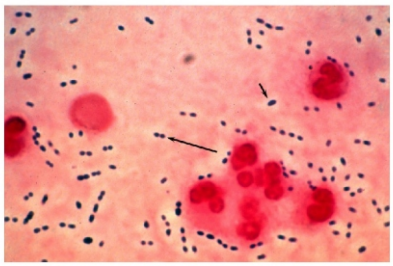
Figure-26



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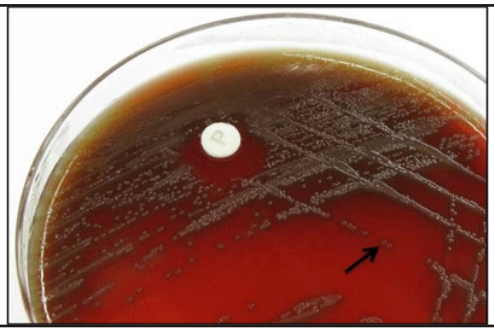

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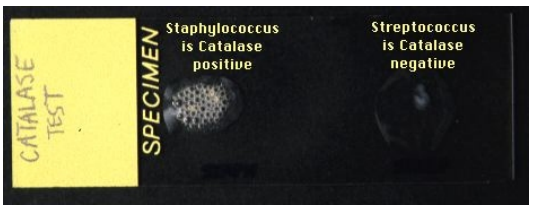
Figure-29



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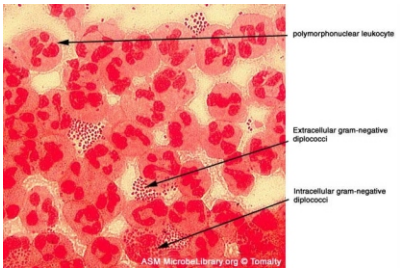

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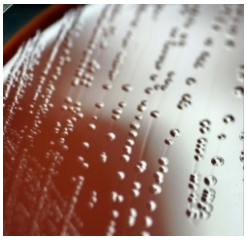

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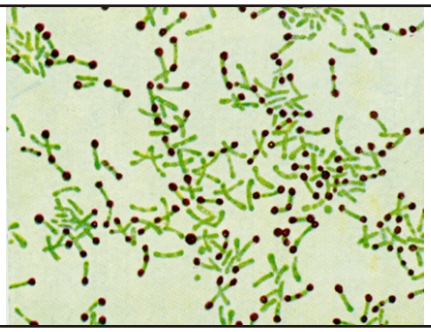

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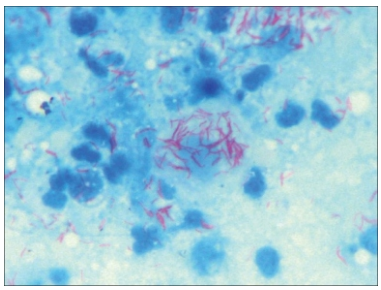

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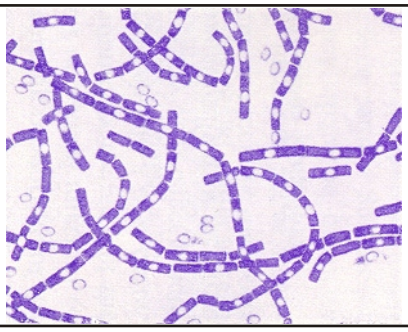

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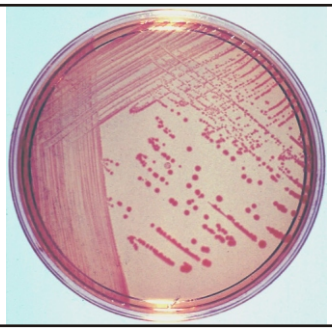

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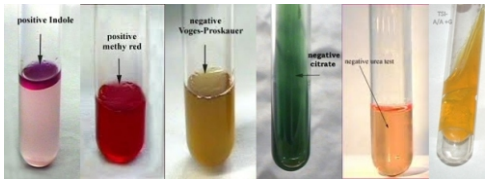

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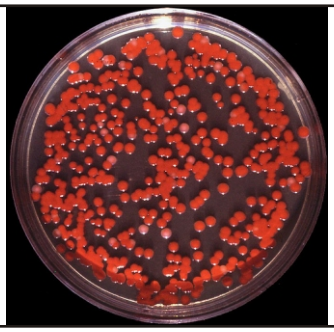

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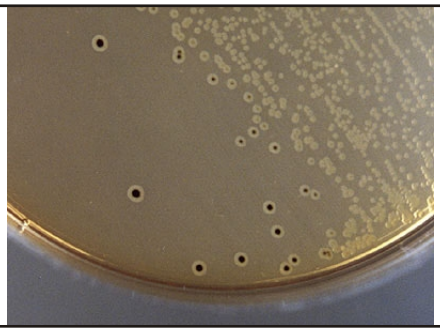

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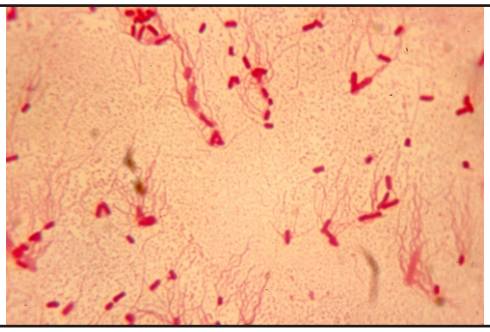

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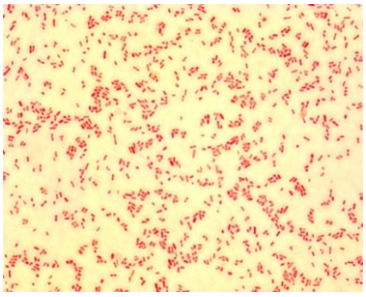

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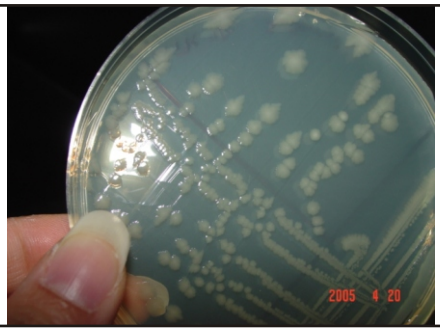

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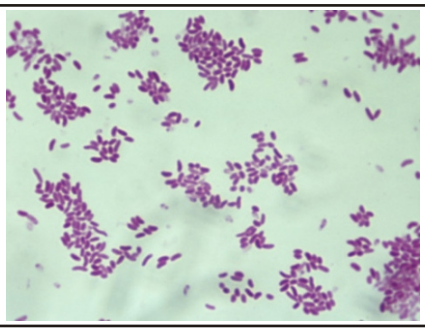

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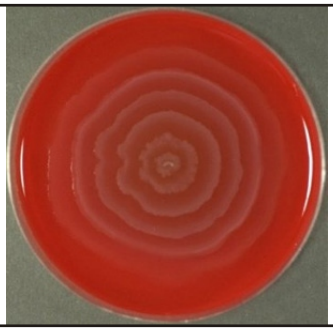

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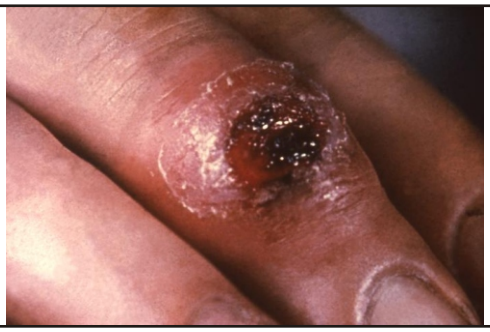

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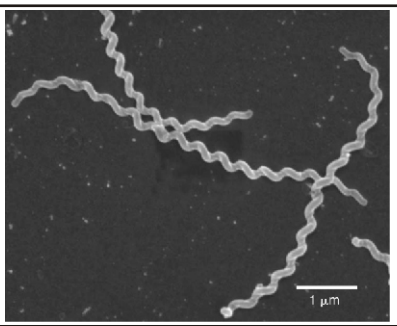

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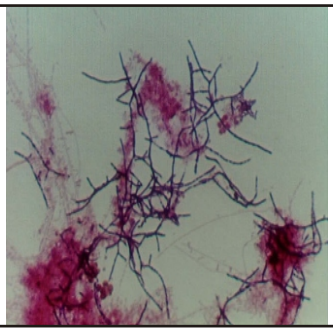

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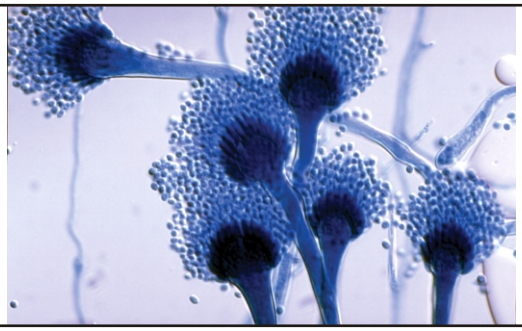
Figure-58



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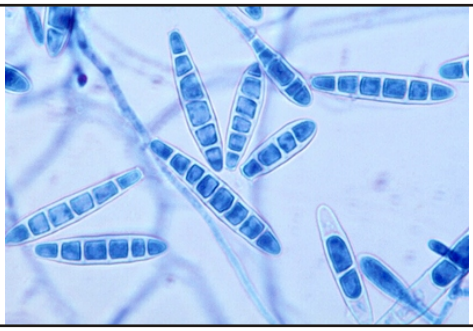

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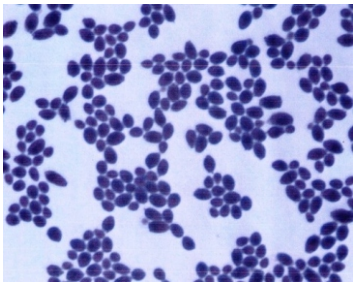

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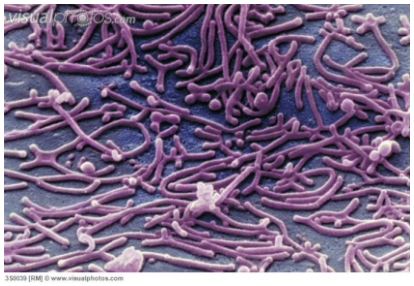

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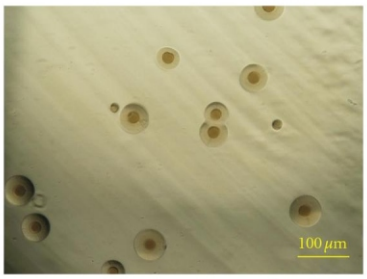

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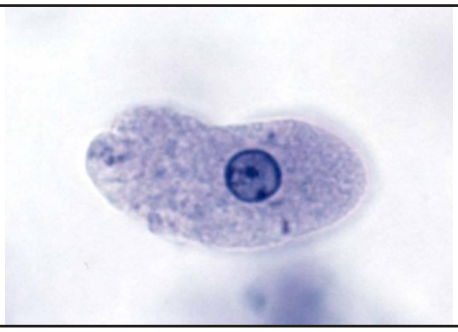

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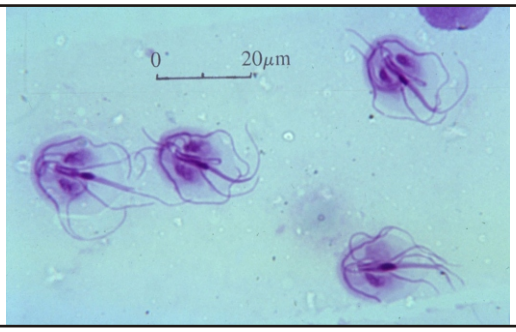

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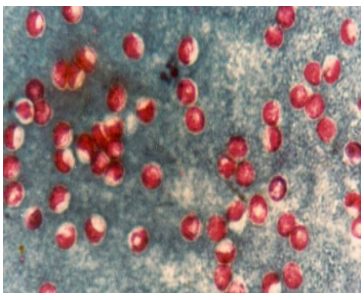

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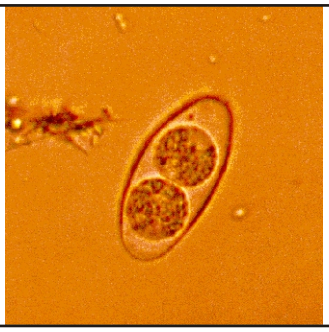

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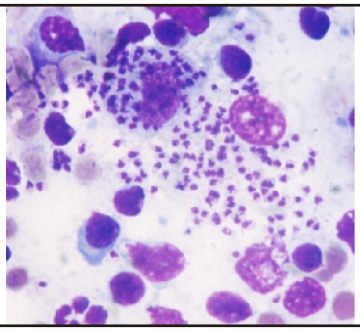

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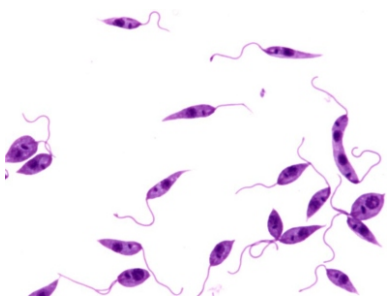

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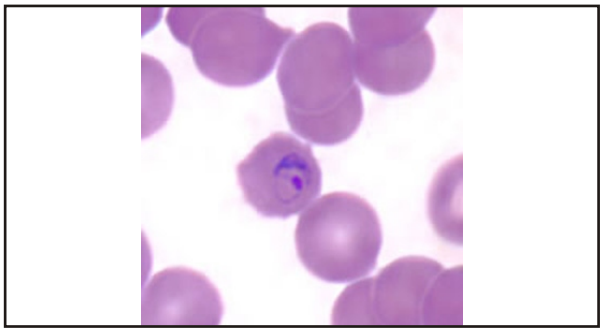

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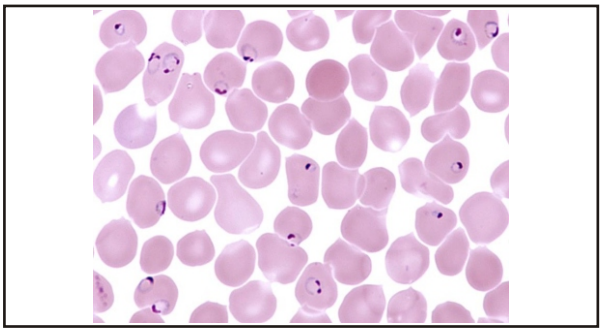

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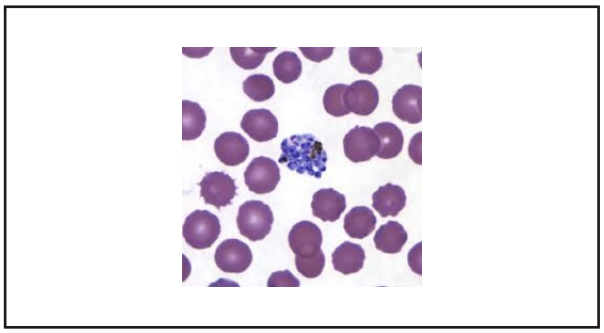

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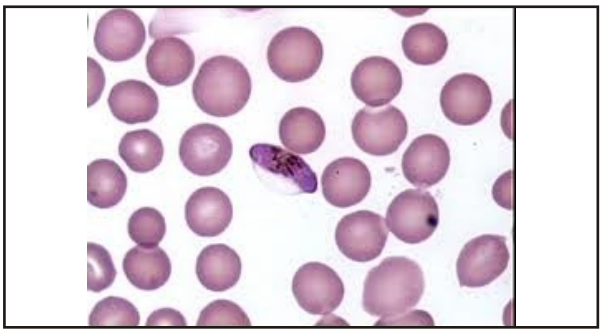

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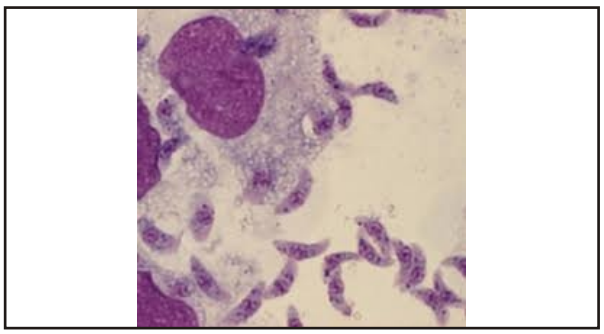

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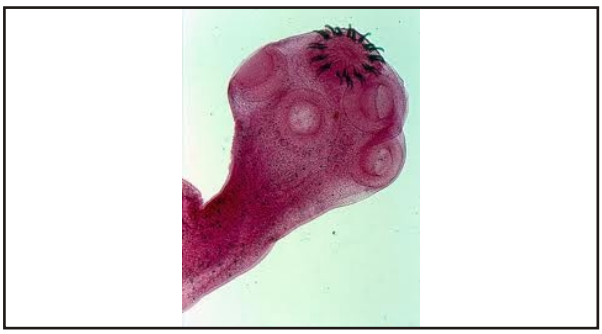

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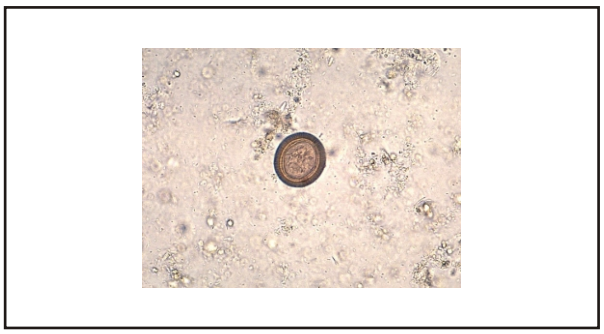

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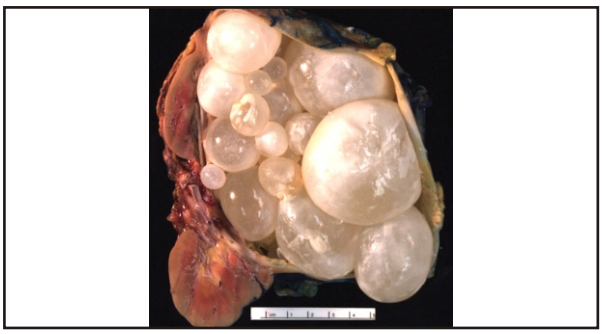

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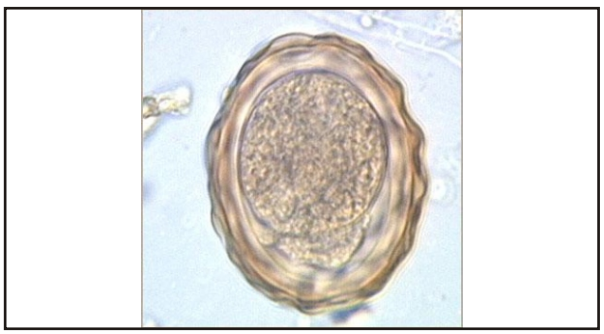

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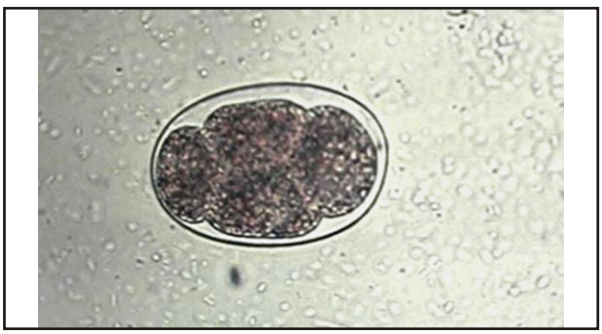

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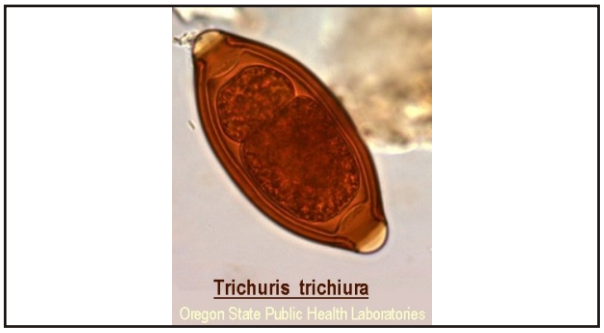

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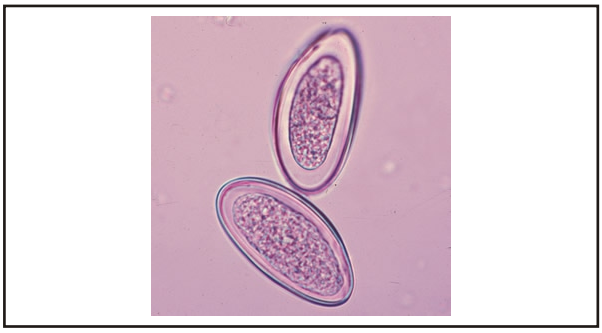

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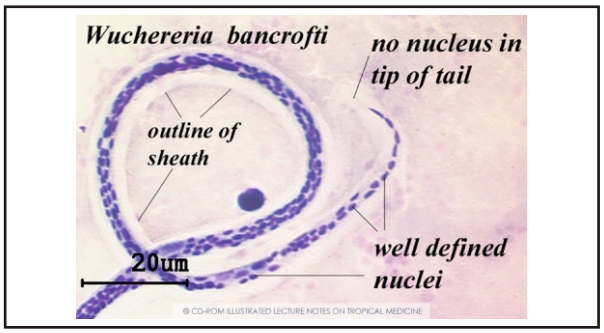

Figure-83

Figure-84

Annexure-I

Biomedical waste categories and treatment/disposal options

"Bio-medical waste" means any waste, which is generated during the diagnosis, treatment or immunisation of human beings or animals or research activities pertaining thereto or in the production or testing of biological or in health camps. Rules for management of biomedical waste were first published in 1998 (given by Govt. of India) and subsequently amended rules were published in 2016.

Segregation, collection, treatment, processing and disposal options of different categories of biomedical waste:

Category	Type of Waste	Type of Bag or Container to be used	Treatment and Disposal options
(1)	(2)	(3)	(4)
Yellow	**(a) Human Anatomical Waste:** Human tissues, organs, body parts and fetus below the viability period (as per the Medical Termination of Pregnancy Act 1971, amended from time to time).	Yellow coloured non-chlorinated plastic bags	Incineration or Plasma Pyrolysis or deep burial*
	(b)Animal Anatomical Waste : Experimental animal carcasses, body parts, organs, tissues, including the waste generated from animals used in experiments or testing in veterinary hospitals or colleges or animal houses.		
	(c) Soiled Waste: Items contaminated with blood, body fluids like dressings, plaster casts, cotton swabs and		Incineration or Plasma Pyrolysis or deep burial* In absence of above facilities, autoclaving or micro-waving/

	bags containing residual or discarded blood and blood components.		hydroclaving followed by shredding or mutilation or combination of sterilization and shredding. Treated waste to be sent for energy recovery.
	(d) Expired or Discarded Medicines: Pharmaceutical waste like antibiotics, cytotoxic drugs including all items contaminated with cytotoxic drugs along with glass or plastic ampoules, vials etc.	Yellow coloured non-chlorinated plastic bags or containers	Expired `cytotoxic drugs and items contaminated with cytotoxic drugs to be returned back to the manufacturer or supplier for incineration at temperature >1200 ^{0}C or to common bio-medical waste treatment facility or hazardous waste treatment, storage and disposal facility for incineration at >1200^{0}C Or Encapsulation or Plasma Pyrolysis at >1200^{0}C. All other discarded medicines shall be either sent back to manufacturer or disposed by incineration.
	(e) Chemical Waste: Chemicals used in production of biological and used or discarded disinfectants.	Yellow coloured containers or non-chlorinated plastic bags	Disposed of by incineration or Plasma Pyrolysis or Encapsulation in hazardous waste treatment, storage and disposal facility.
	(f) Chemical Liquid Waste : Liquid waste generated due to use of chemicals in production of biological and used or discarded disinfectants, Silver X-ray film developing liquid, discarded Formalin, infected secretions, aspirated body fluids, liquid from laboratories and floor washings, cleaning, house-keeping and disinfecting activities etc.	Separate collection system leading to effluent treatment system	After resource recovery, the chemical liquid waste shall be pre-treated before mixing with other wastewater. The combined discharge shall conform to the discharge norms given in Schedule-III.
	(g) Discarded linen, mattresses, beddings contaminated with blood or body fluid.	Non-chlorinated yellow plastic bags or suitable packing material	Non- chlorinated chemical disinfection followed by incineration or Plazma Pyrolysis or for energy recovery. In absence of above facilities, shredding or mutilation or combination of sterilization and shredding. Treated waste to be sent for energy recovery or incineration or Plazma Pyrolysis.

	(h) Microbiology, Biotechnology and other clinical laboratory waste: Blood bags, Laboratory cultures, stocks or specimens of micro-organisms, live or attenuated vaccines, human and animal cell cultures used in research, industrial laboratories, production of biological, residual toxins, dishes and devices used for cultures.	Autoclave safe plastic bags or containers	Pre-treat to sterilize with non-chlorinated chemicals on-site as per National AIDS Control Organisation or World Health Organisation guidelines thereafter for Incineration.
Red	**Contaminated Waste (Recyclable)** (a) Wastes generated from disposable items such as tubing, bottles, intravenous tubes and sets, catheters, urine bags, syringes (without needles and *fixed needle syringes*) and vaccutainers with their needles cut) and gloves.	Red coloured non-chlorinated plastic bags or containers	Autoclaving or micro-waving/ hydroclaving followed by shredding or mutilation or combination of sterilization and shredding. Treated waste to be sent to registered or authorized recyclers or for energy recovery or plastics to diesel or fuel oil or for road making, whichever is possible. Plastic waste should not be sent to landfill sites.
White (Translucent)	**Waste sharps including Metals:** Needles, syringes with fixed needles, needles from needle tip cutter or burner, scalpels, blades, or any other contaminated sharp object that may cause puncture and cuts. This includes both used, discarded and contaminated metal sharps	Puncture proof, Leak proof, tamper proof containers	Autoclaving or Dry Heat Sterilization followed by shredding or mutilation or encapsulation in metal container or cement concrete; combination of shredding cum autoclaving; and sent for final disposal to iron foundries (having consent to operate from the State Pollution Control Boards or Pollution Control Committees) or sanitary landfill or designated concrete waste sharp pit.
Blue	**(a) Glassware:** Broken or discarded and contaminated glass including medicine vials and ampoules except those contaminated with cytotoxic wastes.	Cardboard boxes with blue colored marking	Disinfection (by soaking the washed glass waste after cleaning with detergent and Sodium Hypochlorite treatment) or through autoclaving or microwaving or hydroclaving and then sent for recycling.

	(b) Metallic Body Implants	Cardboard boxes with blue colored marking	

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