MICROBIOLOGY

PRACTICAL MANUAL

Std XII Microbiology Practical Manual

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Higher Secondary – Second Year Practical Examination

Microbiology	
Marking Scheme	
Allotment of Marks	
Internal Assessment	05 marks
External Assesment	15 marks
Total	**20 marks**
Internal Assessment (Practicals) Marks Break Up	
1. Record Note Book	03 marks
2. Skill of performing Experiments	02 marks
Total	**05 marks**
External Assessment Mark Break Up	
1. Major Practical	09 marks
2. Spotters	06 marks
Total	**15 marks**
I. Major Practical (Any one out of 5 questions) 9×1 = 9 marks	
• Aim	01 mark
• Principle	02 marks
• Procedure	03 marks
• Diagram	01 marks
• Observation	01 marks
• Results	01 marks
Total	**09 marks**
II. Spotters (Any three – one from each category) 2×3 = 6 marks	
• Identification	½ marks
• Two salient points	1 mark
• Diagram	½ mark
Total	**02 marks × 3 spotters = 6marks**

Key for Practical Examination

I. Major Practical (Any one) 9×1 = 9 marks

1. Determine the gram nature of microorganism present in the given sample (curd/idly batter/yeast)
2. Identify whether the given fungus is *Aspergillus* or *Mucor* or *Rhizopus* based on its microscopic characteristics.
3. Determine the blood group of the given blood sample.
4. Carry out blood staining using field's stain and observe the erythrocytes and leucocytes.
5. Identify whether the given culture is catalase positive.

II. Spotters

A. Specimen	2 marks
B. Slide	2 marks
C. Spotter	2 marks

1. Gram's staining of curd/idly batter/yeast

Aim: To determine the gram nature of microorganism present in the given sample(curd/ idly batter/yeast) by Gram's staining technique.

Theory and Principle:

Based on gram staining reaction bacteria can be divided into two large groups called gram positive and gram negative. Gram positive bacteria have thicker peptidoglycan compared to the gram negative bacteria. The lipid content of the cell wall of gram negative bacteria is higher than that of gram positive bacteria. Both gram positive and gram negative bacteria take up the primary stain crystal violet and stain red. When decolorized the porosity and permeability of the gram negative bacteria increases due to which the crystal violet iodine complex is given out by the gram negative bacteria. Further the gram negative bacteria takes up the counterstain safranin and stains red. Hence when bacteria are observed under microscope after gram staining the gram positive cells appear violet and gram negative cells appear red.

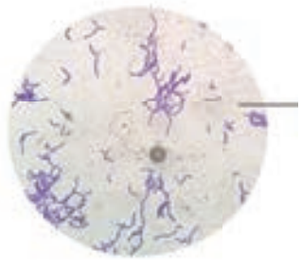
Requirements:

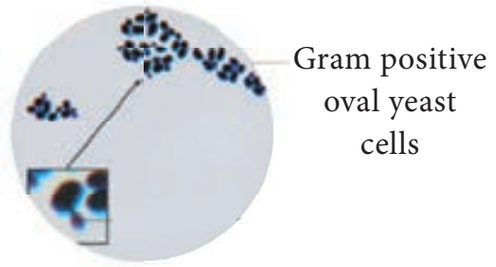
- Clean grease free slide
- Nichrome loop
- Given culture
- Crystal violet
- Grams iodine
- Decolorizer(Acetone Alcohol)
- Safranin
- D/W

Procedure:

1. Take a loopful of the given culture and place on the slide.
2. Prepare a smear and heat fix it.
3. Cover the smear with Crystal Violet for one minute.
4. Wash gently
5. Add Grams iodine for one minute
6. Decolorise with acetone alcohol
7. Wash the slide immediately
8. Cover the smear with safranin for a minute
9. Wash and Air dry.
10. Observe the slide under high power and oil immersion objectives.
11. Record your observations.

Diagram: (Any one Diagram)





Observation Table: (any one shape and stain)

Sr. no	Morphology	Arrangement	Colour of Cytoplasm	Colour of Background	Inference
1.	Rod (bacilli)	Singles, chains	Violet	colourless	Gram positive
2.	Oval yeast cells	Singles, budded	Violet	colourless	Gram positive

Results: Gram staining of the given culture revealed gram positive violet colored rod-shaped bacteria in chains.

2. Identification of the fungus (Aspergillus/Mucor/Rhizopus) by wet mount using LPCB.

Aim: To identify whether the given fungus is *Aspergillus* or *Mucor* or *Rhizopus* based on microscopic characteristics by wet mount method using lactophenol cotton blue stain.

Theory and Principle :

Filamentous fungi are reliably identified by their characteristics microscopic morphology such as shape, size and arrangement of spores and hyphae. Fungi are eukaryotic and range from unicellular yeast to multicellular molds. They reproduce by producing spores.

Common fungi are Aspergillus, Mucor and Rhizopus. They are filamentous and collectively form mycelium. The morphology of the hyphae and spores can be identified using a simple wet mount technique using lactophenol cotton blue stain.

The organism suspended in the stain are killed due to the presence of phenol. Lactic acid preserves fungal structures and cotton blue stains the fungal cell wall.

Fungi	Characteristics of Hyphae	Spores borne in
Aspergillus sp.	Septate	Conidiophore bear conidia
Mucor and Rhizopus sp.	Aseptate	Sporangiosphore bear sporangium containing sporangiospore.

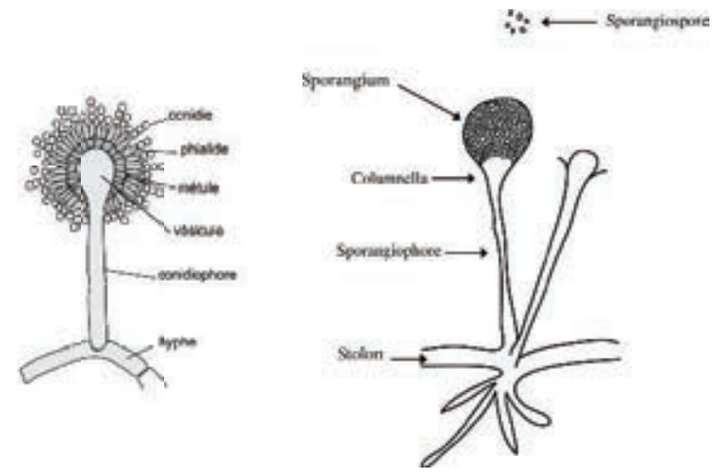
Requirements :

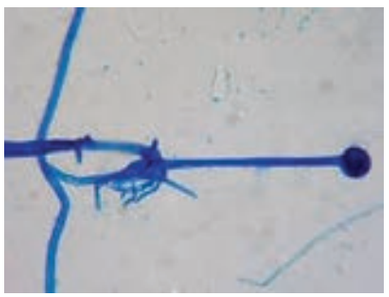
- Clean grease free slide
- Coverslip
- Forcep
- Teasing needle
- Distilled water
- Lactophenol Cotton Blue

Procedure:

1. Take a clean slide.
2. Place a drop of water on the slide.
3. With the help of forceps transfer the fungal mycelium.
4. Tease it with needle to separate the filaments (hyphae).
5. Add a drop of lactophenolcotton blue.
6. Gently place a coverslip avoiding air bubble formation.
7. Observe under low power and high power objective lens.
8. Read the observations and interpret.

Diagram:





Observation;

Filamentous hyphae bearing sporangia were observed.

Results:

Wet mount using lactophenol cotton blue was carried to identify the fungus sample. Hyphae with sporangium bearing sporangiospores were observed. It is likely to be of mucor species.

3. Blood Grouping

Aim: To determine the blood group of the blood sample by the slide agglutination test.

Theory and Principle:

Blood grouping is an essential requirement before blood is transfused from one person to another.It is also useful in settling paternity disputes and medicolegal problems.

Red blood cells contain blood group antigens. Antibodies to the blood group antigens are present in the blood plasma.The antigens are generally determined and are responsible for blood types. When RBCs of a person are mixed with corresponding antiserum, agglutination occurs due to antigen-antibody reactions.

Materials Required

- Blood sample (anticoagulated)
- Sterile cotton
- Sterile lancet
- Clean dry grease free slides or white tile
- Toothpicks
- Marker pen
- Commercially available Anti A sera, Anti B sera and Anti D sera

Procedure

1. Prick the finger under aseptic conditions
2. Place a drop of blood on the slide on each side marked as A, B and D.
3. Add a drop of antiserum A , B and D on A, B and D side respectively.
4. Mix with toothpick using separate toothpicks for each mixture.
5. Wait for 2 mins and observe for clumping reaction if any confirm it by observing under microscope.
6. Interpret the results and report.

Interpretation

If agglutination on A side the blood group is A

If agglutination seen on B side the blood group is B

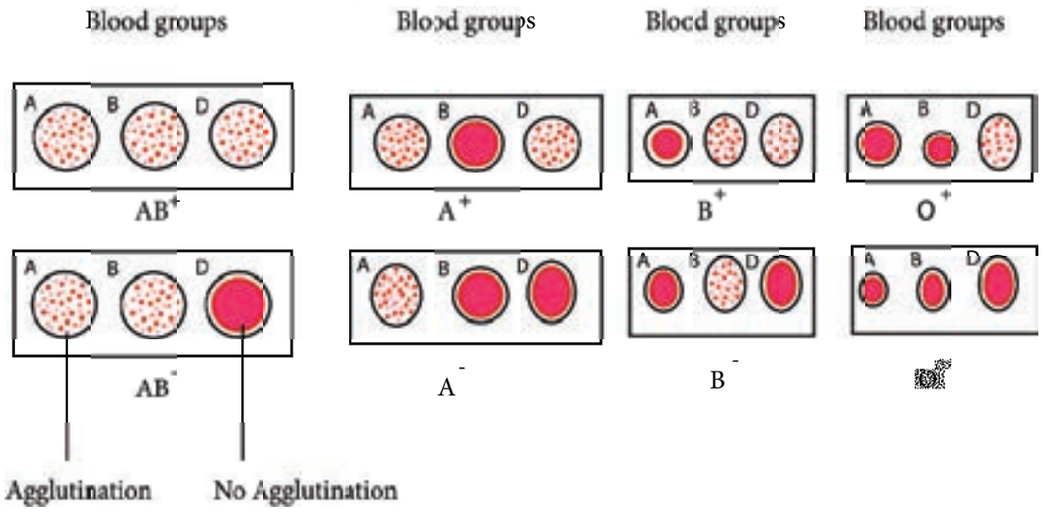
If Agglutination on both A and B side the blood group is AB

If No agglutination on A and B side the blood group is O

If agglutination is seen on D side the blood group is Rh(D) positive

If No agglutination on D side the blood group is Rh(D) negative.

Diagram: (any one depending on the results)



Observation: (will vary with the type of blood group an example is given below)

Agglutination is seen on A, B and D side

Result: The blood group of the blood sample was determined by slide agglutination test and was found to be AB Rh positive.

4. Blood Staining

AIM

To make a blood smear ,stain it using Field's stain and observe the erythrocytes and leucocytes.

Theory and Principle:

Blood smears are used to determine leukocyte differentials, to evaluate erythrocyte, platelet and leukocyte morphology, and, if necessary, to estimate platelet and leukocyte counts. It is also used for diagnosis of parasites like plasmodium in the blood.

Field's Stain is a romanowsky stain, used for rapid processing of blood specimens and is used to stain thick and thin films. It consists of two differential stain.**Field stain A**

which is methylene blue and Azure dissolved in a phosphate buffer solution.It is the basic component of the stain and **Field stain B** made up of Eosin Y in a buffer solution which is the acidic component of the stain.These basic and acidic dyes induce several colours when applied to cells.The fixator, methanol, does not allow any additional changes to the slide. The basic component of peripheral white blood cell(cytoplasm) is stained with acid dye and the acid component that is nucleic acid of the nucleus takes on the basic dye and is stained blue to violet. The neutral components of the cells are stained by both dyes(Field's stain A and B solution).

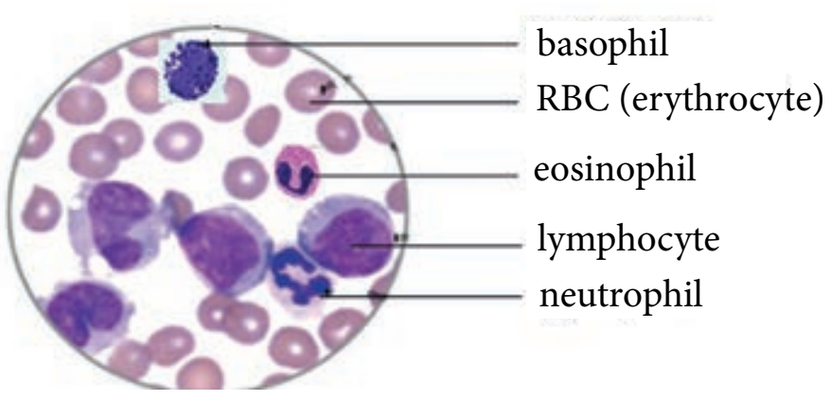
Requirements

- Cotton
- Spirit
- Blood sample
- Clean grease free slides
- Methanol fixative
- Field's stain A and Field's stain B.

Procedure

1. Finger Prick under aseptic condition.
2. Place a small drop of blood, on one side about 1-2 cm from one end of a slide.
3. Without delay place another slide at an angle of 45° to make contact with the drop.
4. Spread it over an area of about 2 cm^2(The film should be distributed so thinly that it appears transparent.
5. After air drying the thin blood film,immerse or fix the smear in methanol for 1 minutes.
6. Flood or dip the slide in Field's Stain A for 2-3 seconds.
7. Wash it with distilled water,
8. Flood or dip the slide in Field's Stain B for 2-3 seconds and wash with distilled water.
9. Now air dry the smear and observe under microscope.

Diagram



Observation

TYPE OF CELL	COLOUR OF CYTOPLASM	COLOUR OF NUCLEUS	COLOUR OF GRANULES
RBC	pink	-	-
WBCs(leucocytes)			
Neutrophil	pink	blue	lilac
Eosinophil	pink	blue	orange
Basophil	pink	blue	Dark blue black
lymphocyte	blue	violet	-

Results

The blood smear was stained using field's stain and erythrocytes and leucocytes were observed under microscope.

5. Test for Catalase

Aim

To test whether the given culture is catalase positive by the catalase test

Theory And Principle

Catalase test demonstrates the presence of catalase, an enzyme that catalyses the release of oxygen from hydrogen peroxide (H_2O_2). It is used to differentiate those bacteria that produces an enzyme catalase, such as *staphylococci*, from non-catalase producing bacteria such as *streptococci*.

The enzyme catalase mediates the breakdown of hydrogen peroxide into oxygen and water. The presence of the enzyme in a bacterial isolate is evident when a small inoculum is introduced into hydrogen peroxide, and the rapid elaboration of oxygen bubbles occurs. The lack of catalase is evident by a lack of or weak bubble production. The culture should not be more than 24 hours old.

Requirements

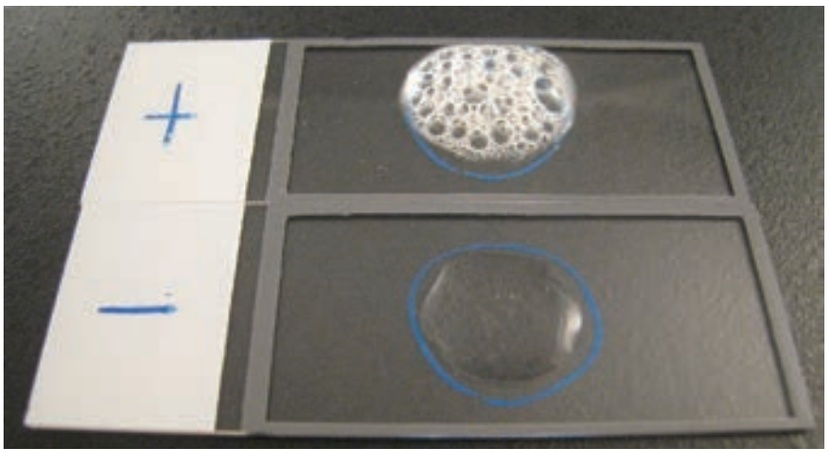
- Slides
- Nichrome loop or toothpick
- 24hour old culture
- 3%hydrogen peroxide
- Dropper

Procedure

Slide Method

1. Use a loop or sterile wooden stick to transfer a small amount of colony growth in the surface of a clean, dry glass slide.
2. Place a drop of 3% H_2O_2 in the glass slide.
3. Observe for the evolution of oxygen bubbles.

Diagram



$2H_2O_2 \xrightarrow[\text{catalase}]{} 2H_2O + O_2$ (gas bubbles)

Observation (any one to be reported depending on the culture)

Positive: Copious bubbles produced, active bubbling

Examples: *Staphylococci, E. coli, Enterobacter, Klebsiella, Shigella, Yersinia, Pseudomonas.*

Negative: No or very few bubbles produced.

Examples: *Streptococcus and Enterococcus* sps.

Result

The given culture was found to be catalase positive as determined by the catalase slide test.

6. Widal Test (Slide Test)

Aim

To carry out the widal test for the given blood sample and to determine the presence of antibodies against salmonella antigens.

Theory And Principle

Widal test is a serological test which is used for the diagnosis of enteric fever or typhoid fever. Typhoid or enteric fever is caused by a gram negative bacteria *Salmonella enterica* (*Salmonella* Typhi or *Salmonella* Paratyphi). *Salmonella* possess O antigen on their cell wall and H antigen on their flagella. On infection, these antigen stimulates the body to produce specific antibodies which are released in the blood. The Widal test is used to detect these specific antibodies in the serum sample of patients suffering from typhoid using antigen-antibody interactions. These specific antibodies can be detected in the patient's serum after 6 days of infection (fever).

Salmonella Typhi possesses O antigen on the cell wall and H antigen on flagella. *Salmonella* Paratyphi A and *S.* Paratyphi B also possess O antigen on their cell wall and but have AH and BH antigen on their flagella respectively.

Widal test is an agglutination test in which specific typhoid fever antibodies are detected by mixing the patient's serum with killed bacterial suspension of Salmonella carrying specific O, H, AH and BH antigens and observed for clumping ie. Antigen-antibody reaction. The main principle of Widal test is that if homologous antibody is present in patient's serum, it will react with respective antigen in the suspension and gives visible clumping on the test slide.

Requirements

Fresh serum

The complete kit containing five vials containing stained Salmonella antigen

- *S.* Typhi ⟶ O antigen
- *S.* Tyhhi ⟶ H antigen
- *S.* Paratyphi ⟶ AH antigen
- *S.* Paratyphi ⟶ BH antigen

Widal positive control

Widal test card or slide

v) Applicator stick

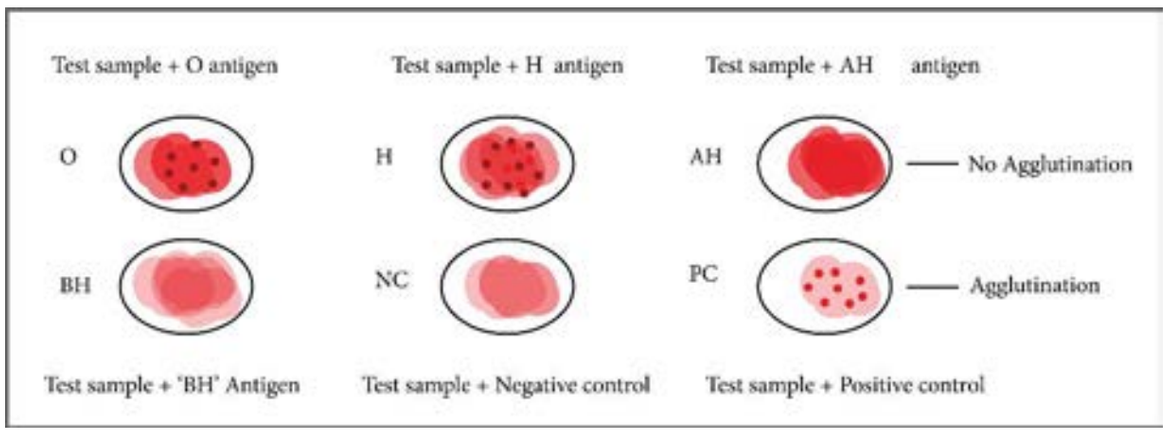
Procedure

- Widal test can be done in two ways-one is rapid test on slide and another is tube test in which result may be obtained after one night of incubation.

Rapid slide test:

1. Clean the glass slide or test card supplied in the kit well and make it dry.
2. Label the circles (1, 2, 3, 4, 5 and 6) in the test card as O, H, AH, BH, Negative control and Positive control
3. Place a drop of undiluted test serum in each of the four labelled circle (1, 2, 3 and 4) ie O, H, AH and BH and place a drop of Negative control serum in circle 5 and Positive control in circle 6.
4. Place a drop of antigen O, H, AH and BH in circle 1, 2, 3, and 4 respectively and no antigen in circle 5 and O/H antigen in circle 6.
5. Mix the content of each circle with a separate wooden applicator stick and spread to fill the whole area of the individual circle.
6. Rock the test card for a minute and observe for agglutination.

Diagram



Observation

Agglutination was observed in O and H side within a minute which indicates the presence of antibodies in the serum sample against Salmonella typhi antigens.

Proceed for quantitative slide test or tube test for the quantitative estimation of the titre of the antibody.

Result

Qualtative widal test was carried out using rapid slide agglutination method. Antibodies against O and H antigens of Salmonella typhi were detected in the serum.

7. Demonstration of rhizobium from root nodules and its isolation

Aim:

To demonstrate the presence of rhizobium in root nodules by gram staining and isolate them on a nutrient medium.

Theory and Principle:

Leguminous plants like cowpea, red gram , black gram contain root nodules formed by rhizobium.Rhizobium in the soil enter into the roots of leguminous plant and form nodules and establish symbiotic association. Bacteria derive nutrients from the plants. The rhizobacteria fix nitrogen which is beneficial to the plant. Rhizobium is a symbiotic N_2 fixer found to occur as bacteroids in the root nodules of leguminous plants. They can be easily isolated and cultured in vitro.

Based on gram staining reaction bacteria can be divided into two large groups called gram positive and gram negative. Gram positive bacteria have thicker peptidoglycan compared to the gram negative bacteria. The lipid content of the cell wall of gram negative bacteria is higher than that of gram positive bacteria. Both gram positive and gram negative bacteria take up the primary stain crystal violet and stain red. When decolorized the porosity and permeability of the gram negative bacteria increases due to which the crystal violet iodine complex is given out by the gram negative bacteria. Further the gram negative bacteria takes up the counterstain safranin and stains red. Hence when bacteria are observed under microscope after gram staining the gram positive cells appear violet and gram negative cells appear red. **Rhizobia are Gram- negative rods which are motile with bi-polar, sub-polar and peritrichous flagella**.

Rhizobium grows well on Yeast Extract Mannitol Agar (YEMA). Congo red added to the medium differentiates rhizobia that stand out as white, translucent, glistening elevated, small colonies with entire margin, in contrast to the red stained colonies of Agrobacterium and other bacteria.

Requirements:

1. Root nodules (pink) of any leguminous plant
2. **Congo red, Yeast Extract, Mannitol Agar (pH 6.8 – 7.0):**

Mannitol	10.0 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.2 g
NaCl	0.1 g
Yeast extract	1.0 g
$CaCO_3$	3.0 g
Agar	25.0 g

Congo red (1% aqueous)

2.5 ml (1.0 g in 100 ml)

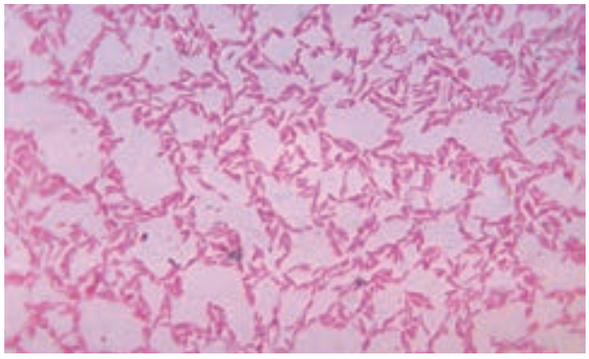
Distilled water 1000.0 ml

3. Inoculation loop
4. Bunsen burner/laminar clean air flow hood.
5. Slides and glass rod.
6. Petri plates with YEMACR medium.
7. Sterile distilled water.
8. 95% alcohol and 0.1% $HgCl_2$.

Procedure:

1. Wash the root system under a slow stream of running tap water, taking care to see that the nodules are intact.
2. Select pink nodules and remove them
3. Wash and keep the nodules in 95% ethanol for a minute, wash and transfer them to 0.1% $HgCl_2$.
4. Remove after five minutes and wash the nodules about four to five times with sterile distilled water.
5. Place the nodule on a sterile slide in a drop of sterile distilled water and crush it either with a sterile glass rod or a flat tipped forceps.
6. Remove a loopful of this cloudy suspension and streak inoculate on YEMACR plates and label.
7. Incubate in dark at 28°-30°C for 2-3 days and observe the colonies.
8. Make a smear of the remaining crushed material and gram stain and observe the gram negative bacilli. Even samples from the colonies can be gram stained.

Diagram:



Observation

Gram's stain

Organism	Morphology	Arrangement	Colour of cytoplasm	Colour of Background	Inference
Rhizobium from root nodule.	Rod (bacilli)	Singles	red	colourless	Gram negative

Colony characteristics of rhizobium on YEMA after incubation for 2-3 days at room temperature

Size – 2-4 mm

Shape- circular

Colour – White

Margin - entire

Elevation – convex, raised

Opacity - semitranslucent

Texture – creamy

Consistency – mucilaginous

Gram nature – gram negative

Motility – actively motile

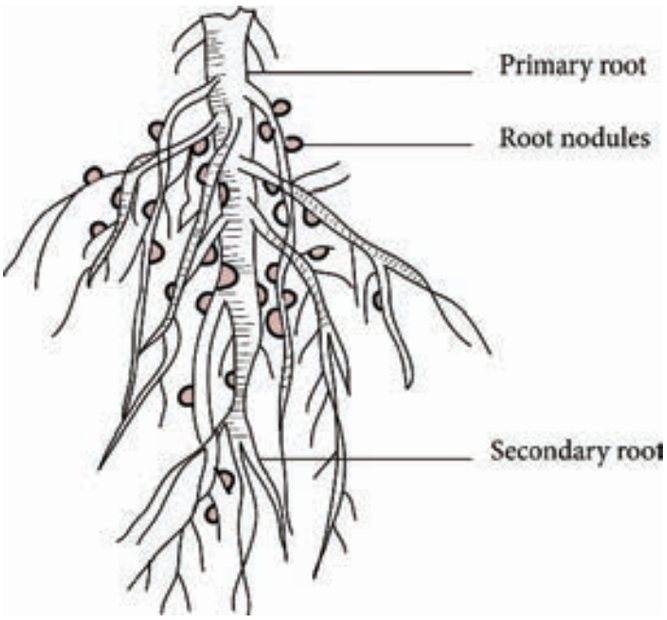
Results: Gram staining of the root nodule exudate revealed the presence of gram negative rods.

The colony characteristics of rhizobia were studied after isolation on YEMA medium. White, creamy, mucoid colonies were obtained.

Spotters

II A) Specimen

1. Root nodules of leguminous plant

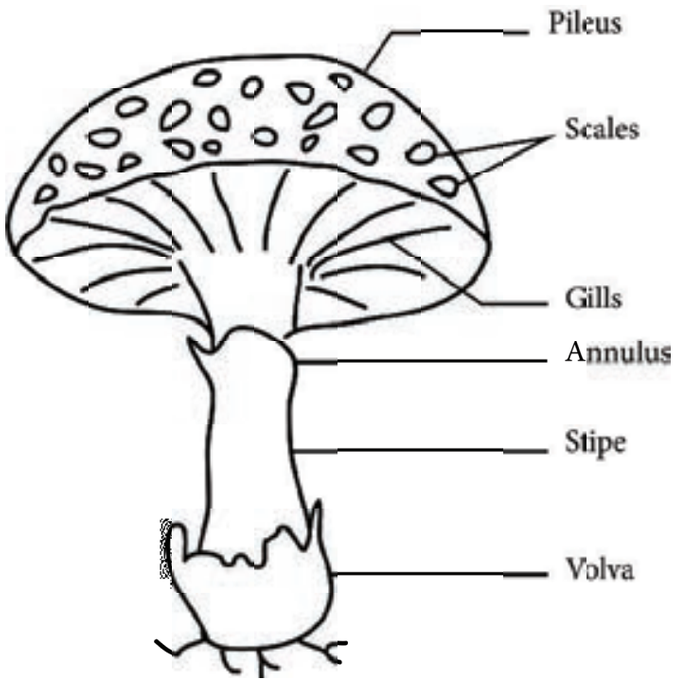


- Leguminous plants like cowpea, red gram contain root nodules formed by rhizobium.
- Rhizobium in the soil enter into the roots of leguminous plant and form nodules and establish symbiotic association.
- Bacteria derive nutrients from the plants.
- The rhizobacteria fix nitrogen which is beneficial to the plant.

2. Tikka leaf spot of groundnut plant

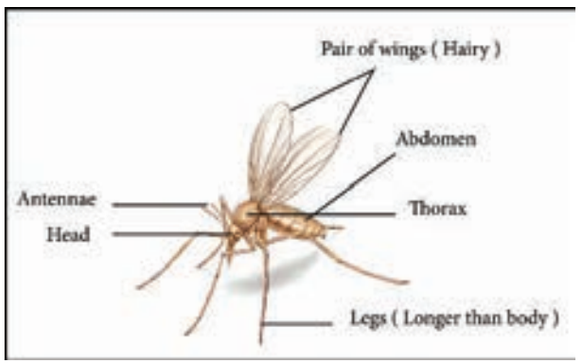
- Tikka leaf spot disease is a kind of fungal disease seen in groundnut leaf.
- This disease is caused by Cercaspora personata.
- Brown spots surrounded by a yellow halo appear on the upper surface of the leaf.
- The fungal spores can be demonstrated if the leaf is processed and observed under microscope.

3. Mushroom



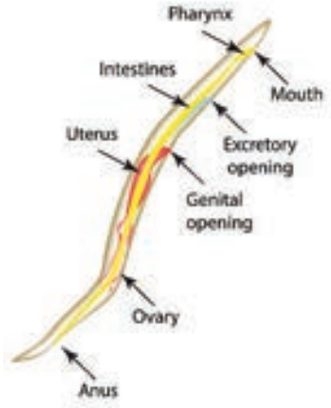
- Mushroom is a saprophytic fungus.
- Primary mycelium grows from basidiospores.
- It has high protein content and edible mushrooms are used as food.
- Example: Agaricus species and Pleurotus species.

4. Sand fly



- Bite of an infected sandfly transmits leishmania donovani infection.
- Female sandfly during a blood meal ingest free as well as intracellular amastigotes in the blood.
- In the midgut these are transformed to flagellated promastigote.

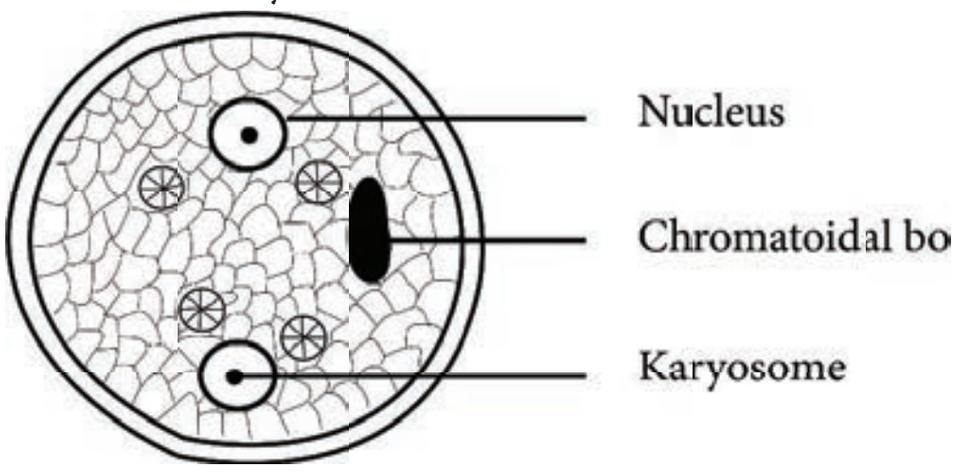
5. Ascaris



- The adult worm of ascaris lives in the small intestine of humans
- They are large cylindrical worms with tapering ends, the anterior end being thinner than the posterior end
- The adult male worm is smaller than female worms.

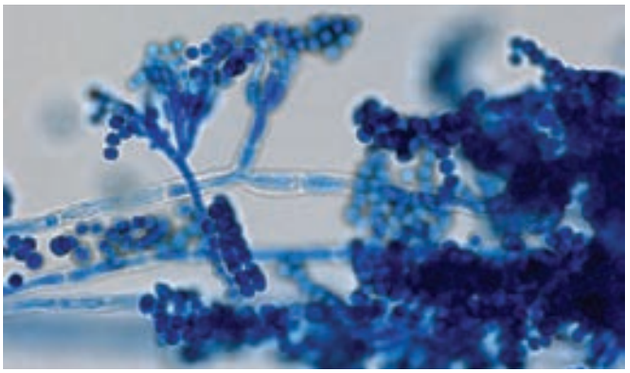
IIB) Slide

6. Cyst of Entamoeba histolytica



- Cyst is one of the three forms of entamoeba histolytica
- A mature cyst is a quadrinucleate spherical body.
- Mature cysts are passed in the stool of infected person
- Direct examination of wet mount of stool for cysts is diagnostic of intestinal amoebiasis

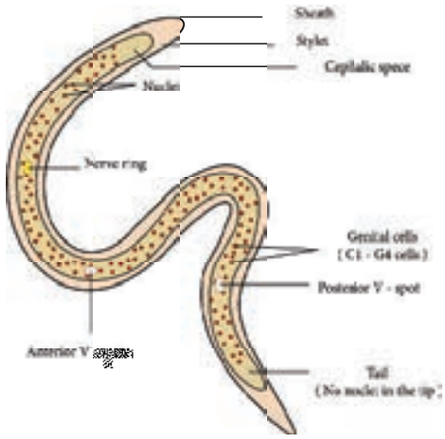
7. Penicillium species



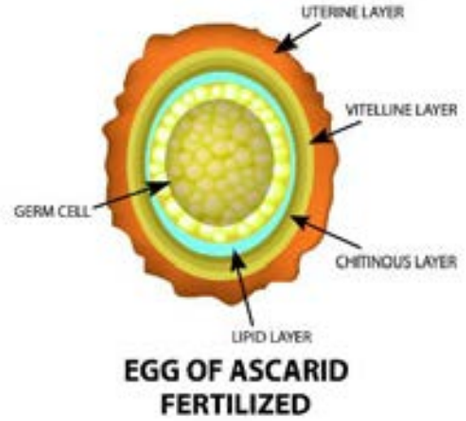
- Colony of penicillium are initially white and fluffy and later produce pigmented spores and turn into shades of green or blue green
- Hyphae are hyaline and septate
- Condiophores are long, give rise to branching phialids
- Phialids branch and give the appearance of brush or penicillins
- They produce sterigmata bearing chain of conidia (spores) which are oval or spherical and measure 1-2micrometer.

8. Microfilariae

- Filariasis is caused by nematodes (roundworms) like *Wuchereria bancrofti* that inhabit the lymphatics and subcutaneous tissues.
- The female worms release the first stage larvae called microfilariae, which are detected in the peripheral blood.
- Identification of microfilariae by microscopic examination is the most practical diagnostic procedure.
- The blood sample can be a thick smear, stained with Giemsa.
- The larva measures about 290microns in length and 6-7micron in breath.

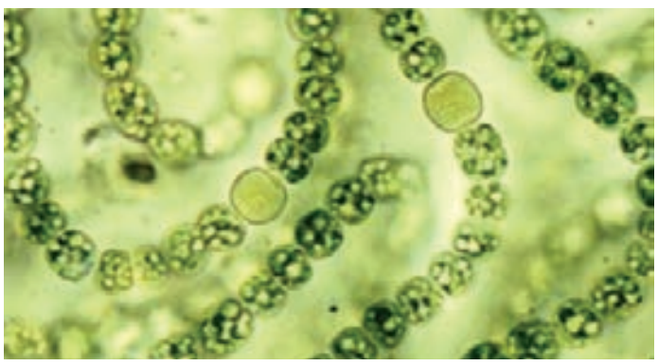


9. Egg of Ascaris lumbricoides



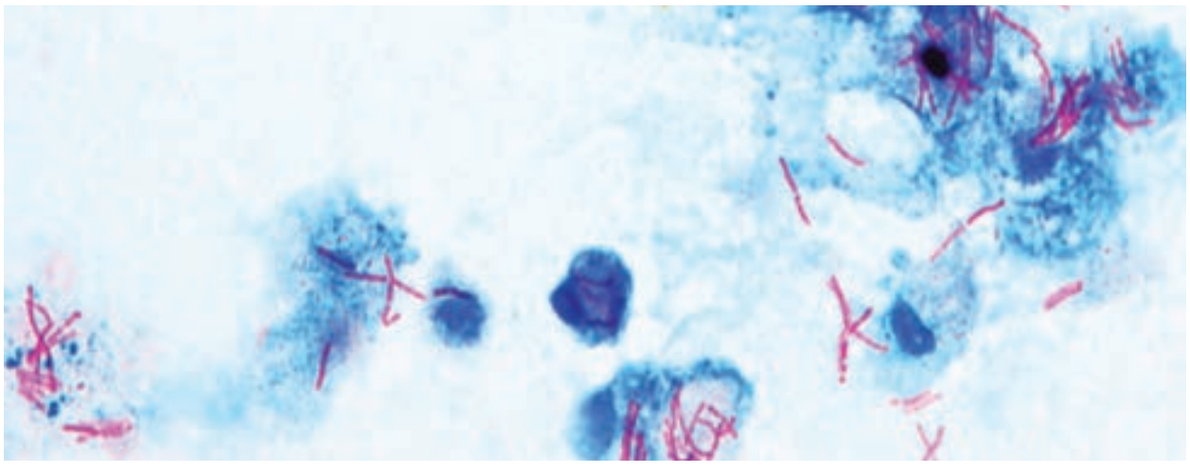
- These are passed in stool of the infected host.
- Brownish due to bile pigment.
- Fertilised eggs are rounded and have a thick shell (chitinous).
- Unfertilised eggs are elongated and larger than fertile eggs.
- When ingested through water or contaminated food by human it causes Ascariasis.
- Microscopic identification of eggs in the stool is the most common method for diagnosing intestinal ascariasis.

10. Heterocysts of Nostoc



- Heterocysts are specialized structures having thick cell wall formed in some filamentous blue green algae like Nostoc, Anabena.
- They may be terminal or found in between the vegetative cells attached to it by means of pores.
- They are sites of atmospheric nitrogen fixation.
- They serve as a store house of food material.

11. Acid fast bacilli

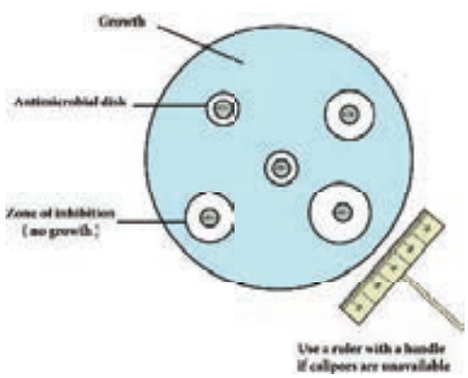


- Acid fast bacilli contains mycolic acid in their cell walls hence do not get stained easily, however once stained cannot be decolourised easily.
- Special method like Ziehl- Neelson's Carbol fuchsin is used to stain acid fast bacilli.
- The acid- fast bacilli are stained red in colour while the non acid fast cells appear blue when counterstained with methylene blue.
- Mycobacterium tuberculosis is and acid fast bacilli.

IIC) Spotter

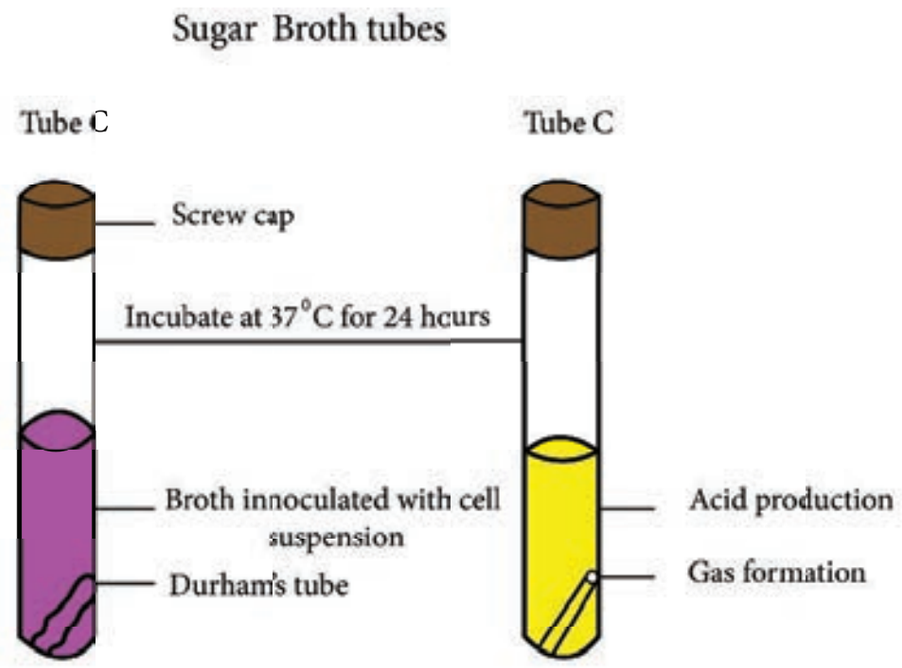
12. Antibiotic sensitivity plate set up by Kirby Bauer technique

- Kirby Bauer technique is used to determine the susceptibility of the organism to various antimicrobial agents.
- Standard suspensions of rapidly growing test bacterium is inoculated on the surface of muller hinton agar plates.
- Antibiotic discs are pressed on the surface of the seeded plates.
- The zone of inhibition or the zone of growth determines the degree of susceptibility of the organism towards antibiotic.



13. Sugar fermentation tube showing acid and gas production

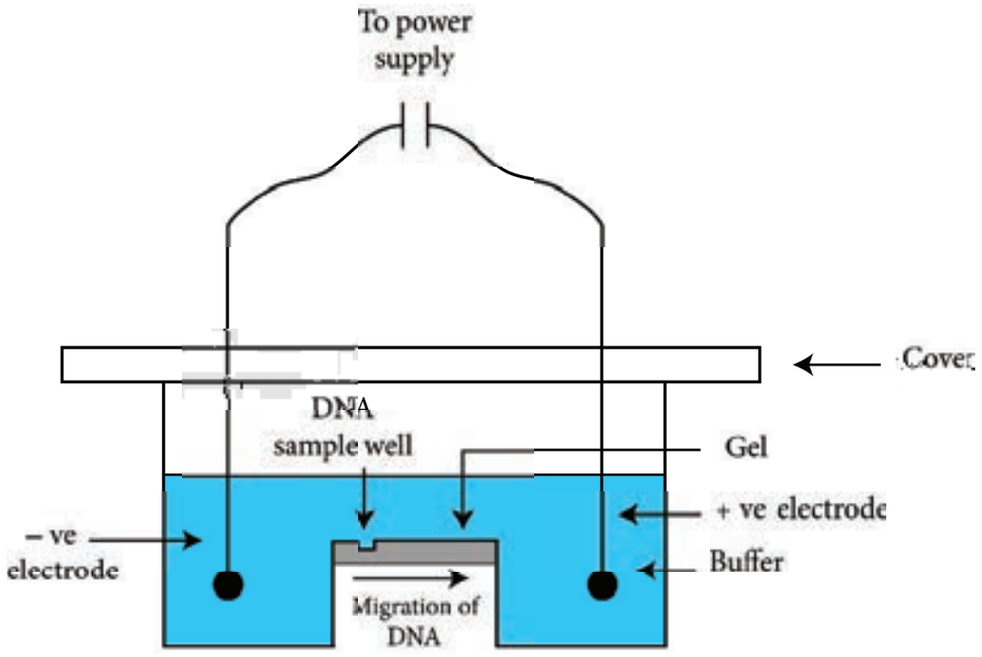
- Carbohydrate broth with bromocresol purple as indicator is used for testing the ability of pure bacterial culture to ferment a specific sugar like lactose, xylose, mannitol and other sugars.
- Acid production is indicated by colour change of the indicator from purple to yellow
- Gas production is indicated by an air bubble in the durham's tube.
- Escherichia coli ferments lactose producing acid and gas.



In the above image Test tube C was innoculated with cell suspension and incubated at 37 C for 24 hours and after innoculation the purple colour of broth was changed to yellow colour with gas bubble formation in durham's tube.

14. Agarose gel electrophoresis apparatus.

- Electrophoresis refers to the movement of charged molecules in an electric field.
- The negatively charged molecules move towards the positive electrode while the positively charged molecules migrate towards the negative electrode.
- Gel electrophoresis is a routinely used analytical technique for the separation and purification of specific DNA fragments.
- As the DNA is negatively charged, DNA fragments move through the gel towards the positive electrode. The rate of migration of DNA is dependent on the size and shape.



15. Spoiled food

- Spoilage is a process in which food deteriorates such that its quality of edibility is reduced.
- Food poisoning may result on eating contaminated or spoiled food.
- Foods spoil due to attacks from enzymes, oxidation and microorganisms.
- These include bacteria, mold, yeast, moisture, temperature and chemical reaction.