



University of Baghdad
The College of Veterinary Medicine
Dept. of microbiology



PRACTICAL MICROBIOLOGY

First term
Third class
Prepared by

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First Lab.

Safety Procedures and Precautions

1. Laboratory coats are worn.
2. Long hair is tied back away from the shoulders.
3. Working areas are kept clear of all unnecessary items.
4. Hand and bench tops are kept clean with disinfectant.
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 afresh, unhealed cut, scratch, burn or other injury on either hand should notify the instructor before beginning or continuing with the laboratory work.
8. If you should spill or drop culture or if any types of accident occur, call the instructor immediately.
- 9 .Unnecessary activities can cause accidents and promote contamination.
10. Before leaving the laboratory, carefully wash and disinfect your hands.



Microscope

Microscope: it is an optical instrument that magnifies images of objects invisible to the naked eye by means of lens or lens system.

Types of microscopes

1- Light microscope.

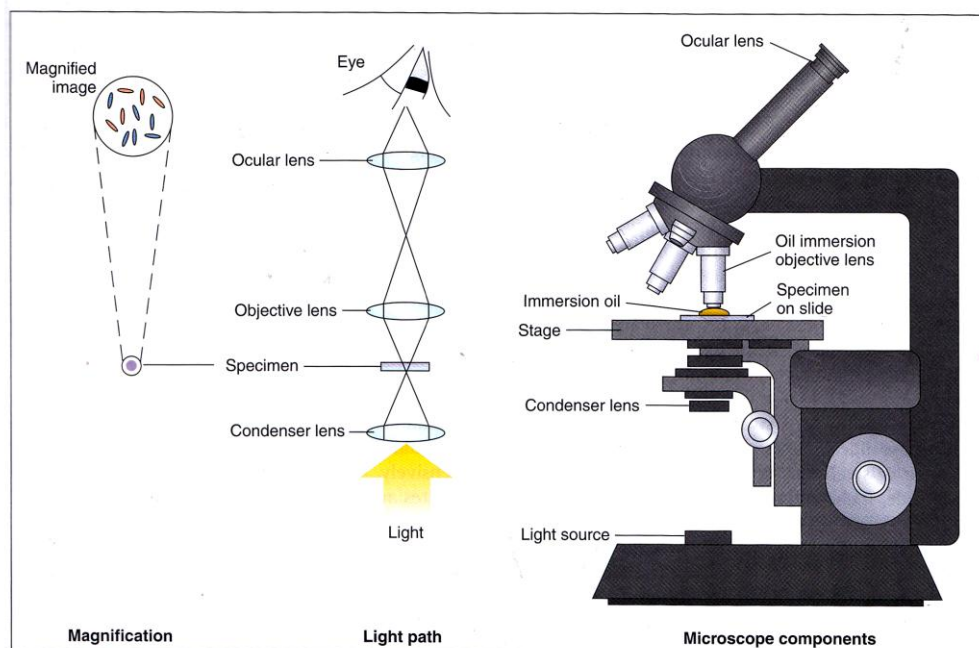
- a. bright field light microscope. (As in our lab.)
- b. phase contrast microscope .
- c. dark field microscope .
- e. fluorescence microscope.

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



Principles of bright-field (light) microscopy. (Modified from Atlas RM: *Principles of microbiology*, St Louis, 1995, Mosby.)

How can you measure magnification power?

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

40×10 (ocular lens) =400.

Types of oil are:

- a- oil of cedar –wood.
- b- Canada –balsam oil.

Why we use oil with oil immersion lens?

We use oil to prevent shattering of light rays.

As light rays refracted when passing from media with high density (glass slide) to media with low density (air) and as the used oil had a refraction index equal to that of glass slide we can reduce this refraction and obtain a full spectrum of light reached to lens.

Microscopes identify invisible microorganisms (bacteria) depending on their morphological characteristics.

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

1-Shape: bacteria could be

- a. cocci (spherical)
- b.bacilli(rod-like)
- c. coccobacilli (between cocci and bacilli)
- d. spiral (curved rod).

2-size: small or large.

3-cell arrangements: bacteria could be arranged in many forms like: a) single.

- b) Pairs (called diplococci or diplobacilli).
- c) Chains (arranged in series, strept) like Streptococcus
- d)cluster or grape like arrangement like Staphylococcus.

Second Lab.

Tool, instrument & equipment

- **Loop:** use for transfer of bacterial cells from medium to another (as colony or drop "0.01ml"), sterilized by the flame of burner after and before using.
 - **Slide:** use for the examination .placed on microscope stage.
 - **Cover- sips:** placed on the slide, the sample will be between the cover and the slide.
 - **Test tube:** use to place the broth, solid, or semi-solid medium for stabbing, or place as slant for bacterial culturing. The empty tubes or with uncultured broth sterilized by autoclave (15 min) but with cultured broth by autoclave (30 min).
 - **Petri-dish** (petri-plate): use for place the solid medium in it. Glass petri-plate use for many times &sterilized by oven or by autoclave .sterilized plastic plate use for one time.
 - **Flask:** used for place cultured or uncultured broth in it. Sterilized after plugs with cotton by autoclave.
 - **Beaker:** use for graduate the volume of fluid. Sterilized by oven.
 - **Cylinder** (graduated cylinder): use for graduate the volume of liquid. Sterilized by oven.
 - **Washing bottle:** use to filled with liquids (specially distilled water) for washing and homogenizing the glass wares and washing the slide during the staining, don't need for sterilization.
 - **Burner:** may be gaseous or alcoholic ,use for sterilization of the loop ,needle and other metal tools by the fame (dry heat sterilization)
 - **Autoclave:** equipment with high temperature, pressure and steam to sterilize the culture media and some of metal tools and glass wares.
 - Temperature=121 C⁰
 - Pressure=1 atm (15 pound/inch²)
 - Time=10-30 min.
 - 10 min. for media with sugar
 - 15 min for uncultured media
 - 30 min. for cultured media &contaminated tools &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.=180c⁰
 - Time= one hour and half
 - **Incubator:** for the availability of suitable temperature for growth of microorganism by place the culture media in it, for example pathogenic

bacteria growth in optimal temperature 37°C for 18-48 hours (the optimum 24hrs.)

- **Refrigerator:** use to place the sterilized media and broth and not use to avoid the contamination, and also to preserve the bacterial cultures for long time by preventing the growth in 4°C .

Preparation of smear:

What you need 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 spreading the material across 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 form of growth media :-

- 1-Liquid or called (broth) usually put in test tube.
- 2-Solid or called (agar) usually put in petri-dish.

Specimens:

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

Preparation of smear

How you will prepare a smear?

A-From fluid material:

Such as broth culture Urine, sputum, pus, purulent, exudates.....etc.

1-Sterilize the loop in Bunsen flame and let it to cool.

2-Shake the specimen container (broth tube) takes loopful of the specimen and spread it on the center of a clean slide to form a somewhat thick film of (1-2 cm) in diameter then re 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 micro-organism.

2-Make the micro-organism stuck to the surface of the slide.

3-Make the MICROORGANISM more permeable to the stain.

4-Prevent MICROORGANISM from going autolytic changes.

Third Lab.

Staining of bacteria

Stains are employed in 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.
- Neutral stains as Giemsa.

As bacterial cells are rich in nucleic acid (which has a negative charge) it will follow that (basic stain), bearing its coloring matter in the positive cat ions, will be attract to the organism and stain it. Acid stain, however, will not stain the bacteria, they are used mainly for staining, the background material a counter staining color.

a – Simple stain: using one stain only and so appears in one color. Used to get the following information:

- 1-Size of micro – organism.
- 2-Shape : could be one of the following : spherical (cocci), rods (bacilli), pleomorphic (coccobacilli)spiral (spirillum) .
- 3-Grouping or called arrangement: could be single, pairs, chain, cluster, or square arrangement. Bacteria groups because of a process of multiplying or division.

Method of simple staining:

- 1-Flood the slide with crystal violet or carbol fuchsine or methylene blue for 1 min.
- 2-Wash off the stain with slowly running tap water.
- 3-Allow the slide to dry in air or placed it between two sheets of filter paper (called blotting the slide).
- 4-Examine under oil immersion lens, see the shape and arrangement of the bacteria.
- 5-Draw in your book what you seen.

b-Differential staining: using two or more types of dye successfully to differentiate between the bacteria according to their response to these dyes.

Example for these stains:

- 1-Gram stain.
- 2- Acid – fast stain (Zeihl – Nielsen stain)

1- Gram staining:

Is one of the most important methods widely used in bacteriology 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- Those that retained the first dye (crystal violet) throughout the staining procedure, are known as "gram – positive".

b- Those that lost the first dye (crystal violet) after washing with a decolorizing solution and stained with the second dye (diluted carbol fuchsin) are known as "gram – negative".

In conclusion, the gram – positive bacteria appear violet, while gram – negative bacteria are red in colour.

Therefore, it is possible to differentiate between bacteria of the same morphology.

Further more, it can be used to determine the relative number and morphology of bacteria in smear.

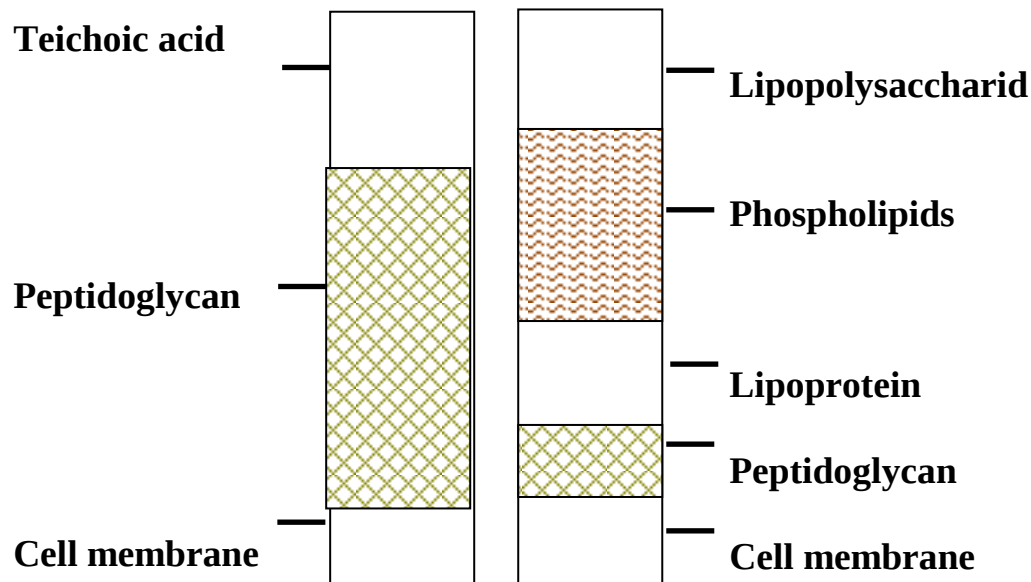
Gram staining method:

- 1- Prepare the bacterial smear.
- 2- Flood the slide with crystal violet leave to all for 1-2 min. Wash with tap water.
- 3- Apply gram's iodine (lugol's iodine) . Leave to all one minute. Wash with tap water.
- 4- Apply 95% ethyl alcohol (a decolorizer). Leave to all for 20-30 seconds. Wash with tap water.
- 5- Apply diluted carbol – fuchsin (the counter stain) leave to all for 1 min. wash with tap water.
- 6- Blot, dry in air and examine with oil immersion lens.

Base of gram – stain: there are several theories .

1- mg+2 ribonucleate theory : the most accepted theory it state that gram (+ve) bacteria had mg+2 ribonuclease enzyme in their protoplasm which bind with (crystal violet + iodine) to form a strong complex difficult to destroyed by alcohol . Gram (-ve) bacteria dose not have this enzyme. (Note: tiechoic acid in gram +ve is the source of mg+2) .

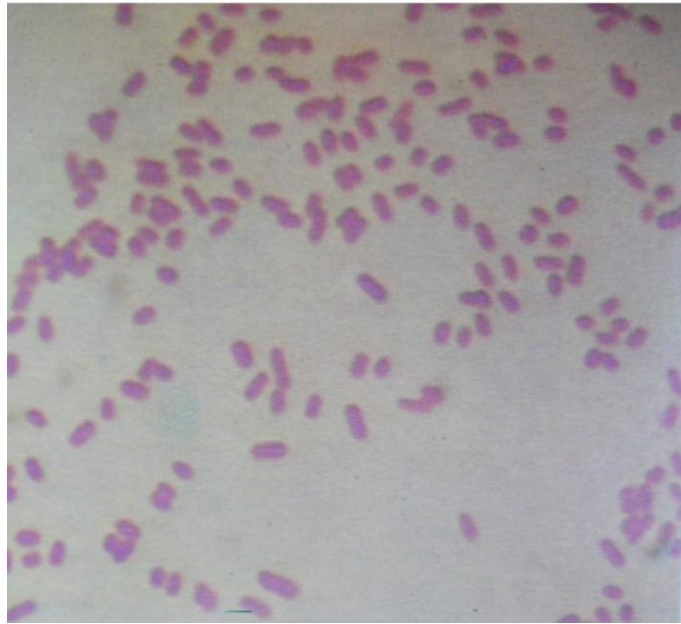
2-Cell wall permeability theory: it suggests that in gram +ve bacteria when you add the crystal violet immediately the pores in the cell wall will be locked while in gram -ve it remain opened. Why? Because of the chemical composition of cell wall are different between gram +ve and gram -ve in which (see figure):



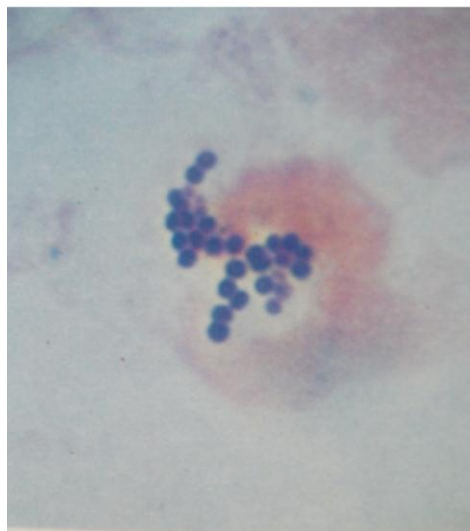
- Gram +ve has a thick layer of Peptidoglycan which gives rigidity and strong integrity to the cell wall and is not affected by alcohol. Also, it contains teichoic acids which are the source of Mg^{+2} .
 - Gram -ve has a thin layer of Peptidoglycan with a thick layer of lipid-containing layer (lipoprotein, Lipopolysaccharide and phospholipids) and these are affected more by alcohol.
- 2- Acidity of the protoplasm theory: protoplasm of the gram +ve bacteria is more acidic than in gram -ve bacteria, so it attracts and retains more basic stain (crystal violet) inside the cell.

Not: we can study in the gram stain:

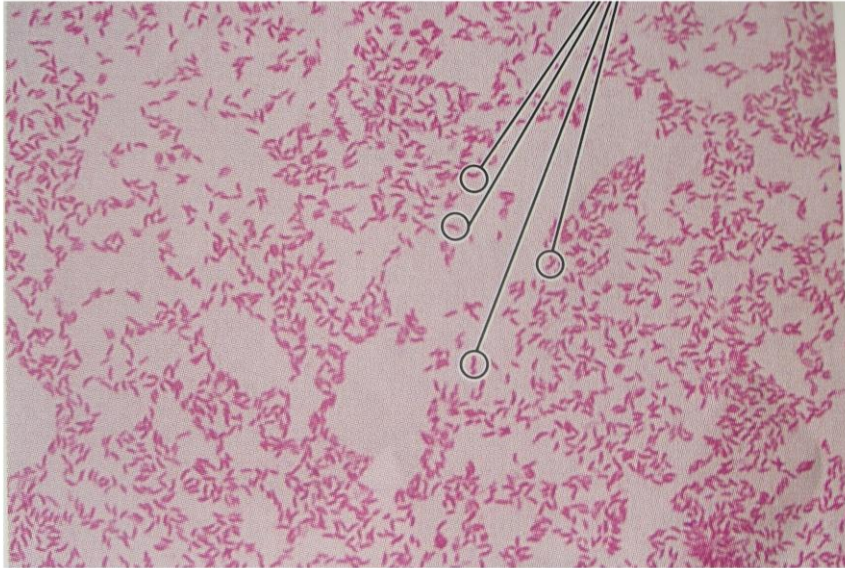
- 1- Gram staining, gram +ve or gram -ve.
- 2- Morphology of bacteria which include:
 - a- Shape.
 - b- Size.
 - c- Arrangement.



Gram strain of *E.coli*
The *E.coli* Appear as gram negative bacilli



Gram stain of Staphylococcal pus. The typical gram positive coccus in grape like clusters is well shown x4308



Simple stain of the Gram-negative rod
Vibrio cholerae, the causative agent of cholera.

Fourth Lab.

2) Acid-Fast stain (Ziehl- Nielsen stain)

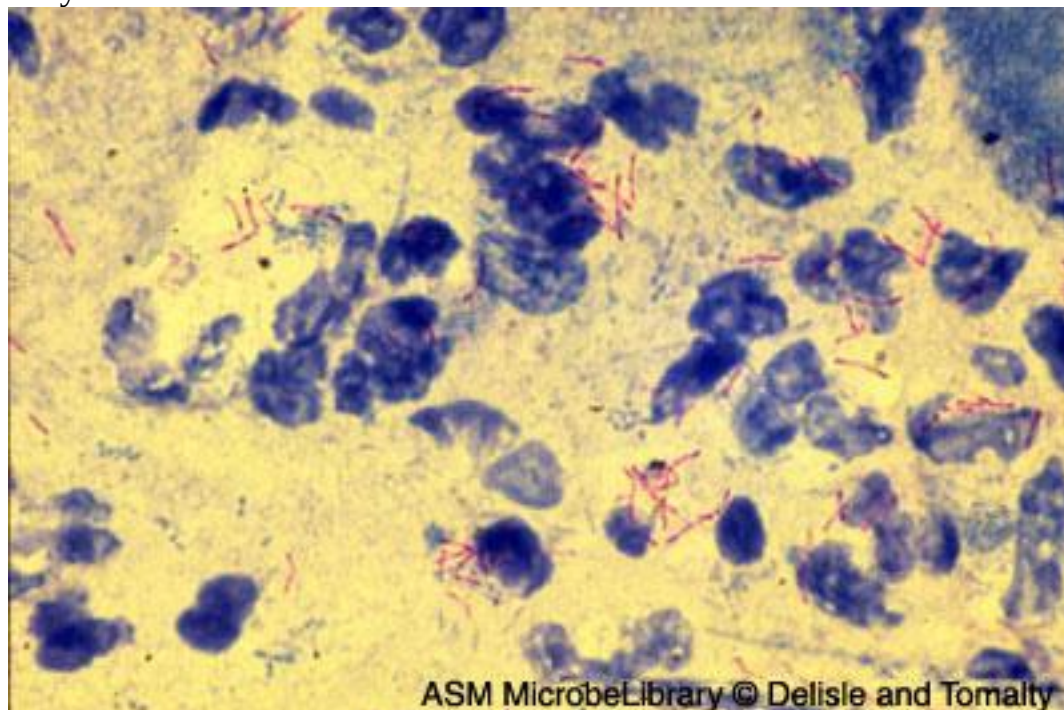
Acid – fastness, a characteristic is the Mycobacterium is associated with their lipid and mycolic acid contents. These organisms retain the carbol fuchsin and resist decolorization by acid and alcohol.

The Zeihl – Neelsen stain is used in direct examination if specimens for the presence of acid – fast bacilli. The property of acid – fastness is corrected with high fat and lipid content if Mycobacterium and Actinomyces. The high lipid content requires staining with hot solution if dyes having very strong affinity for the bacterial cell.

Method:

1. Prepare bacterial film (smear).
2. Flood the slide with concentrated carbol fuchsin, heat until steaming for 5 min. (Avoid boiling of stain, and avoid dryness stain by adding few drops of stain).
3. Cool the slide wash with water.
4. Dipping the slide in Acid – alcohol for 20-30 sec (3%HCL)
5. Wash with tap water.
6. Add counter stain (methylene blue) and leave for 1 min.
7. Wash with tap water.
8. Blot and dry in air.
9. Examine under oil immersion.

Mycobacterium stain with red color other stains with blue color.



Acid fast stain

C – Special stains: - stains for special morphological structures such as flagella, spores capsule.

1-) spore stain: it is a special stain for spores which are common in the aerobic genus *Bacillus* and the anaerobic genus *Clostridium*.

Endospores differ in their shape and situation according to the type of bacteria:

- Circular and central in *B. anthracis*.
- Circular and sub terminal in *Cl. chauvoei*.
- Circular and terminal in *Cl. tetani*.
- Oval and terminal in *Cl. tertium*.

Method of spore stain (modified Ziehl – Neelsen):

1. Prepare bacterial film (smear).
2. Flood the slide with C.C.F, heat until steaming for 5 min (avoid boiling and drying of stain).
3. Cool the slide then wash with tap water.
4. Dipping the slide in 3% Acetic acid for 20-30 sec.
5. Wash with tap water.
6. Flood the slide with counter stain (methylene blue or malachite green) and leave for 1 min.
7. Wash with tap water.
8. Blot and dry in air.
9. Examine under oil immersion.

Spores colored red other part of MICROORGANISM colored blue or green.



Gram stain of the Gram-positive rod *Clostridium tetani*, the causative agent of tetanus.

Fifth Lab.

2) Capsule stain:

Capsule: it is extracellular material surrounding bacterial cell wall; consist of polysaccharide i.e. (Strep. Pneumoniae) or polypeptides (E. coli and klebsiella) or polysaccharide and polypeptide (B. anthracis).

Its function:-

Its form as protective covering against phagocytosis.

Inhibit the killing factors in the serum of the host.

Presence of capsule is related to virulence and pathogenicity of MICROORGANISM.

Capsule seen by:

1-) negative stain

(Indian ink, Nigrosin, Congo red etc.) as white hallow around the unstained Bacterial cell.

2-) Hiss's stain:

1.Prepare the bacterial smear and not fix.

2.Flood the slide by concentrated carbol – fuchsin 1/5% with heating for (5) second or until the steam is appear.

3.Wash and decolorize with 20% copper sulfate for (20) sec.

4.Dry in air.

5.Examine under oil immersion.

3-) Anthony's method or stain:

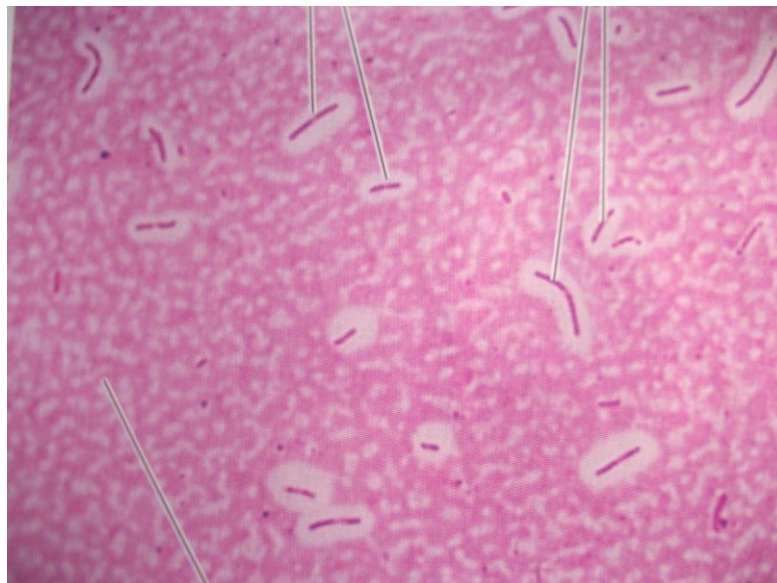
6.Prepare the bacterial smear with out fixing.

7.Flood the slide with crystal violet for 3-7 min.

8.Wash and decolorize with 20% copper sulphate for (20) sec.

9.Dry in air.

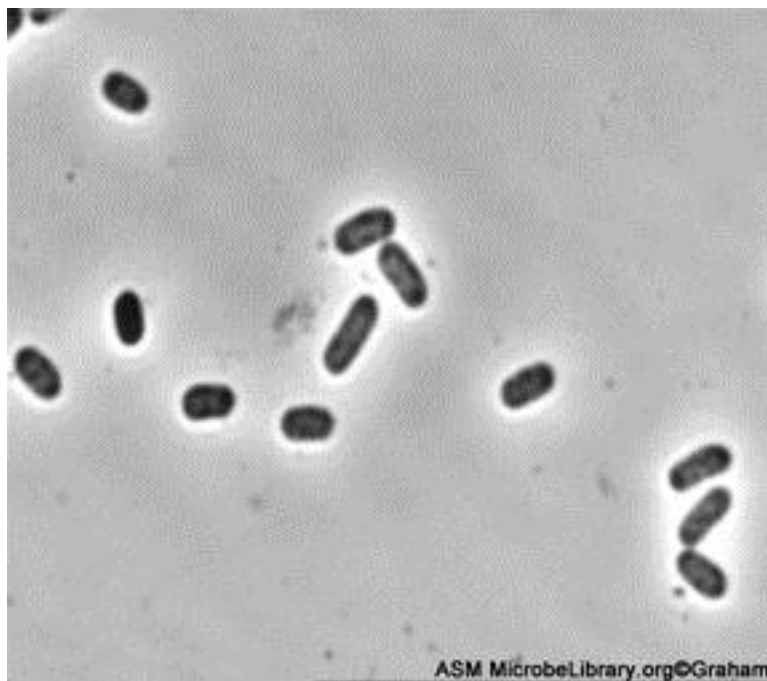
10.5-examine under oil immersion.



Hiss's stain



Negative stain



Anthony's stain

Motility of bacteria

There are 2 types of movements:

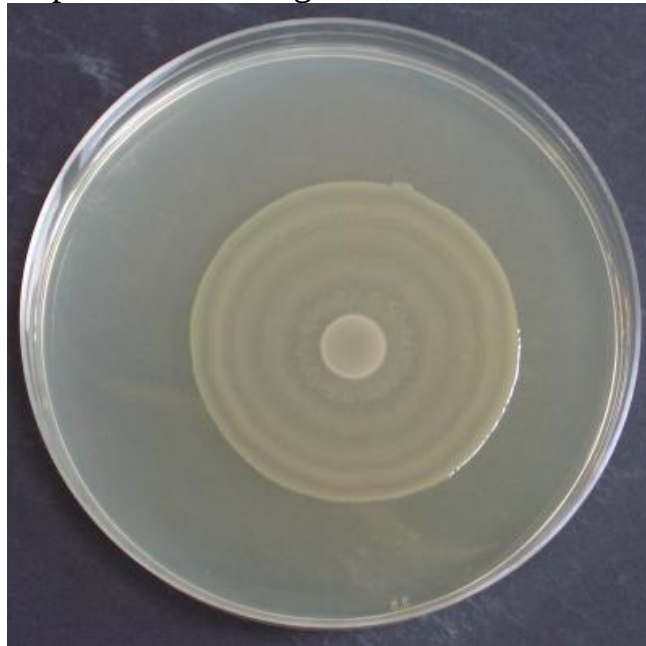
True movement: the bacteria can change its position by means of flagella and it is directional Locomotion often quite rapidly.

Brownian movement : is oscillatory movement possessed by organisms or other particles suspended in a fluid and it caused by the continuous ,

rapid oscillation of molecule of the fluid , such movement is irregular and non directional (up and down , back and forth) but not change position with respect to other objects around them .
This is not movement the MICROORGANISM considered non – motile.



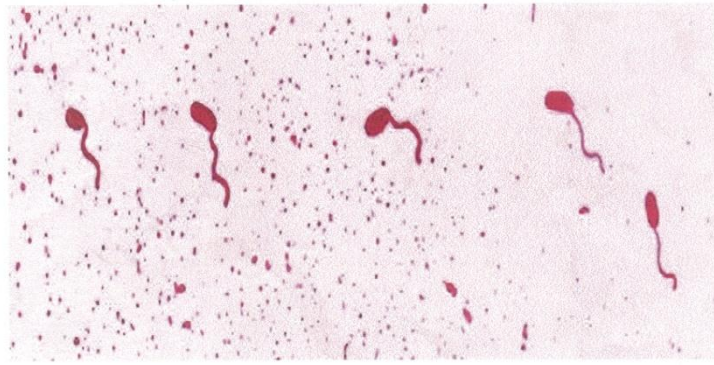
Result of H₂S Production in SIM medium tubes
(Left to right)H₂S positive, H₂S negative, uninoculated control



Swarming of *proteus* on basal agar

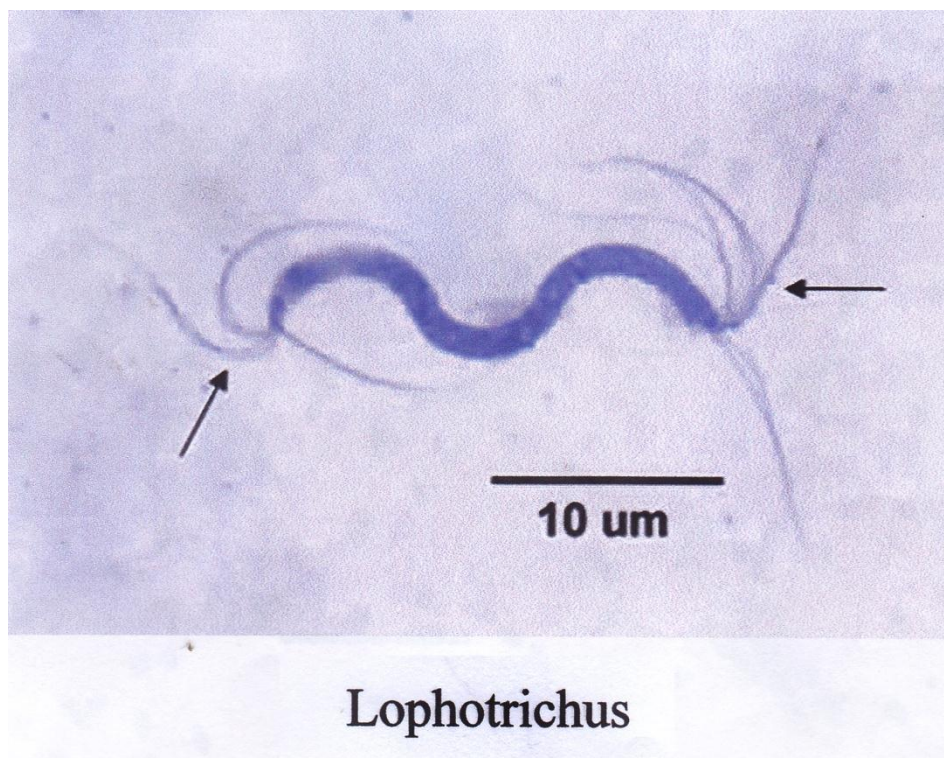
Bacteria move by flagella, Number and position of flagella depend on species of bacteria

1- Monotrichous.



Monotrichus

2- Lophotrichous.

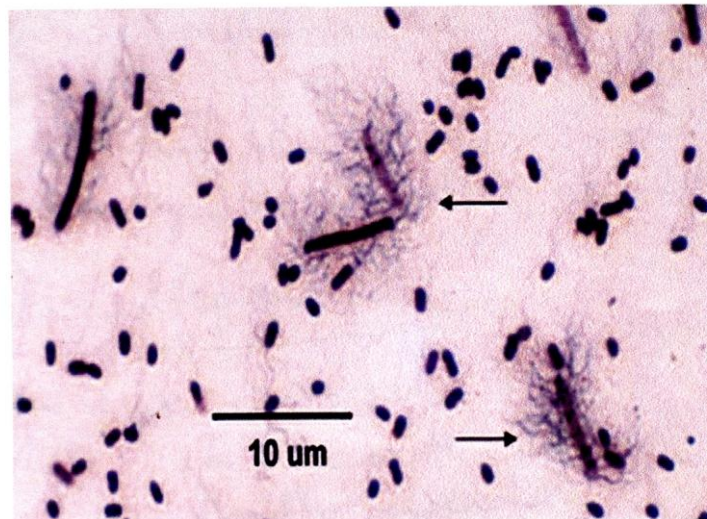


3-Amphitrichous



Amphitrichus

4-Peritrichous



Peritrichus

Study of motility can be carried out by:

- microscope :
 - 1-Wet – mount method.
 - 2-Hanging drop method.
- culture media :
 - 1-Graigies method (semi – solid media called SIM) cultivated by stabbing technique.
 - 2-Swarm movement on solid media.

Flagella stain: It is special stain for motile bacteria in order to stain the flagella which are the organs of locomotion.

Wet – mount method:

- Take clean slide, place loopful of broth culture in the center of slide.
- Cover the loopful of broth with cover glass.
- Place the slide on the microscope stage, cover glass up.
- Examine under low and high power objective lens.

Note: lowering the condenser to reduce the light when you exam motility of bacteria.

Hanging drop method:

- Take clean cover glass.
- Gently shake the broth culture of bacteria until it is evenly suspended.
- Sterilize the wire loop, remove the cap of the tube and take up a loopful of culture. Be certain the loop has cooled before inserting it into the broth, close and return the tube to the rack.
- Place the loopful of culture in the center of the cover glass, sterilize the loop and put it down.
- Take clean hollow – ground slide, place a thin film of paraffin around the concave well on the slide.
- Hold the hollow – ground slide inverted with the well down over the cover glass, and then press it down gently so the paraffin adheres to the cover glass.
- Now turn the slide over the drop of culture hanging in the well.
- Place the slide on the microscope stage cover glass up, examine with low and high power objective lenses.

Graigies method (semi – solid medium, SIM):

Flaming the straight wire of the loop.

Take with cooled loop small port of culture to inoculate it in a tube of semi – solid medium (SIM) (Sulfide Indole motility medium, making a single stab down the center of the tube to a bout half the depth of the medium .

Incubate under the conditions favoring motility.

Examine at intervals, e.g. after 6 hr and 1, 2 and 6 days when incubating at 37 C°.

- Motile bacteria give diffuse growths that spread throughout the medium rendering it slightly opaque.
- Non- motile bacteria generally give growths that are confined to the stab – line and leave the surrounding medium clearly transparent.

Sixth lab.

Cultivation of Bacteria

- Colony: A macroscopically visible growth of microorganisms on solid culture medium.
- Culture: Any growth, population or cultivation of mo.
- Subculture: the streaking of isolated colonics of differential media to obtain pure culture.
- Stock culture: species or strains of mo. (pure culture), known a maintained in the laboratory for various test and study.

Methods of cultivation:

A - Cultivation of bacteria in fluid media (broth).

- Transfer of a bacterial colony on a plate culture (solid medium in Petri-dish) to a broth (fluid medium in a tube).

1- Take up the inoculating loop by the hand and hold it as you would a pencil, loop down, hold the wire in the flame of the Bunsen burner until it glows red, remove loop from flame and hold it quietly a few moments until cool. Do not put it down or touch it to anything.

2- Hold the sterile , cooling loop in one hand and with the other hand turn the plate .culture with bottom part of the dish up , so that you can clearly see isolated colonies growing on the surface of the plated agar when we remove the cover partly .

3- With sterile cool loop scrap a small surface area of bacteria colony gently and remove a loop full of culture, with draw the loop and replace the cover of the Petri – dish 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 loop 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 (don't over – heat, if it glass, it could crack, if it is plastic it could melt) this flaming sterilize the air in and immediately around the month of the tube.

6-insert the loop into the open tube and inoculate a sterile broth with the charged loop gently rubs the loop against the wall of the tube.

7-with draw the loop slowly, being careful not to touch it to the month of the tube and do not touch it to anything, replace the tube closure and put the tube back in the rack .

8-Now carefully flam the loop until it glows red (Be sure all the wire is sterilized), when the wire has cooled the loop can be placed on the bench top.

9-label the tube you have just inoculated with name of the provided organisms and the date.

10-incubate the tube at 37°C for 24 hour.

B- Cultivation of bacteria on solid media.

1-) streaking method:

- Streaking a broth culture for colony isolation on solid media in Petri - dish.

1- place a loop full of broth culture on the surface of agar in the Petri – dish , near but not touching the edge , lightly streak the inoculum back and forth over an area about 1 1/2 cm, do not dip up the agar .

2- Flame the loop and let it cool in air.

3-Rotate the plate in your left hand so that you can streak a series of four parallel lines, each passing through the inoculum and extending across one side of the plate.

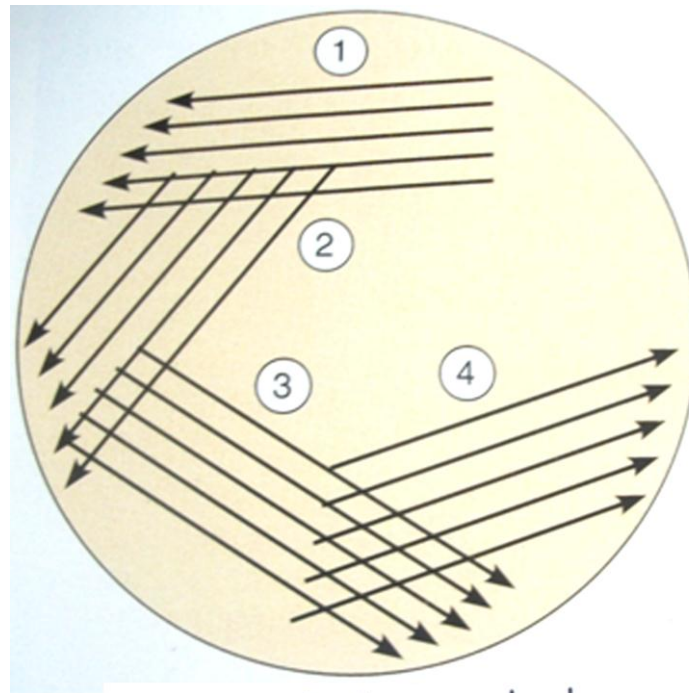
4-flam the loop again and let it cool in air.

5- Rotate the plate and streak another series of four parallel lines, each crossing the end of the last four streaks and extending across the adjacent side of the plate.

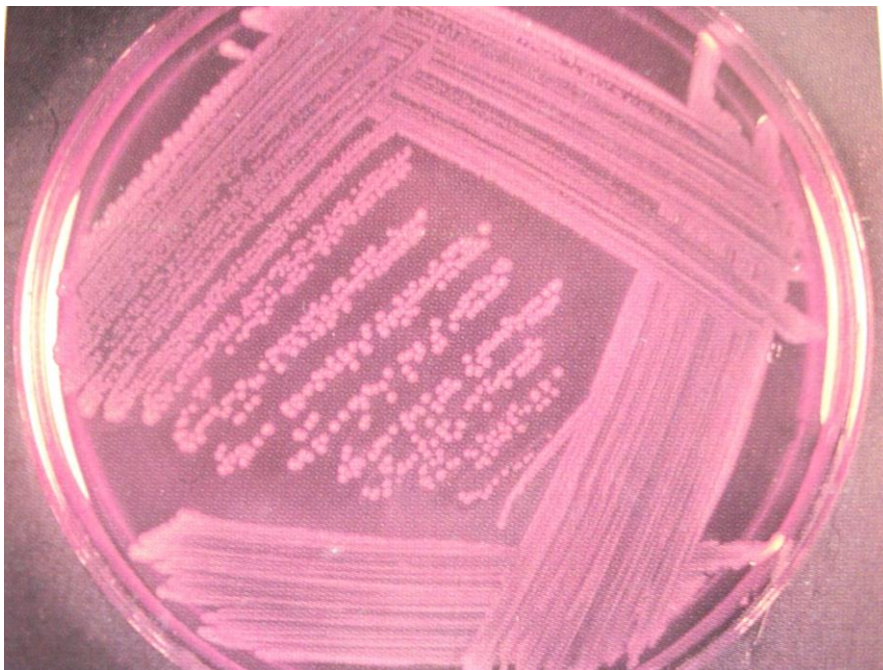
6-Rotate the plate and repeat this parallel streaking once more.

7- Finally, make a few streaks in the untouched center of the plate, do not touch the original inoculum .

8-Incubate the plate (inverted) at 37°C for 24 hours.



The streak-plate method.



2-) 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 the 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.

C-) Cultivation of semi – solid media:

Semi – solid media 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 semi- solid medium as SIM or gelatin medium making a single stab down the center of the tube to about half the depth of the medium.

3- Incubate at 37°C for 24 hr.

Cultural characteristics:

To study the macroscopic characteristics of pure culture on a solid medium, the following points have to be considered in describing a colony: (see fig .2).

-Size: measured in mm.

-Shape: spindle, circular, filamentous irregular.

-Elevation: flat, raised, convex, umbonate .

-Margin: entire, lobate .

-Consistency: dry, mucoid .

-Surface texture: smooth, rough.

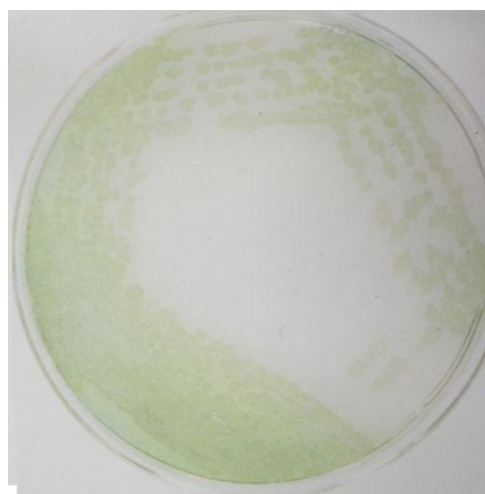
-Color or pigmentation: yellow, green.

-Optical density: opaque, transparent, translucent.

-odour: bad , musty .

-Changes in the inoculated media(hemolysis) .

Whole colony:	Edge:
Punctiform 	Entire 
Circular 	Undulate 
Rhizoid 	Lobate 
Irregular 	Filamentous 
Filamentous 	Curled 
Surface:	Elevation:
Smooth, glistening	Flat 
Rough	Raised 
Wrinkled	Convex 
Dry, powdery	Pulvinate 
	Umbonate 



***P. aeruginosa* on nutrient agar.** The characteristic green diffusible pigment and the metallic sheen can be seen.

Seventh Lab.

Culture Media

Culture or growth media: an artificial media contains basic requirements needed for micro organisms' growth. Used for recognition and identification of micro organisms. the then the media which dissolved in the flask must be sterilized by heat(i.e. autoclave) are contained either in test tubes , plates (Petri-dishes).

Common or basic ingredient for culture media:

Bacteria like any living cell it needs organic and inorganic material for their live, so in order to propagate these bacteria we should provide them with these material by.

- Peptone: source of protein.
- Meat extract: source for N₂.
- NaCl: for isotonic environment.
- H₂O.
- Agar – Agar: used for solidifying purposes and have no nutritional benefits it is obtained from sea weeds melting point is 92-95C°, solidifying point 42-45C°, CONC . (1.5-2%) .
- PH: most pathogenic bacteria needs a pH of 7.2 -7.4 this obtained by addition of NaOH or HCL.

Types of culture media:

Culture media can be divided according to:

A) Physical state (consistency) of media to:

1. Liquid (fluid) media: used for primary cultivation. Its disadvantage is the inability to obtain colony morphology, e.g., nutrient broth, peptone water, brain heart infusion broth.

1. Solid media: the concentration of agar is 1.5-2% used for identification of colony morphology e.g. nutrient agar, MacConkey's agar, Blood agar ...etc.

Blood agar if heated for about 40C° it will yields Chocolate agar.

2. Semisolid media: used for cultivation of spirochetes and to study motility such as (SIM) contain 0.4 -0.8% of agar agar.

B) Uses of media:

- Simple media: this media contains only common ingredients used for cultivation of non-fastidious microorganism. Such as nutrient agar and nutrient broth.
- Special purpose media: these media contain common ingredients plus addition another type material

There are seven types of this media include:

- Enriched media: simple media enriched with appropriate substances, e.g. blood 5-10%, glucose 1-2% serum fluid 10%.
E.g. Blood agar and Chocolate agar.
- Selective media: containing inhibitory substance (e.g. bile salts, antibiotic, dyes ... etc.) which favor the growth of the concerned microorganism and inhibit the growth of the others. E.g. MacConkey agar and Mannitol salt agar.
- Differential media: certain bacterial species produce characteristic growth then can easily recognized or can produce certain effects in the medium. e.g. MacConkey agar which regarded as both selective (because bile salt inhibit gram +ve bacteria and let gram -ve bacteria to grow and differential (because it contains lactose which could differential between gram-ve bacteria as two groups lactose fermentor and non fermentor .
- Enrichment media: this media allow the growth and Enrichment for one type of bacteria (fastidious one) and inhibits other (by competition on food) e.g. selenite f – broth.
- Transport media: certain mo. is weak and dies rapidly between the times of taken of the specimens and examination so it needs a special media for transport. E.g. Stuart's media.
- Indicator media: this is use the visual change in the color of an indicator due to mo. metabolism as a diagnosis feature e.g. sugar media.
- Sensitivity media: a special media used to tested antibiotic sensitivity for given mo. e.g. Muller Hinton media.

Enriched media: such as blood agar and chocolate agar which ore supplement with blood, one routinely used to culture pathogenic bacteria from clinical samples.

Blood agar: contains a basal medium and 5-10% sheep, horse or rabbit blood. Blood agar is also useful as differential agar according to blood cell hemolytic.

There are 3 types of hemolysis:

1. Beta haemolysis (β - hemolysis): complete lysis or destruction of red blood cells resulting in clear area around colonies. This haemolysis is displayed by *Streptococcus pyogenes* .
2. Alpha hemolysis (α - hemolysis): partial lysis of RBC, resulting in greenish discoloration around colonies this type is demonstrated by *Streptococcus pneumonia*.
3. Gamma – haemolysis (γ -haemolysis): not lyse RBCs this type is typical of *Enterococcus faecalis* .

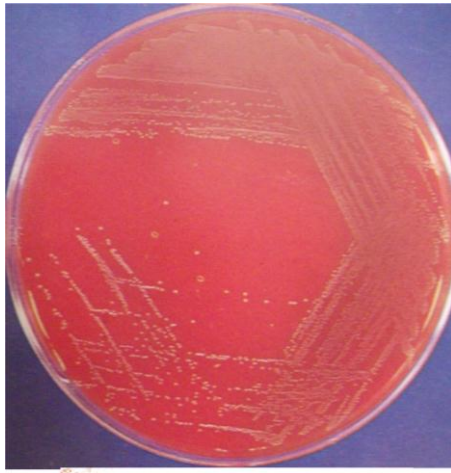
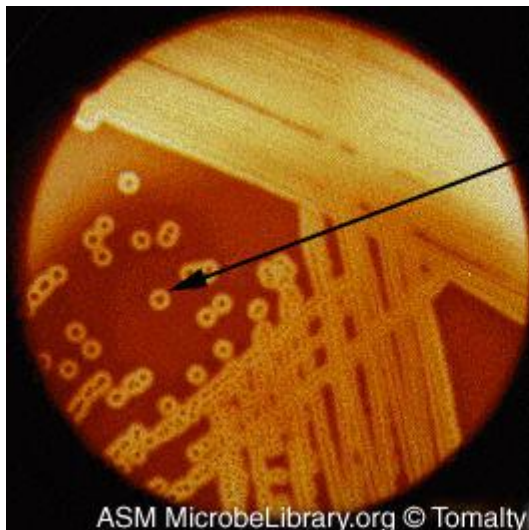


Fig. III.14. Gamma haemolytic streptococcus on BA. Note the absence of all haemolysis and the typical small opaque colonies of *S. faecalis* in the example shown here.



Note the clear zone of beta-hemolysis surrounding the *Streptococcus* colonies when grown on blood agar.

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Chocolate agar: is used to culture *Neisseria gonorrhoeae* and *Haemophilus influenzae*, it is prepared by heating blood agar to 70-80°C to lyse RBCs and release growth factors as X and V factors.

Eight Lab.

Mannitol salt agar:

contain 7.5% NaCl which is inhibitory to most bacteria. However, staphylococci grow on this agar and can be differential on the basis of mannitol fermentation.

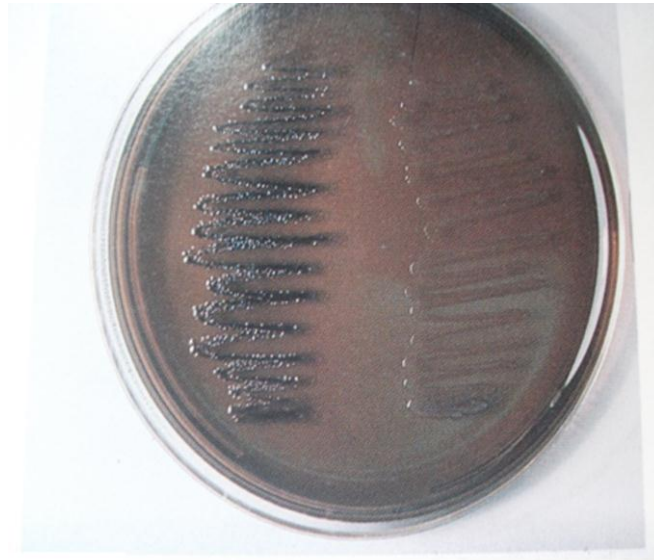
Pathogenic staphylococci, such as *Staphylococcus aureus* ferment mannitol to form acidic products that lower the pH of the medium. The phenol red pH indicator in the medium turns from red to yellow.

Non pathogenic staphylococci, such as *Staphylococcus epidermidis*, do not ferment mannitol, so no color change occurs.



MSA

Eosin – methylene blue (EMB) agar: contains bile salt and the dyes eosin and methylene blue, all inhibitory to Gram – positive bacteria. Therefore this media select for Gram- negative bacteria as *Escherichia coli*. EMB agar also differentiates lactose fermenting bacteria from non lactose fermenting bacteria; lactose fermenting bacteria such as *E. coli*, often appear dark with green metallic sheen, while non lactose fermenting bacteria such as *Shigella* or *salmonella* appear colorless.



Eosin-methylene blue agar inoculated with lactose-fermenting *Escherichia coli* (at left)

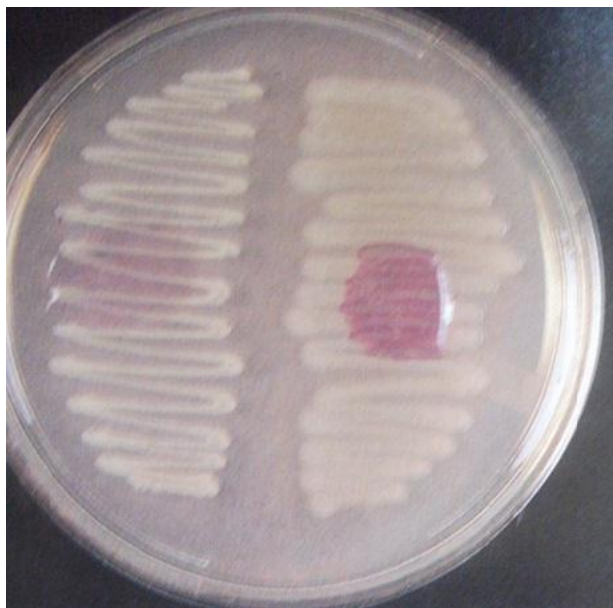
MacConkey agar: contain bile salt and crystal violet both inhibitory to Gram – positive bacteria.

Therefore this medium select for Gram – negative bacteria such as *E.coli* while inhibiting Gram – positive bacteria such as staphylococcus. This medium also differentiates lactose fermenting bacteria from non – lactose as fermenting bacteria. Lactose fermenting bacteria, such *E. coli* appear pink or red, while non – lactose fermenting bacteria such as shigella or salmonella appear colorless.



MacConkey agar inoculated with lactose-fermenting *Escherichia coli* (at left) and non-lactose fermenting *Shigella flexneri* (at right). Lactose-fermenting bacteria appear pink or red, while non-lactose-fermenting

Nutrient agar: is used to demonstrate bacteria in the environment. This medium contains beef extract and peptone. It supports a wide variety of bacteria.



Result of an oxidase test on nutrient plate

Salmonella shigella agar (S.S. Agar) Medium.

Considered as selective and differential medium for Salmonella and Shigella from other bacteria. Salmonella and shigella grow on this medium but other bacteria not.

This medium contain yeast extract, peptone, lactose , bile salt, sodium citrate, sodium thiosulfate, ferric citrate , neutral red , brilliant green and agar .

Lactose and neutral red for differentiate lactose fermenting bacteria from non lactose fermenting bacteria. Bile salt and Brilliant green for inhibiting Gram – positive bacteria, sodium thiosulfate prevent the growth of coliform bacteria such as E. coli. Ferric citrate reacts with sodium citrate and produce H₂S (black precipitate).

Salmonella and shigella colonies appear colorless because these bacteria were non lactose fermenting. Salmonella colonies contain dark ppt. in their centers (H₂S production) while shigella colonies not.

Biochemical test

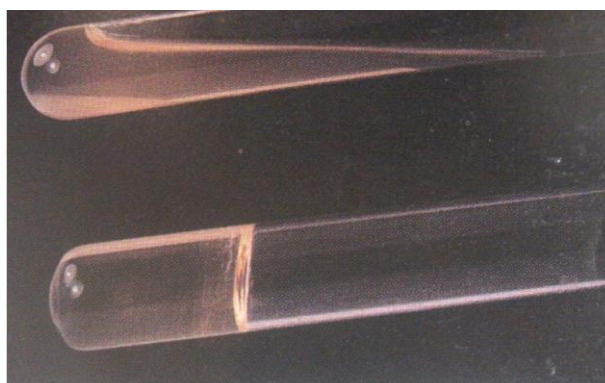
Some important Biochemical tests for identification of Bacteria:

Gelatin hydrolysis (gelatinase test) :

- Medium used: Nutrient broth + 12-15% gelatin.
- Inoculation: by stabbing, and incubation at 37°C for 24-48 hours.
- Result: liquefaction of gelatin after exposure of the inoculated medium to refrigeration temperature, i.e. the semi solid medium becomes liquefied in positive cases due to the action of gelatinase enzyme produced by the bacteria.

Gelatin → **gelatinase** → polypeptides → **polypeptidase** → Amino acids.

E.g. E. coli is negative, staphylococcus aureus is positive.



Nutrient gelatin tubes inoculated with *Pseudomonas aeruginosa* at the top (gelatin positive) and with *Alcaligenes faecalis* at the bottom (gelatin negative). Nutrient gelatin tubes must be thoroughly chilled before examination.

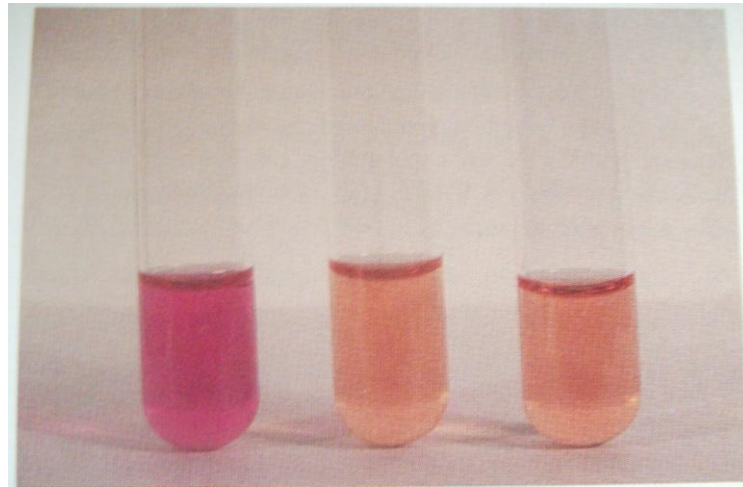
2-Production of ammonia from urea (Urease test):

- Medium: urea broth or urea agar containing phenol red indicator
- Inoculation: as for broth media, incubation for 24 - 48 hr. at 37°C.

Result: change of color (faint orange) to pink in +ve cases due to the hydrolysis of urea to ammonia by the action of urease enzyme produced by the bacteria and the medium because alkaline affecting PH of the indicator .



In – ve cases the color of the medium not change.
e.g. *E. coli* is ureases –ve , proteous spp + ve .



Results of urea broth tubes inoculated with *Proteus vulgaris* on the left (urease positive) and *Escherichia coli* in the center (urease negative).

3-Triple sugar iron (TSI) test.

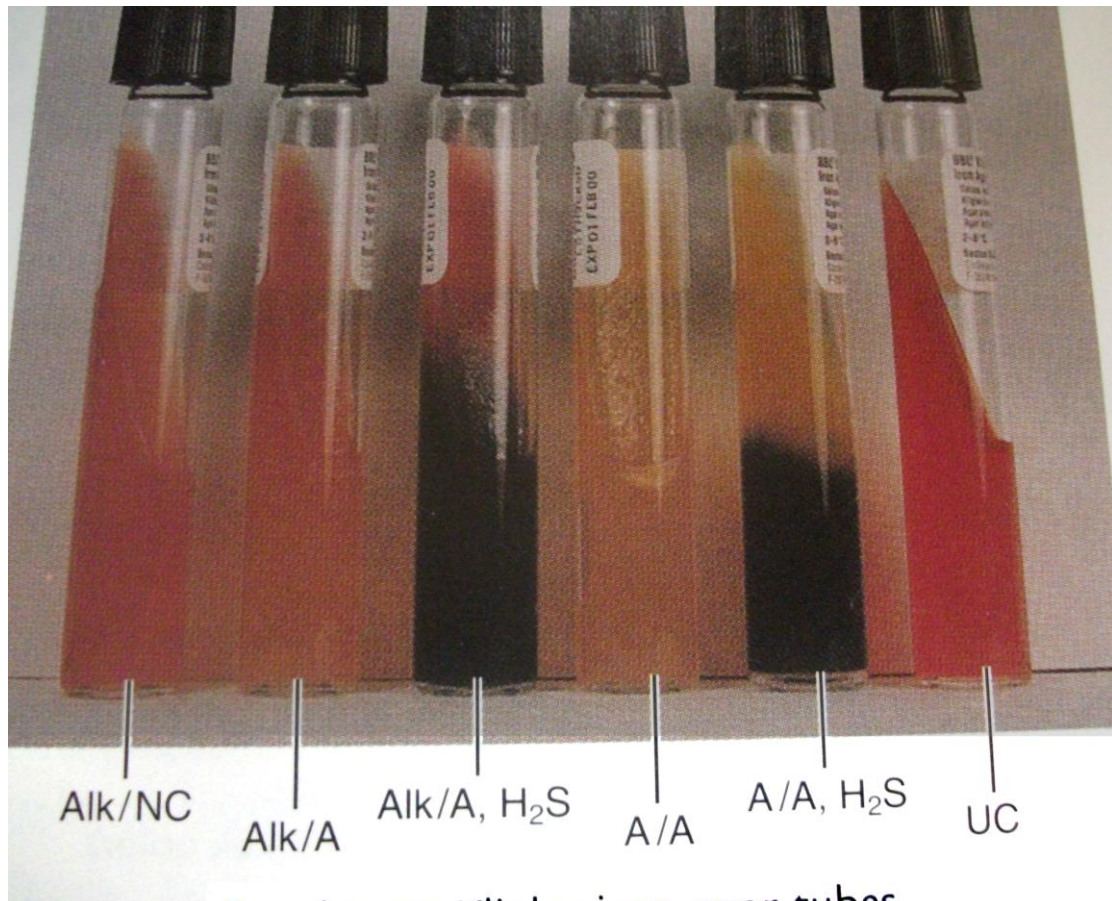
- Aim: to demonstrate fermentation of sugars and production of H₂S and gas.
 - Medium: consist of 0.1% glucose, 0.1% lactose, 1% sucrose, phenol red indicator, peptone, ammonium thiosulfate and ferrous sulfate, made as slant and button.
 - Inoculation: by stabbing the bottom followed by streaking of the slant incubation overnight at 37°C.
 - Result: read the result as slant / button.
- 1-Acid (A)/Acid (A) or Yellow (Y)/Yellow (Y) with or without gas (CO₂ and H₂) due to fermentation of glucose with lactose (or sucrose).
e.g . *E. coli*.
 - 2-Alkaline (K)/ Acid (A) or pink (p)/Yellow (A) with or without gas (CO₂ and H₂) due to fermentation of glucose only. e.g. *Shigella sonnei* .
 - 3-Alkaline k/Acid (A) or pink (P/Y)+H₂O , and sometimes CO₂ and H₂ due to fermentation of glucose only and a black precipitate formation from H₂S .
e.g *Salmonella paratyphi* .
 - 4-Alkaline (K)/Alkaline (K) (P/P) here no sugar is fermented only peptones
Pseudomonas aeruginosa.

Mechanism: pink color of the slant results from utilization of peptones by the organism and release of NH_3 yielding an alkaline pH which affects the indicator (phenol red).

Yellow colour of the medium or bottom only result from the change in the color of indicator (phenol red) due to acid formation from sugar fermentation.

Black colour develops due to production of H_2S ppt.

From ammonium thiosulfate + iron salt (ferrous sulfate).



Results on Kligler iron agar tubes.

Tenth Lab.

4-) Indole production from tryptophan:

- Medium: nutrient broth containing peptone which is rich in tryptophan.
- Inoculation: as for broth cultures.
- Indicator: add kovac's reagent.
- Result: a deep red colored ring develops in the presence of indole and this is a positive result.

Tryptophan → indole + pyruvic acid + ammonia

In – ve result the ring stays yellow.

E.g. *E. coli* is indole +ve , *Salmonella* spp – ve



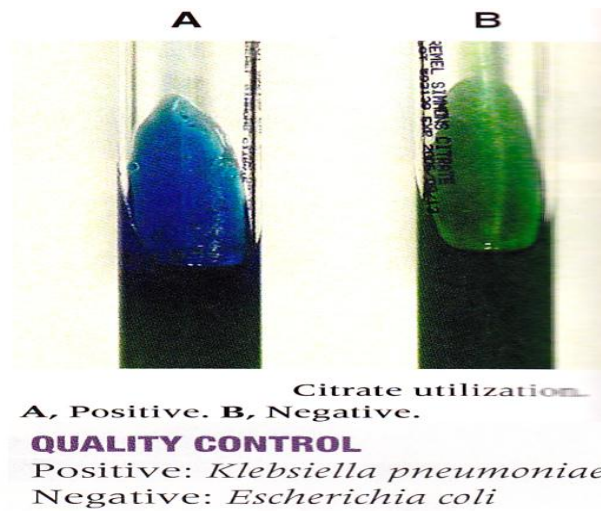
Results for indole production in SIM medium tubes. Inoculated tubes received five drops of Kovac's reagent after incubation. The reagent appears as a layer on the agar surface. Left: *Escherichia coli* (indole +ve).

5-) Citrate utilization test:

- Medium: Simon's citrate agar containing Bromothymol blue (indicator).
- Inoculation: streaking of the slant after stabbing of the bottom incubation at 37°C for 24 - 48 hr.
- Result: utilization of citrate as the source of carbon for energy and growth of the organism.

Growth of organism on the citrate agar result in an alkaline reaction which makes the bromothymol blue change from green to blue color in positive cases, in negative cases there is no change in color of the

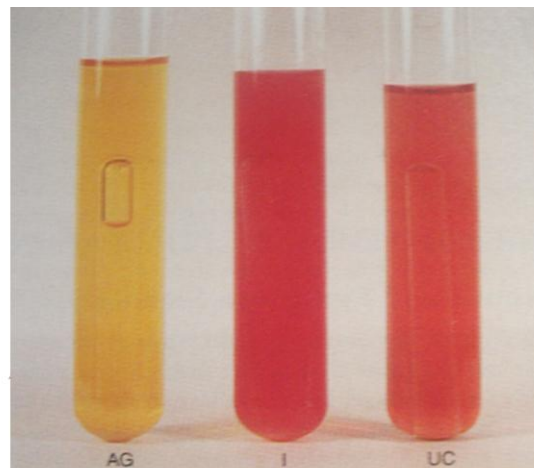
medium and no growth of the organism. e.g *E. coli* is – ve
Salmonella spp + ve .



6-) sugars fermentation test:

- Medium: peptone water medium containing (0.5 -1%) sugar (glucose or lactose or sucrose etc) and phenol red indicator in tubes containing Durham tube (converted) for demonstration of gas.
- Inoculation: as for broth medium incubation for 18-24 hr. at 37°C.
- Result: change of color (faint orange or red) to yellow in +ve cases and the bottom of Durham tube is empty if gas is found. In +ve cases there is no change in color (no fermentation of sugar).

E.g. *E. coli* lactose fermentation + ve



Carbohydrate utilization test results in phenol red lactose broth tubes. (Left to right): *Escherichia coli*, acid and gas (AG); *Alcaligenes faecalis*, inert (I); uninoculated control (UC).

Salmonella spp –ve .

7-) oxidation / fermentation (O/F) test.

- Medium: Hugh and Leifson's medium containing 1% glucose and bromothymol blue indicator.
- Inoculation: by stabbing 2 tubes for the same organism and then adding paraffin to one of them only.
- Result: Acid production is shown by a change in the color of the medium from green to yellow due to fermentation of glucose and change in color of the indicator (bromothymol blue) in acidic pH.

Fermentative organism (bacteria that ferment glucose in the absence of oxygen) will produce acid in the paraffin coated tube only while oxidative organisms (bacteria that ferment glucose in presence of O₂) will produce acid in the open tube only.

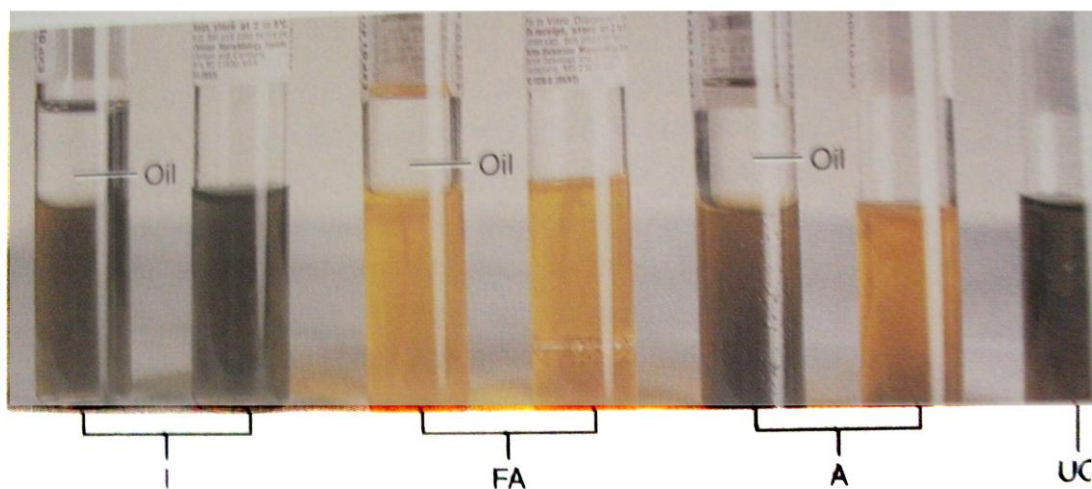
So the results are read as follows:

O/F = oxidative / fermentative organism.

O/-= oxidative organism.

-/F= fermentative organism.

-/- = Inert (not active) organism.



Results in O-F glucose tubes. (Left to right): *Alcaligenes faecalis* with and without oil, inert (I); *Escherichia coli* with and without oil, facultative anaerobe (FA); *Pseudomonas aeruginosa* with and without oil, aerobe (A); uninoculated control (UC).

Eleventh Lab.

8-) phenylalanine deaminase test:

- Aim: to detect oxidative deamination of phenylalanine to phenyl pyruvic acid by phenylalanine deaminase enzyme produced by organism.
- Medium: phenyl alanine slant medium.
- Inoculation: by stabbing the bottom and streaking the slant incubation of 37°C for 18-24 hr.
- Indicator: add ferric chloride (10% FeCl₃) reagent.
- Result: development of green color after addition of the reagent indicates a +ve result.

Yellow color presence is – ve.

Phenylalanine $\xrightarrow{\text{phenylalanine deaminase}}$ phenyl pyruvic acid + NH₃ + H₂O

Phenyl pyruvic acid + 10% FeCl₃ $\rightarrow$ green complex.

e.g. proteus spp + ve , E . coli - ve



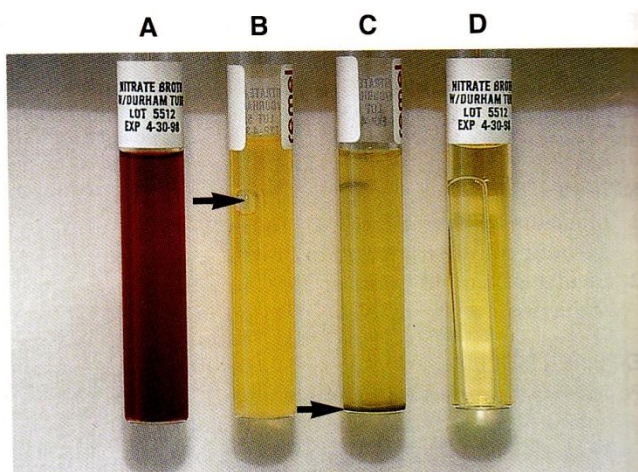
Results in phenylalanine agar tubes.
(Left to right): *Proteus vulgaris*, phenylalanine deaminase positive; *Escherichia coli*, phenylalanine deaminase negative; uninoculated control. A positive test turns green after the addition of ferric chloride.

9-) Nitrate reduction test:

- Medium: nitrate peptone water.
- Inoculation: as for broth medium incubation at 37°C for 18-24 hr.
- Indicator (reagent): add α - naphthylamin and sulfanilic acid .
- result: red color develops within few minutes in + ve cases (NO₃ reduced to NO₂), if no red color develops, small quantity of zinc dust are added the development of a red colour indicates – ve result because the reduction occur by Zinc dust and not by bacteria, but if no red colour develops (grey or brown colour) this indicate that the bacteria is reduce the NO₃ to NO₂ to NH₃ to N₂ and the result considered + ve.

e.g. *E. coli* is nitrate + ve ,
Clostridium botulinum – ve .

Nitrate reduction.
A, Positive, no gas.
B, Positive, gas
 (arrow). **C**, Positive,
 no color after
 addition of zinc
 (arrow). **D**, Uninoculated
 tube.



QUALITY CONTROL

NO₃⁺, no gas: *Escherichia coli*
 NO₃⁺, gas: *Pseudomonas aeruginosa*
 Negative: *Acinetobacter* spp.

10-) Catalase test:

Aim: to detect the production of catalase enzyme by the microorganism (growing aerobically).

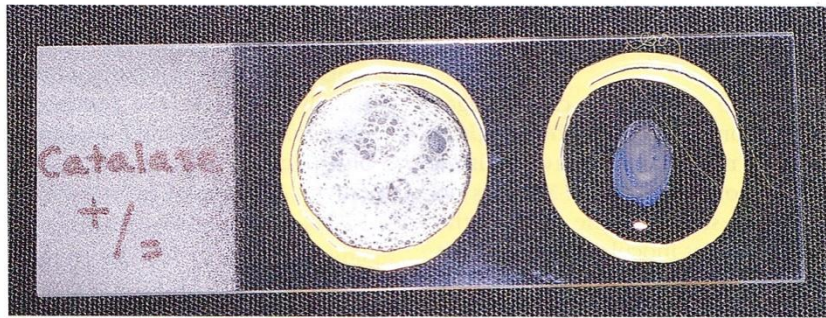
Reagent (indicator): 3-6% hydrogen peroxide (H₂O₂).

Result: a loopful of bacteria growth is emulsified with a loopful of H₂O on clean slide, the production of Effervescence or foam caused by liberation of free O₂ as gas bubbles indicates the presence of catalase.



E.g. *Staphylococcus* spp + ve .

Streptococcus spp – ve .



Catalase test. **A**, Positive. **B**, Negative.

QUALITY CONTROL

Positive: *Staphylococcus aureus*

Negative: *Streptococcus pyogenes*

11-) phosphatase test:

Aim: to detect the production of phosphatase enzyme by bacteria.

Medium: nutrient agar containing phenolphthalein phosphate.

Reagent (indicator): concentrated ammonia solution (ammonium hydroxide).

Result: plates inoculated by stabbing and incubated overnight at 37°C are exposed to ammonia vapour by adding a few drops ammonia to filter paper inserted in the lid of the Petri – dish. Pink or red colonies indicate the presence of free phenolphthalein.

Note: Phosphatase enzyme produced by bacteria destroys the phosphate in the medium.

Leaving the phenolphthalein free and then react with the ammonia giving the pink color of colonies.

If the colors of colonies not change this indicate the – ve result.

E.g. *Staphylococcus aureus* + ve *Staphylococcus epidermidis* – ve .

Twelfth Lab.

12- deoxy ribonuclease test (DNASE)

12-) oxidase test:

Aim: to detect production of oxidase enzyme by bacteria.

Reagent: 1% a queous solution of tetramethyl – p- phenylene diamine – HCL.

Result: few drops of the reagent are added to a filter paper and using wooden stick or glass rod, some of the bacterial growth is transferred to the impregnated filter paper; a purple coloration is produced within 5-10 sec indicating the presence of oxidase enzyme. Delayed + ve result may appear with in 10-60 sec. but more than that (or a colorless result) is – ve .

E.g. Staphylococcus spp. And Enterobacteriaceae spp. – ve .

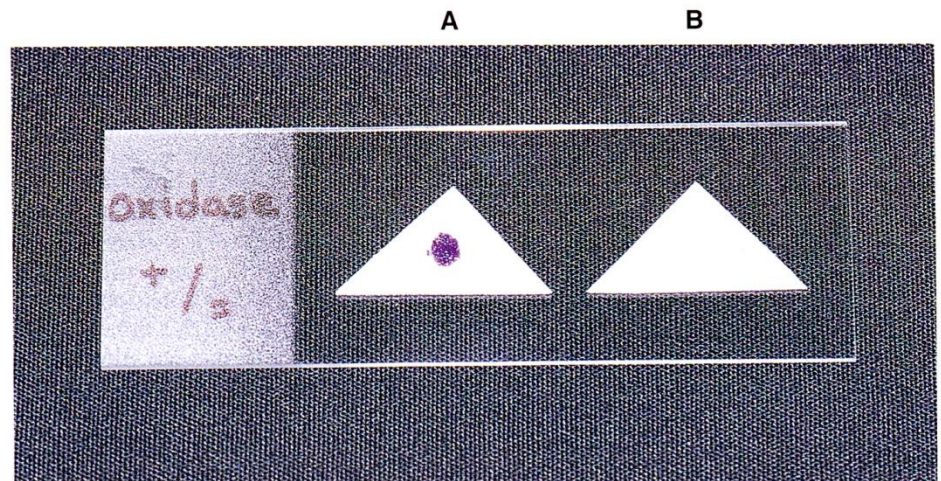
Streptococcus spp and pseudomonas spp + ve .

Oxidase test. A,
Positive. B, Negative.

QUALITY CONTROL

Positive: *Neisseria gonorrhoeae*

Negative: *Escherichia coli*



13-) Deoxy ribonuclease test (DNASE).

Aim: to detect the production of DNase enzyme by staphylococcus aureus.

Medium: nutrient agar containing DNA.

Reagent (indicator): (1N) HCL.

Result: plates inoculated by stabbing and incubated for 18-24 hour at 37c° then adding a few drops of (1N) HCL and after 15-30 minute.

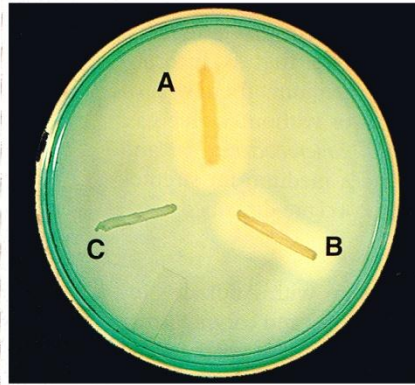
Read the result, if there are clear zone around the colonics this indicate + ve result (DNA hydrolysis by DNA se enzyme).

If not this is – ve result.

E.g. staphylococcus aureus + ve

Staphylococcus epidermidis – ve .

DNA hydrolysis.
 A, Positive,
Staphylococcus aureus. B, Positive,
Serratia marcescens.
 C, Negative.



QUALITY CONTROL

Positive: *Staphylococcus aureus*

Negative: *Staphylococcus epidermidis*

14-) methyl red / voges proskauer (MR/ VP) test.

Medium: glucose phosphate broth.

Inoculation: as for broth media incubation at 37°C for 2-7 days.

Reagent (indicator): methyl red added for MR test.

α -naphthol and 40% KOH or NaOH added for VP test .

Result: the inoculated broth is divided into 2 portions, in the MR part, a red color indicates acidic pH (4.5 or less) and positive result, while yellow color is negative.

In + ve cases, glucose is fermented and end products of fermentation are acids.

E.g. lactic acid and butyric acid.

In the VP part development of a red colour within 5 minute, constitutes a + ve reaction due to formation of Acetyl methyl carbinol (Acetone) from glucose fermentation. Yellow color appearance is – ve.

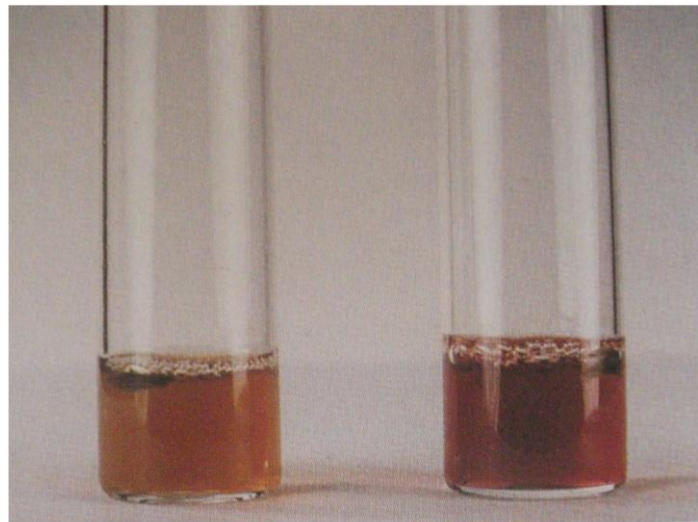
e.g. *E. coli* is MR+ / VP –

Klebsiella spp is MR -/VP +

Note: some organisms may have both tests – ve, but very rare and not find organisms with both tests + ve.



Methyl red test results in MR-VP medium tubes. The inoculated tubes received five drops of methyl red after incubation. Left: *Escherichia coli* (methyl-red-positive); center: *Enterobacter aerogenes* (methyl-red-negative); right: uninoculated.



Results of the Voges-Proskauer test in MR-VP tubes. Tubes have received six drops of Barritt's Reagent A and two drops of Barritt's Reagent B. *Escherichia coli* (left) is VP negative, and *Enterobacter aerogenes* (right) is VP positive.

15-) Starch hydrolysis:

Medium: starch agar.

Inoculation: by stabbing and incubation at 37°C for 3-5 days.

Reagent (indicator): Gram's iodine (logal iodine) added to the growth on plates.

Result: clear zone around colonies indicate hydrolysis of starch due to production of β - amylase enzyme by the bacteria, reddish – brown zones around the colonies indicate partial hydrolysis of starch to dextrin.

If clear zone not appear around colonies and the medium stay blue – brown this indicate a – ve result (the starch not hydrolyzed)

E.g. *Bacillus* spp + ve . *E. coli* – ve

Starch (Polysaccharide) → α -**amylase** → dextrin (trisaccharide) β -amylase
maltose (disaccharide) → **maltase** → Glucoses (monosaccharide)

16-) Sulfide Indole Motility test (SIM test):

Aim: to detect the production of H₂S and indole by the bacteria and the motility of bacteria.

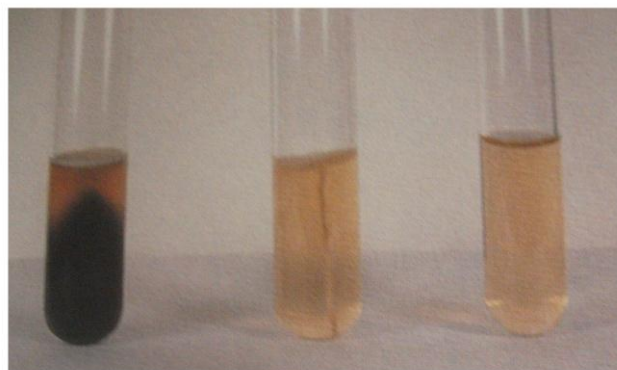
Medium: Sulfide Indole Motility agar (semi solid) containing peptones as (cysteine), sodium thiosulfate and Fe (NH₃) SO₄ as indicator.

Result: Tubes inoculated by stabbing and incubated for 18-24 hour at 37°C if a black precipitate appears in the medium this indicates the production of H₂S.

And appearance of red ring after adding of Kovac's reagent indicates the +ve result of Indole production and the spreading of growth all over the tube indicates that the bacteria is motile while the growth only on the stabbing line indicates the bacteria is non-motile.

Note: the result read as in the table:

S	I	M	
+	+	+	<i>Proteus vulgaris</i>
-	+	-	<i>Klebsiella pneumonia</i>
+	-	+	<i>Salmonella typhimurium</i>



Results for H₂S production in SIM medium tubes. (Left to right): *Proteus vulgaris*, H₂S positive; *Escherichia coli*, H₂S negative; uninoculated control.