قسم التقنيات الاحيائية

مادة الاحياء المجهرية1 (العملي)

المرحلة الثانية

الكورس الاول

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2021-2022

Lab. one

First lab.

Biosafety procedure and precautions:

1. At first the laboratory coats and medical gloves must be worn.
2. Long hair must be tied back away from the shoulders.
3. Working areas must be kept clear of all unnecessary items or tools.
4. Hand and bench tops must be kept clean and sterilized by sung disinfectants.
5. Nothing is placed in the mouth such as fingers, pencils or any subject.
6. Do not smoke, eat or drink in the laboratory.
7. Any student with a fresh, unhealed cut, scratch, burn or other injury on either hand should notify the instructor before beginning or continuing with the laboratory work.
8. Notify supervisor immediately of any accidents (such as spelling of a dangerous cultured infections materials) or unsafe conditions in the lab.
9. Avoid Unnecessary activities which can cause accidents and promote contamination.
10. Before leaving the laboratory must be organized and wash and disinfect your hands with soap.

Microscope

Microscope : Is an optical instrument that magnifies images of objects that invisible to the naked eye by having a magnifying lens or a combination of lenses .

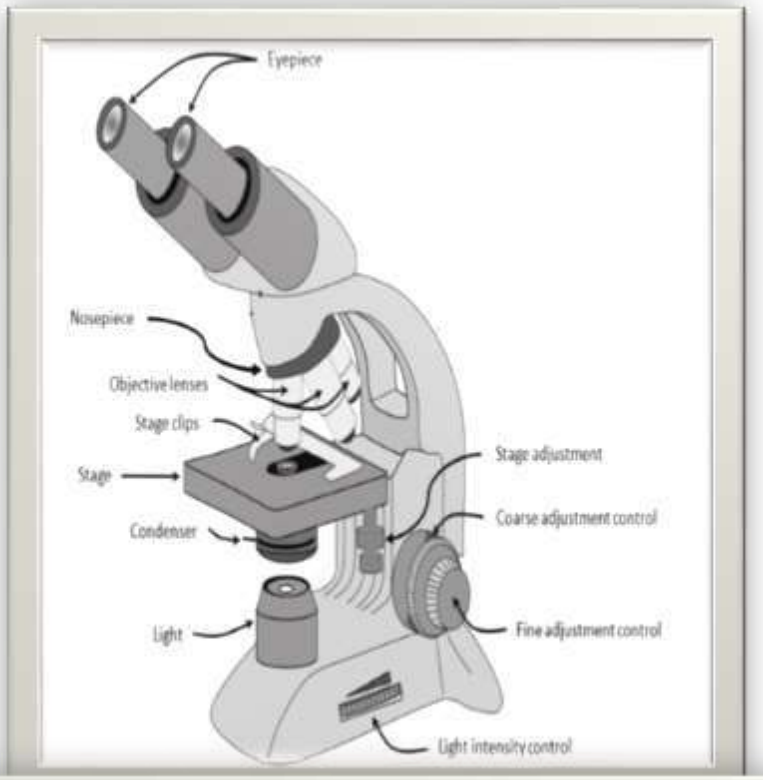
Types of microscope:

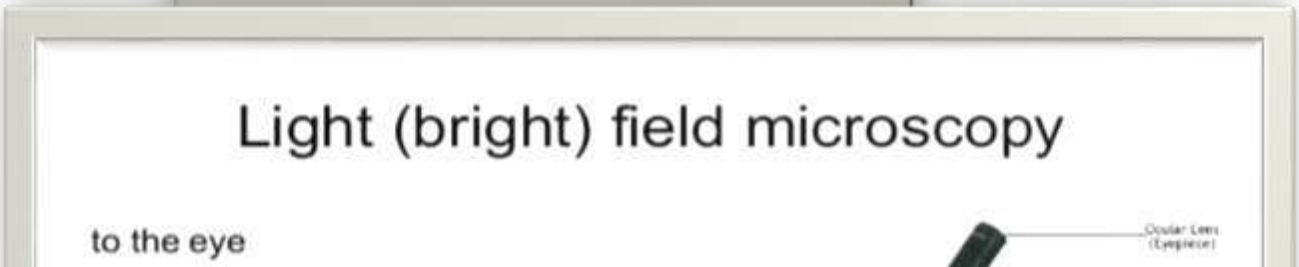
1. Light microscope:
   a. Bright field light microscope (as in our lab).
   b. Phase contrast microscope
   c. Dark field microscope.
   d. Fluorescence microscope.
2. Electron microscope.
   a. Transmission electron microscope (TEM).
   b. Scanning electron microscope (SEM).

Bright field light microscope component :

1. Base . 2. Arm. 3. Illuminator. 4. Condenser. 5. Stage. And clip.

6. Objective lenses :
a. Scanning lens (4 X).
b. Low power lens (10 X).
c. High power lens (40 X).
d. Oil immersion lens (100 X).
7. Coarse and fine adjustment.
8. Ocular or eye piece lens (10 X).





How can you measure magnification power ?

By multiply power of objective lens X power of ocular lens example if the objective lens is on light (40 X) so the magnification power equal to :

40 X 10 (ocular lens) = 400

Types of oil using for oil immersion lens during examination by using microscope:

1. **Oil of cedar – wood.**
2. **Canada – balsam.**

Why we use oil with oil immersion lens ?

We use oil to prevent shattering of light ways.

What are the morphological characteristics that can be seen by microscopes to identify the bacteria?

1. **Shape of the bacteria either :**
   - **Cocci (spherical).**
   - **Bacilli (rod like).**

- **Coccobacilli (between cocci and bacilli).**
- **Spiral (curved rod).**

2. **Size : either small or large.**
3. **Cell arrangements : bacteria could be arranged in many forms like :**
   - **A single.**
   - **Pairs (known as diplococci or diplobacilli).**
   - **Chains (arranged in series such as genus streptococcus).**
   - **Cluster or grape like arrangement such as staphylococcus.**

Lab. two

Tools, instruments and equipment:

- **Loop** : An inoculation loop, is a simple tool used mainly by microbiologists to pick up and transfer a small sample (either specimen or inoculum (at a diameter of drop or 0.01 ml.) from a culture media to another , e.g. for streaking on a culture plate. Sterilized by flaming of burner and before using.
- **Slide :** is a thin flat piece of *glass* used for the examination of the bacterial smear by placing it on the microscope stage.
- **Cover slip :** glass piece placed on the slide in order to covering the bacterial smear.
- **Test tube**: This object is of Pyrex glass. The test tube is one of the most commonly used pieces of laboratory ware used to placing the broth, solid medium, or semi solid medium for stabbing the bacterial inoculum.
- **Petri- dishe:** used for putting the solid medium in it, and available at different size.
- **Flask** : used for place cultured or uncultured broth in in it . Sterilized after plugs with cotton by autoclave.
- Beaker : used for graduate the volume of fluid. Sterilized by oven.
- **Cylinder** : used for graduate the volume of liquid. Also sterilized by oven.
- **Washing bottle** : used for filing with liquids (especially distilled water) for washing ex. the slides during the staining.
- **Burner** : may be gaseous or alcoholic, used for sterilization of the loop , needles and other metal.
- **Autoclave** : equipment with high temperature, pressure and steam to sterilize the culture media and some of tools and glass wares.
    - Temperature = 121 C°.
    - Pressure = 15 pound / inch^2 .
    - Time = 10-30 min.
        - 10 min. for media with sugar.
        - 15 min. for uncultured media.

- 30 min. for cultured media and contaminated tools and glass wares.

Sterilization by autoclave named (wet heat sterilization, the death of bacteria take place by protein denaturation.

- **Oven** : the sterilization is dry heat sterilization , the death of bacteria take place by oxidation, use for sterilization of glass wares and some metal tools.
    - Temp = 180 C°.
    - Time = one hour and half.
- **Incubator** : for the availability of suitable temperature for growth of microorganisms by place the culture media in it for example pathogenic bacteria growth in optimal temperature 37 C° for 18-48 hours (the optimum 24 hours).
- **Refrigerator** : used to kept and preserve the sterilized media and broth, for a different times may be long or short time at 4 C°.

What you needed to prepare a smear ?

1. Broth or solid medium.
2. Bacteriological loop.
3. Clean glass slide,
4. Bunsen burner.

Smear : a specimen for microscopic study prepared by the spreading the material a cross the glass slide.

Culture : propagation of microorganisms in a growth media.

Growth media : an artificial media contains basic requirements needed for microorganism's growth.

Forms of media:

We have two main forms of cultural or growth media :

1. Liquid or called (broth) usually put in the test tube .

2. Solid or called (agar) usually put in petri - dish.

What are the types of specimens:

1. Blood.
2. Stool.
3. Urine.
4. Pus.
5. Sputum.
6. Cerebrospinal fluid (CSF).
7. Pleural fluid or peritoneal fluid.
8. Any discharge.
9. Broth or agar.

Preparation of smear :

How you will prepare a smear ?

A. From fluid material :

Such as broth culture, urine, sputum ,pus, purulent and exudates.

1. Sterilize the loop in Bunsen flame and let it to cool.
2. Shake the specimens container (broth tube) takes loopful of the specimen and spread it on the center of a clean slide to form a somewhat a thick film of (1-2) cm in diameter then are sterilize the loop.
3. Allow the film to dry by air (avoid heating to avoid shrinkage and missing of clear form).
4. The film is fixed on the slide by passing it (3-4) times through the Bunsen flame allow the slide to cool before staining.

B. From solid material : (such as a culture on agar i.e. colonies)

1. Sterilize the loop in Bunsen flame and let it to cool.
2. Place a loopful of clean water (tap water can be used) on the center of a clean slide.
3. Re sterilize the loop, transfer a small portion of the colony to the drop of water, emulsify thoroughly and spread the mixture evenly on the slide to form a thin film of 1-2 cm in diameter.
4. Dry and fix as mentioned above.

Aim of fixation :

1. Kill the microorganism.
2. Make the microorganism stuck to the surface of the slid.
3. Make the microorganisms more permeable to the stains.
4. Prevent microorganism from going autolytic changes.

Lab. three

Staining of bacteria

Stains or dyes are used in the bacteriology for making organisms visible. Dyes usually aniline derivatives which can be divided into three groups:

- Basic as crystal violet and methylene blue.
- Acidic as Nigrosin stain.
- Neutral stains as Gimsa's stain.

As the bacterial cells are rich in nucleic acid (which has a negative charge) so the stain must be basic stain (which bears positive charge) and the attraction between the bacteria cells and the stain with be occur and leading to staining of whole bacterial body. Acidic stain, however, will not stain the bacterial cells (as both the bacterial cells and acidic stain bearing the negative charge) so, only the background of the smear will be stained by counter stain such as India ink.

Types of staining :

I. Simple staining : (involvs one stain) Simple staining involves directly staining the bacterial cell with a positively charged dye in order to see bacterial detail (the bacterial cell appear in one color), in contrast to negative staining where the bacteria remain unstained against a dark background.

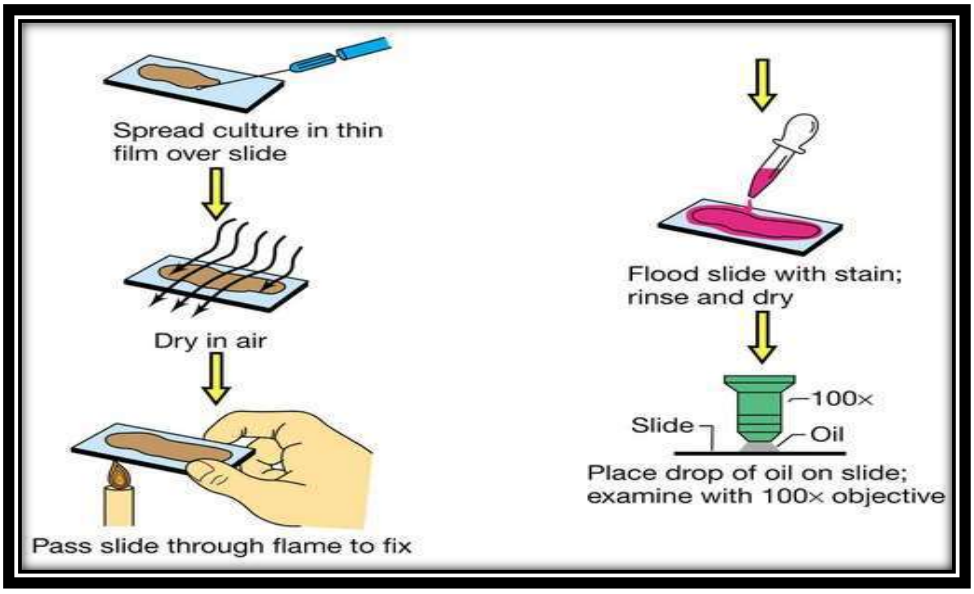
Aims of the simple stain :

- To see the size of microorganisms.

- To see the shape of the bacteria either cocci (spherical), bacilli (rod in shape), pleomorphic (coccobacilli) and spiral (spirillum).
- Grouping or arrangement: bacteria groups because of a process of multiplying or arrangement.

Method of simple staining:

1. Cover (flood) the fixed smear (culture) with either methylene blue or crystal violet (or any available stain) for one minute.
2. Gently wash off the stain with slowly running tap water.
3. Let the slide to dry in air or placed it between two sheets of filter paper by a process called blotting the slide.
4. Examine under oil immersion (100X).



Simple staining procedure

II. **Differential staining**: using 2 or more types of dye successfully to differentiate between the bacteria according to these dyes.

Examples for these stains:

1. **<u>Gram's stain :</u>**

Is one of the most important methods widely used in bacteriology, was discovered in 1884 by Christian Gram (a Danish physician), using two dyes in sequence, each of different color; he found bacteria fall into two different categories:

a. The bacteria which retained the first or primary stain such as (crystal violet) throughout the staining procedure, are known as "gram – positive" and will appear purple in color .
b. While the bacteria which lost the first or primary stain such as (crystal violet) after washing with a decolorizing solution and stained with the second stain (diluted carbol fuchsin) are known as gram" Gram – negative" and appear pink in color .

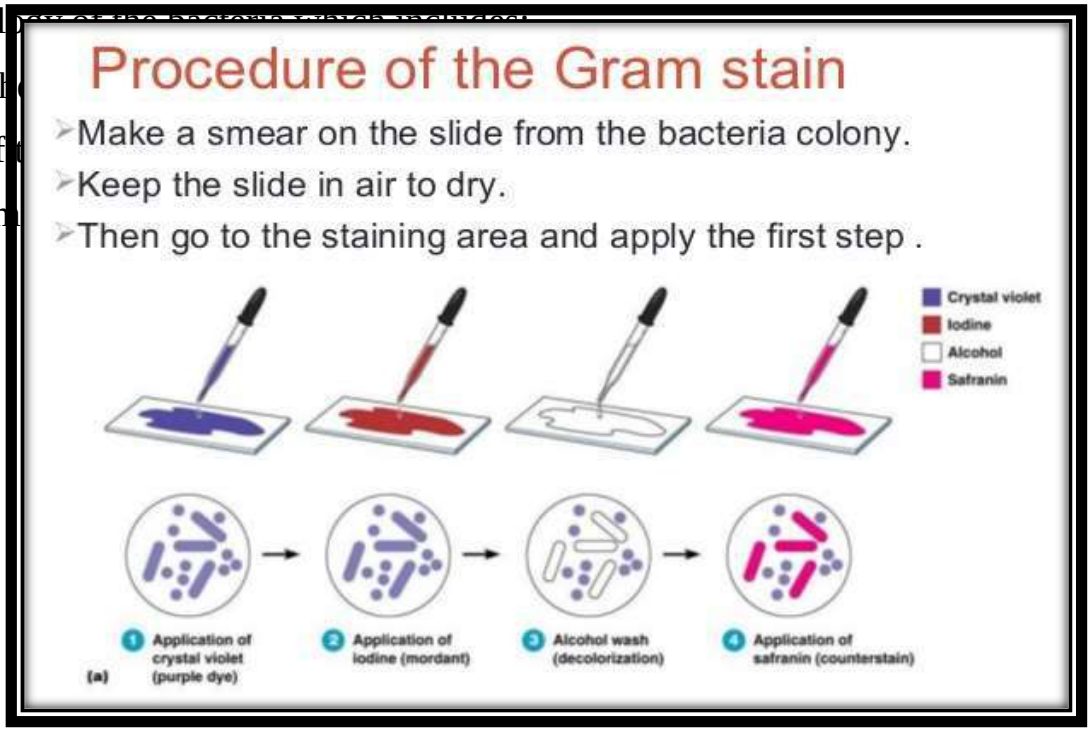
On the basis of the difference of the color of the bacteria that stained by Gram's stain it can be very possible to differentiate between them. In addition to determine the morphology of any bacteria.

Gram Staining procedure:

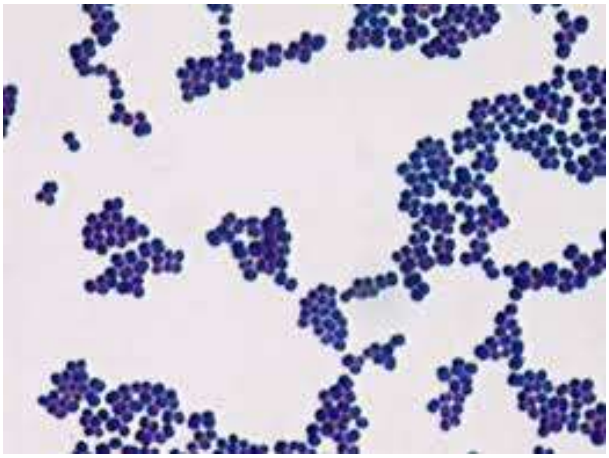
1. Add the primary stain (crystal violet) over the fixed bacterial smear. Let stand for 60 seconds.
2. Pour off the stain then gently rinse the stained slide either directly by tap water or by using plastic water bottle.
3. Then add the iodine solution on the smear, enough to cover the fixed smear. Let stand for 60 seconds.
4. Pour off the iodine solution and rinse the slide with running water. Shake off the excess water from the surface.
5. Then add a few drops of decolorizer so the solution trickles down the slide. Rinse it off with water after 5 seconds. The exact time to stop is when the solvent is no longer colored as it flows over the slide. Further delay will cause excess decolorization in the gram-positive cells, and the purpose of staining will be defeated.
6. Counterstain with basic fuchsine solution or (safranin) for 40 to 60 seconds. Wash off the solution with water. Blot with bibulous paper to remove the excess water. Alternatively, the slide may shake to remove most of the water and air-dried.

7. Examined the stained smear under oil immersion lens (100X) to determine the following items :

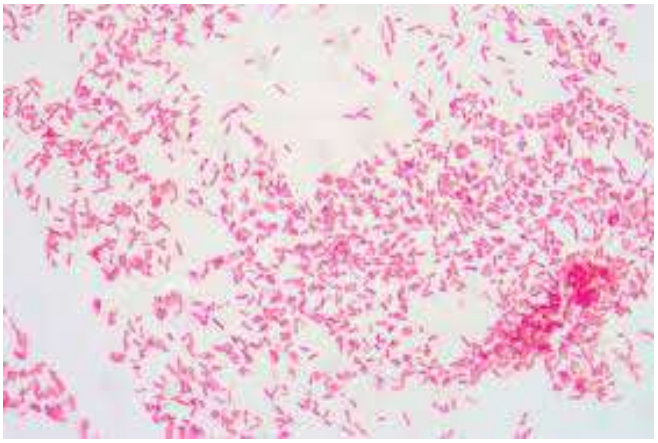
- Type of Gram staining (either gram positive or gram negative).
- The morphol
- The size of t
- The shape of
- The arrangem



Gram's staining procedure



Gram- positive bacteria (cocci)



Gram- negative bacteria (bacilli)

Lab. four

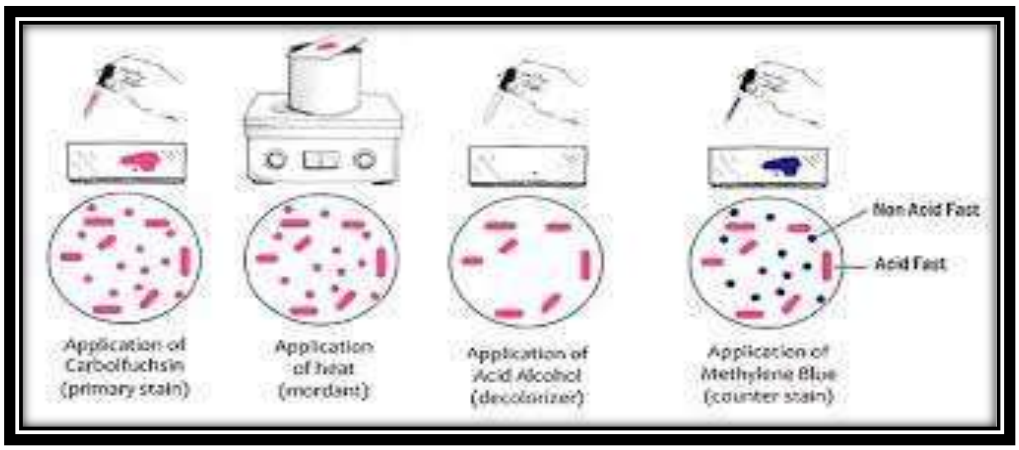
2. Acid fast stains (Ziehl – Nielson technique):

Acid fast stains are used to differentiate and detect acid fast bacilli (acid fastness) such as *Mycobacterium tuberculosis* which the causative agent of tuberculosis.tuberculosis (TB). Acid fast bacteria have a high content of mycolic acids and lipids in their cell walls for this reason these organisms resist the decolonization by acids and alcohol and retain the primary stain (carbol fuschin). Acid fast bacteria will be red, while non - acid fast bacteria will stain blue with the counterstain with the methylene blue stain.

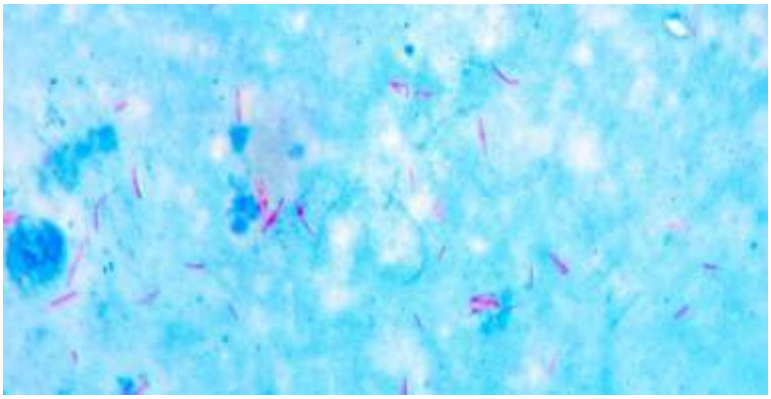
The procedure of Acid fast stain include:

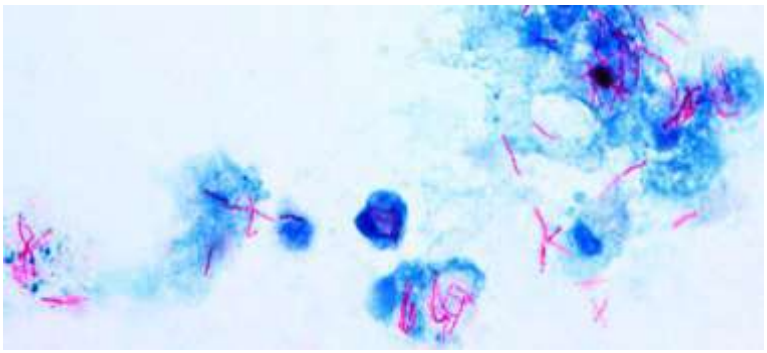
1. *Prepare bacterial film (smear).*
2. Flood the slid with concentrated carbol fucshin, heating the smear until steaming for 5 min. (avoid boiling of stain, and avoid dryness stain by adding few drops of stain).
3. Cool the slide and wash by tap water.
4. Dipping the slide in acid – alcohol (3%HCL) (decolorizer agent) for 20 -30 second and rinse with tap water to remove the primary stain if present.
5. Apply the counterstain, methylene blue for 2 minutes then rinse with tap water.
6. Allow to air dry or by using blotting.
7. Observe and examine stained slide with a light microscope under oil immersion lens (X100).

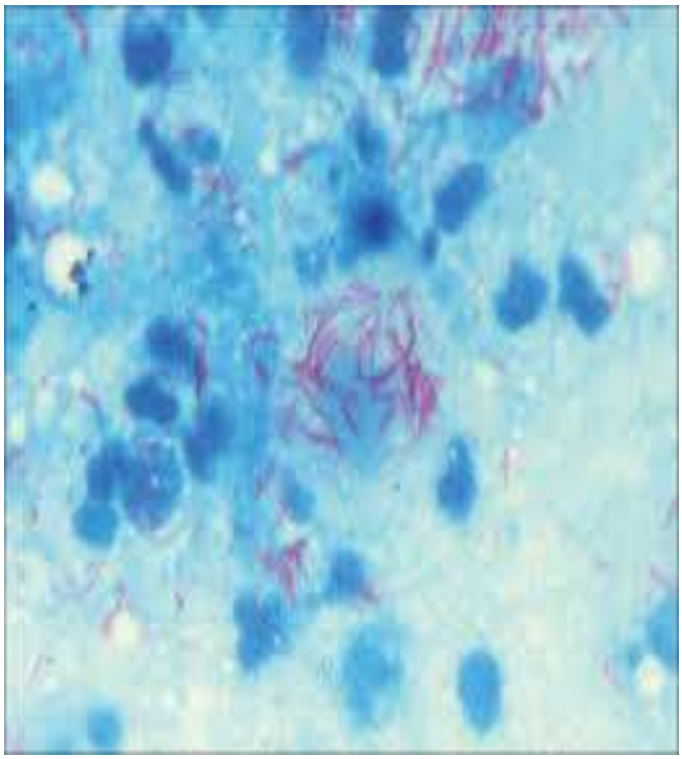
Mycobacterium tuberculosis will appear as a red – bacilli while other organisms and or background will appear with blue color see below figure.



Procedure of Acid fast stain (Ziehl – Nielson technique).







Mycobacterium bacilli (red in color).

III. Special staining: Stains used for special bacterial structures such as flagella, spores and capsule.

A. Spore stain: A special stain technique is used to examine bacterial spores which are exist in the aerobic bacteria (genus Bacillus) and an aerobic (genus Clostridium)**.** The Primary stain malachite green or methylene blue are used with heat to force the stain into the cells and give them color. A counterstain, safranin

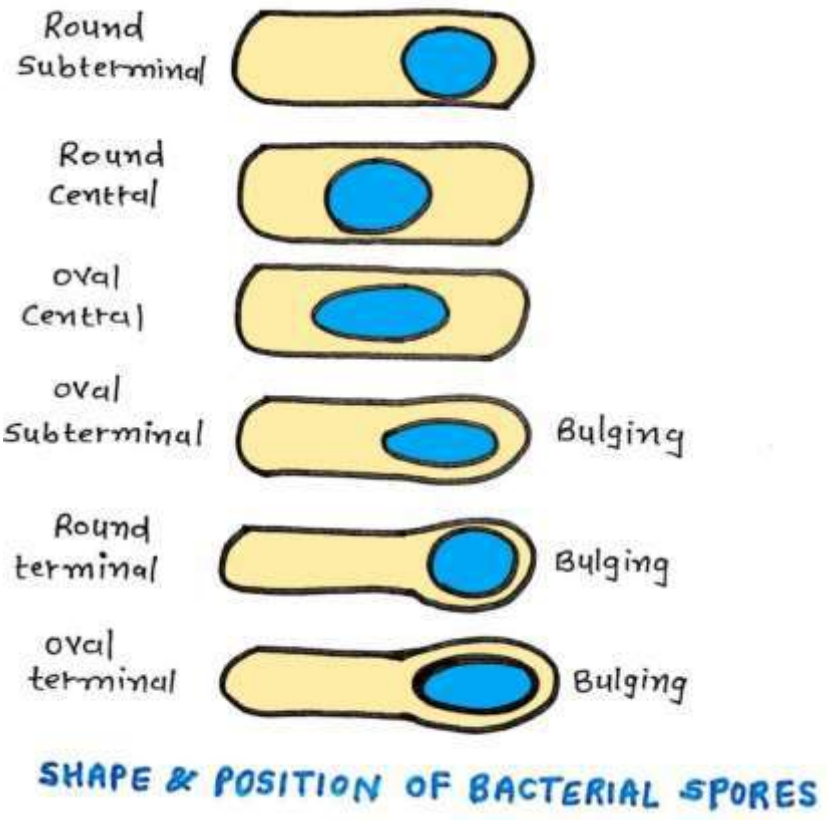
or carbol fucshin, is then used to give color to the non - sporeforming bacteria. At the end of the procedure, spores stained green and other cells stain red.

The shapes and position of the endospore :

The shapes of the endospores either rounded or oval in shape.

The position of the endospore differs among bacterial species and is useful in identification. The main types of endospores within the cell are:

- Terminal (Terminal endospores are seen at the poles of cells).
- Subterminal (endospores are those between these two extremes, usually seen far enough towards the poles).
- Centrally placed endospores (central endospores are more or less in the middle).



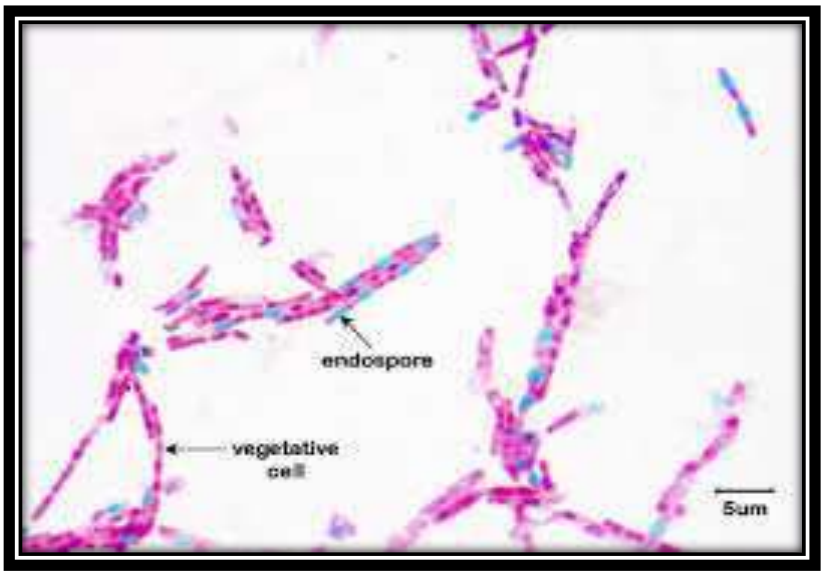
Lab. five

Spore stating procedure:

Modified acid fast stain include:

1. Flood the slid with methylene blue or malachite green, heating the smear until steaming for 5 min. (avoid boiling of stain, and avoid dryness stain by adding few drops of stain).
2. Cool the slide and wash by tap water.
3. Dipping the slide in 3% acetic acid (decolorizer agent) for 20 -30 second and rinse with tap water to remove the primary stain if present.
4. Apply the counterstain, Carbol fucshin or safranin, for 2 minutes then rinse with tap water.
5. Allow to air dry or by using blotting.
6. Observe and examine stained slide with a light microscope under oil immersion lens (X100).

Result : The vegetative forms will take up the pink/red stain from counter stain while the endospores will stain green, from primary stain .



Lab. six

B. Capsule stain :

The **bacterial capsule** is a large structure of many bacteria. It is a polysaccharide layer that lies outside the cell envelope, and is thus deemed part of the outer envelope of a bacterial cell. It is a well-organized layer, not easily washed off, and it can be the cause of various diseases.

Function of bacterial capsule:

The capsule is considered a virulence factor because it:

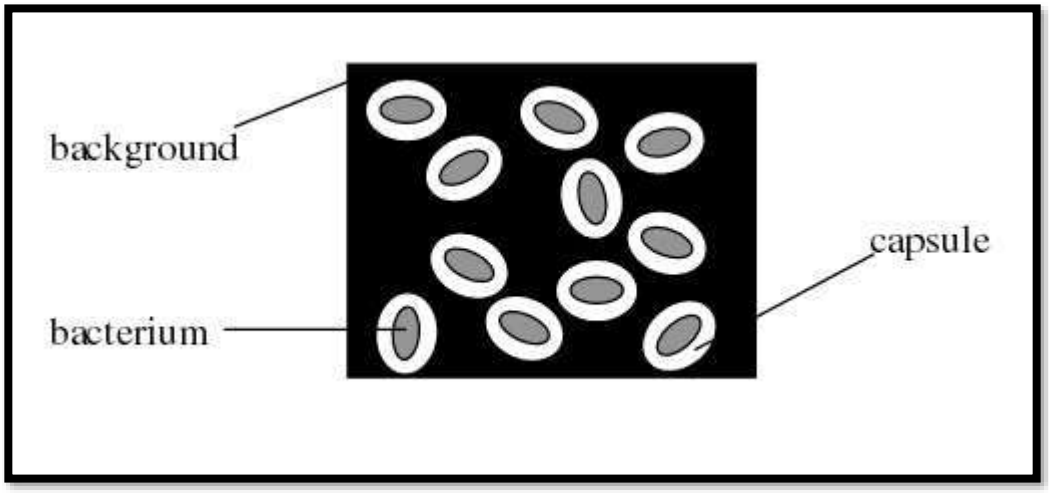
1. Enhances the ability of bacteria to cause disease (e.g. prevents phagocytosis).
2. The capsule can protect cells from engulfment by eukaryotic cells, such as macrophages.
3. Capsules also contain water which protects the bacteria against desiccation.
4. They also exclude bacterial viruses and most hydrophobic toxic materials such as detergents.
5. Capsules also help cells adhere to surfaces.

Principle of Capsule Stain:

Bacterial capsules are non-ionic, so neither acidic nor basic stains will adhere to their surfaces. Therefore, the best way to visualize them is to **stain the background using an acidic stain** *(e.g., Nigrosine, congo red)* and to **stain the cell itself using a basic stain** *(e.g.,crystal violet, safranin, basic fuchsin, and methylene blue).*

Capsule is seen by :

Various types of methods are available for the demonstration of the presence of a capsule. The results (stain of the cells, background, and capsule) depend on the type of method used.



Two commonly used methods are discussed here:

1. Negative stain :

In this method, two dyes, crystal violet, and India ink are used. The capsule is seen as a clear halo around the microorganism against the black background. The background will be dark (color of India ink).

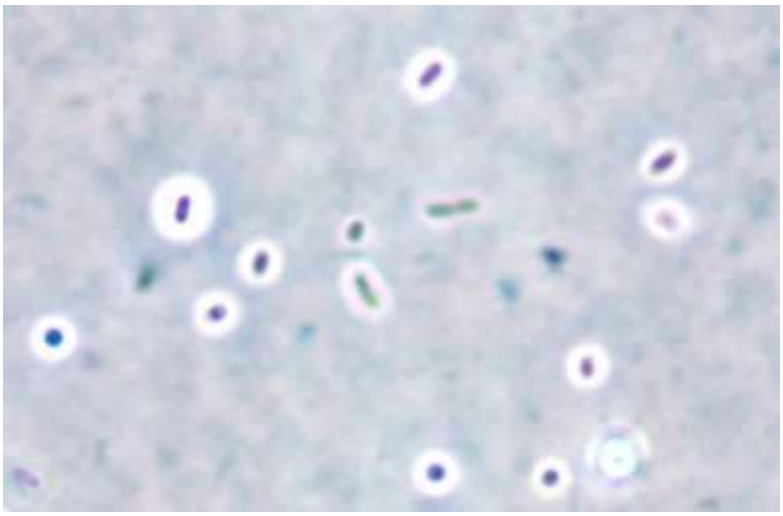
The bacterial cells will be stained purple (bacterial cells take crystal violet-basic dyes as they are negatively charged).

The capsule (if present) will appear clear against the dark background (capsule does not take any stain). Another negative stains are : negrosin , congo red …..ect .

Procedure of Capsule Staining :

1. Place a small drop of a negative stain (India Ink, Congo Red, Nigrosin, or Eosin) on the slide.
   Congo Red is easier to see, but it does not work well with some strains. India ink generally works well and Nigrosin may need to be kept very thin or diluted.
2. Using sterile technique, add a loopful of bacterial culture to slide, smearing it in the dye.
3. Use the other slide to drag the ink-cell mixture into a thin film along the first slide and let stand for 5-7 minutes.

4. Allow to air dry (do not heat fix).
5. Flood the smear with crystal violet stain (this will stain the cells but not the capsules) for about 1 minutes. Drain the crystal violet stain and let stain run off until it air dries.
6. Examine the smear microscopically (100X) for the presence of encapsulated cells as indicated by clear zones surrounding the cells see figure bellow.

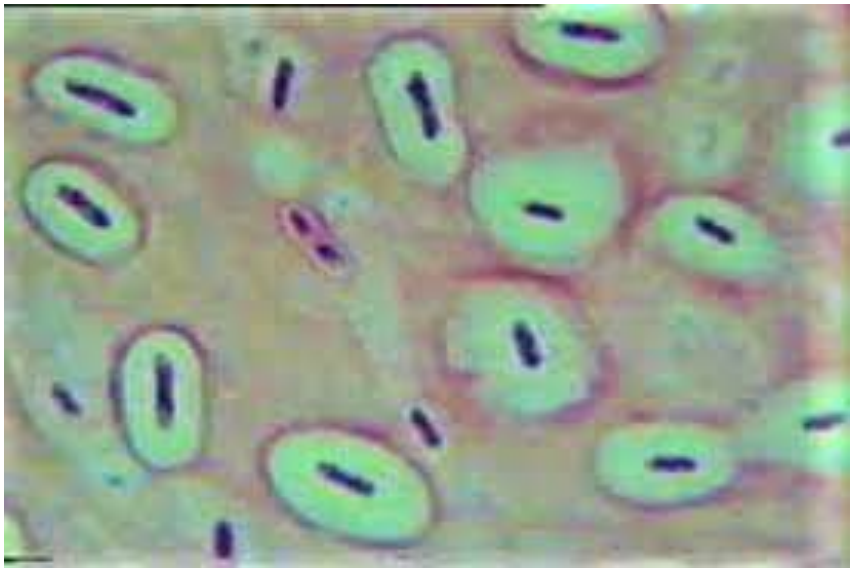


Bacterial capsule stained by negative stain

2. ***Anthony's*** **stain method**

1. Prepare the bacterial film and allow to dry without fixing it ((will take 5-7 minutes). DO NOT heat or blot dry! Heat will melt the capsule!
2. Flood the bacterial film with crystal violet for 3-7 min.
3. Then decolorized the slide and rinse with 20% copper sulfate solution. DO NOT RINSE WITH WATER! Water will remove the capsule from the cell.
4. Let the slide air dry for a few minutes. DO NOT blot the slide! Blotting will remove the bacteria from the slide and/or distort the capsule.
5. Observe the slide under oil immersion.

Results: Look for purple cells surrounded by a clear or faint blue halo on transparent background. The halo is the capsule. You may need to decrease the amount of light in order to make the capsule easier to see. See figure bellow.



Lab. seven

Culture media , preparation and their types :

Culture media: also known as growth media, are artificial and specific mixtures of nutrients and other substances that used for supporting the growth and detection of microorganisms such as bacteria and fungi (yeasts and molds).

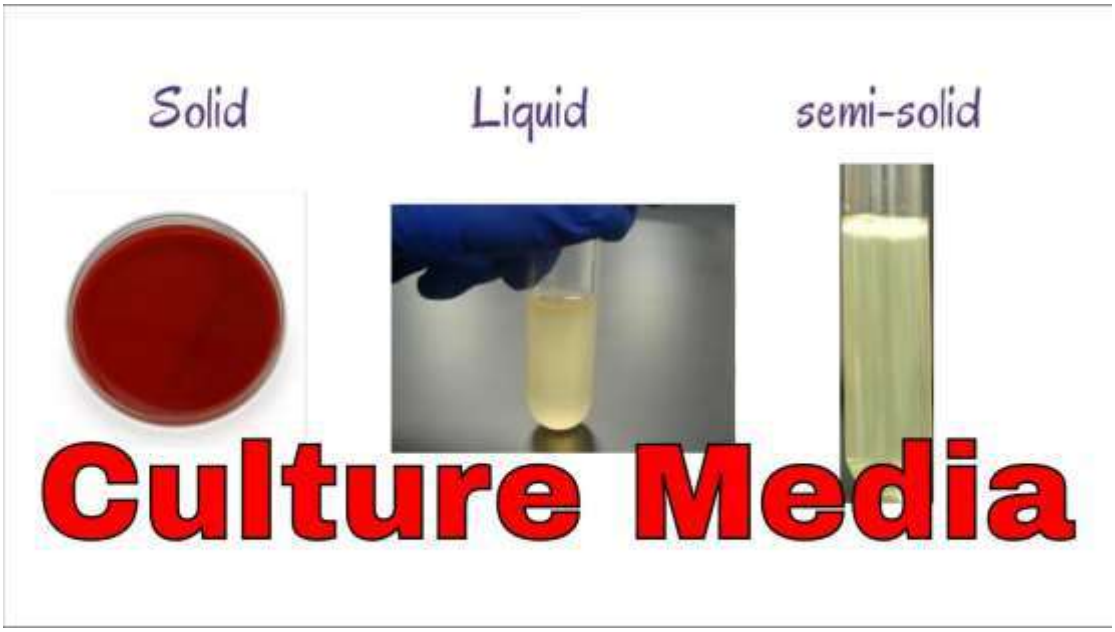
Common ingredients of culture media:

- Water: essential for bacterial growth, use deionized or distilled water.
- Peptone: from hydrolised animal or plant protein, it provides nitrogen and amino acid.
- Meat extract: provides amino acid, vitamins, mineral salts (phosphate and sulphate).
- Yeast extract: used to stimulate the growth of bacteria.
- Mineral salts: traces of magnesium, potassium, iron and calcium which are essential for bacterial enzyme activity.
- Carbohydrates: to provide bacteria with energy and carbon source.
- Agar: inert polysaccharide from sea weed or marine algae, it is solidifying agent with concentration of 1-2%, dissolves at 90-100 °C, solidify at 45 °C.

Forms of media:

- **Liquid form (broth):** without agar (no solidifying agent), used to grow bacteria in large quantity, the growth appear as turbidity and if no growth it appear clear.
- **Solid form**: by adding agar, it can be slant or deep agar which is used to keep bacteria for long time (up to 3 months), agar plate can be used to have isolated colonies that help identification.

- **Semisolid form:** is prepared with agar at concentrations of 0.5% or less. Semisolid medium has a soft custard-like consistency and is useful for the cultivation of microaerophilic bacteria or for the determination of bacterial motility.



<u>Pure culture</u>: culture containing only one type of bacteria to study them. It is impossible to study the bacteria when other organisms are present.

Preparation of media: All constituents of media should be weighed and mixed as indicated in instruction on the bottle. Example: calculate how many grams needed for 100ml media? 20g in 1000ml (stated in instruction) So, for 100ml 100*20/1000= 2 g - When we want to add material sensitive to heat, we add them after sterilization. Example is the blood that should be added to the cooled media after sterilization.

Pure culture technique the act of organism culturing into the media is called inoculation or streaking. - The common method to obtain pure culture (isolated colony) is dry dilution that should be done under septic conditions to prevent growth of contaminants.

Preparation of Culture Media
Agar Plate Medium

- Weight dehydrated media.
- Dissolve in distilled H_2O to 1L volume.
- Adjust pH to 7.0

Types of media and their functions:

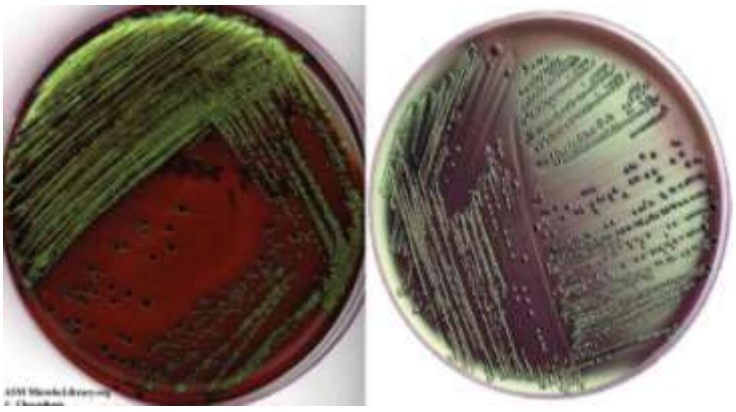
1- **Basal media**: allows growth of most nonpathogenic bacteria. E.g. nutrient agar.

2- **Enriched media**: when the basal agar has been enriched through adding blood or serum. To allow the growth of pathogenic bacteria. E.g. blood agar.

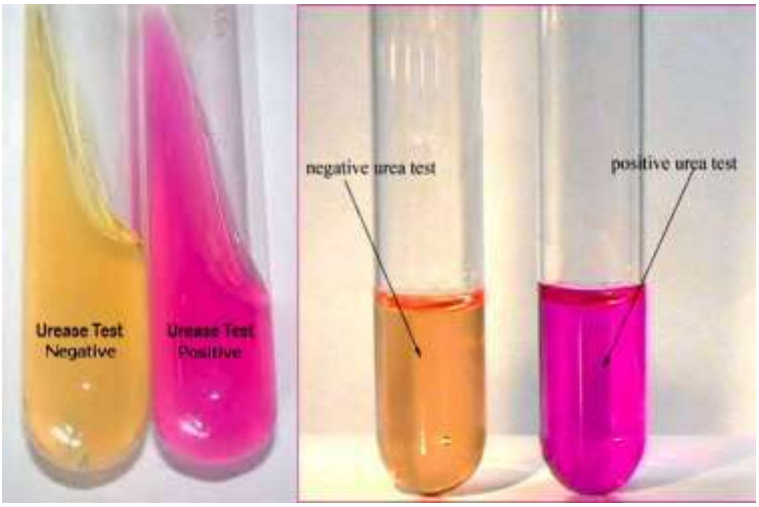
3- **Selective media**: has certain inhibiting agent to inhibit the growth of some bacteria and allow growth of others. Example: macconkey agar (Mac): contains bile salt and crystal violet as inhibiting agent. It allows growth of gram negative bacteria and inhibits growth of gram positive ones.

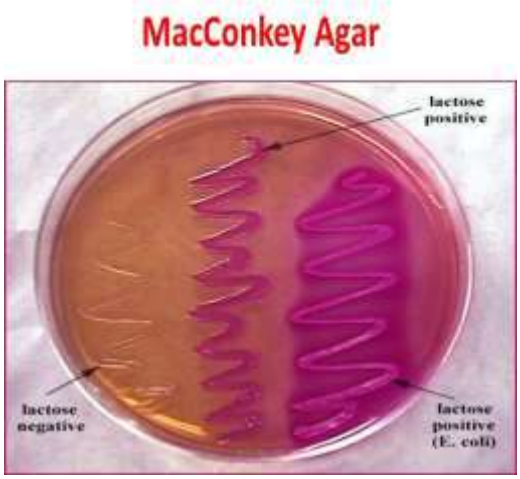
4- **Differential media**: contains indicator that can differentiate between two types of bacteria. Examples – Macconkey (Mac): to differentiate between lactose fermenting bacteria (LF) and non-lactose fermenting ones (NLF). The media contains sugar (lactose) and indicator (neutral red). LF bacteria (such as E.coli) ferment lactose and produce acid + indicator……pink color. NLF bacteria (such as proteus) are not able to ferment lactose +indicator……colorless. - EMB: differentiate between LF and NLF. It has sugar (lactose) and indicator (eosin + methylen blue). E.coli on EMB gives green metallic sheen. - Mac and EMB are selective and differential media. - CLED (cystine lactose electrolyte deficient): differentiate between LF and NLF. It has sugar (lactose) and indicator (bromo thymol blue). LF appears yellow and NLF appears colorless. - CLED is only differential but not selective.

EMB



5. **Indicator (DIFFERENTIAL) MEDIA**. An indicator is included in the medium. A particular organism causes change in the indicator Examples: urease and MacConkey agar are indicator media.





6. **Transport media**. These media are used when specie-men cannot be cultured soon after collection. Examples: Cary-Blair medium, Amies medium, Stuart medium.

7. **Storage media**. Media used for storing the bacteria for a long period of time. Examples: Egg saline medium, chalk cooked meat broth.

8. **Sensitivity media:** a special media used to tested antibiotic sensitivity for given microorganism e.g. Muller Hinton.

Lab eight

Growing and Cultivation of the bacterial species in the lab.

A population of bacteria grown in the laboratory is referred to as a culture. A microbial culture: is a method of multiplying microorganisms by letting them reproduce in predetermined culture media under controlled laboratory conditions. Microbial cultures are foundational and basic diagnostic methods used extensively as a research tool in molecular biology.

A pure culture : contains only one single type; a mixed culture contains two or more different bacteria. If a bacterial culture is left in the same media for too long, the cells use up the available nutrients, excrete toxic metabolites, and eventually the entire population will die. Thus bacterial cultures must be periodically transferred, or subcultured, to new media to keep the bacterial population growing.

Bacteriological culture media can be prepared as a liquid (broth), a solid (plate media or slant media), or as a semi-solid (deeps). Solid and semi-solid media contain a solidifying agent such as agar or gelatin.

Colony: A macroscopically visible growth of microorganisms on solid culture medium.

Agar: A gelatinous material and is a polysaccharide derived from red seaweed (Rhodophyceae) belong to the marine algae, used as a bacterial culture medium, in electrophoresis and as a food additive.

Why the agar is preferred? because it is an inert, non-nutritive substance. The agar provides a solid growth surface for the bacteria, upon which bacteria reproduce until the distinctive lumps of cells that we call colonies form.

Cultivation methods:

A. Streak plate method:

Streak plate technique is used for the isolation into a pure culture of the organisms (mostly bacteria), from a mixed population. The inoculum is streaked over the agar surface in such a way that it “thins out” the bacteria. Some individual bacterial cells are separated and well-spaced from each other. As the original sample is diluted by streaking it over successive quadrants, the number of organism's decreases. Usually, by the third or fourth quadrant, only a few organisms are transferred which will give discrete colony forming units (CFUs).

Materials required:

 A source of bacteria (stock culture, previously streaked agar plate or any other inoculum)

 Inoculation loop.

 Bunsen burner.

 Ethyl alcohol (70%).

 Agar plate (nutrient agar or any other agar medium).

 Paper towels.

Tips for the best results:

 Use only a small amount of inoculum.

 Streak lightly so that you do not gouge the agar.

 Flame the loop after you streak each quadrant.

 Make sure the surface of the plate is free of droplets of condensed moisture.

Purpose of streaking on the solid media:

 To produce isolated colonies of an organism (mostly bacteria) on an agar plate. This is useful when we need to separate organisms in a mixed culture (to purify/isolate particular strain from contaminants) or when we need to study the colony morphology of an organism.

 To identify the organism: biochemical tests to identify bacteria are only valid when performed on pure cultures.

Procedure of streaking:

1. Sterilize the inoculating loop in the bunsen burner by putting the loop into the flame until it is red hot. Allow it to cool.

2. Pick an isolated colony from the agar plate culture and spread it over the first quadrant (approximately 1/4 of the plate) using close parallel streaks or Insert your

loop into the tube/culture bottle and remove some inoculum. You don't need a huge chunk.

3. Immediately streak the inoculating loop very gently over a quarter of the plate using a back and forth motion.

4. Flame the loop again and allow it to cool. Going back to the edge of area 1 that you just streaked, extend the streaks into the second quarter of the plate (area 2).

5. Flame the loop again and allow it to cool. Going back to the area that you just streaked (area 2), extend the streaks into the third quarter of the plate (area 3).

6. Flame the loop again and allow it to cool. Going back to the area that you just streaked (area 3), extend the streaks into the center fourth of the plate (area 4).

7. Flame your loop once more.

The streaked plate is incubated at 37°C for 24 hours. Examine the colonies grown in the plate carefully. All colonies should have the same general appearance. If there is more than one type of colony, each type should be streaked again on a separate plate to obtain a pure culture.

B. Streaking and stabbing on slant solid media:

1. Flaming the loop with straight wire.

2. By the sterile cooling loop take a small part of provided culture.

3. Use the other hand to pick up a tube of sterile slant media and remove the tube closure with little finger of the loop hand.

4. Flame the neck of the tube inserts the charged loop into bottom of the tube and make stab down then withdraw the loop and streak the slant surface of the media.

5. With draw the loop out tube closure and return the tube to the neck.

6. Flame until the loop wire glows red and when the wire has cooled put the loop down.

7. Label the tube with name of the provided organism and the date.

8. Incubate the slant tube at 37 C° for 24 – 48 hr.

The purpose of using the stabbing on the solid media:

1. To identify the organism: by using biochemical tests to identify bacteria.

2. To storage the pure culture of isolated colonies for approximately 6 months.

Lab nine

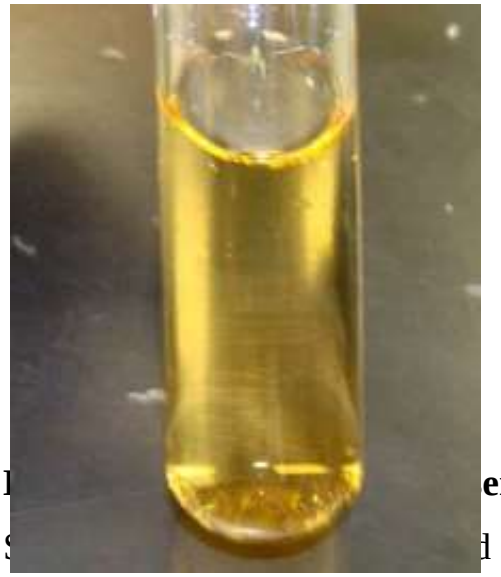
C. Cultivation of the bacteria in the liquid media (broth):

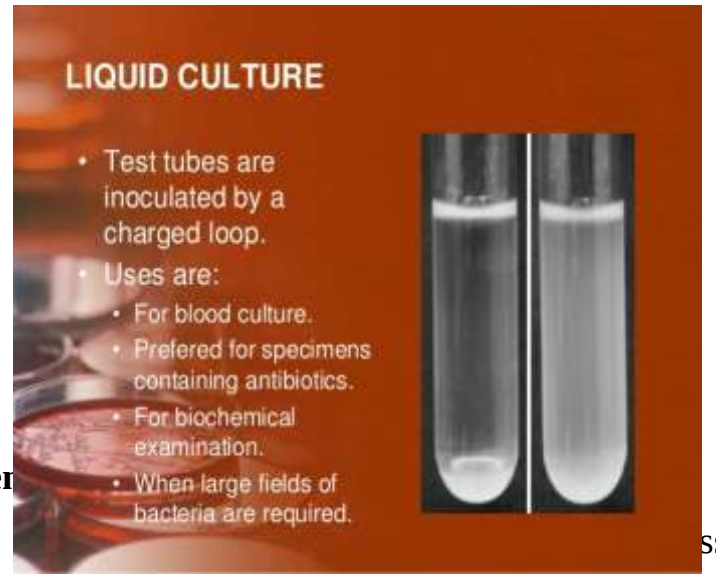
These media contain specific amounts of nutrients but don't have a trace of gelling agents such as gelatin or agar. Broth medium serves various purposes such as propagation of a large number of organisms, fermentation studies, and various other tests. e.g. sugar fermentation tests, MR-VP broth**.**

Procedure :

1. Transfer of a bacterial colony on a plate culture (solid media especially which preserved for up to one month) to a broth such as nutrient broth (liquid medium putting in the tube).
2. Take up the sterilized (by passing it over the flame of the Bunsen burner) inoculating loop by the hand and let it to cool for many seconds and at the same time be care touching of the loop with anything.
3. Remain holding the sterilized the inoculating loop by the hand and the other hand turn the cultured plate and scrapped gently a little amount of bacterial colony and remove it by loop full then draw the loop and replace the cover petri dishes and put the dish on the table.
4. Still holding the loop like pencil but more horizontally in your right hand, use the little finger of the same hand to remove the closure (cotton plug slip or screw cap) of the culture tube (broth tube), keep your little finger curled around this closure when it is free do not place it on the table.
5. Passing the neck of the open tube rapidly through the Bunsen flame 2-3 times (not over – heat, if it glass, it could crack, if it is a plastic it could melt).this flaming sterilize the air in and immediately around the nozzle of the tube.
6. Insert the loop (which carrying the inoculate) through the nozzle of tube and inoculate the sterilized broth with inoculum of the bacteria and gently rubs against walls of the tube.

7. Then draw the loop slowly, be careful not to touch it to the nozzle of the tube and do not touch it to anything
8. Carefully, flame the loop until glows red to prevent the contamination.
9. Label the tube with special data of the inoculated organism.
10. Incubate the tube at 37C° for 24 hour.





Semisolid medium has a soft custard-like consistency and is useful for the cultivation of microaerophilic bacteria or for the determination of bacterial motility.

Procedure:

Semisolid cultivated by stabbing technique:

1. Flaming the straight wire of the loop.
2. Take by cooled loop a small part of provided culture to inoculate it in a tube of semisolid as SIM (sulphate indol medium) or gelatin medium making a single stab down the center of the tube to about half the depth of medium.
3. Incubate at 37C° for 24hour.

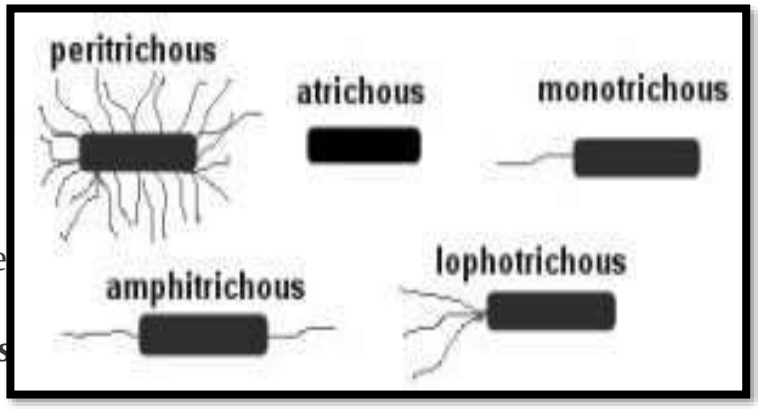
Tests for Bacterial Motility:

Motile bacteria move with structures called **flagella** (a few exceptional bacteria move with the help of axial filaments, which cannot be seen in the microscope). In semi-solid agar media, motile bacteria ‘swarm’ and give a diffuse spreading growth that is easily recognized by the naked eye.

Flagella : **Flagella** are microscopic hair-like structures involved in the locomotion of a cell. The word "**flagellum**" means "whip". The **flagella** have a whip-like appearance that helps to propel a cell through the liquid.

Types and Examples of Flagella

There are 4 types of flagellar distribution on bacteria-



1. Monotrichous
– Single polar flagellum

– Example: ***Vibrio cholerae***
2. Amphitrichous
– Single flagellum on both side

– Example: ***Alkaligens faecalis***
3. Lophotrichous
– Tufts of flagella at one or both sides

– Example: ***Spirillum***
4. Peritrichous
– Numerous falgella all over the bacterial body

– Example: ***Salmonella Typhi***

There are a variety of ways to determine the motility of a bacterium -biochemical tests as well as microscopic analysis. If a fresh culture of bacteria is available, microscopy is the most accurate way to determine bacterial motility, and '**hanging drop method**' is a commonly used microscopic technique.

1. **Microscopically methods :**

a. Wet mount method: (**Distilled water motility test) :** It is a simple and very useful test to differentiate *Vibrio species* (gram-negative motile curved rod) and *Aeromonas species* (gram-negative motile rod). *Aeromonas* species will grow on **MacConkey agar** and sometimes on **TCBS**, producing yellow colonies. Both of them are **oxidase** positive.

Procedure:

1. Mix a loopful of growth from a nutrient agar subculture in a drop of sterile distilled water on one end of a slide. On the other end of the slide, mix another loopful of growth in a drop of peptone water.
2. Cover each preparation with a cover glass.
3. Examine microscopically using the 40x objective.

b. Hanging drop method :

The **hanging drop technique** is a well-established **method** for examining living, unstained, very small organisms. The traditional **procedure** employs a glass slide with a circular concavity in the center into which a **drop** of fluid, containing the 'microorganisms', hangs from a coverslip

Lab. ten

LABORATORY DIAGNOSIS OF FUNGI

INTRODUCTION

We have learned in earlier chapters about various fungal infections. This chapter deals with the diagnosis and in particular laboratory diagnosis of fungal infection As processed with bacterial infection, laboratory diagnosis of fungal infection starts with appropriate specimen collection & transport. And in most fungal infections the identifications are based primarily on the assessment of colony

morphology & microscopic features. Key biochemical tests may be required to differentiate between the genes & species. Also molecular techniques like Nucleic acid probe assays are being used with increased frequency to provide early confirmation in suspected cases of deep seated mycoses. Serological studies are required in some instances to establish differential diagnosis. Non culture methods & automated system too are available for diagnosis of fungal infections.

OBJECTIVES

After reading this lesson, you will be able to:

- list the steps involved in the diagnosis of fungal infection
- describe the Specimen collection and transport
- explain the Direct examination and mount preparation
- describe the Selection & innoculation of culture media
- explain Incubation of fungal cultures
- describe the Presumptive diagnosis of fungal isolates

Laboratory Diagnosis of Fungi

SPECIMEN COLLECTION AND TRANSPORT

For laboratory diagnosis of fungal infections various specimens can be received in the laboratory; Physicians, Nurses, ward personnel & Laboratory technologists needs to work together in developing protocols that ensure the proper collection and prompt collection of specimen.

Transport condition for diagnosis of fungal infection:

Specimen Transport condition

Sputum Sterile Screw capped container

Bronchoscopy Fluid Sterile Screw capped container

CSF If delay anticipated, specimen should be left at room temperature

Urine If delay beyond 2hrs is anticipated, refrigerate at 4°C

Blood Biphasic agar broth bottles designed especially for fungal cultures Tissue biopsy the specimen should not be frozen or allowed to dehydrate prior to culture. In all cases the specimen should be transported as early as possible to the laboratory. In general the specimen that are not processed immediately are held at room temperature (for urine if delay more that 2 hrs refrigerate at 4°C). Cryptococcus neoformans, cystoplasma capsulatum & Blastomyces dermatitidis do not survive well in frozen or iced specimen.

Criteria for specimen rejections

1. Absence of patient identification on the container or discrepancy between

the information.

2. Sputum specimen with >25 squamous epithelial cells as per low power field

3. A dried out swab or if the material collected is insufficient

4. The sample submitted in an improper container

5. The 24hr sputum or urine specimen for fungal culture is received

SPECIMEN PROCESSING

On receiving the specimen, it should be promptly processed. The direct wet mounts or smears are prepared and for culture the specimen is inoculated on culture media.

Direct Examination

Almost all the specimens are processed for direct microscopic examination. This provides the presumptive diagnosis for the physician and also aid in the selection of appropriate culture media.

Various methods for direct examinations are

- Direct wet mount of specimen
- India Ink
- KOH/calcoflurol mounts
- Lactophenol cotton blue (LPCB) mounts
- Frozen section of tissue biopsies

 Modified Kinyoun Acid Fast Stain for Nocardia

Direct Microscopic Observations Presumptive Identification

Hypae relatively small (3-6 μm) and Aspergillus spp regular in size, dichotomously branching at 45° angles with distinct cross-septa Hypae irregular in size (6-50 μm), Zygomycetes (Phycoycetes) ribbonlike, and devoid of septa. rhizopus-Mucor Hypae small (2-3 μm) and regular, Dermatophyte group some branching with rectangular Microsporum spp arthrospores sometimes seen, found Trichophytoon spp only in skin, nail scrapings and hair Epidermophyton spp.

Laboratory Diagnosis of Fungi

Hyphae regular in diameter (3-6 μm), Phaeohyphomyces spp parallel walls, irregular branching, Hyalohyphomyces spp septate, dark yellow, brown or hyaline. Hyphae, distinct points of constriction Candida spp simulating link sausages (pseudohyphae),

with budding yeast forms (blastospores) often seen. Yeast forms, cell spherical and irregular Cryptococcus neoformans in size (5-20 μm) classically with a Cryprococcus spp, nonencapsualted thick polysaccharide capsule (not all cells are encapsulated), with one or more buds attached by a narrow constriction Small budding yeast, relatively uniform Histoplasma capsulatum in size (3-5 μm) with a single bud attached by Yeast forms, large (8-20 μm) with cells Blastomyces dermatitidis appearing to have a thick, doublecontoured wall, with a single bud attached by a broad base Large, irregularly sized (10-50 μm) thick Coccidiodes immitis walled spherules, many of which contain

small (2-4 μm) round endospores.

Preparation of Mounts

The tease mount, transparency tape method and microslide techniques are

commonly used methods for microscopic examination.

The mold colony is mounted in a drop of Lactophenol cotton blue stain on a glass slide and examined microscopically

The specimen are directly mounted in 40% & 10% KOH for skin and nail specimens respectively. The skin and nail samples are mounted on the glass slide to which two drops of KOH preparation is added and kept for sometime. KOH helps in dissolving the epithelial cells and thus aid in fungal visibility.

India Ink - India ink can be added to specimens such as spinal fluids or exudates to provide a dark background that will highlight hyaline yeast cells and capsular material (halo effect). Hence, it should be used to examine specimens suspected of containing Cryptococcus neoformans. White blood cells may be distinguished from Cryptococcus neoformans because of the irregular edge of the halo and the pale cell wash. The India ink preparation is not routinely offered by the laboratory. If a request is received for it, the laboratory should call the physician and offer a Cryptococcus Antigen Test instead. The procedure will be performed only in particular instances with the approval of the director or supervisor.

Fig. 52.3: Cryptococcus neoformans using a light India ink staining preparation

Gram Stain

Gram stain is usually a poor stain to use when examining a specimen for a

fungus. Gram stain may be used when examining smears of Candida, Malassezia, and Sporothrix but should not be relied upon to demonstrate the yeast of the other dimorphic fungi. A gram stain will demonstrate the filaments of Nocardia and Actinomyces which may produce clinical signs resembling mycotic infections.

Modified Kinyoun Acid Fast Stain for Nocardia

a. Make a thin smear of the specimen to be stained; fix in methanol. A positive control smear (Nocardia asteroides) and a negative control smear (Streptomyces sp.) must be included.

b. Kinyoun carbolfushcin; 5 minutes, no heat.

c. Rinse with water.

d. 50% ethanol rinse; flood and pour off until excess carbolfuchsin is removed.

e. Rinse with water.

f. Decolorize with 0.5% (aqueous) H SO ; 3 minutes.

g. Rinse with water

h. 1% (aqueous) methylene blue; 1 minute.

i. Rinse with water.

Selection and Inoculation of culture media

Generally two types of culture media are used, nonselective (such as brain heart

infusion heart) it permits growth of virtually all clinically relevant fungi. The use of sabourauds dextrose agar as primary recovery medium is discouraged as it is insufficiently rich to recover certain fastidious pathogenic species, particularly dimorphic fungi. Rather, the use of Potato flake agar (PFA), inhibitory mold agar (IMA), or combination of sabouraud's dextrose agar with heart infusion (SABHI) agar is recommended.

Sabouraud's agar is sufficient for the recovery of dematophytes from cutaneous samples or yeasts from vaginal culture. Czepak's agar can be used for the subculture of aspergillus species if colony morphology is an important identifying criteria for any given unknown isolate. For more fastidious dimorphic fungi such as Blastomyces dermatiditis & soistoplasma capsulatum an enrich agar like IMA or SABHI is used and in particular for Histoplasma capsulatum media with the addition of 5-10% sheep blood is recommended. Cryptococcus neoformans, aspergillus fumigatus may be partially or totally inhibited by cycloheximide, therefore a nonselective media must always be used in parallel.

Incubation of fungal culture

Each sample is cultured in two set of culture media and is incubated at two

different temperatures at 30oC (Room temperature) and at 35oC

All fungal cultures are incubated for a minimum of 30 days before discarding as negative.

The choice between the use of culture tubes or plate is optional. For tube, the media is poured in thick slants to prevent dehydration during prolonged incubation period. After the medium is inoculated, do not screw down the cap too tightly because fungi require breathing.