Ministry of Higher Education and Scientific Research

Al- Mamoun University

Anesthesia techniques Department

قسم تقنيات التخدير Anesthesia techniques Department

المرحلة الأولى للعام الدراسي 2024-2023

الفصل الدراسي الثاني

Microbiology –practical Lab. section

تدريسي المادة :

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Microbiology

AN INTRODUCTION TO MICROBIOLOGY, ASEPTIC TECHNIQUE AND SAFETY

- **The teaching of such an important subject as microbiology cannot be achieved effectively without enhancing the theory with 'hands on' experience in the laboratory.**

- **The purpose of this laboratory is to provide you with good techniques in practical microbiology to ensure that investigations proceed safely and achieve the required educational aims successfully.**

General Lab Safety:

- **There are many potential hazards when working with microorganisms. Potential safety hazards can be avoided with the appropriate precautions.**

- **When working with microbiological agents you will need to be aware of standard laboratory safety procedures, protective wear, and chemical safety.**

- **Safety in the lab is everyone's responsibility.**

- **Before starting any experiment, you should understand the entire procedure that you will be following. You need to make sure that you have the proper equipment, and that you know how to use it.**

Microbiology Lab Practices and Safety Rules:

1. **Wear a lab coat in the lab and do not wear it to other non-lab areas.**

2. **Wash your hands. Use a disinfectant soap to wash your hands before and after working with microorganisms.**

3. **Absolutely no food, drinks, chewing gum, or smoking is allowed in the laboratory. Do not store food in areas where microorganisms are stored. Do not put anything in your mouth such as pencils, pens, labels, or fingers.**

4. **Cover any cuts on your hands with a bandage. Gloves may be worn as extra protection.**

5. **Avoid loose fitting items of clothing. Wear appropriate shoes (sandals are not allowed) in the laboratory.**

6. **Keep your workspace free of all unnecessary materials. Backpacks, purses, and coats should be placed in the cubbyholes by the front door of the lab. Place needed items on the floor near your feet, but not in the aisle.**

7. **Disinfect work areas before and after use. Laboratory equipment and work surfaces should be decontaminated with an appropriate disinfectant, such as 10% bleach or 70% ethanol solution.**

8. **Label everything clearly. All cultures, chemicals, disinfectants, and media should be clearly and securely labeled with their names and dates.**

9. **Do not open Petri dishes in the lab unless absolutely necessary.**

10. **Inoculating loops and needles should be flame sterilized in a Bunsen burner before you lay them down.**

11. **Turn off Bunsen burners when not in use. Long hair must be restrained if Bunsen burners are in use.**

12. **When you flame sterilize with alcohol, be sure that you do not have any papers under you.**

13. Treat all microorganisms (especially unknown cultures) as potential pathogens. Use appropriate care and do not take cultures out of the laboratory.

14. Wear disposable gloves when working with potentially infectious microbes or samples.

15.Sterilize equipment and materials. All materials, media, tubes, plates, loops, needles, pipettes, and other items used for culturing microorganisms should be sterilized.

16. Never pipette by mouth. Use pipette bulbs or pipetting devices for the aspiration and dispensing of liquid cultures.

17. Consider everything a biohazard. Do not pour anything down the sink. Autoclave liquids and broth cultures to sterilize them before discarding.

18. Autoclave or disinfect all waste material. All items to be discarded after a class, such as culture tubes, culture plates, swabs, toothpicks, wipes, disposable transfer needles, and gloves, should be placed in a biohazard autoclave bag and autoclaved 30 to 40 minutes at 121° C at 20 pounds of pressure. If no autoclave is available and you are not working with pathogens, the materials can be covered with a 10% bleach solution and allowed to soak for at least 1 to 2 hours.

19. Familiarize yourself with the location of safety equipment in the lab (e.g., sinks, fire extinguisher, and first aid kit).

20. Dispose of broken glass in the broken glass container.

21. Dispose of razor blades, syringe needles, and sharp metal objects in the "sharps" container.

22. Report all injuries or accidents immediately to the instructor, no matter how small they seem.

23. Report spills and accidents immediately to your instructor. Clean small spills with care. Seek help for large spills.

Microbiology: is the study of microorganisms (mo.s) which are microscopic organisms; include:

A- The Eukaryotes: such as Fungi and Protists .

The Fungi includes: (Yeasts) and (Molds).

The Protists include: (unicellular protozoa) and (unicellular algae).

B- The Prokaryotes: including the (Eubacteria) and the (Primitive bacteria).

C- Chlamydiae; Rickettsiae ; Viruses.

Bacteriology: is the study of bacteria.

Medical microbiology: studying pathogenic bacteria that causing infections and diseases to human and animals.

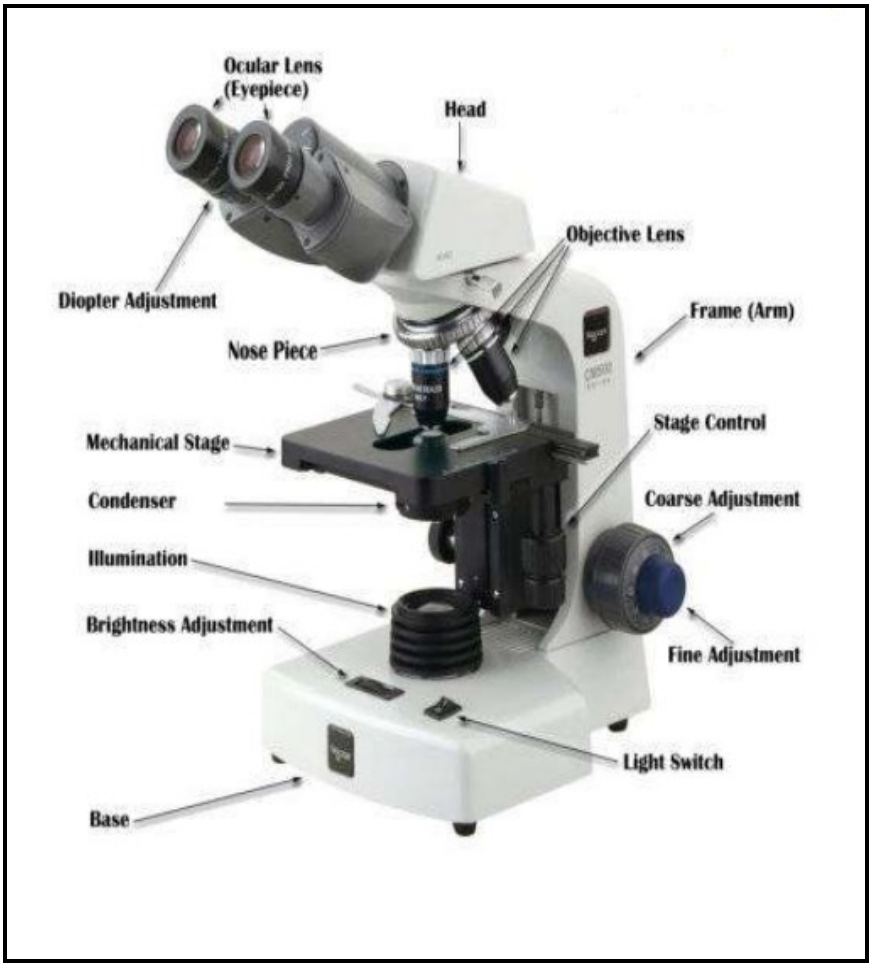
Practical medical bacteriology help to identify bacterial pathogens in clinical specimens; e.g. pus, blood, sputum, spinal fluid , swabs of skin, throat swab, rectum swab or urogenital surfaces.

Bacterial identification involve; Staining, microscopic examination, isolation of bacteria, and characterization; to identify pathogens from harmless microorganisms mo.s

Equipments and Tools that involved in Microbiological studies:

Equipments:

1- The Microscope: it is used to examine (directly) the microorganisms by naked eyes



Optical Microscope

2- Autoclave

Wet heat sterilization= death by protein denaturation

It's an equipment with: High temperature(121C°), High pressure 1 atm (15 pound/inch^2) used to sterilize media (with sugar for 10min) (uncultured media for 15min), (cultured media and contaminated glass wares for 30 min) , especially used to sterilize cultures media

3- Oven

Dry heat sterilization=death by oxidation

Equipment with high temperature only (180C°) for (90 min) used to sterilized some of metallic tools and glass wares

4- Incubator

Is a device used to grow and maintain microbiological cultures or cell cultures. The incubator maintains optimal temperature ,humidity and other conditions such as the carbon dioxide (CO2) and oxygen content of the atmosphere inside

5- Refrigerator

Used to maintain the sterilized media and broth when not used to avoid the contamination , and also to preserve the bacterial culture for long time by preventing the growth at 4C°.

6- Biosafety cabinet (BSC) or Laminar air flow

Also called a biological safety cabinet or is an enclosed, ventilated laboratory workspace for safely working with materials contaminated with (or potentially contaminated with) pathogens

7- Water bath

Is laboratory equipment made from a container filled with heated water. It is used to incubate samples in water at a constant temperature over a long period of time

8- Centrifuge

A machine with a rapidly rotating container that applies centrifugal force to its contents.

vertical and horizontal Autoclaves

Incubator

Laminar air flow

Oven

Centrifuge

Tools:

1- Loop

The loop is used in the cultivation of microbes on plates by transferring inoculums for streaking. The inoculation loop is sterilized with flame or another heat source.

2- Can

Used for preserve the pipette from any contamination, sterilized with pipettes by autoclave

3-Pipette

Used for transfer of cultured and uncultured broth from tube or flask to other and placed in can sterilized by autoclave inside the can.

4-Spreader (L-shape)

Used for spreading bacterial cell on surface of solid medium in petri plate ,before using placed in alcohol and then sterilized by flame of burner.

5- Petri-Dish (Petri-plate)

Used for place the solid medium in it, glass petri-dish used for many times and sterilized by oven or autoclave , while sterilized plastic plates used for one time.

6- Swab

Used for swabbing bacterial cells on the surface of solid medium in Petri plate, must be placed in test tube and sterilized by autoclave, it used for one time.

7- Test tube

Used to place the liquid or solid or semisolid medium for stabbing or placed as slant for culture of bacteria ,it sterilized by autoclave.

8- Needle

Used for transfer of bacterial cells to a solid medium or semi-solid medium by stabbing , sterilized by the flame of burner before and after use.

9- Slide

Used for examination of bacterial smear under microscope , it used for one time.

10- Cover-Slips

Placed on the slide , the bacterial smear may be between the cover and the slide, it used for one time.

11- Flask

Used for place cultured and uncultured broth in it , sterilized after plugs with cotton by autoclave.

12- Cotton plugs

A piece of coiled cotton used to close the upper part of flasks and tubes.

13- Beaker

Used for graduated the volume of liquids. Sterilized by oven.

14- Cylinder (Graduated Cylinder)

Used for graduated the volume of liquids, sterilized by oven.

15- Washing bottle

Used to fill with liquid (specially distilled water)for washing and homogenizing the glass wares and washing the slide during staining , don't need sterilization.

16- pH paper

Used to know the pH of the medium or any liquids

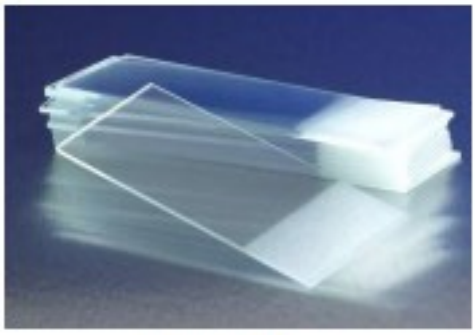
17- Rack

May be wooden , metallic or plastic used to stand and hold the tube.

18- Burner

May be gaseous or alcoholic , used for sterilized the loop , needle and other metallic tools by flame (dry heat sterilization).

Loops and needles

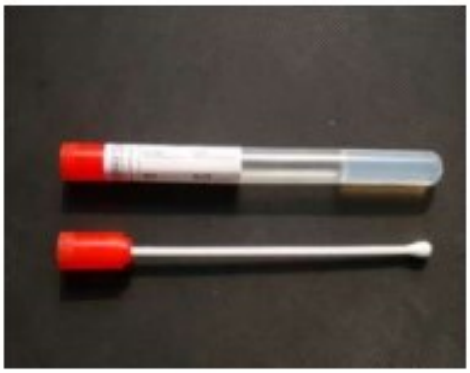


Glass slides

Petri dishes

Flask

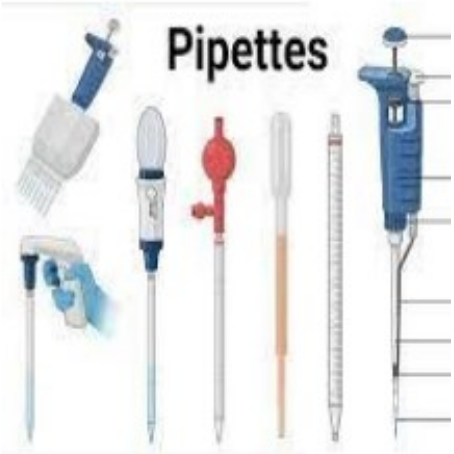
Beaker



Swab

Spreader (L-shape)

Bunsen and alcoholic burner



Cylinder

Washing bottle

pH paper

Rack

STERILIZATION

Sterilization refers to the anti-microbial process during which all microorganisms are killed or eliminated in or on a substance by applying different processes.

1. Sterilization by heat:

1.1. Flaming:

By incinerating metal objects and tools in flame e.g. knife, inoculating loop or needle.

1.2. Dry heat :

The use of dry heat is based on the removal of water content from microbial cells and subsequent oxidation, using the hot air oven. Different laboratory glassware can be sterilized by means of oven.

1.3. Moist heat:

The heat conductivity of water is several times higher than that of the air, therefore heat sterilizes more quickly and effectively in the presence of hot water or steam than dry heat.

1.3.1. Boiling: is the simplest and oldest way of using moist heat. The temperature of boiling water does not exceed 100°C at normal atmospheric pressure. Heat resistant, endospore-forming bacteria can survive the 10-30 minute heat treatment of boiling, so no sterilizing effect can be expected with boiling.

1.3.2. Pasteurization: is a widespread method, named after Louis Pasteur – to reduce the number of microorganisms found in different heat sensitive liquids. Milk can be pasteurized by heating to 65°C for 30 minutes or to 85°C for 5 minutes, thereafter sudden cooling to apply thermal shock, to kill all vegetative microbial cells. During ultra-pasteurization milk is heat-treated at 135-150°C for 2 minutes in a heat exchanger.

1.3.3. Autoclaving: nutrient media, cotton, gauze, dressings, clothes, sheets and bed covers, sterilized by autoclave, applying steam pressure and temperature to kill all microbial forms including bacterial spores, within a certain time. Conditions of autoclaving process are: : 121°C (temp.), 15 psi (pressure) and 15 min (time) (variable).

1.4. Tyndallisation (intermittent sterilization):is an old and lengthy method of heat sterilization. During this method, a medium or solution is heated to a temperature over 90°C for 30 minutes for four successive days, and the substances are placed in an incubator at 37°C or stored at room temperature

in the intermittent periods. Vegetative forms are destroyed during the heat treatments. Endospores which can germinate during the incubation period are destroyed during the consecutive heat treatments. This way, after the fourth day of heat treatment, no living cells remain in the substance. Soil samples can be sterilized by this method.

2. Sterilization by radiation

The full spectrum of UV radiation can damage microbes, Among the high-energy ionizing radiation, γ-rays is used for sterilization of disposable needles, syringes, bandages, medicinal and certain foods (e.g. spices). The advantage of gamma radiation is its deep penetration through the packaging. Its disadvantage is the scattering in all directions, which requires special circumstances for application.

3. Filter sterilization

The most commonly used mechanical method of sterilization is filtration. During filtration, liquids or gases are pressed through a filter, which (depending on its pore size) retains or adsorbs (e.g. asbestos filter pads) microbes, thereby the filtrate becomes sterile. The pore diameter of filters should be chosen carefully so that bacteria and other cellular components cannot penetrate.

4. Sterilization by chemicals

A wide range of chemicals are suitable to inhibit or kill microbes. Some of the antimicrobial agents only inhibit the growth of microorganisms (e.g. bacteriostatic, fungistatic, and virostatic compounds) while others kill them.

ASEPTIC TECHNIQUES

Microorganisms usually exist as mixed populations. However, if we are aiming to study, characterize, and identify microorganisms, we must study it in the form of a pure culture (members of single culture).

Finally, in working with microorganisms, we must have a method of transferring growing organisms from a pure culture to a sterile medium without introducing any unwanted outside contaminants. This method of preventing unwanted microorganisms from gaining access is termed aseptic technique.

Five Basic Techniques of Culturing :

- **Inoculation: Producing a pure culture**
- **Isolation: Colony on media, one kind of microbe, pure culture**
- **Incubation: growing microbes under proper conditions**
- **Inspection: Observation of characteristics (data)**
- **Identification: use of data, correlation, to ID organism to exact species**

Inoculation methods :

1. Broth tube: Are tubes containing a liquid medium. A typical nutrient containing broth medium such as nutrient broth. After incubation, growth may be observed as one or a combination of three forms:

a. Pellicle: A mass of organisms is floating on top of the broth.

b. Turbidity: The organisms appear as a general cloudiness throughout the broth .

c. Sediment: A mass of organisms appears as a deposit at the bottom of the tube.

2. Solid tube media :

a- Slant tubes: Are tubes containing a nutrient medium plus a solidifying agent, agar-agar. The medium has been allowed to solidify at an angle in order to get a flat inoculating surface (aerobic) .

b- Agar deep tubes (stabbing): are tubes of hardened agar medium which are inoculated by "stabbing" the inoculum into the agar (anaerobic).

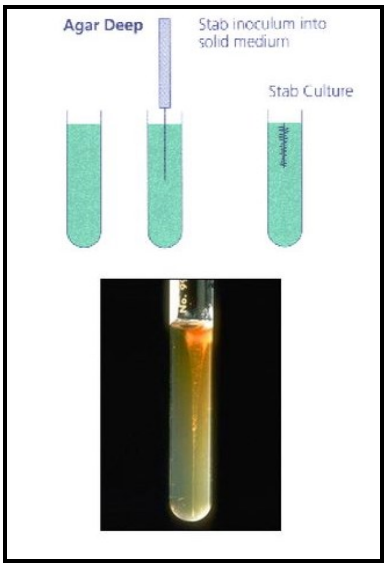
3. Agar plates: Are sterile petri plates that are aseptically filled with a melted sterile agar medium and allowed to solidify. Plates are much less confining than slants and stabs and are commonly used in the culturing, separating, and counting of microorganisms.

a-Pour plate technique: Is based on dilution of microbes and pouring the tested material with gelatin. After cooling the gelatin isolated colonies of microorganisms are found on both the surface and bottom of the media (aerobic and anaerobic).

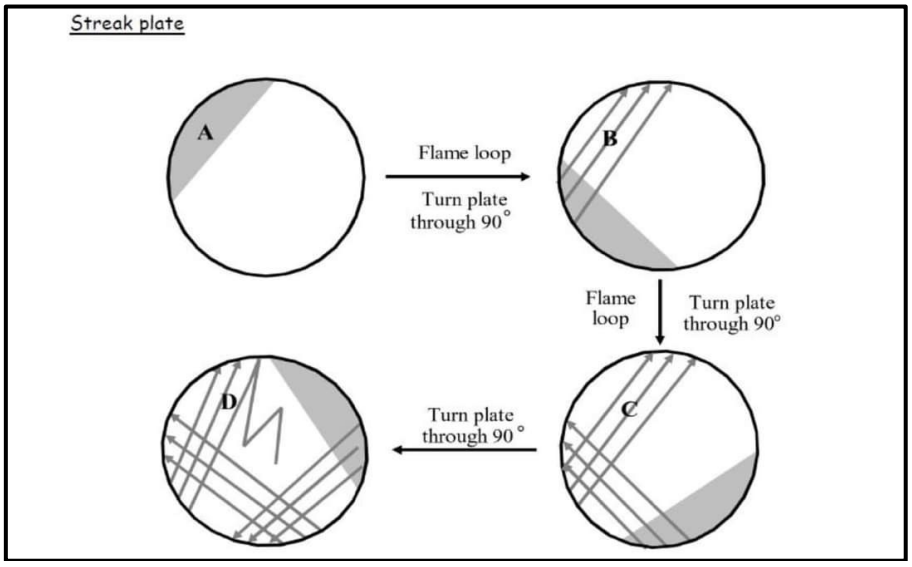
b-Spread plate technique: It is quantitative technique that allows the determination of the number of bacteria in a sample. The cultures are found only on the surface of the media (aerobic).

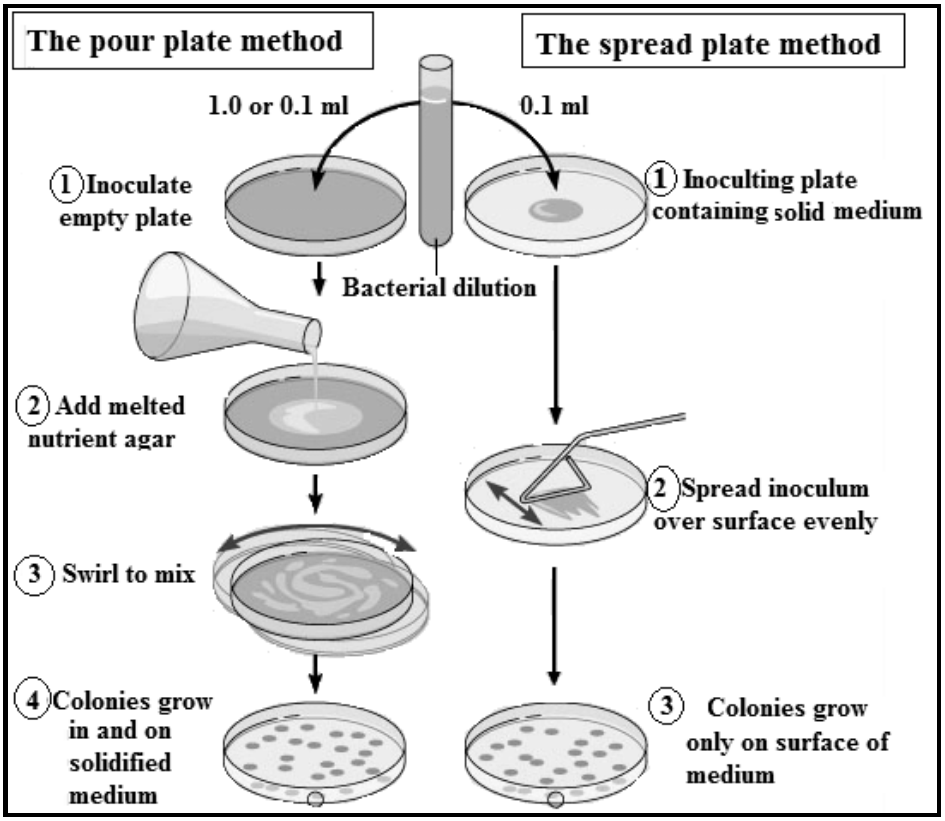
c-Streaking method : The Streak Plate Method: sterile wire loop streaked in different patterns on agar .

slant tube tech.



stab tube tech.





MORPHOLOGY OF BACTERIAL COLONY

Bacteria grow on solid media as colonies. A colony is defined as a visible mass of microorganisms all originating from a single mother cell or a group of cells, therefore they are all genetically alike.

Different species of bacteria can produce very different colonies.

FEATURES OF COLONY : the largest colony

1-SHAPE OF COLONY.

2-SIZE OF COLONY: (measure with a millimeter ruler).

3-EDGE/MARGIN OF COLONY: magnified edge shape (use a dissecting microscope to see the margin edge)

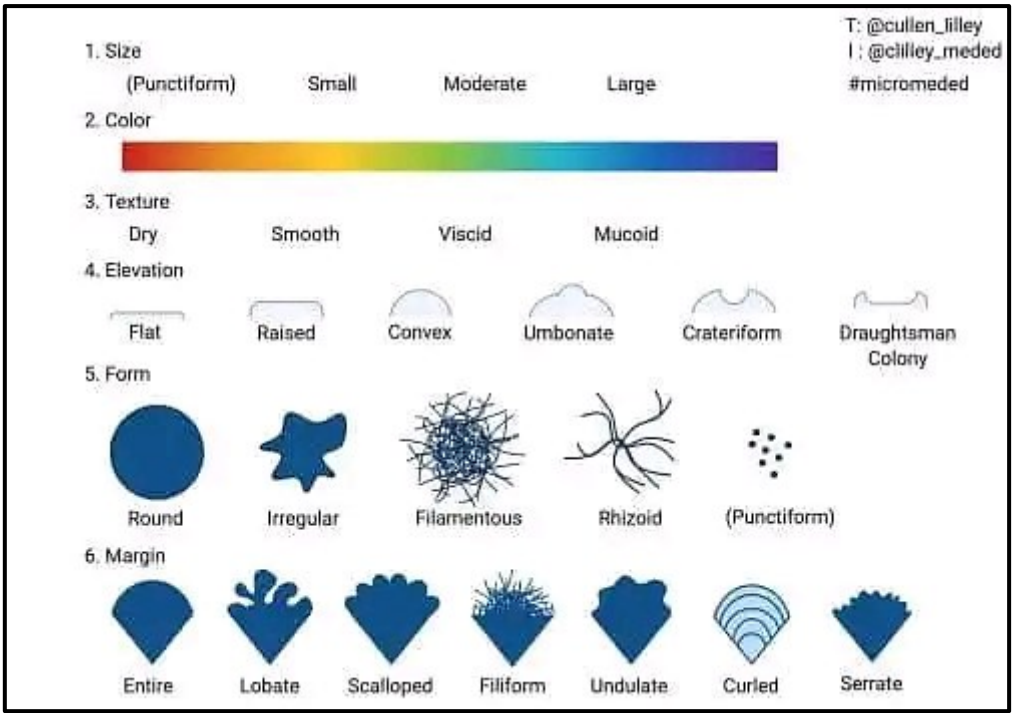
4-CHROMOGENESIS: (color of colonies, pigmentation): white, buff, red, purple.

5-OPACITY OF COLONY: Is the colony transparent (clear), opaque (not transparent or clear), translucent (almost clear, but distorted vision–like looking through frosted glass), iridescent (changing colors in reflected light).

6-ELEVATION OF COLONY: the rising above the agar (turn the plate on end to determine height)

7-SURFACE OF COLONY: smooth, glistening, rough, dull (opposite of glistening), rugose (wrinkled)

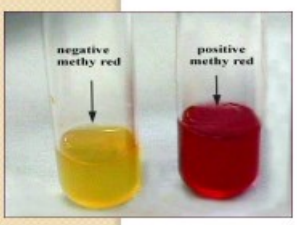
8-CONSISTENCY or TEXTURE: butyrous (buttery), viscid (sticks to loop, hard to get off), brittle/friable (dry, breaks apart), mucoid (sticky, mucus-like) (use your inoculating loop)



CULTURE MEDIA

Advantages of culture media:

- **To culture microorganisms.**
- **For pure culture isolation.**
- **For storage of stock cultures.**
- **To observe specific biochemical reactions.**
- **As transport media to preserve bacteria during transportation to the laboratory**
- **For preparation of antigens (vaccines and diagnostic kits)**



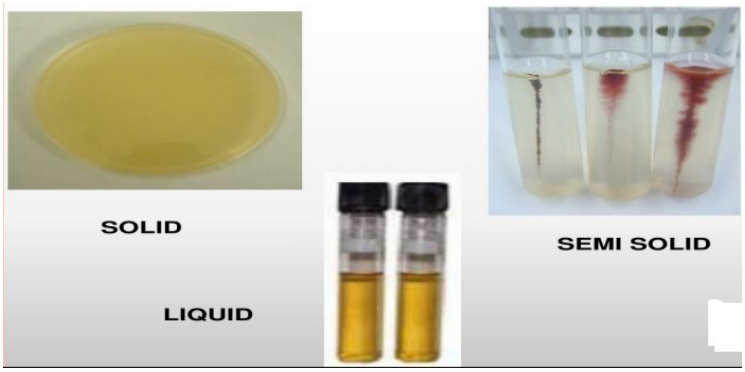
Types of media according to their solidity;

1. solid medium (2 % of agar):

agar plate /slant medium

2. semi-solid medium (1 % of agar)

3. liquid medium (broth)(do not contain agar)



The benefit of the broth is to propagate large number of microorganism for various biochemical test& fermentation studies.

The benefit of the Solid media is to obtain surface (or depth) growth in order to observe colony appearance

The benefit of the semi solid media is to:

1.fermentation studies.

2.determining bacterial motility

3.promoting anaerobic growth.

Types of Culture media according to their composition:

1. Basic Media:

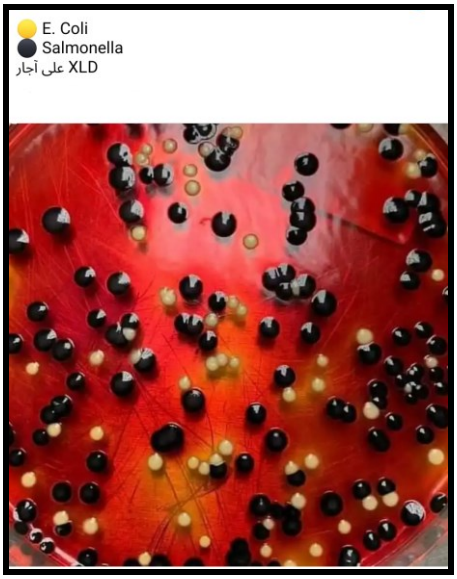
It is simple media such as nutrient agar, nutrient broth and peptone water that support the growth of microorganisms that don't have special nutritional requirement. They are often used in the preparation of enriched media.

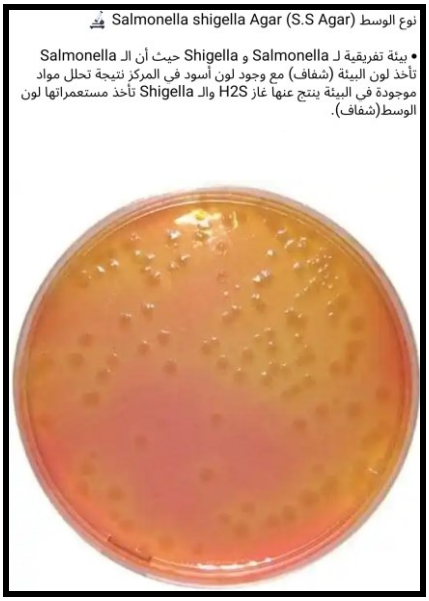
2. Enriched Media:

It is a broth or solid medium containing a rich supply of special nutrients (e.g. Blood, serum or egg) that promotes the growth of particular organisms. It is made by adding extra nutrients to a nutrient agar (basic medium). Commonly used examples are Blood agar and Chocolate agar, both of which are rich in nutrients and free from inhibitory substances.

3 . Selective Media:

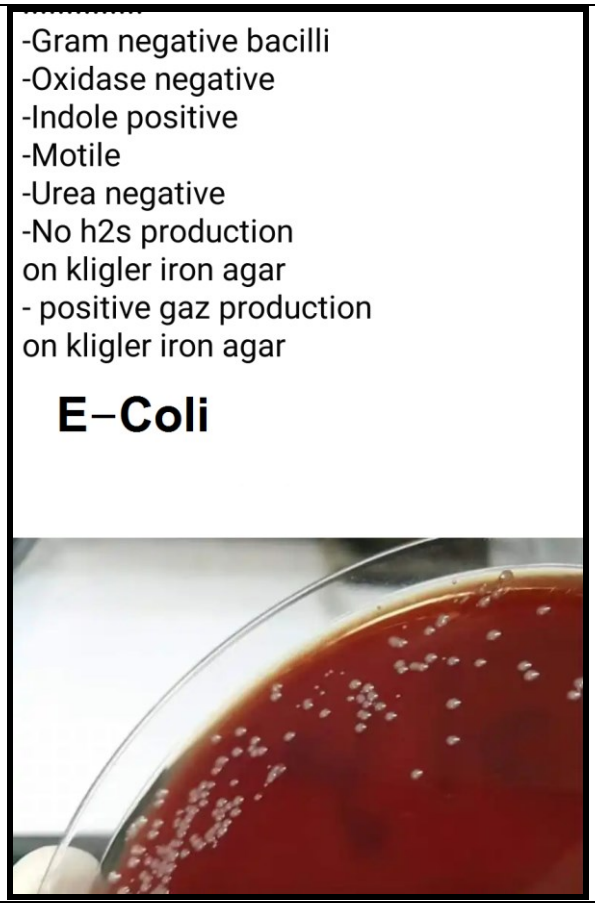
These media contain substances (e.g. bile salt or other chemicals, dyes, antibiotics) which inhibit the growth of one organism to allow the growth of another to be more clearly demonstrated e.g. XLD (Xylose Lysine Deoxycholate agar) and DCA (Deoxycholate Citrate Agar) are used for the isolation of Salmonella and Shigella species.





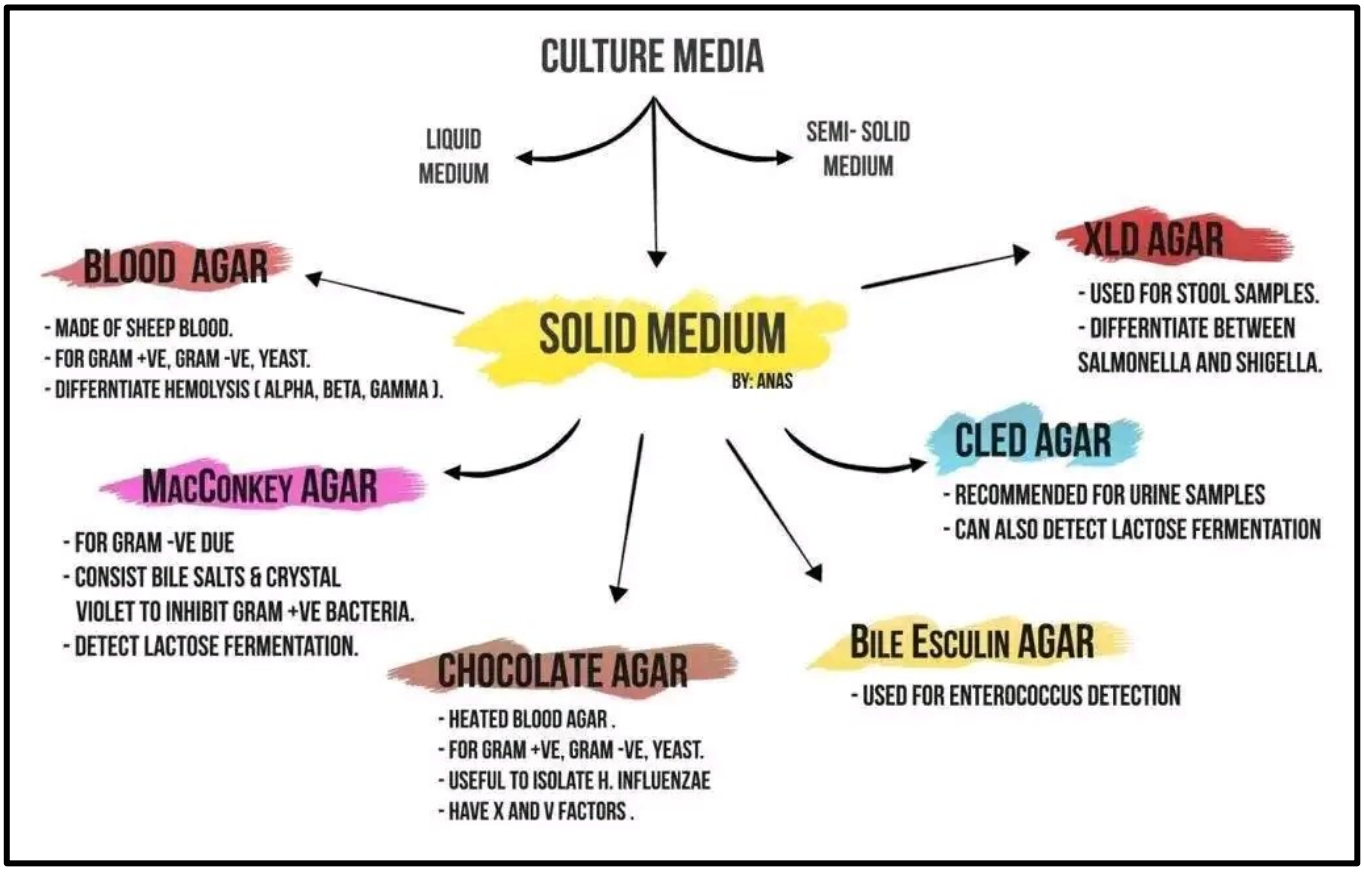
4. Differential (Indicator) Media:

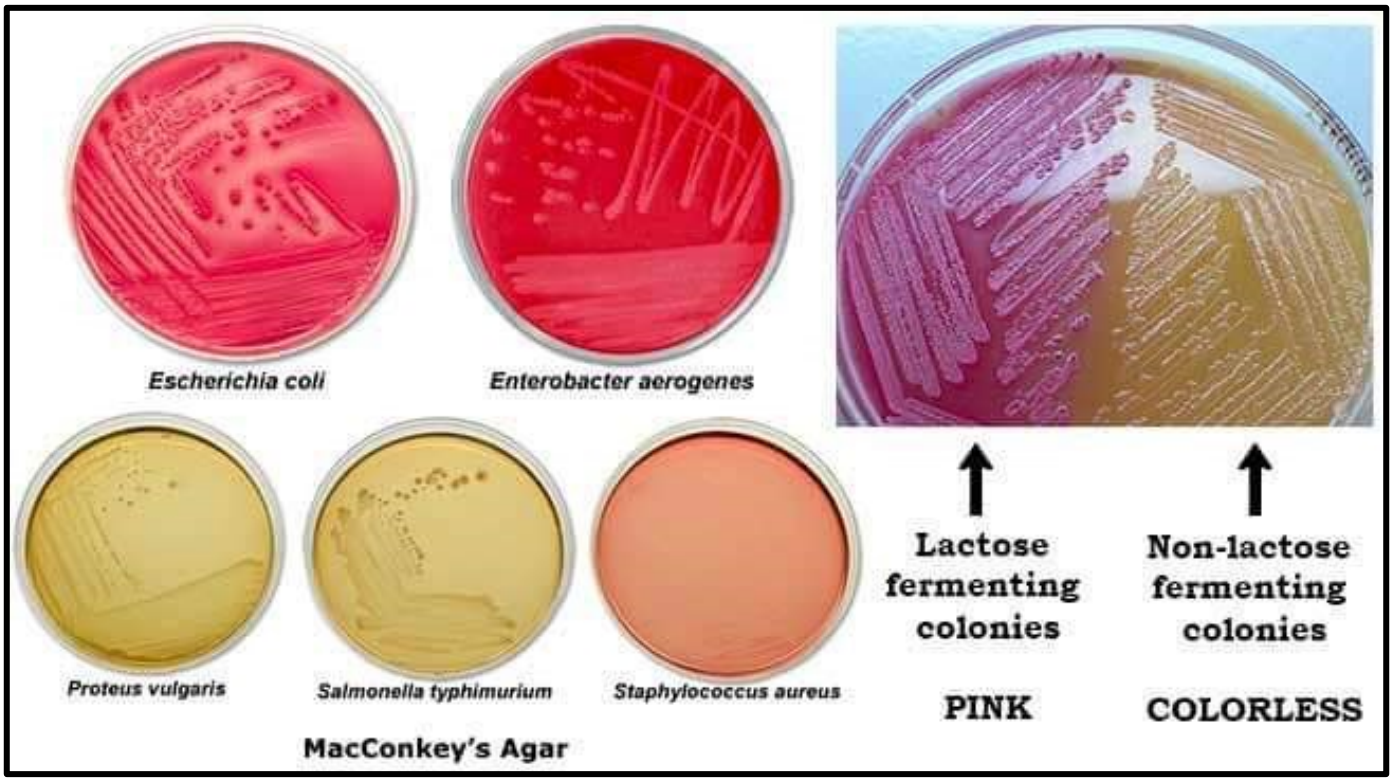
These media contain a substance that is changed visibly as a result of the metabolism of particular organism : Kliger Iron Agar (KIA) and Triple Sugar Iron agar (TSI), both differentiate between different types of Enterobacteriaceae on the basis of their ability to ferment carbohydrates.



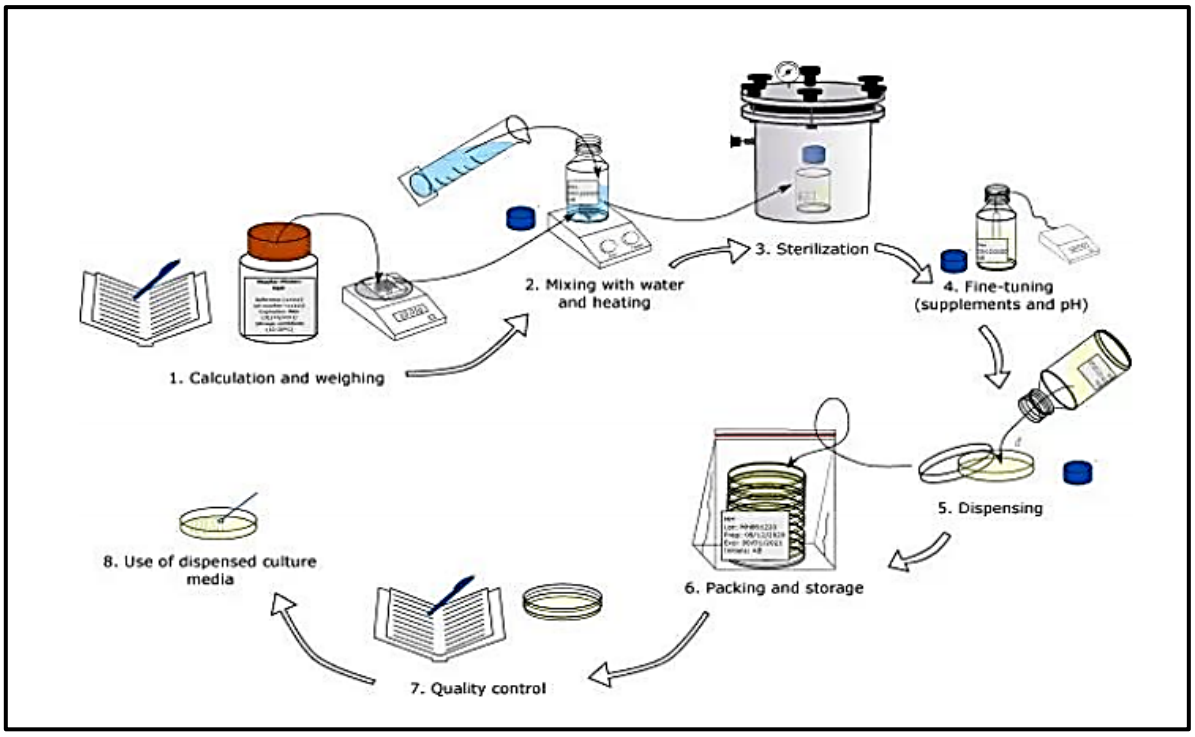
5. Selective and Differential Media:

An example of this is MacConkey Agar which is selective for Gram negative(-) bacteria while inhibits gram positive(+) bacteria, and has an indicator (neutral red) to differentiate between lactose and non-lactose fermenting organisms. Another example, MSA (Mannitol Salt Agar) is selective and differential to grow Staphylococcus aureus.





The preparation steps of culture media:



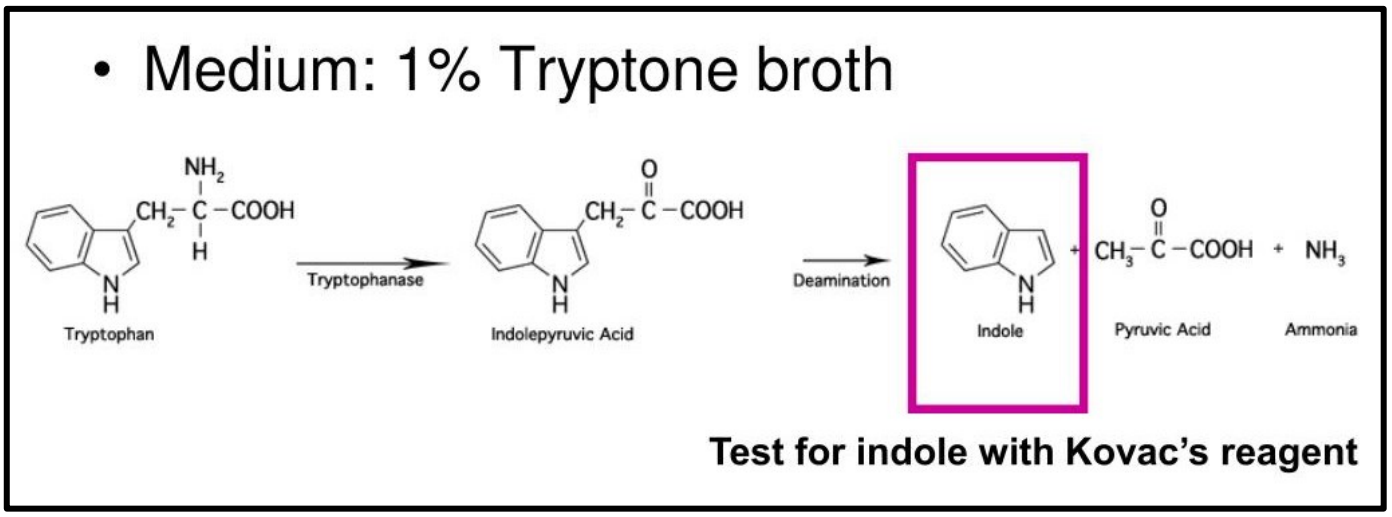
TESTS THAT DIFFERENTIATE STRAINS

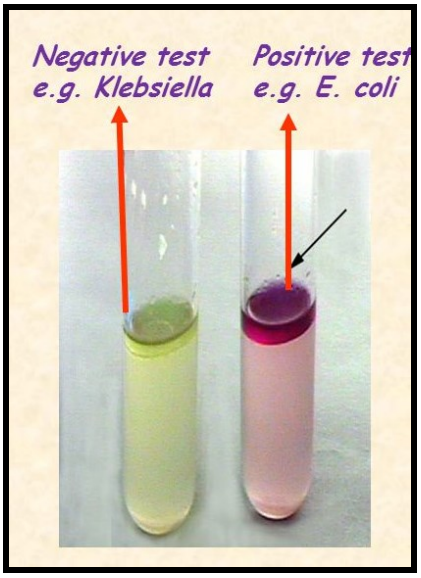
IMVIC TESTS: It is 4 tests that differentiate the intestinal tract pathogenies strains:

1. INDOLE TEST

Indole is a component of the amino acid tryptophan. Some bacteria have the ability to break down tryptophan for nutritional needs using the enzyme tryptophanase. When tryptophan is broken down, the presence of indole can be detected through the use of Kovacs' reagent (yellow), reacts with indole and produces a red color on the surface of the test tube.

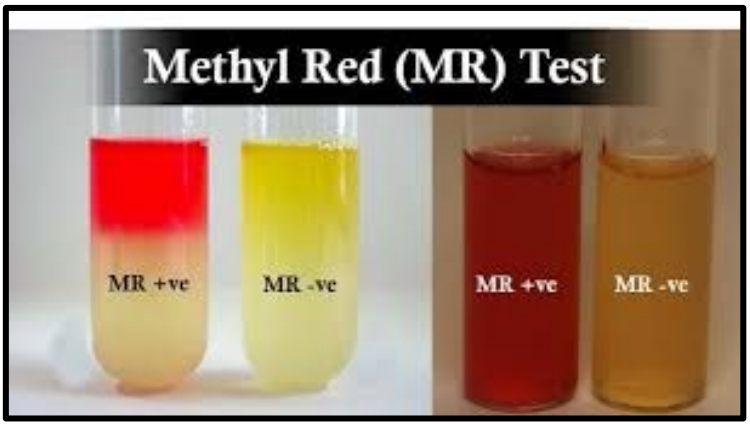
Results: Positive (+): red color ex. E.coli; Negative (-): yellow color ex. Klebsiella





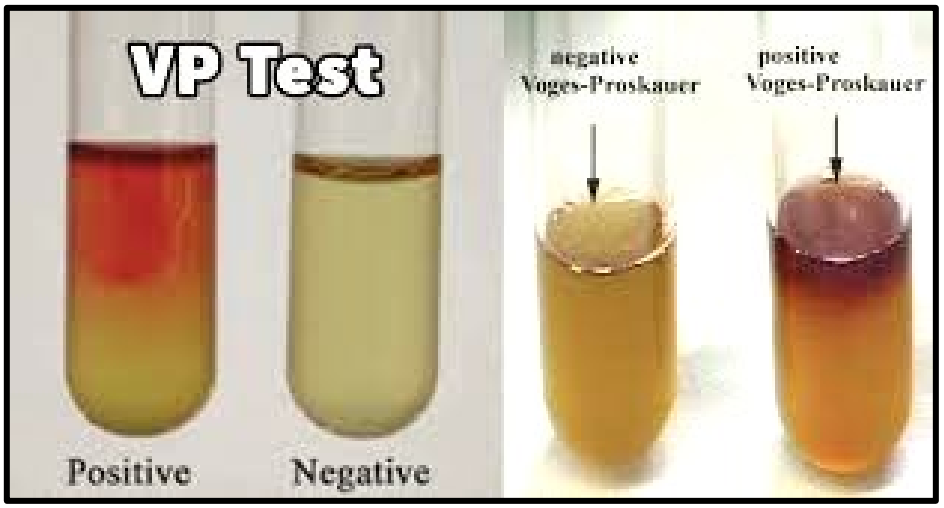
2- MR TEST (METHYL RED TEST):

some microorganisms produce acid from glucose fermentation, this is a qualitative test for acid production.



3- VP TEST (VOGES-PROSKAUER) :

some microorganisms produce (acetoin) from glucose fermentation.



Procedure

1. Inoculate the tested organism into 2 tubes of MR-VP broth

2. Incubate the tubes at 37°C for 24 hours

3. After incubation: Run the MR test in the tube 1, and the VP test in tube 2.

– For methyl red: Add 6-8 drops of methyl red reagent.

– For Voges-Proskauer: Add 12 drops of Barritt's A (α-naphthol), mix, 4 drops of Barritt's B (40% KOH), mix.

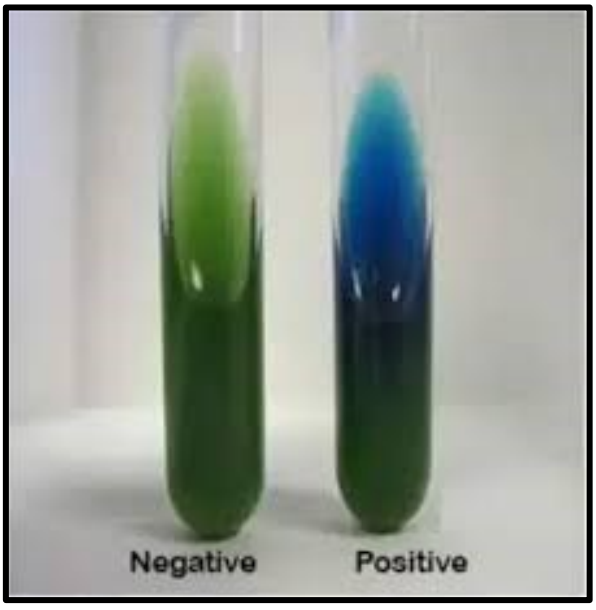
4- CITRATE UTILIZATION TEST:

Simmons Citrate agar is a defined medium containing sodium citrate as the carbon source. Some bacteria can use citrate to grow in the absence of another carbon source due to production of enzyme Citrase or Citrate permease.

The pH indicator, bromothymol blue, will turn from green at neutral pH (6.9) to blue when a pH higher than 7.6 is reached (alkaline). If the citrate is utilized, the resulting growth will produce alkaline products changing the color of the medium from green to blue.

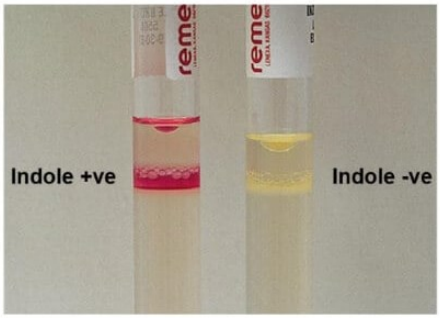
(Blue color= positive reaction eg; Klebsiella)

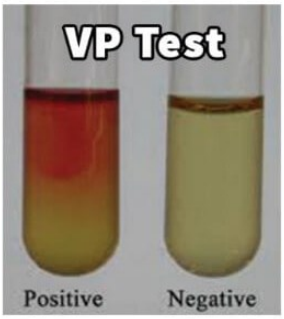
(Green color=negative reaction eg; E.coli)

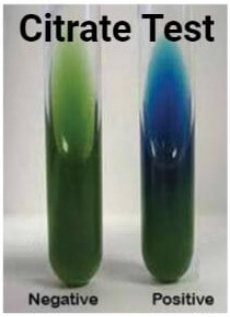


test	+ ve result	-ve result
Indol Test	red	yellow
Methyl Red Test	red	yellow
Voges Proskure Test	red	yellow
Simmon Citrate Test	blue	green

Species	Indole	Methyl Red	Voges-Proskauer	Citrate
Escherichia coli	+	+	-	-
Shigella spp.	-	+	-	-
Salmonella spp.	-	+	-	+
Klebsiella spp.	-	-	+	+







UREASE TEST

The test is used to determine the ability of an organism to split urea, through the production of the enzyme urease.

Medium used for urease test: Any urea medium, agar or broth.

Indicator (Phenol red), Color change:

- **Original: orange yellow color**
- **Final color (in positive test): Bright pink**

Urease test principle

- **Urea is a diamide of carbonic acid. It is hydrolyzed with the release of ammonia and carbon dioxide.**
- **Many organisms especially those that infect the urinary tract, have an urease enzyme which is able to split urea in the presence of water to release ammonia and carbon dioxide. The ammonia combines with carbon dioxide and water to form ammonium carbonate which turns the medium alkaline, turning the indicator phenol red from its original orange yellow color to bright pink.**

Procedure for urease test

1. The broth medium is inoculated with a loopful of a pure culture of the test organism; the surface of the agar slant is streaked with the test organism.

2. Leave the cap on loosely and incubate the test tube at 35 °C in ambient air for 18 to 24 hours; unless specified for longer incubation.

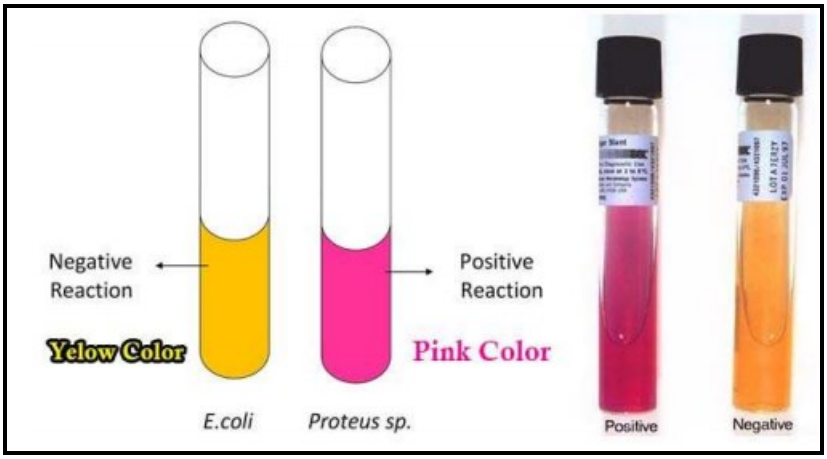
Result and Interpretation

Organisms that hydrolyze urea rapidly (e.g. Proteus spp) may produce positive reactions within 1 or 2 hours; less active species (e.g. Klebsiella spp) may require 3 or more days. In routine diagnostic laboratories the urease test result is read within 24 hours.

If organism produces urease enzyme, the color of the slant changes from light orange to magenta.

If organism do not produce urease the agar slant and butt remain light orange (medium retains original color).

Name of urease positive Bacteria:
1. **Proteus spp**
2. **Cryptococcus spp**
3. **Corynebacterium spp**
4. **Helicobacter pylori**
5. **Brucella spp**



CATALASE TEST

H_2O_2 is a poisonous substance for several strains of Bacteris, thus, to get rid of it, some Bacteria have the ability to produce catalase (enzyme that convert H_2O_2 into O_2), this action produce bubbles.

By catalase test we can distinguish between :

- G +ve cocci : (Staphylococcus) is (+) whereas Streptococcus is (-).

- G +ve bacilli : (Bacillus) is (+) whereas (Clostridium) is (-).

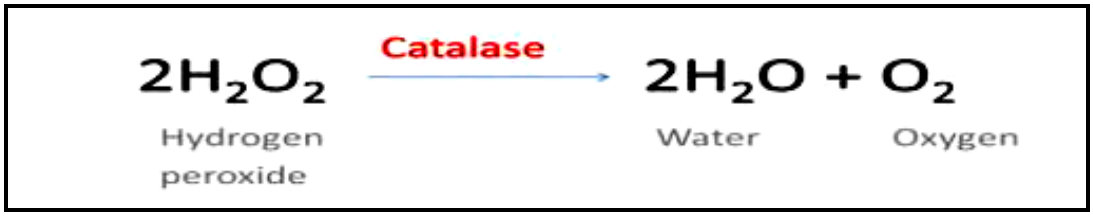
- All Enterobacteriaceae (G -ve bacilli) are catalase (+).

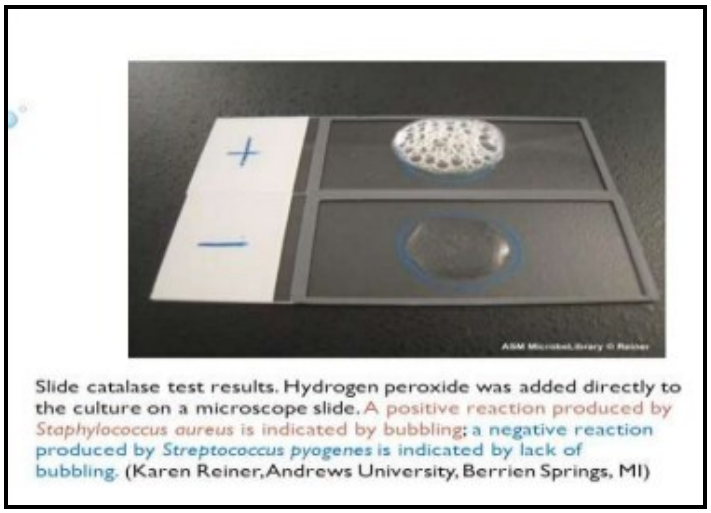
PROCEDURE

1- Place a small amount of culture onto a clean slide.(If using colonies from a blood agar plate, be very careful not to scrape up any of the blood agar— blood cells are catalase positive and any contaminating agar could give a false positive).

2. Add a few drops of H_2O_2 onto the smear. If needed, mix with a toothpick (DO NOT use a metal loop or needle with H_2O_2; it will give a false positive and degrade the metal).

3. A positive result is the rapid evolution of O_2 as evidenced by bubbling.





Slide catalase test results. Hydrogen peroxide was added directly to the culture on a microscope slide. A positive reaction produced by *Staphylococcus aureus* is indicated by bubbling; a negative reaction produced by *Streptococcus pyogenes* is indicated by lack of bubbling. (Karen Reiner, Andrews University, Berrien Springs, MI)

OXIDASE TEST

This test is performed to determine whether the bacteria is aerobic (live in the presence of O2) or not.

Normally, oxygen is the final electron acceptor for this system. In the oxidase test, an artificial final electron acceptor (N,N,N',N'-tetramethyl phenylenediamine dihydrochloride) (BioHazard material) is used in the place of oxygen. This acceptor is a chemical that changes color to a dark blue/purple when it takes the electron from the last element (cytochrome oxidase) in the electron transport chain.

PROCEDURE

a. Hold reagent dropper upright and point tip away from yourself. Hold the middle with thumb and forefinger and squeeze gently to crush the glass container inside the dropper. Tap the bottom on the tabletop .a few times, Invert the container and squeeze gently for drop-by-drop dispensing.

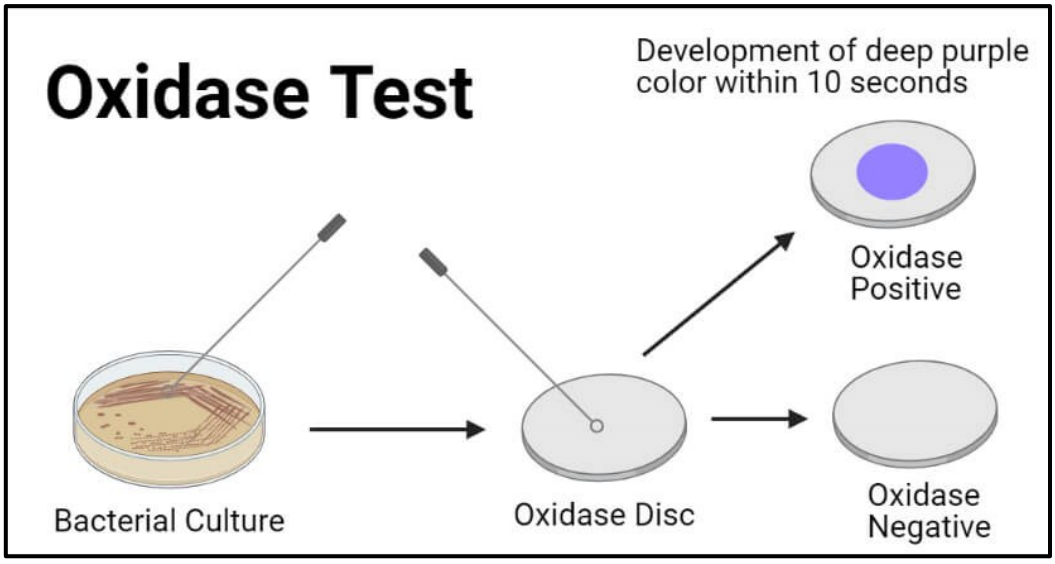
b. With a sterile swab, obtain a small amount of organism from an agar slant or plate.

c. Place one drop of reagent onto the culture on the swab or filter paper.

d. Positive reactions turn the bacteria violet to purple immediately or within 10 to 30 seconds. Delayed reactions should be ignored.

OXIDASE POSITIVE ORGANISMS:

- Pseudomonas
- Neisseria
- Vibrio
- Campylobacter
- Aeromonas
- Alcaligenes
- Brucella
- Pasturella
- Eikenella
- Kingella
- Moraxella
- Legionella
- Helicobacter
- Chromobacter (oxidase variable)



COAGULASE TEST

Staphylococcus aureus is known to produce coagulase, which can clot plasma 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 is used to detect the presence of S.aureus and tube coagulase is 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 clumping factor crosses-links 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. (individuals clot to each other)

Procedure: Dense suspensions of Staphylococci from culture are made on two ends of clean glass slide (test T and control C). 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

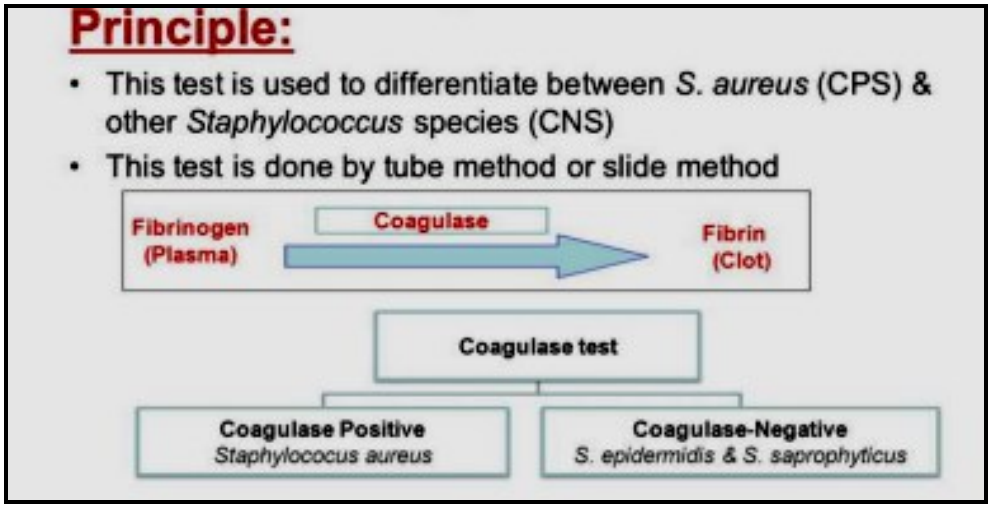
TUBE COAGULASE TEST:

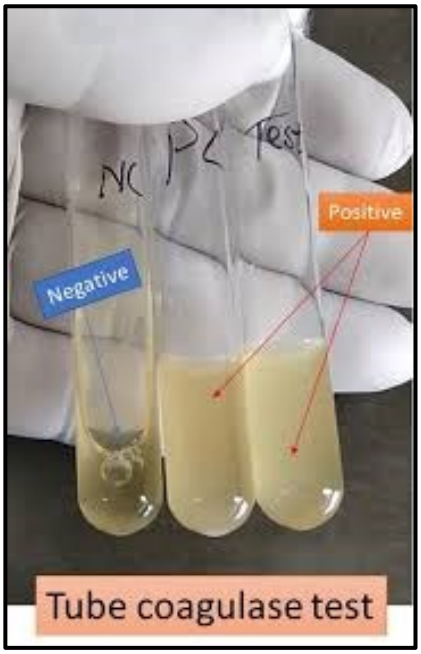
Principle: The free coagulase secreted by S.aureus reacts with coagulase reacting factor (CRF) in plasma to form a complex, which is thrombin. This converts fibrinogen to fibrin resulting in clotting of plasma. (clotting plasma)

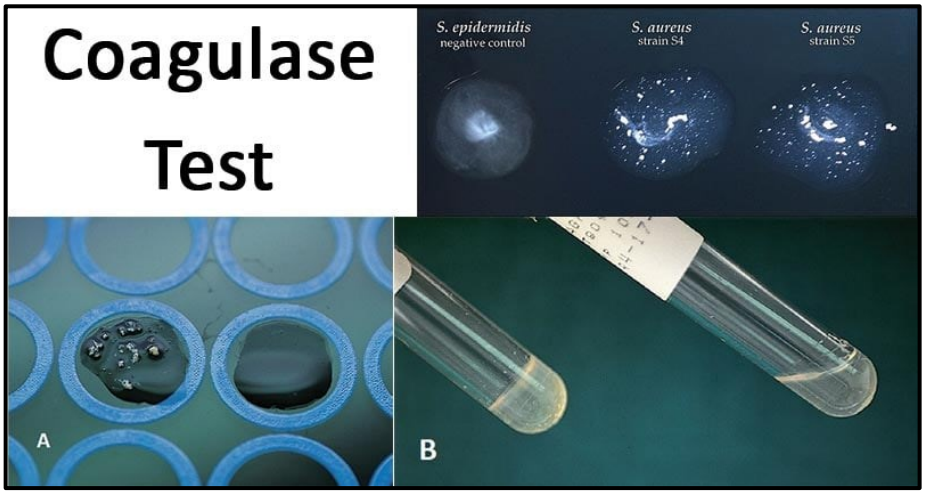
Procedure: Three test tubes are taken and labeled (negative control NC , positive control PC , test tube T) Each tube is filled with 0.5 ml of 1 in 10 diluted rabbit plasma.

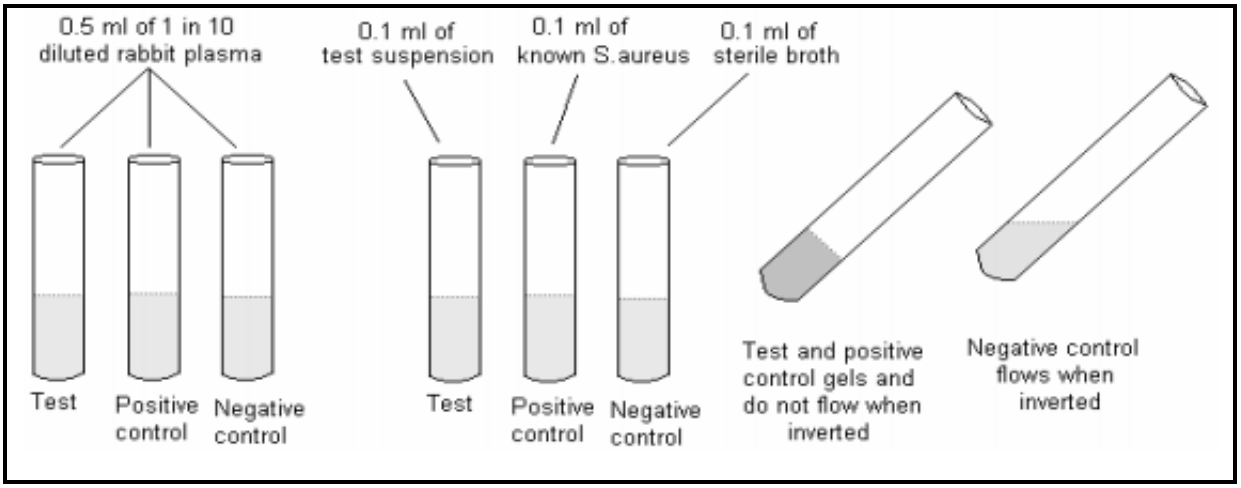
To the T tube, 0.1 ml of overnight broth culture of test bacteria is added. To the PC tube, 0.1 ml of overnight broth culture of known S.aureus is added and to the NC tube, 0.1 ml of sterile broth is added. All the tubes are

incubated at 37°C for 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.









TRIPLE SUGAR IRON AGAR TEST

Triple sugar iron agar test is used to determine whether gram negative bacilli (-) ferment glucose and lactose or sucrose and produce hydrogen sulfide (H2S). It contains 10 parts of lactose: 10 parts of sucrose: 1 part of glucose and peptone. Phenol red and ferrous sulphate serves as indicators for acidification of medium and H2S production respectively.

Procedure:

1. Touch a colony with a sterile straight wire.

2. Inoculate TSI by first stabbing through the center of the medium to the bottom of the tube and then streak the surface of the slant.

3. Leave the cap loose and incubate the tube at 35 ^{o}C in ambient air for 18 to 24 hours.

4. Observe the reaction Analysis of Triple Sugar Iron Agar

Results:

1. If lactose (or sucrose) is fermented, a large amount of acid is produced, which turns the phenol red indicator to yellow both in butt and in the slant (A/A). Some organisms generate gases, which produces bubbles/cracks on the medium.

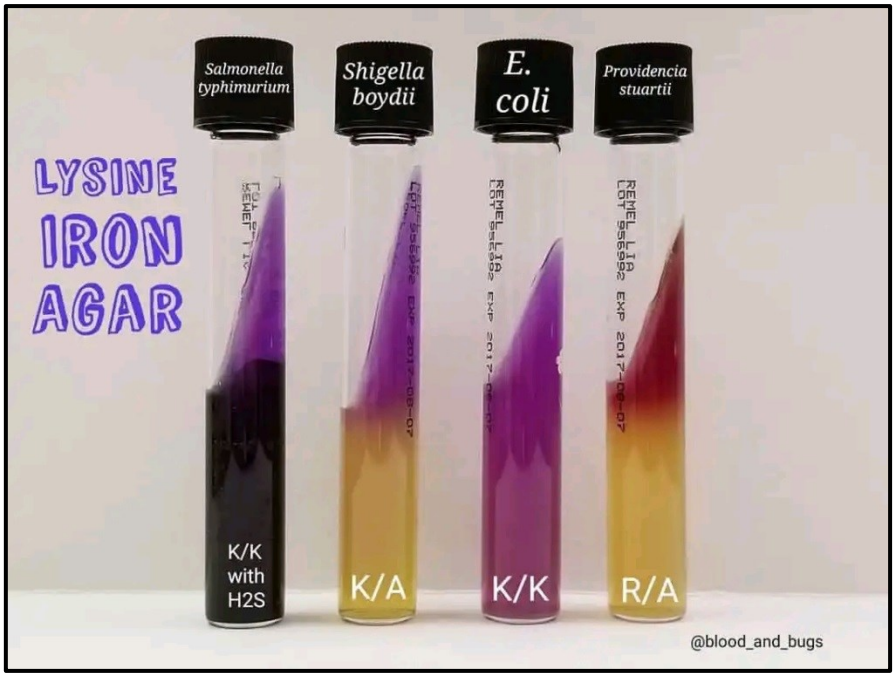
2. If lactose is not fermented but the small amount of glucose is, the oxygen-deficient butt will be yellow (remember that butt comparatively have more glucose compared to slant), but on the slant the acid (less acid as media in slant is very less) will be oxidized to carbon dioxide and water by the organism and the slant will be red (alkaline) (K/A).

3. If neither lactose/sucrose nor glucose is fermented, both the butt and the slant will be red. The slant can become a deeper red-purple (more alkaline) as a result of production of ammonia from the oxidative deamination of amino acids (remember peoptone is a major component of TSI Agar). (K/K).

4. if H2S is produced, the black color of ferrous sulfide is seen.

So the expected results of TSI Agar test are:

- **Alkaline slant/Alkaline butt (K/K) Red/Red = glucose, lactose and sucrose non-fermenter**
- **Alkaline slant/acidic butt (K/A); Red/Yellow = glucose fermentation only, gas (+ or -), H2s (+ or -)**
- **Acidic slant/acidic butt (A/A); Yellow/Yellow = glucose, lactose and/or sucrose fermenter gas (+ or -), H2s (+ or -).**



Name of the organism	Slant	Butt	Gas	H2S
Escherichia, Klebsiella, Enterobacter	Acid (A)	Acid (A)	Pos (+) bubbles	Neg (-)
Shigella, Serratia	Alkaline (K)	Acid (A)	Neg (-)	Neg (-)
Salmonella, Proteus	Alkaline (K)	Acid (A)	Pos (+) bubbles	Pos (+)
Pseudomonas	Alkaline (K)	Alkaline (K)	Neg (-)	Neg (-)

ANTIBIOTIC SENSITIVITY TEST

There is a large number of antimicrobial agents available for treating diseases caused by microorganisms. To be of therapeutic use, an antimicrobial agent must exhibit selective toxicity; it must exhibit greater toxicity to the infecting pathogens than to the host organism.

THE KIRBY-BAUER DISC METHOD (AGAR DIFFUSION OR DISK DIFFUSION METHOD)

A filter disk filled with an antibiotic is applied to the surface of an agar plate containing the pathogenic microorganism and the plate is incubated at 37°C for 24-48 hours. The antibiotic in disc is diluted to the point that it no longer inhibits microbial growth. The effectiveness of an antibiotic substance is shown by the presence of growth-inhibition zones. The diameter of the zones of inhibition can be measured with a ruler and saved for both antibiotic and the microorganism (antibiogram).

MATERIALS AND METHOD

Antibiotic discs, Dispenser, Trypticase Soy Broth, 5 ml (or MHB), Mueller Hinton Agar (MHA) pH 7.3 ±0.1 , Sterile Saline or Broth.

TEST PROCEDURE

1. Preparation of plates

Sterile Muller Hinton Agar is poured into Petri dishes, The depth of the medium should be 4 mm. after the medium has solidified, dry the plates for 30 minutes in an incubator (35-37 °C).

2. Preparation of inoculum:

a) Use only pure culture for sensitivity test. Perform Gram staining before preparing an inoculum.

b) Select 4 to 5 similar colonies and transfer (using flamed wire loop) them into tube containing 5 ml of Trypticase Soy Broth (or MHB).

c) Incubate the broth culture at 35-37 °C for 2 to 5 hours to obtain moderate turbidity.

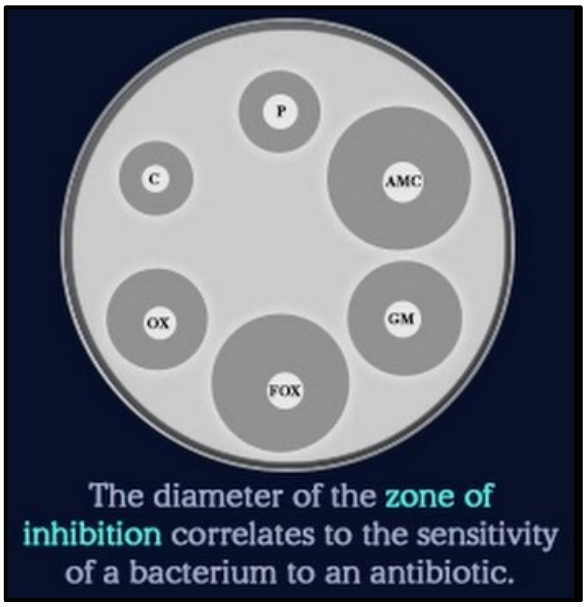
3. Inoculation :

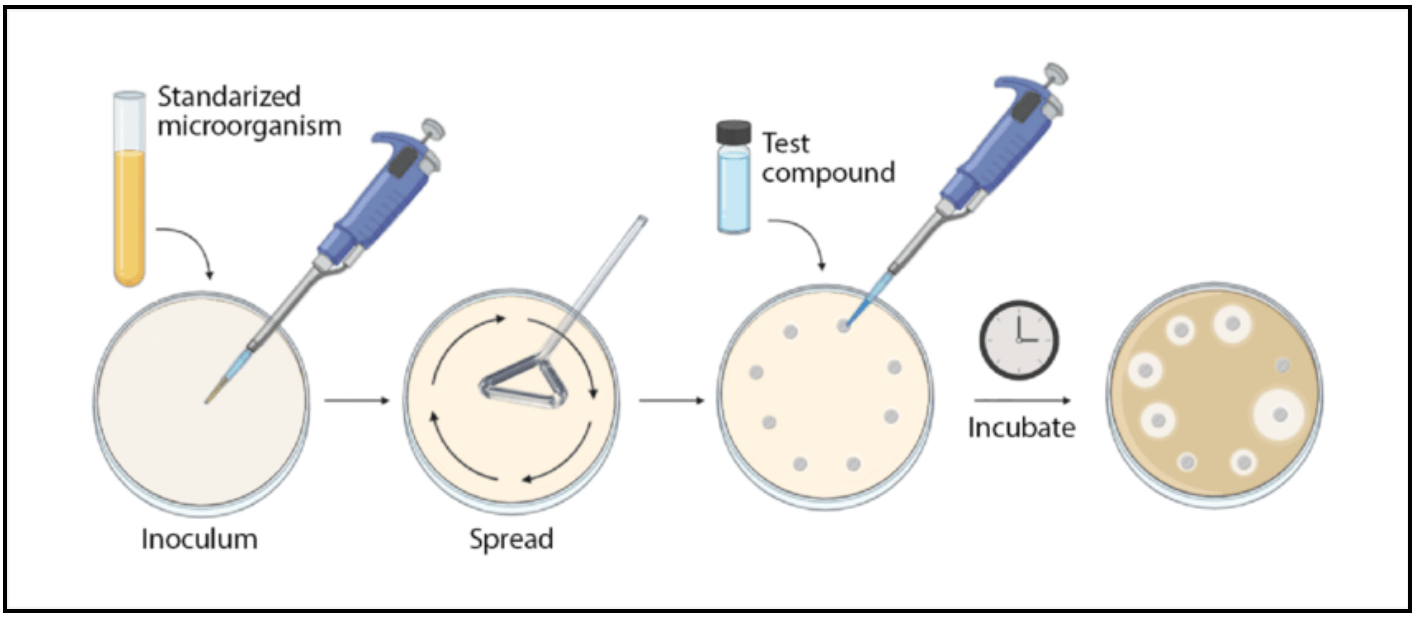
a) Dip a sterile cotton swab into diluted culture.

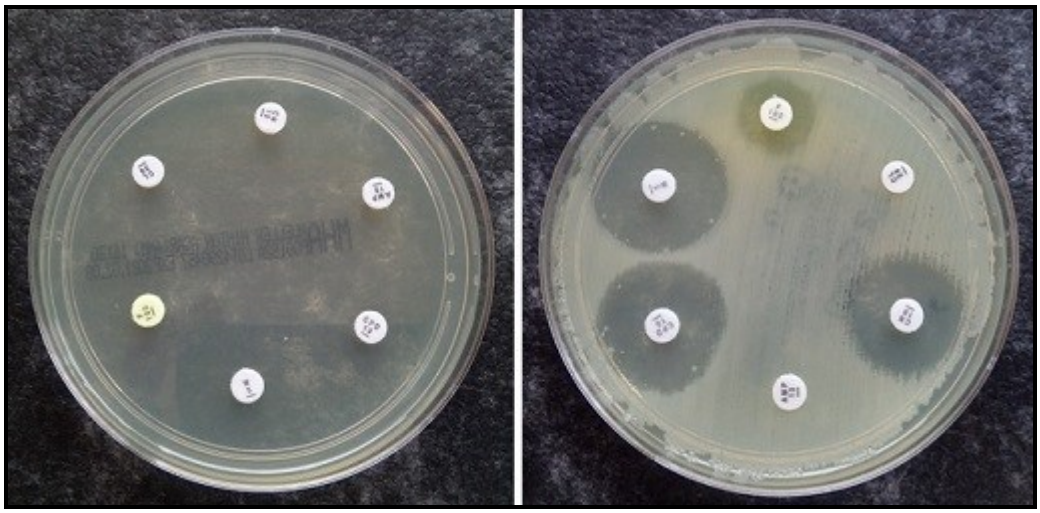
b) Streak the agar surface of the plate in three directions, tuning the plate by 60° between each streaking.

c) keep it at room temperature for 5 to 10 minutes, but no longer than 15 minutes to dry the inoculum.

4. Application of sensitivity-discs : take a disc from the container with a flamed forceps and place it on the surface of the medium. press it tightly with the forceps.
5. Incubate the plates at 35-37 °C for 16 to 18 hours.
6. Reading the zones
Measure the diameter of inhibition zone at the end of the incubation period with a ruler. If plates are showing proper growth, they may be read after 6 to 8 hours in clinically urgent situations.
- If the zone of inhibition is equal to or greater than the standard, the organism is considered to be sensitive to the antibiotic.
- If the zone of inhibition is less than the standard, the organism is considered to be resistant.

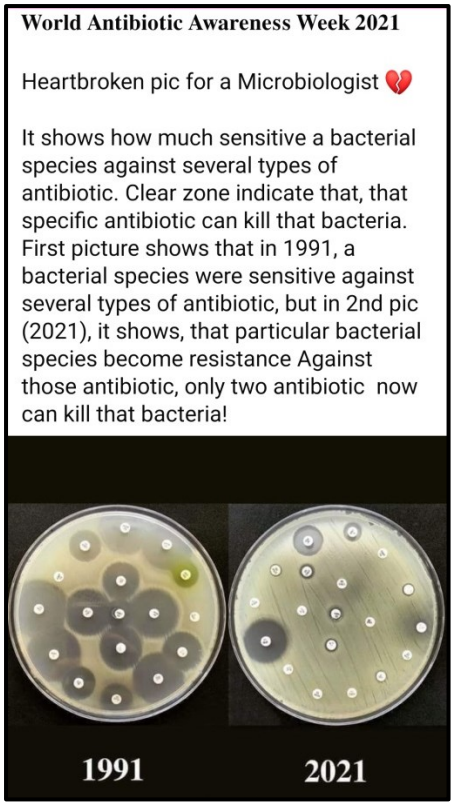






INTERPRETATION OF RESULTS

Although there is some correlation between the size of the zone of inhibition and sensitivity of the organisms to the antibiotic, the former is vary by: density of the inoculum, depth of the medium, infusibility of antibiotic etc.



STAINING AND SLIDE PREPARATION

Staining is a procedure used to color either the microorganism (whole of it or parts) or the background.

There are several types of stains:

1-The first is a <u>simple stain</u>

<u>A- Positive stain</u> : uses only one reagents which makes background uncolored and the heat-fixed bacterium colored.

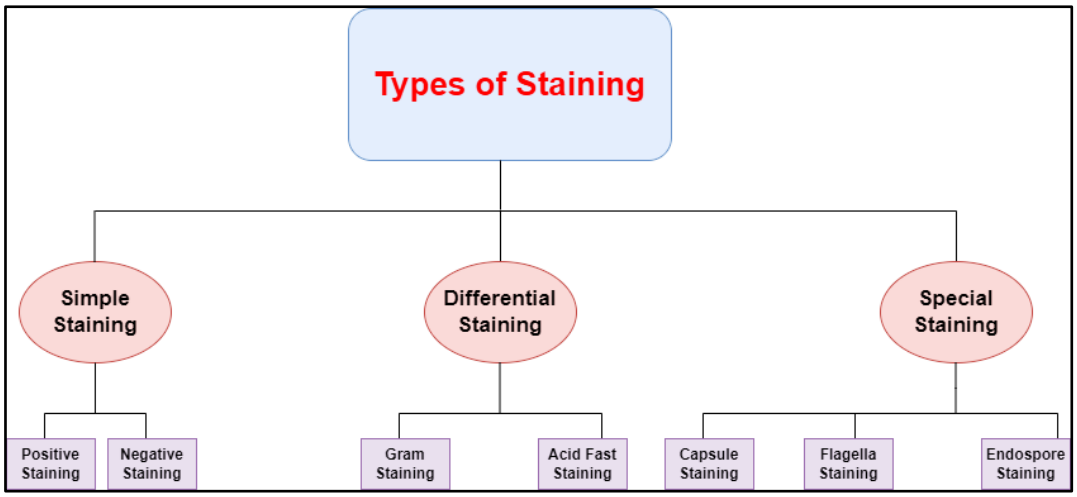
<u>B- negative stain</u> : uses only one reagents which makes background colored and the heat-fixed bacterium uncolored.

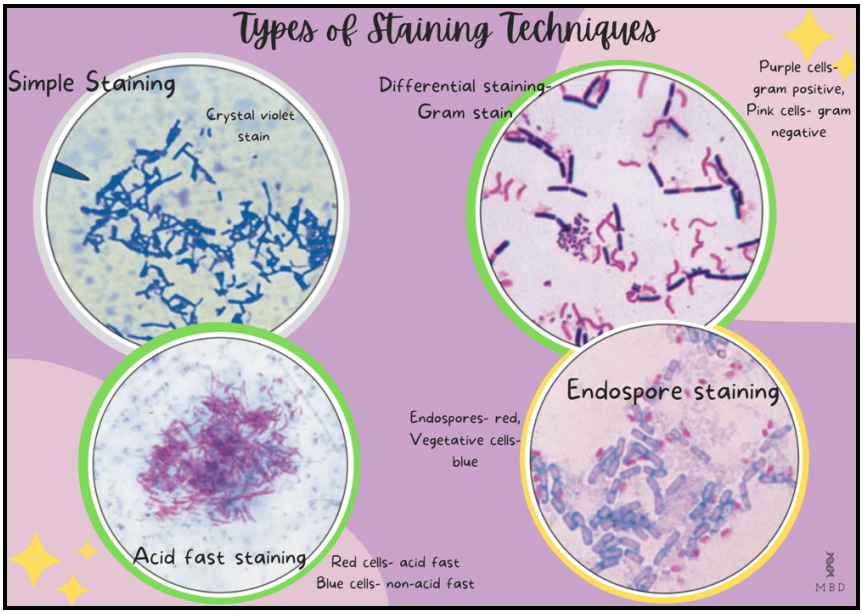
2 - differential stain: it allows to distinguish between types of bacteria or between specific structures in a bacterium. It uses two or more reagents – a primary stain and a counter stain.

Chemically, there are two main types of stains: basic stains, which have a positive charge (cationic) and acidic stains, which have a negative charge (anionic).

Basic stains have an affinity for negative components of cells, and include dyes such as methylene blue, crystal violet, and carbol fuchsin.

Acidic stains have an affinity for positive components of cells, and include dyes such as nigrosin, India ink, and picric acid. Since cell walls are negatively charged, a positive dye will be attracted to and stain the cell wall, whereas a negative dye will be repulsed by the cell wall and not directly stain the cell.





PREPARATION OF BACTERIAL SPECIMENS:

A smear is a small volume (a loopful) of sample containing medium that is spread (smeared) and fixed on to a microscope slide, in order to stain it.

Heat fixation accomplishes three things: (1) it kills the organisms; (2) it causes the organisms to adhere to the slide; and (3) it alters the organisms so that they more readily accept stains (dyes).

PROCEDURE FOR PREPARING A BACTERIAL SMEAR:

1. Using a broth culture:

a. Sterilize the loop using Bunsen burner, and let cool for 20-30 seconds.

b. Place loop in the bacterial broth and put the loopful of the broth onto the glass slide , and rub it.

c. Sterilize the loop again to kill any remaining bacteria.

d. Let the smear air dry completely.

e. Do not use heat to dry your smear.

2. Using an agar plate:

a. Place a small drop of water in the center of the slide.

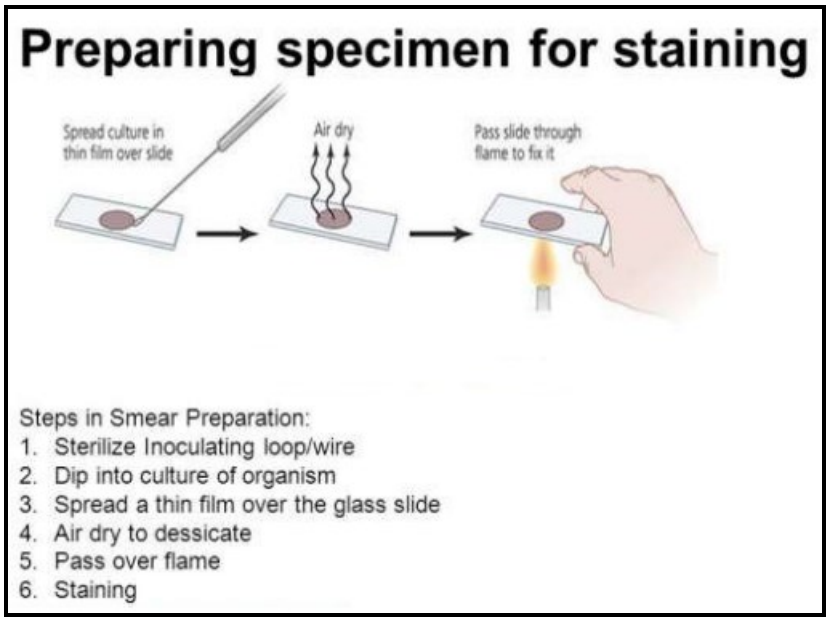
b. Sterilize the loop using Bunsen burner, and let cool for 20-30 seconds.

c. Use the sterile loop to pick up a small amount of bacterial growth from the surface of the plate. Do not dig into the agar.

d. Put the loopful of bacteria into the drop of water on the glass slide, and rub it.

e. Sterilize the loop again to kill any remaining bacteria.

f. Let the smear air dry completely. Do not use heat to dry your smear



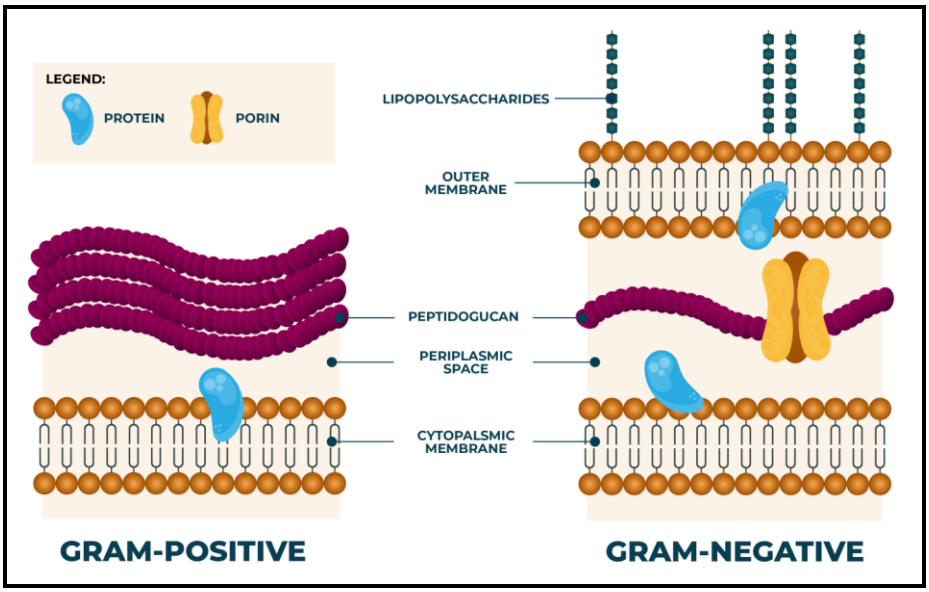
GRAM's STAIN

 The Gram stain is a differential stain which distinguishes bacteria based on cell wall properties.

 Bacterial cell walls are composed primarily of peptidoglycan and bacteria can be classified into two main groups dependent on the amount of peptidoglycan present in their cell wall.

 Gram-positive (+) organisms have a thick layer of peptidoglycan.

- **Gram-Negative (-) organisms have a thin layer of peptidoglycan.**
- **Gram-Negative (-) have an additional outer membrane layer that is absent in Gram-positive organisms.**



PROCEDURE:

1. Prepare a bacterial smear (1-2) loop full and heat fix

2. Place the slide on a staining tray, and cover the smear with primary stain called crystal violet (In this step all cells take up the purple color) and Allow to stain for 60 seconds.

3. Tilt the slide and gently rinse with distilled water until the stain is removed.

4. Cover the smear with Iodine, and allow to sit for 60 seconds (The iodine acts as a mordant, enhancing the ability of the stain to enter and bind to the bacteria) .

5. Tilt the slide and gently rinse with distilled water.

6. IMPORTANT STEP: Tilt the slide and let 2-3 drops of decolorizer run over the slide (The decolorizing agent used in the gram staining procedure is 95% ethanol, which is a lipid solvent that melts the Gram negative outer membrane. and leads to decolorization of Gram-negative cells), .

If the last drop is still purple, continue decolorizing, 2-3 drops at a time, until the decolorizer runs clear. Rinse with distilled water.

7. Cover the smear with a stain called Safranin (which stains the decolorized Gram-negative cells pink), and stain for 45 seconds.

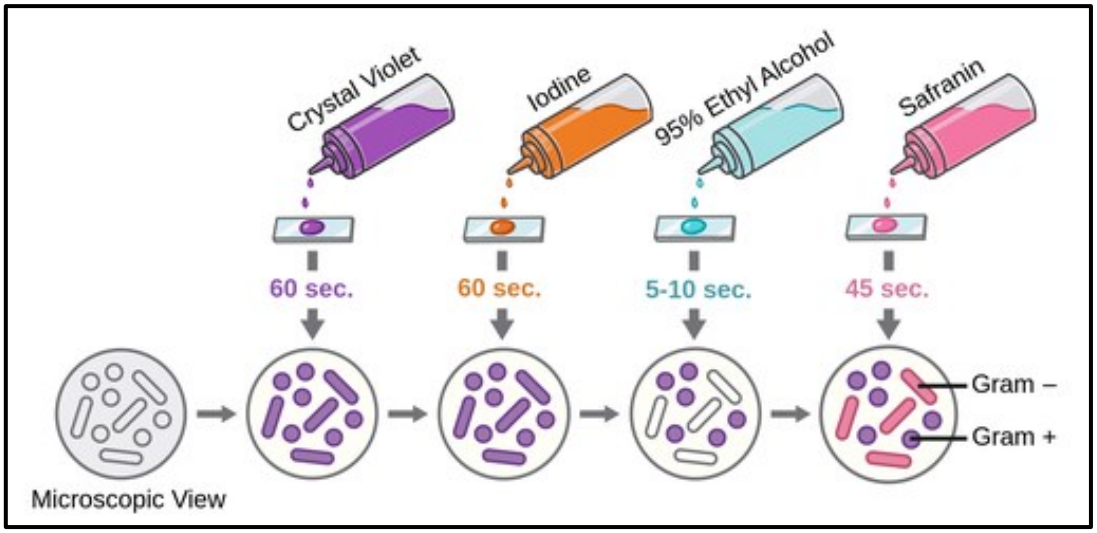
8. Tilt the slide and rinse with distilled water.

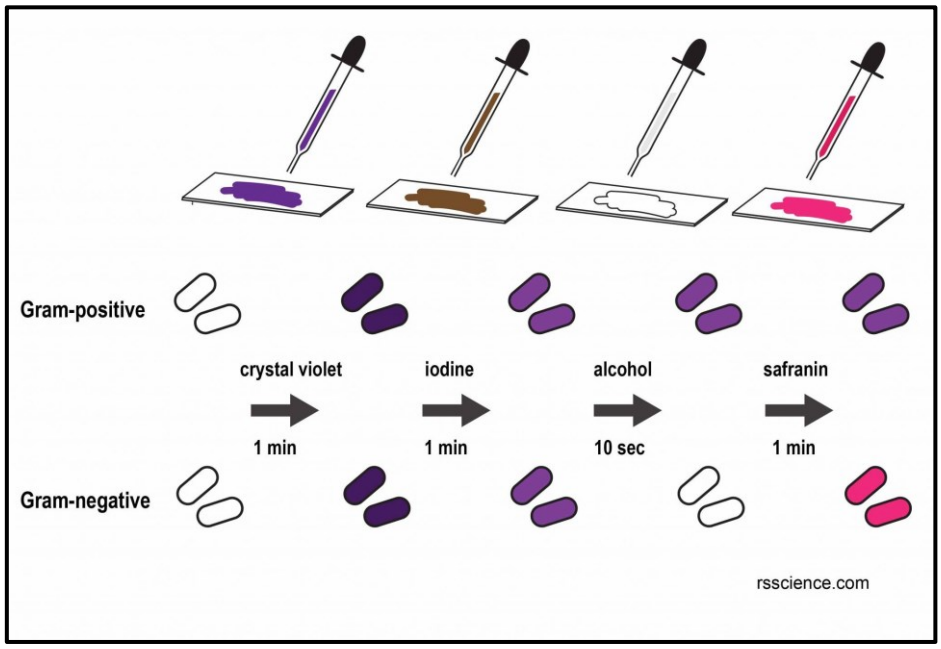
9. Place the slide on filter paper till dry. do not need a cover slip.

10. Observe the slide under oil immersion. You should see Gram(+) cells purple and Gram (-) cells pink.

11. Clean your microscope with lens cleaner, check on the 40X and 100X objectives.

12. Dispose of staining waste in designated waste container. Dispose of slides in red SHARPS container.





ACID-FAST STAIN

Bacteria which are acid-fast (+) are microorganisms that are resistant to the Gram stain (-).

Acid-fast bacteria have a waxy substance called mycolic acid in their cell walls, which comprises up to 60% of the cell wall components.

Genera which are acid-fast (+) include Mycobacterium and Nocardia, which include pathogenic organisms such as *M. Leprae* and *M. tuberculosis*.

PROCEDURE:

1. Fill a beaker about 1/2 full of tap water and place on a hot plate. Turn hot plate on, and allow water to boil. Once the water comes to a boil, reduce the heat to low. (The procedure requires the application of heat in order for the stain to penetrate the waxy cell wall)

2. Prepare a bacterial smear with a mixture of 2-3 loopfuls of *M.smegmatis* + 1 loopful *S.xylosus* and heat fix.

3. Place the slide on top of the beaker of water and let steam for 5 minutes.

4. Place a paper towel square on top of the smear area. This will hold the stain in place and keep it from running off the slide.

5. Apply enough amount of primary stain Carbol Fuchsin (which contains phenol to solubilize the cell wall and allow the stain to enter the cell) to soak the paper towel square and allow to sit for 5minutes, keeping the paper towel moist with stain. Do not let it dry . All cells will take up the reddish-pink primary stain

6. Carefully remove the slide with a clothes pin, and place on the staining tray to cool. Remove paper towel square.

7. Tilt the slide and rinse with distilled water.

8. Tilt the slide and rinse with the decolorizer acid alcohol, drop by drop, until the acid alcohol runs clear (which decolorizes all cells except acid fast cells. The acid alcohol is unable to penetrate the waxy cell wall of acid fast microorganisms) .

9. Tilt the slide and rinse with distilled water.

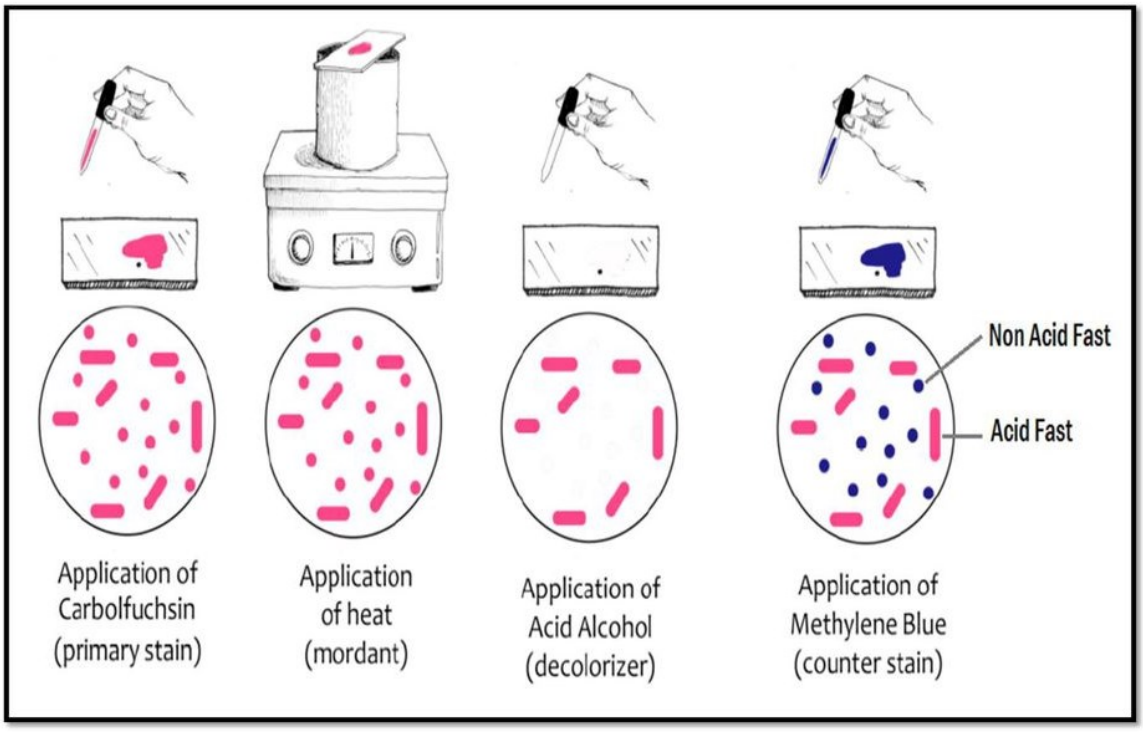
10. Cover the slide with the counter stain Methylene Blue and allow it to sit for 2 minutes (which stains the decolorized non acid fast cells blue)

11. Tilt the slide and rinse with distilled water.

12. Blot your slide dry with bibulous paper and observe your slide under oil immersion using proper microscope techniques. at the end of the staining procedure, acid fast cells (+) are reddish pink and all other cells (-) are blue.

13. Clean your microscope with lens paper and lens cleaner, check on the 40X and 100X objectives.

14. Dispose of staining waste in designated waste receptacle. Dispose of slides in red SHARPS container.



DETECTION OF ENDOSPORE AND CAPSULE

1-ENDOSPORE STAIN

The endospore stain is a differential stain which stains bacterial endospores or spores. Some bacteria, including those belonging to the genera Clostridium and Bacillus, have the capacity to produce metabolically inactive cells called spores.

- **The primary stain in the endospore staining procedure is malachite green, which stains both vegetative cells and endospores.**
- **Heat is required to penetrate the endospore coat.**
- **The cells are decolorized with water, which selectively removes the malachite green from all vegetative cells but not from endospores.**
- **The counter stain then applied is safranin, which stains the decolorized vegetative cells pink.**
- **At the end of the staining procedure, the endospores are green, and vegetative cells are pink.**

PROCEDURE:

1. Fill a beaker about semi-full of tap water and place on a hot plate.

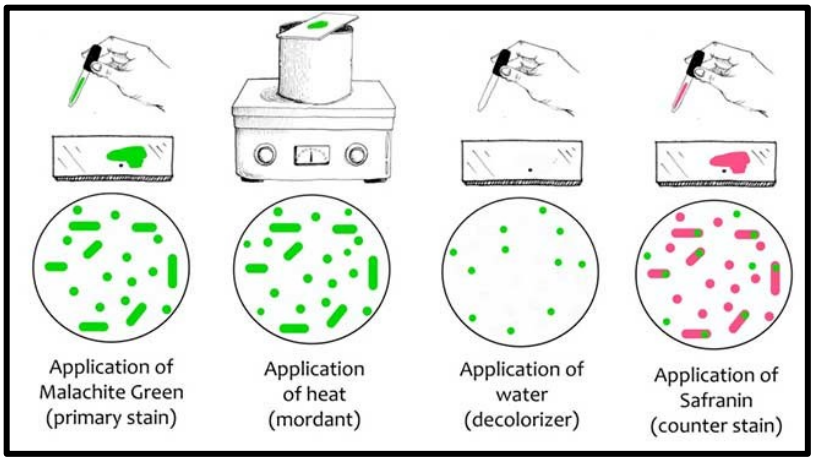
2. Obtain a clean glass slide and prepare a bacterial smear as described. Air dry and heat fix the slide.

3. Place the slide on top of the beaker of water and let steam for 5 minutes.

4. Place a paper towel on top of the smear area. This will hold the stain in place and keep it from running off the slide.

5. Apply enough Malachite Green to soak the paper towel square and allow to sit for 2-3 minutes, keeping the paper towel moist with stain. Do not let it dry.

6. Carefully remove the slide with a clothes pin (slide will be hot) and place on the staining tray to cool. Remove paper towel square.
7. Tilt the slide and rinse with distilled water.
8. Cover the slide with Safranin and allow to sit for 30 seconds.
9. Tilt the slide and rinse with distilled water.
10. Blot your slide dry with bibulous paper and observe your slide under oil immersion using proper microscope techniques. Record your results below. Endospores will appear as dark green or clear circles or ovals, whereas vegetative cells will be pink rods.



2- CAPSULE STAIN

The capsule stain is a type of differential stain which selectively stains bacterial capsules. A capsule is a substance that is synthesized in the cytoplasm and secreted to the outside of the cell where it surrounds the bacterium. Capsules can be polysaccharide, polypeptide, or glycoprotein.

Because of their structure and composition, heat and water will dislodge capsules from bacteria during laboratory procedures.

- **The primary stain is crystal violet, and all parts of the cell take up the purple crystal violet stain.**

- **A 20% copper sulfate solution serves a dual role as both the decolorizing agent and counter stain. It decolorizes the capsule by washing out the crystal violet, but will not decolorize the cell. As the copper sulfate decolorizes the capsule, it also counter stains the capsule. Thus, the capsule appears as a faint blue halo around a purple cell.**

PROCEDURE:

1. Obtain a clean glass slide.

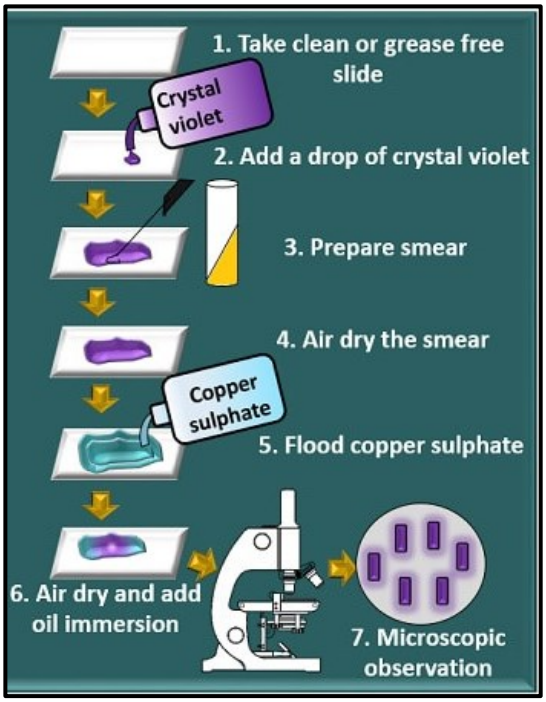
2. Prepare a smear using 2-3 loopfuls of the broth culture. Allow the smear to air-dry, but do not fix slide by heat because heat will cause the capsule to dislodge.

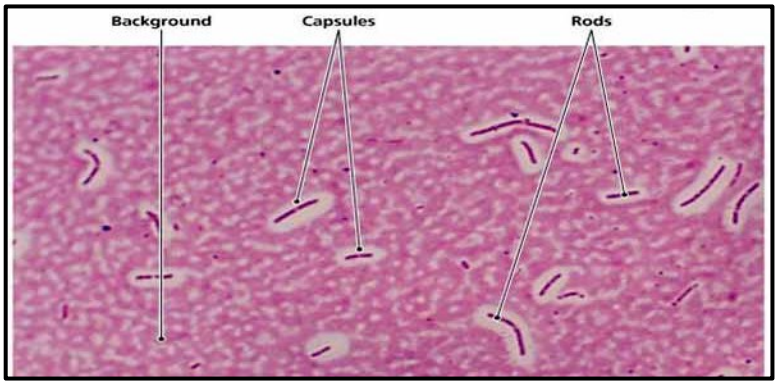
3. Cover the smear with crystal violet, and stain the smear for 2 minutes.

4. Tilt the slide and rinse with 20% copper sulfate solution. DO NOT rinse with water because water will remove the capsule from the cell.

5. 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.

6. Observe the slide under oil immersion, and draw what you see in the results section below. Look for purple cells surrounded by a clear or faint blue halo on a purple background. (The halo is the capsule) .





THE END

GOOD LUCK