LAB 1:

Bacteria: Bacteria are one-celled microscopic living organisms (ranged from 0.5-2.0 micron in diameter) it can be seen just under light microscope with the aid of oil immersion lenses (100x).

Bacteria could be found everywhere. They can be either

- **Beneficial bacteria**, as in the process of fermentation (such as in vinegar and dairy production), also many bacteria play important role in decomposition.

  or

- **Pathogenic bacteria** that could cause a disease when enters any living body (human or animal) and it can spread through water, air, soil and through physical contact

Classification of Bacteria

1-Shape: The bacteria can classify according to their shape

***Cocci (Spherical)**

Diplococci e.g.: *Streptococcus pneumoniae*

Chain (Cocci) e.g.: *Streptococcus pyogenes*

Cluster or Grape like shape e.g.: *Staphylococcus aureus*

***Bacilli**

Short Bacilli e.g.: *Bacillus subtilis.*

Long Bacilli e.g.: Lactobacillus. spp.

Coccobacilli e.g.: like members of Enterobacteriaceae family (Escherichia *coli, Shigella, Salmonella*).

***Kidney shape**: e.g.: *Neisseria gonorrhea.*

***comma shape:** e.g.: *Vibrio cholera.*

*** Spiral shape:** e.g.: *Helicobacter pylori*

2- Ability to form spores:

The bacteria are divided to two groups according to their ability to form spores.

***Non-spore - former Bacteria:** e.g. - *Staphylococcus* spp., *Escherichia coli, Streptococcus* spp.

***spore - former Bacteria:** e.g.: *Bacillus, Clostridium and Sporolactobacillus.*

3- Oxygen requirements:

***Obligates (strict) aerobes bacteria:** e.g.: *Bacillus*, *Pseudomonas*.

***Obligates (strict) anaerobes bacteria:** e.g.: *Clostridium*.

***Facultative anaerobes** e.g.: Enterobacteriaceae e.g.: *Escherichia coli,* Shigella, Salmonella and *Staphylococcus spp.*

***Microaerophiles** e.g. *Helicobacter pylori.*

***Aerotolerant** e.g. *Streptococcus*

4-Reaction to the Gram stain: The Bacteria are divided in two groups according to the reaction with Gram stain.

- Gram positive bacteria. e.g.: *Streptococcus, Staphylococcus, Bacillus* and *Clostridium.*
- Gram Negative Bacteria. e.g. All the members of Enterobacteriaceae *(Escherichia coli, Shigella,Salmonella*,…..)

5- Bacterial Nutrition

A) Autotrophic bacteria: These bacteria synthesize all their food from inorganic substances (CO_2 and hydrogen donor), the autotrophic bacteria are including two types:

(i) Photosynthetic bacteria.

(ii) Chemosynthetic bacteria.

B) Heterotrophic bacteria: The heterotrophic bacteria obtain their-ready made food from organic substances, living or dead. These bacteria including three types:

(i) Saprophytic bacteria.

(ii) Parasitic bacteria.

(iii) Symbiotic bacteria.

6-Mode of energy production: (glycolysis, cellular respiration).

Laboratory diagnosis of bacterial disease:

It depended on clinical specimens reaching to the lab

1-Blood: septicemia.

2- Urine: urinary tract infections.

3- Stool: gastrointestinal infections.

4- Sputum: respiratory infection

5- Vaginal swabs: Vaginal infections.

6- Nose &ear swabs: Nose &ear infection

7- Cerebral spinal fluid. CNS infections.

8-Food &vomit: food poisoning.

9- Pus: Acne, wounds, burns.

10-Seminal fluid, urethral discharge.

Methods for bacterial identification:

1- Phenotypic characters:

a- Microscopic morphology- Gram Staining, shapes, arrangements, motility.

b- Macroscopic morphology - colony appearance, motility.

2- Physiological/ biochemical characteristics (Growth requirement) aerobic, anaerobic, photosynthetic, growth on selective media
3- Chemical analysis- e.g. peptides and lipids in cell membranes
4- Phage Typing- which phage infects the bacterium
5- Serological analysis - what antibodies are produced against the bacterium
6- Genetic and molecular analysis
7- Growth requirement.

Lab. safety Directions

1-Wear lab Coat before start working and safety glasses if it necessary.
2-Wash your hands with disinfectant soap when you arrive at the lab and again before you leave.
3-Disinfect work areas before and afterwork by using 70% ethanol or fresh 10% bleach.
4-Don't Eat or Drink in the Lab also don't taste or Sniff Chemicals
3- Dispose Lab Waste Properly.
4- Inoculating loops and needles should be flame sterilized in a Bunsen burner before you lay them down.
5- Sterilize equipment and materials.
6- Never pipette by mouth.

LAB2: culture Media

- Microorganisms, like all other living organisms, require basic nutrients for sustaining their life. All microorganisms have the same basic requirements but they are diverging in inorganic and organic compounds needs. By providing environmental and nutritional factors it is often possible to provide the appropriate conditions for their cultivation. On this basis we can define the
- Culture media as a fellow: An artificial environment simulating natural conditions that necessary for bacteria to grow in laboratory.

- Components of the Typical Culture Medium:
- 1- Energy source
- 2- Carbon source
- 3- Nitrogen source
- 4- Salts like phosphate, chlorides, sodium carbonates, potassium, magnesium, ferric, calcium and trace element like copper.
- 5- Source of different minerals e.g. iron, magnesium, sodium, potassium and traces of zinc and manganese.
- Note: Some microorganisms may need a source of vitamins and amino acids which are important in building cellular components of microorganisms

•Culture Media Importance:

1. Isolation and preservation of microorganisms.
2. Reproducing a microorganism and studying its characteristics.
3. Encouragement and induction of the microorganisms to produce materials that have industrial importance like antibiotics and some organic acids.

Classification of Culture Media:

A) Depending on its consistency:

- **1- Liquid media or broth**: these are media that do not contain any solidifying agent like agar. Liquid media are suitable to grow bacteria when the numbers in the inoculum is suspected to be low, also they are usually used in the extraction of active compounds produced by microorganisms (e.g. toxins).
- **2- Solid media:** these are media that contain (1.5 – 2 %) agar. Solid medium they are important for isolation of microorganisms in pure form especially when there is more than one species in one sample.
- **3) Semisolid media**: it contains less than 1% (about 0.7 - 0.8) % of agar. Such media are soft and are useful in demonstrating bacterial motility and separating motile from non-motile strains, also their requirements for O2 to know if these microorganisms are aerobic, anaerobic, micro aerobic, or facultative anaerobic.

B) Depending on its nature or components or contents:

- **1) Natural media:** these are media that contain natural materials e.g. plant or animal tissues, milk, blood, fruit and vegetables juice and meat extract. The components of these media are accurately unknown.
- **2) Artificial media:** these are divided into:
  - **a) Synthetic or defined media:**
    these are media which contain chemical substances that we know their composition and their concentration accurately. These media are used for a wide variety of physiological studies.

- **b) Semi synthetic or complex media:**
  these are synthetic media supplemented with natural components of unknown chemical composition like the addition of meat extract, yeast extract, peptone or serum. Complex media are usually used for cultivation of bacterial pathogens and other fastidious bacteria.
- **3) Living media: these are media in which living cells are used as culture media like using chicken embryo and Hela cell for cultivation of viruses.**

- **C) Depending on the purpose of uses**
- **1) General purpose media: these are media in which many are grown. They are used for many purposes e.g. nutrient broth.**
- **2) Selective media: these are media that are used for the cultivation and isolation of certain species of microorganisms from a mixture of different species. These media are divided into two kinds:**
- **A) Suppressive selective media:**
  Selective media contain a component that helps to grow of a microorganism and suppresses the growth of other undesirable (un wanted) species.

B)Enrichment selective media:

these are media which are used for the selection of the desirable species of microorganisms by induction their growth rather than other species which are grown in the same medium, this done by adding stimulatory materials which enrich the media like blood to nutrient agar medium to form blood agar medium. These media are used for the cultivation of fastidious bacteria.

3) Differential media: these are media which differentiate between two different groups of microorganisms and allow to diagnosis of microorganisms depending on its biological characters. Differential media contain certain material allows to detection of certain microorganisms depending on their metabolic activity.

- **There are several ways to suppress microorganisms like:**

1- Addition of some suppressive materials to the medium like:

* **The addition of certain dyes e.g. crystal violet, methylene blue, basic fuchsine which inhibit the growth of G +ve bacteria without affecting the G -ve growth.**
* **The addition of certain antibiotics e.g. cycloheximide which inhibits the growth of saprophytic fungi and allows the growth of fungi that have medical important like the Dermatophytes when it is added to Sabouraud agar.**

2- By manipulation of certain growth conditions according to the growth conditions of the desirable species e.g. temperature, aeriation, and pH.

- **3) Maintenance media:** These media are used for maintenance and storage of microorganisms for long time by adding materials in a certain ratio. These materials maintain the persistence and viability of microorganisms for a longer time e.g. glycerol or tween-80 which leads to the slow growth rate of microorganisms (fast growth is followed by fast death). Maintenance media is preserved in the freezer. e.g. nutrient broth, brain-heart infusion broth.

4) Transport medium:

Transport media are special media formulated to preserve a specimen and minimize bacterial overgrowth from the time of collection (human, soil, water, etc.) to the time it is received at the laboratory to be processed. Depending on the type of organisms suspected in the sample, transport media may vary. However, in general, transport media are classified based on physical state as semi solid and liquid and in basis of their utility as bacterial or viral transport media. Transport media contain only buffers and salt and doesn't contain any nutritional ingredients such as carbon, nitrogen, and organic growth factors to prevent microbial multiplication.

Example of transport medium:

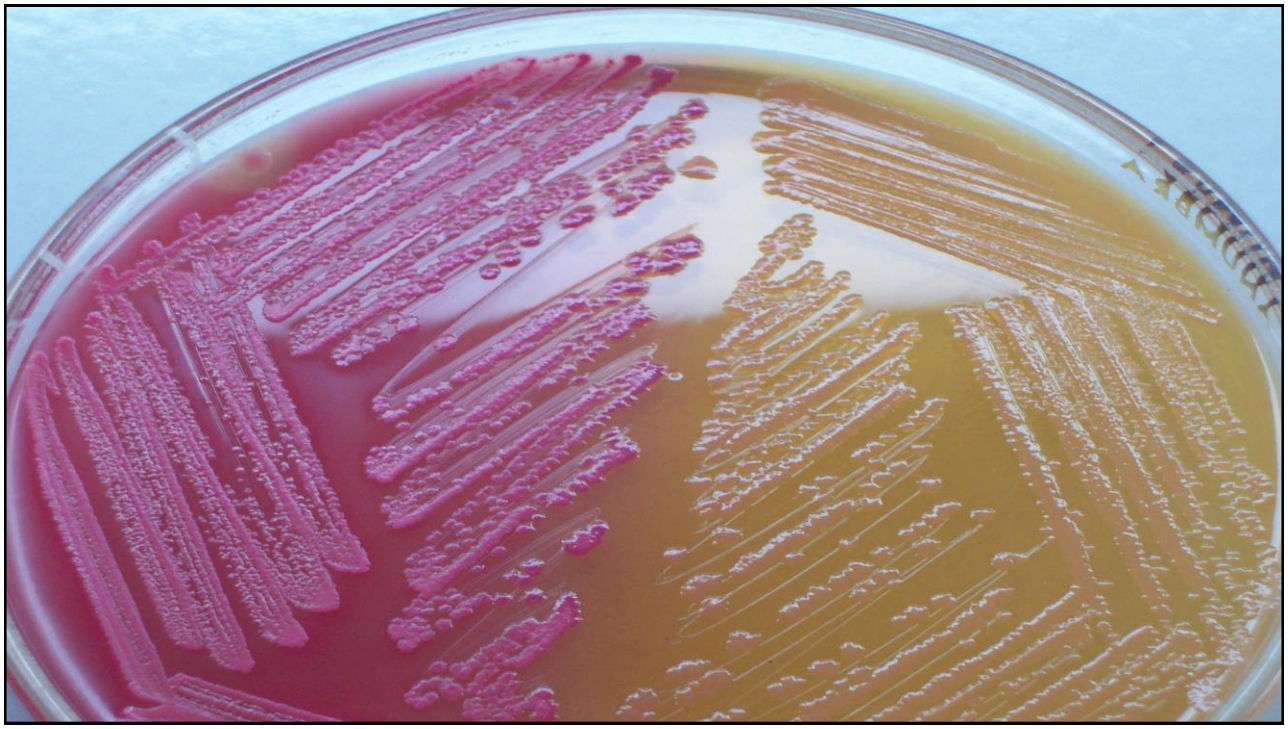
- **Stuarts medium:** Commonly used for transporting specimens suspected of having gonococci. Also used for transporting Throat, vaginal, wound and skin swabs that may contain fastidious organisms
- **Cary and Blair Medium:** semi-solid, white colored transport medium for faeces that may contain Salmonella, Shigella, Vibrio or Campylobacter.

5) Assay media:

- these media are used for performing assay like the medium that used for performing antibiotic sensitivity test which called Muller- Hinton agar.

6) Stimulatory media:

- these are media that stimulate the production of certain materials or structures inside the microorganism's cell like: toxins, pigments and endospores.



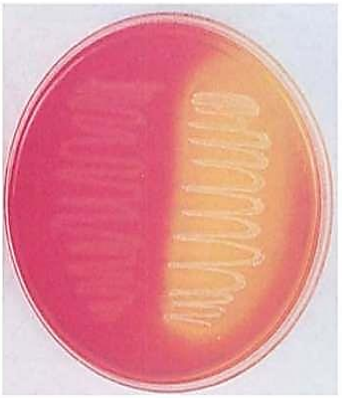
MacConkey agar (selective differential media for gram negative bacteria)

- Sugar: Lactose
- PH-indicator: Neutral red
- crystal violet: inhibitor for gram positive bacteria
- bile salt: inhibitor for enterococci
- Lactose fermenter = pink colony
- Lactose non fermenter = pale colony

- **Principle of MacConkey Agar: MacConkey agar is considered as a suppressive selective medium it permits the growth of Gr-ve enteric bacteria and inhibits the growth of Gr+ve non-enteric bacteria. The selective action of this medium is attributed to the (crystal violet) which inhibits the Gr+ve bacteria, also the medium contains (bile salts) that inhibit non-enteric bacteria and both (crystal violet and bile salts) do not affect the growth of enteric Gr-ve bacteria because these bacteria is adaptable to live with the presence of bile salts in the intestine.**
- **Note: - Many reagents or indicators are added to differential media to differentiate between different bacterial species that grow on same media. Usually, these reagents are dyes which detect the changes in the media acidity that result from microbial metabolic activity, the changing in acidity manifested by changes in the dye color. Thus, these reagents or dyes are called as pH-indicators.**

Mannitol Salt Agar

- Mannitol fermenters includes: Staphylococcus aureus
- Non-mannitol fermenters includes: Staphylococcus epidermidis
- Positive growth but non-mannitol fermenters includes: Micrococcus luteus
- Negative growth includes: Escherichia coli, Pseudomonas aeruginosa



Mannitol salt agar (selective differential media for gram positive bacteria :*Staphylococcus spp*)

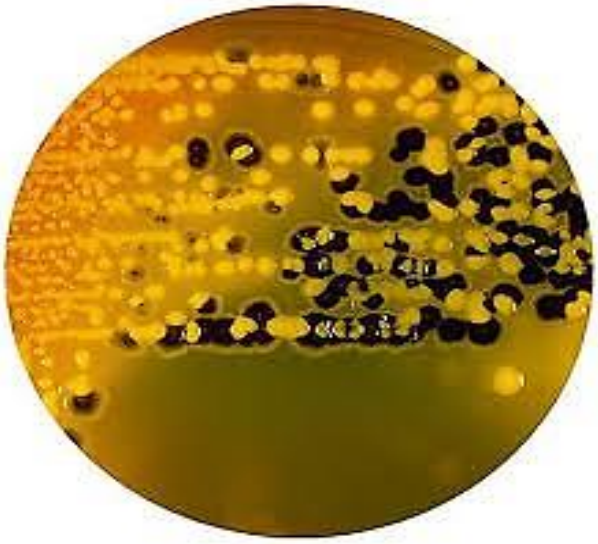
- **Sugar:** Mannitol
- **Nacl 7.5%:** The inhibitor for gram positive & negative bacteria
- **PH-indicator:** phenol red
- **Mannitol fermenter** = yellow colony
- **Mannitol non fermenter** = red colony

XLD agar (Xylose Lysine Deoxycholate) selective differential media for Shigella And Salmonella

- **Sugar :** Xylose
- **PH-indicator:** phenol red
- **H2S indicator**: Na-thiosulphate
- **Na-Deoxycholate:** Gram positive & gram negative inhibitor
- **Shigella non fermenter Xylose** = red colony
- **Salmonella fermenter Xylose** = yellow colony with black center

Xylose lysine Deoxycholate (XLD) Principle...

- *Sodium desoxycholate* inhibits contaminating Gram-positive flora.
- *Xylose* is fermented by practically all coliforms bacteria and *Salmonella*, except for *Shigella* which are thus differentiated from the other species.
- After exhausting xylose, *Salmonella* decarboxylate *lysine* (via lysine decarboxylase) to cadaverine, causing the pH to rise.
- Colonies of *Salmonella* resemble those of *Shigella* in the medium having become basic.
- *Phenol red* is the pH indicator.
- The addition of *lactose* and *sucrose* to the medium enable coliform bacteria to decarboxylate lysine and thereby produce excess acidity, making the indicator turn yellow, favoring their differentiation.
- *Sodium thiosulfate* and *Ferric ammonium citrate* allow the detection of the H_2S producing bacteria.



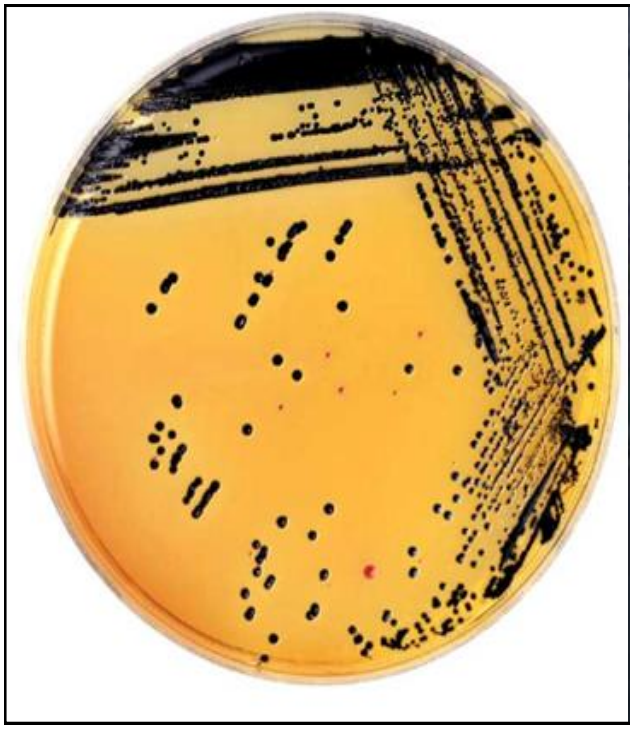
Shigella on XLD. *Salmonella on XLD.*

Image Source: Faculty of Health and Medical Sciences - University of Copenhagen, Denmark

S-S agar (Shigella Salmonella agar)

- **Sugar:** lactose
- **PH-indicator:** Neutral red
- **H2S indicator:** Na-thiosulphate
- **Brilliant green:** Gram positive & gram negative inhibitor
- **Shigella non fermenter lactose =** pale colony

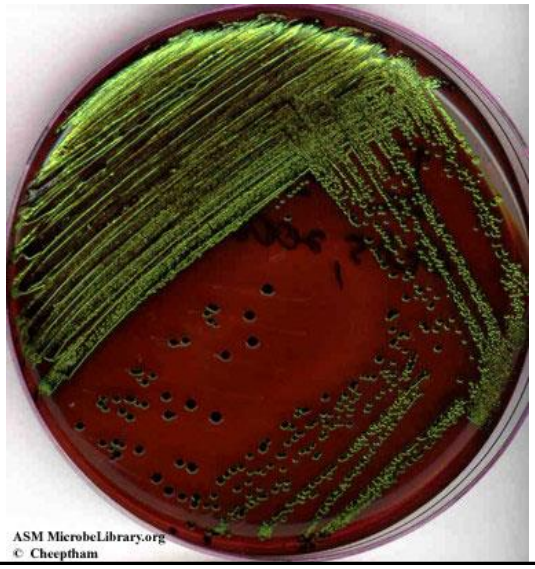
- **Salmonella non fermenter lactose =** pale colony with black center



Eosin-methylene blue agar:

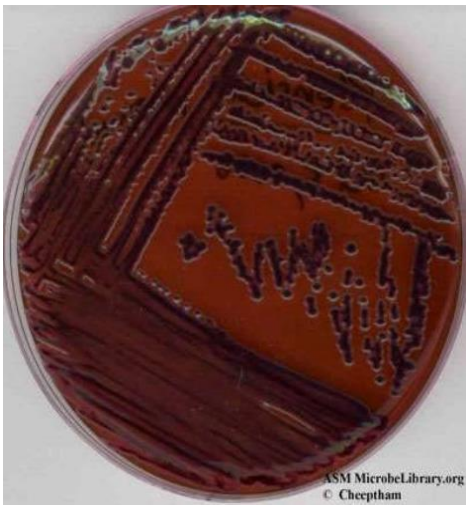
selective for gram-negative bacteria against gram-positive bacteria .In addition, EMB agar is useful in isolation and differentiation of the various gram-negative bacilli and enteric bacilli, generally known as coliforms and fecal coliforms respectively The bacteria which ferment lactose in the medium form colored colonies, while those that do not ferment lactose appear as colorless colonies EMB agar is used in water quality tests to distinguish coliforms and fecal coliforms that signal possible pathogenic microorganism contamination in water samples .EMB agar is also used to differentiate the organisms in the colon-typhoid-dysentery group:*Escherichia coli* colonies grow with a green metallic sheen with a dark center.

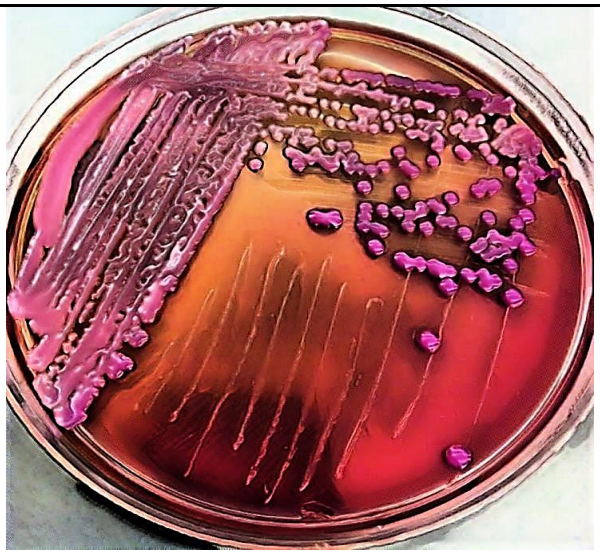
Eosin methylene blue



EMB

Klebsiella species produces; large, mucoid, pink to purple colonies with no metallic green sheen on EMB agar.





Klebsiella on EMB agar

EMB – Eosin Methylene Blue Agar,
isolate Gram neg. bacteria

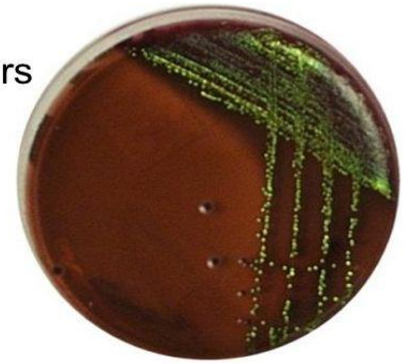
Selective – eosin; methylene blue; inhibitory to Gram + organisms

Differential – lactose fermentation

Metallic green/blue black colonies- (+) vigorous lactose fermentors, acidic

Dark Purple – (+) slower fermentors acidic

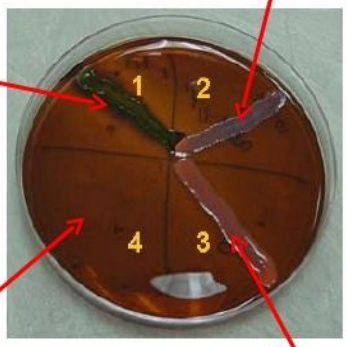
Light pink/colorless – (-) not fermenting



Levine Eosin-Methylene Blue (**EMB**) Agar

Lactose fermenter
(high acid producer: black colonies)

Non-lactose fermenter



Gram positive (growth inhibited)

Lactose fermenter
(low acid producer: pink colonies)

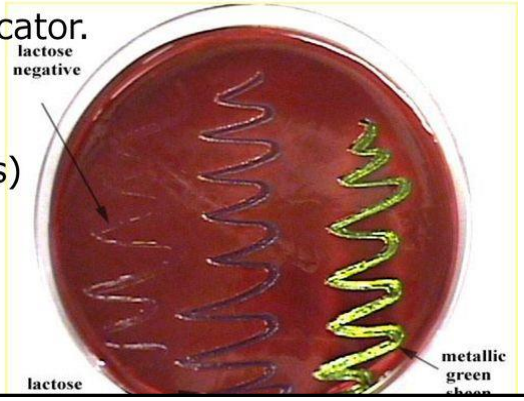
EMB (Eosin Methylene Blue) Agar

Contains peptones, lactose, sucrose, and the **dyes eosin** and **methylene blue**.

Eosin dye inhibits growth of Gram+ bacteria.

Methylene blue acts as indicator.

Lactose and sucrose are nutrients (fermentable carbohydrates)



- **Blood agar (Enrichment differential media)**
- Enrichment : for fastidious bacteria
- Differential : alpha hemolysis & beta hemolysis
- Add 5 ml blood \100 ml base of blood agar

LAB 3:

BACTERIAL SAINTS

Stains (Dye) is any colored, organic compound, that used to stain tissues, cells, cell components, cell contents, or microorganisms. Stains are an important tool for biological researchers, particularly microbiologists. The stain may be natural or synthetic. The object stained is called the substrate. The small size and transparency of microorganisms and cells make them difficult to see even with the aid of a high-power microscope. Staining facilitates the observation of a substrate by introducing differences in optical density or in light absorption between the substrate and its surroundings or between different parts of the same substrate.

Bacterial smear:

The preparation of a smear is required for many laboratory procedures, including the staining. A smear can be prepared from a solid or broth medium.

Bacterial smear is a thin layer of bacteria placed on a slide for staining. Preparing the smear requires attention to several details that help to prevent contamination of the culture and ensure safety to the preparer.

Bacterial smear preparation:

1- Handle a clean slide from the edge, label the slid at the bottom part (use wax or diamond pencil)

2- Hold the loop as you would hold a pencil, and place it nearly vertically into the blue tip portion of a Bunsen burner flame for a few seconds or until it glows red hot. Allow the loop to cool several seconds.

3- Bacterial sample:

a- From broth culture

shake the tube well to mix the culture, remove the cap and place the tip of the tube in the flame for a few seconds, this will burn off any dust kill any airborne organisms that might happen to fall into the tube.

Dip the loop carefully into the liquid, remove the loop, re-flame the tip of the tube briefly, then replace the cap, transfer the loopful of broth to the center of the slide and spread over the target circle.

b- From solid culture

Place loopful of water on the slide then transfer inoculums to the water and homogenize the smear.

*Note: It is preferred that the microbial culture used in the staining process is a recent one, since the old one gives false results.

4-Sterile the loop.

5-Leave the smear to dry at room temperature (by air).

6-Heat fixation: After drying, Pass the slide over the flame to fix the smear (avoid over heating of the slide). This procedure will kill any bacteria that may still be alive, facilitates stain penetration, and fixes cells to the slide and prevent removal of these cells during washing between two sequent steps of staining.

Types of stains:

Stains are selected for staining based on the chemical properties of the dye and the specimen being observed, which determine how the dye will interact with the specimen. stain consisting of a colored ion (a chromophore) and a counter ion to balance the charge. Attachment of the chromophore part of the stain complex to a cellular component represents the staining reaction.

Depending upon the stain, the chromophore can be either positively charged (cationic) and have an affinity for negative ions or negatively charged (anionic) with an affinity for positive ions.

Thus, stains are divided into three groups according to the ions charge which carry the color of the stain.
These two groups are:

1-Basic (cationic) stain: these stain act well on bacteria because they have color bearing ions that are positively charged. These ions will attract to the bacterial cell wall which is negatively charged, combine with it and stain the cell. e.g. methylene blue, safranin, crystal violet, malachite green.

2-Acidic (anionic) stain: these stain that are negatively charged (have anionic chromophore) do not attract to the bacterial cell wall (which is negatively charged}, therefore they do not stain the cell (do not penetrate the bacterial cell but they make the background area around a cell opaque or dark) and this process is called "negative staining. e.g. Eosin, Nigrosine, India ink, eosin, rose Bengal (These stains show or clarify the shape and size of the cell and extracellular structure such as capsules).

3-Neutral stain:

In neutral stain, both cation and anion are colored, such that net charge is neutral. Neutral stain is a salt of acidic and basic stain. e.g. giemsa stain (combining the basic dye methylene blue and the acid dye eosin). Giemsa stain when it is combined with Wright stain form Wright-Giemsa stain. It can be used to study the adherence of pathogenic bacteria to human cells.

Classification of stains according to the purpose of use:

1-Simple stains:

Simple staining implies the use of only a single stain, which is usually enough to reveal the morphological features of most bacterial cells, including relative size, shape, and characteristic arrangements for groups of cells. e.g. crystal violet, methylene blue and carbolfuchsin.

1- Apply a couple drops from crystal violet to a fixed smear.
2- Let it for 1 min.
3- Washed off with tap water gently.
4- Let the slide to dry at room temperature.
5- Examine under $100X$ (oil immersion lens).

2-Differential stains:

Stains that react differently with different cell types are known as differential stains. In the differential stain more than one stains are involved in the staining proses.

The most important and widely used differential stain for bacteria is the Gram stain (named according to the name of its discoverer scientist "Hans Christian Gram" in 1884). Based on their reaction to the Gram stain, bacteria can be divided into two groups: Gram positive and Gram negative. The differential response to the Gram stain is based on fundamental differences in the cell wall structure and composition of cells. In a manner quite like the Gram stain, the Acid-Fast stain differentiates an important group of bacteria, the mycobacteria, based on lipid content of their cell wall.

Gram staining:

1- apply crystal violet (primary stain) to heat fix bacterial smear for 1 min.
   *Crystal violet will stain all vegetative cells with purple color.
2- Wash off gently the stain by distal water.
3- apply Gram's Iodine solution for 1 min.
   *Gram's Iodine as "mordant solution" that form an insoluble complex by binding to primary stain. The resultant crystal violet-iodine (CV-I) complex which cannot be removed easily after treating with alcohol in decolorization step form G +ve bacteria due to composition of their cell wall, in contrast this complex is washed out from G -ve bacteria. The color of all cells will appear purple-black at this point.
4- Wash off gently the stain by distal water.

5- Cover the smear with 95% alcohol by a couple drops and let it stand for 30 sec.

*Ethyl alcohol, 95%: an organic solvent serves a dual function as a lipid solvent and as a protein dehydrating agent. Its action is determined by the lipid concentration of the bacterial cell wall. it removes or decolorizes the purple color of the primary stain from G -ve cells but not from G +ve cells, this process is called "decolorization" and ethanol is called decolorizer agent. Acetone can be used as alternative decolorizer.

7- Wash off gently by distal water.

8- Cover the smear with Safranin for 30 sec.

* Safranin stain is the counter stain "secondary stain" that used to stain the cells that lost the primary stain after treating with alcohol. These cells are colored with red color.

9- Wash off gently by distal water

10- Let it dry at room temperature.

Factors affecting the efficiency of Gram staining process

1- Bacterial smear thickness: a smear with an appropriate amount must be prepared, because thick smear does not allow to determine the cells shape, arrangement and other details. Furthermore, the thick smear cannot be removed by washing. On the other hand, the thin smear may lose stains easily, therefore it may lead to false results.

2- Smear fixation: if the smear overheated during heat fixing, the cell walls will be ruptured and that may lead to fails results.

3- Concentration and freshness of reagents may affect the quality of the stain: these solutions should be fresh (recently prepared) or must be filtered before using.

4- The nature and age of the bacterial culture: Gram stain is reliable only on cells from cultures that are in the exponential phase of growth. Older cultures contain more ruptured and dead cells. So, cells from old cultures may stain Gram negative even if the bacteria are Gram positive.

5- The washing water: since the slide must be washed during subsequent steps of staining, it must be take attention to avoid excessive washing because water that left on the slide will dilute reagents, particularly Gram's iodine.

*NOTE: False results that may resulted from staining are: -
1- False positive staining where the G -ve may appear as G +ve
Because ethanol is used less than 30 sec and the (CV-I) complex has not been removed.
2- False negative staining G+ ve may appear as G-ve.
Because iodine solution may be used less than 1min and the (CV-I) complex has not been formed perfectly and not contacted to the cell wall for that the crystal violet has been easily removed by alcohol and the cells stained with safranin and appear as G- ve.

Theories explaining the Gram staining principles:
Gram positive cells take up the crystal violet, which is then fixed in the cell with the iodine mordant. This forms a crystal-violet iodine complex which remains in the cell even after decolorizing. It is thought that this happens because the cell walls of G +ve organisms include a thick layer of protein-sugar complexes called peptidoglycans. This layer makes up 60-90% of the G +ve cell wall. Decolorizing the cell causes this thick cell wall to dehydrate and shrink, which closes the pores in the cell wall and prevents the stain from exiting the cell. At the end of the gram staining procedure, gram positive cells will be stained a purplish-blue color.

Gram negative: cells also take up crystal violet, and the iodine forms a crystal violet-iodine complex in the cells as it did in the G +ve cells. However, the cell walls of G -ve organisms do not retain this complex when decolorized. Peptidoglycans are present in the cell walls of G -ve organisms, but they only comprise 10-20% of the cell wall. Gram negative cells also have an outer layer which gram

positive organisms do not have; this layer is made up of lipids, polysaccharides, and proteins. Exposing gram negative cells to the decolorizer dissolves the lipids in the cell walls, which allows the crystal violet-iodine complex to leach out of the cells. This allows the cells to subsequently be stained with safranin. At the end of the gram staining procedure, gram negative cells will be stained a reddish-pink color.

Acid Fast Stain:

The acid-fast stain is another differential stain that used to identify the acid-fast organisms such as members of the genus *Mycobacterium*.

Acid-fast organisms are characterized by wax-like, nearly impermeable cell walls; they contain mycolic acid and large amounts of fatty acids, waxes, and complex lipids. Acid-fast organisms are highly resistant to disinfectants and dry conditions.

Because the cell wall is so resistant to most compounds, acid-fast organisms require a special staining technique. The primary stain used in acid-fast staining, carbolfuchsin, is lipid-soluble and contains phenol, which helps the stain penetrate the cell wall. This is further assisted by the addition of heat. The smear is then rinsed with a very strong decolorizer, which strips the stain from all non-acid-fast cells but does not permeate the cell wall of acid-fast organisms. The decolorized non-acid-fast cells then take up the counterstain.

1- Cover the smear with carbol fuchsin and steam it over a boiling water bath for 8 minutes. Add additional stain if stain boils off.

2- Cooldown the slide then decolorizes with acid alcohol for 15 to 20 sec.

3- Stop decolorization action of acid-rinsing briefly with water.

4- Apply the counter stain (methylene blue) for 30 sec.

5- Rinse briefly with water to remove excess methylene blue.

6- Blot dry with bibulous paper. Examine under oil immersion.

3- Special stains:

special stains are used to stain specific structure of bacteria, such as spore or flagella and helping to identify the presence of capsule. The three most common special stain are negative staining for Capsule, spore staining, and flagella stain.

1- Negative stain:

like nigrosine, India ink, eosin, Rose Bengal do not penetrate the bacterial cell but they make the background area around a cell opaque or dark. So, these stains show or clarify the shape and size of the cell and extracellular structure such as: capsules.

The acidic dye nigrosine will be used to visualize the capsular or sheath that surrounds some bacteria. In general, the size and shape of microorganisms is often less distorted with indirect staining procedures, especially when sampled from a broth culture. Therefore, negative staining is useful whenever their need for morphology of individual bacteria is in question.

1- Preparing a clean, greaseless slide, put a small drop of nigrosine and mix it with a small drop from a broth culture or part of colony from solid culture.

2- Spread the drop across the slide using the edge of another slide as a spreader.

3- After air drying, the smear is observed using the high power lens 40*x*, or oil immersion if desired. The back ground should be blue - gray and the bacteria will be evident by the absence of any color.

2- Spore stain (Schaeffer-Fulton Stain):

Endospores are formed by a few genera of bacteria, such as *Bacillus* and *Clostridium*. By forming spores, bacteria can survive in hostile conditions. Spores are resistant to heat, dryness,

chemicals agent, and radiation. Bacteria can form endospores in approximately 6 to 8 hours after being exposed to adverse conditions.

Because of their tough protein coats made of keratin, spores are highly resistant to normal staining procedures. The primary stain in the endospore stain procedure is malachite green, is driven into the cells with aid of heating. Since malachite green is water-soluble and does not adhere well to the vegetative which disrupted by heat, the malachite green rinses easily from the vegetative cells, allowing them to readily take up the counterstain.

1- Primary stain: malachite green (steam over boiling water for 5 mints) heating allow malachite green to enter spore coat (like acid -fast cell walls are resistant to most staining reagents). Vegetative cells take up malachite green as well.

2- Rinse gently with water.

3- Spore coats of endospores retain stain (endospores remain green). Meanwhile water washes malachite green from vegetative cells (become clear).

5- Add counter stain (safranin) for 20 sec.

6- Rinse the slide gently with water.

7- Carefully blot the slide dry.

8- Observe the slide under the microscope, using proper microscope technique. Endospores will stain green. Vegetative cells will stain red.

3-Flagella stain (Leifson's stain):

The flagella stain allows observation of bacterial flagella under the light microscope. Bacterial flagella are normally too thin to be seen under such conditions. The flagella stains employ a mordant to coat the flagella with stain until they are thick enough to be seen. These staining techniques are typically very difficult.

1- First of all, take two hours old flagellated cell culture slant and

Lab 4 Staphylococcus

Introduction

Family: Micrococceae (consists of Gram-positive cocci, arranged in tetrads, clusters)

Genus: Staphylococcus Term "staphylococcus" derived from Greek: Staphyle = bunch of grapes and Kokkos = berry, meaning bacteria occurring in grapelike clusters or berry.

Classification

Based on pathogenicity:

Pathogenic: includes only one i.e., *S. aureus*

Non-pathogenic: includes *S. epidermidis, S. saprophyticus, S. albus, S. citrus, S. hominis,* etc.

Based on coagulase production:

Coagulase positive: S. aureus

Coagulase negative: *S. epidermidis, S. saprophyticus S. aureus*

Based on pigment production:

- ***S. aureus*:** golden-yellow pigmented colonies
- ***S. albus*:** white colonies
- ***S. citrus*:** lemon yellow colonies

S. aureus

Natural habitat: Nostril and skin

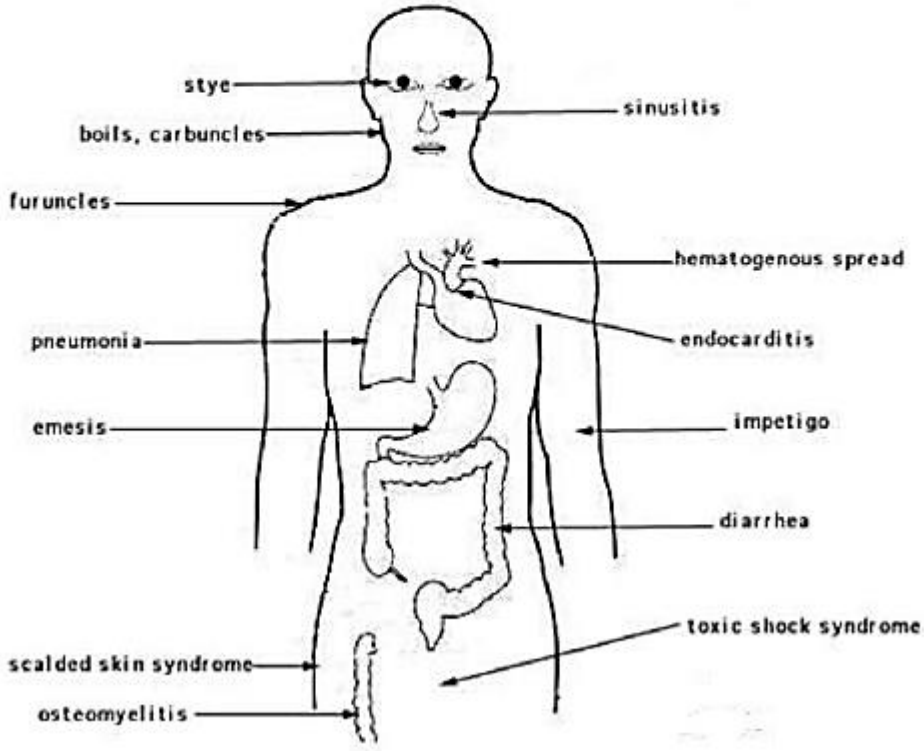
Morphology:

- Gram-positive, cocci, 0.5-1.5µm in diameter; occur characteristically in group, also singly and in pairs.

- Form irregular grapelike clusters (since divide in 3 planes).

- Non-motile, non- sporing and few strains are capsulated.

Clinical Syndromes



Laboratory Diagnosis

Specimens: -

- **Pus:** from wound or abscess or burns.
- **Nasal Swab:** from suspected carrier.
- **Food:** to diagnose staphylococcal intoxication.
- **Blood:** to diagnose endocarditis and bacteremia.
- **Sputum:** to diagnose lower respiratory tract infection.
- **Milk:** To detect mastitis.

STAPHYLOCOCI

Gram's Stain:

- These are spherical cocci.
- Approximately 1 um in diameter.
- Arranged characteristically in grape like clusters.
- They are non-motile and non-sporing.
- A few strains possess capsules.

Culture

- Aerobes and facultative anaerobes.
- Opt. Temp. For growth= 37°C.
- Opt. pH for growth= 7.5
- On Nutrient agar, - golden yellow and opaque colonies with smooth glistening surface, 1-2 mm in diameter.

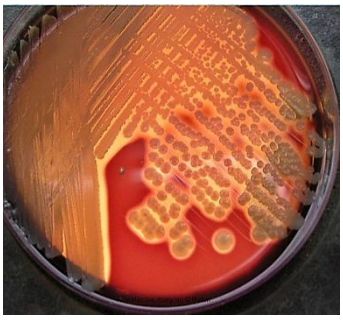
On nutrient agar

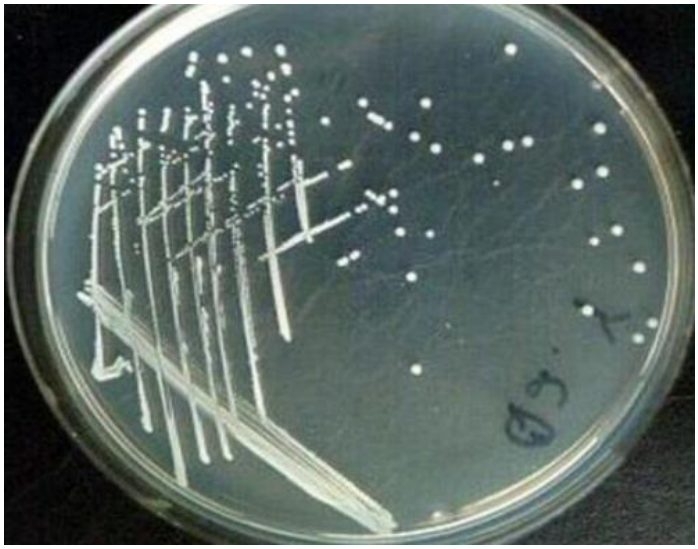
Staph. auerus form golden yellow colonies.

On blood agar

Staph. auerus produce complete haemolysis
(clear zone around the growth)

Staph epidermidis
Staph saprophyticus } non-haemolytic
(no change on blood agar)





Staph epidermidis white colonies on nutrient agar

Staphylococcus saprophyticus

Culture

- Aerobes and facultative anaerobes
- Optimum temp. for growth= 37°C
- Optimum pH for growth= 7.4-7-5

On Nutrient agar

Golden yellow and opaque colonies with smooth glistening surface, 1-2 mm in diameter (max. pigment production 22 °C).

On Blood agar

Golden yellow colonies, surrounded by a clear zone of hemolysis (beta-hemolysis), esp. when incubated in sheep or rabbit blood agar in atmosphere of 20% CO2.

On MacConkey agar

Smaller colonies than those on NA (o.1-0.5 mm) and are pink colored due to lactose fermentation.

On Mannitol salt agar

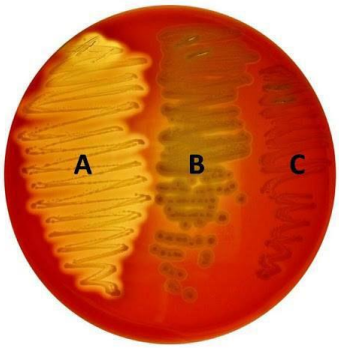
- S. aureus ferments mannitol and appear as yellow colonies.

- MSA is a useful selective medium for recovering *S. aureus* from fecal specimens, when investigating food poisoning.

* Beta-hemolysis (B): complete hemolysis. It is characterized by a clear (transparent) zone surrounding the colonies. *Staphylococcus aureus, Streptococcus pyogenes* and *Streptococcus agalactiae* are beta-hemolytic

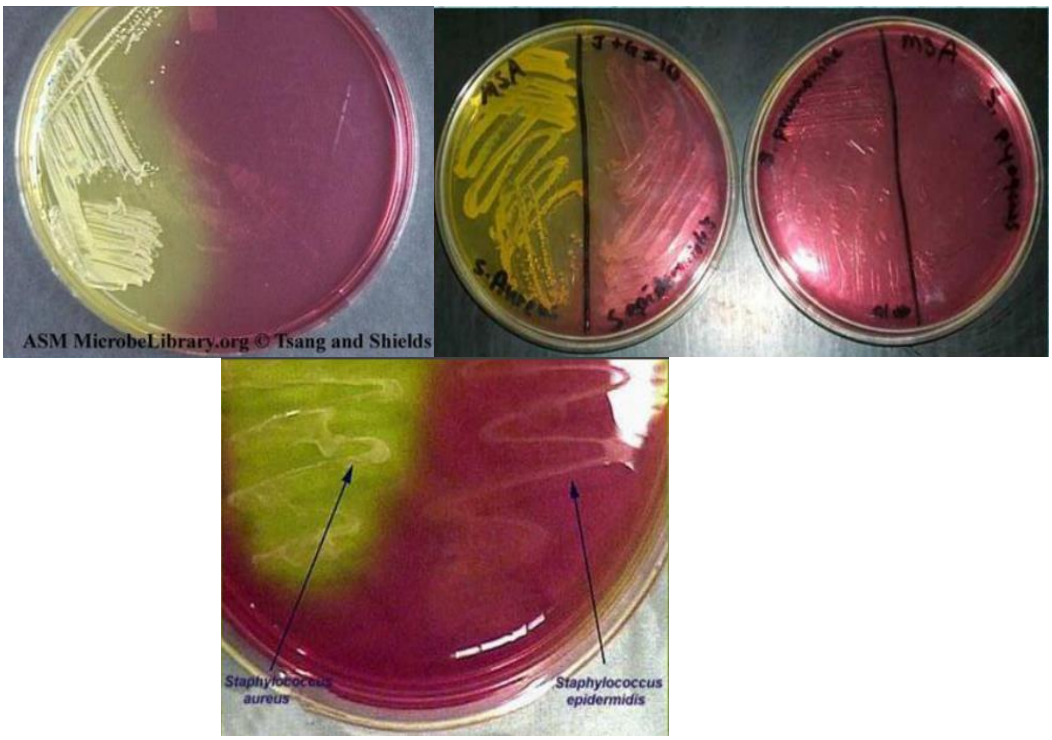
* Alpha-hemolysis (a): Partial hemolysis. Colonies typically are surrounded by a green, opaque zone. *Streptococcus pneumoniae* and *Streptococcus mitis* are alpha-hemolytic

* Gamma-hemolysis (y): no hemolysis occurs. There are no notable zones around the colonies. *Staphylococcus epidermidis* is gamma-hemolytic.

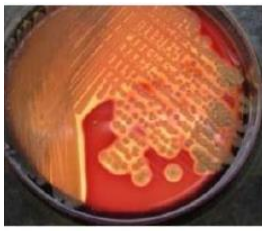


Growth on mannitol salt agar and mannitol fermentation

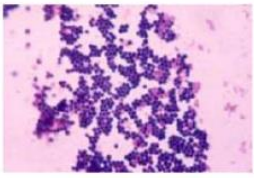
- MSA is a selective and differential medium in microbiology It encourages the growth of a group of certain bacteria while inhibiting the growth of others. It contains a high concentration (7.5%-10%) of salt (NaCl), making it selective for gram positive bacterium Staphylococci since this level of NaCl is inhibitory to most other bacteria.
- It is also a differential medium for mannitol-fermenting staphylococci, containing carbohydrate mannitol and the indicator phenol red a pH indicator for detecting acid produced by mannitol-fermenting Staphylococci. Staphylococcus aureus produce yellow colonies with yellow zones, whereas other Staphylococci produce small pink or red colonies with no color change to the medium.



Laboratory Diagnosis



- Culture and isolation: Specimens are cultured on BA plate and are incubated After incubation, BA plate is observed for significant bacterial growth (> 2mm in diameter)
- Then, Gram-staining is performed of the isolated organisms
- Then, subcultured on NA plate for further biochemical tests



Tube coagulase test:

- Mix 0.5ml of human plasma with 0.1ml of an overnight broth culture of *S. aureus*.
- Incubate the mix in a water bath 37°C for 3-6 hours.
- Result: plasma clots and doesn't flow if the tube is inverted.

Biochemical Properties

Indole test = negative

Catalase = positive

Oxidase = negative

Urease test = positive

Hydrolyze gelatin

Reduces nitrate to nitrite

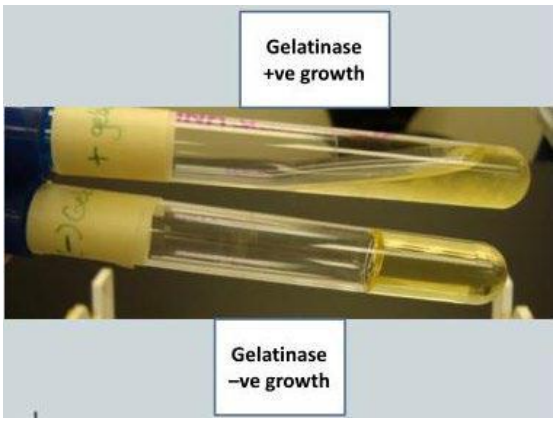
Phosphatase = positive

DNase test = positive

Coagulase test= positive

Gelatin hydrolysis test:

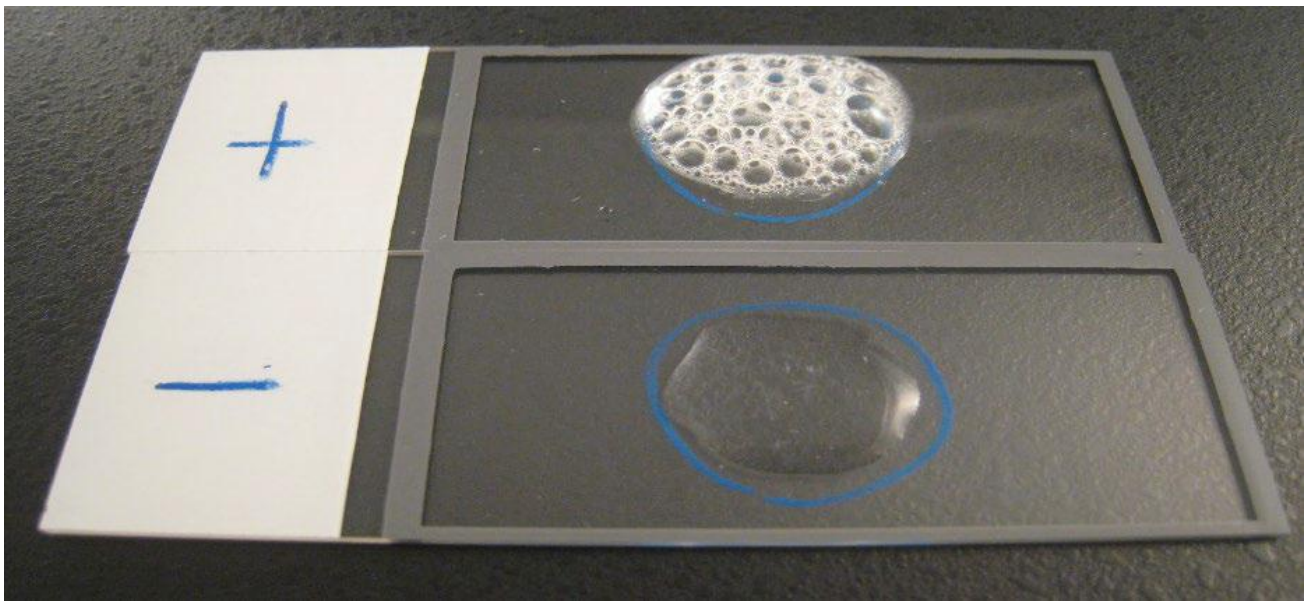
liquefaction of gelatin is used to detect the ability of an organism to produce gelatinase (proteolytic enzyme) that liquefy gelatin. Hydrolysis of gelatin indicates the presence of gelatinases. It distinguishes the gelatinase-positive, pathogenic *Staphylococcus aureus* from the gelatinase- negative, non-pathogenic *S. epidermidis.*



Biochemical activities

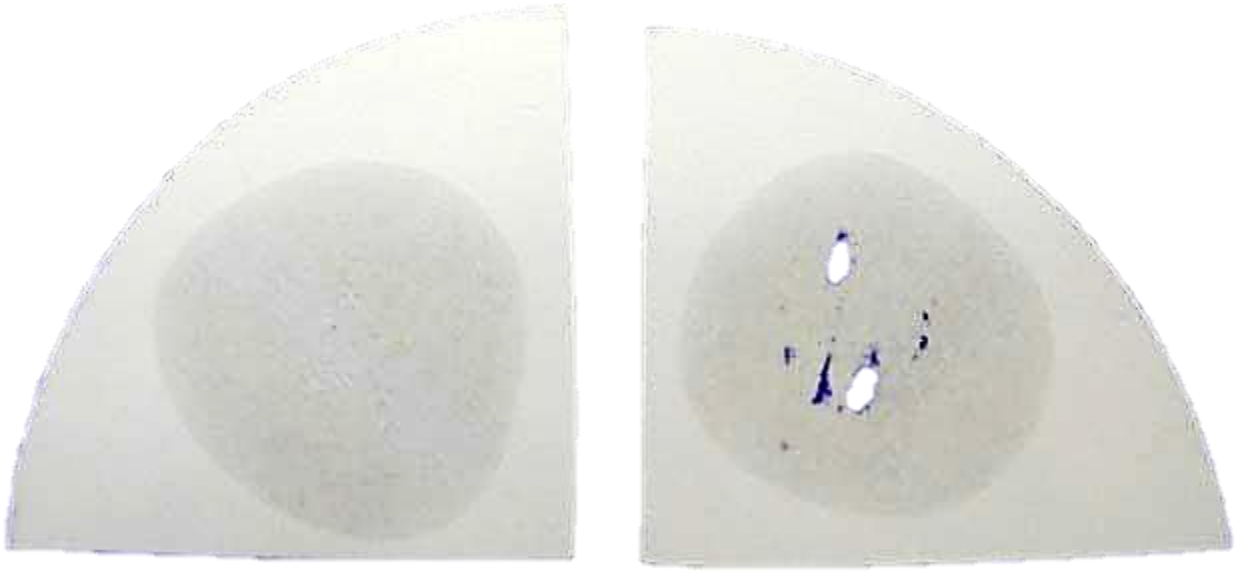
Catalase test: all spp of Staphyloccoci give positive catalase which differentiate it from Streptococci (catalase negative).

$$H2O2 \xrightarrow{catalase} H_2O + O_2$$



Note: catalase test not done on blood agar

Oxidase Test: The oxidase test identifies organisms that produce the enzyme cytochrome oxidase. Cytochrome oxidase participates in the electron transport chain by transferring electrons from a donor molecule to oxygen. The oxidase reagent contains a chromogenic reducing agent, which is a compound that changes color when it becomes oxidized.



-ve +ve

CONS

Coagulase Negative Staphylococci (CONS) that are commonly implicated as pathogens include:

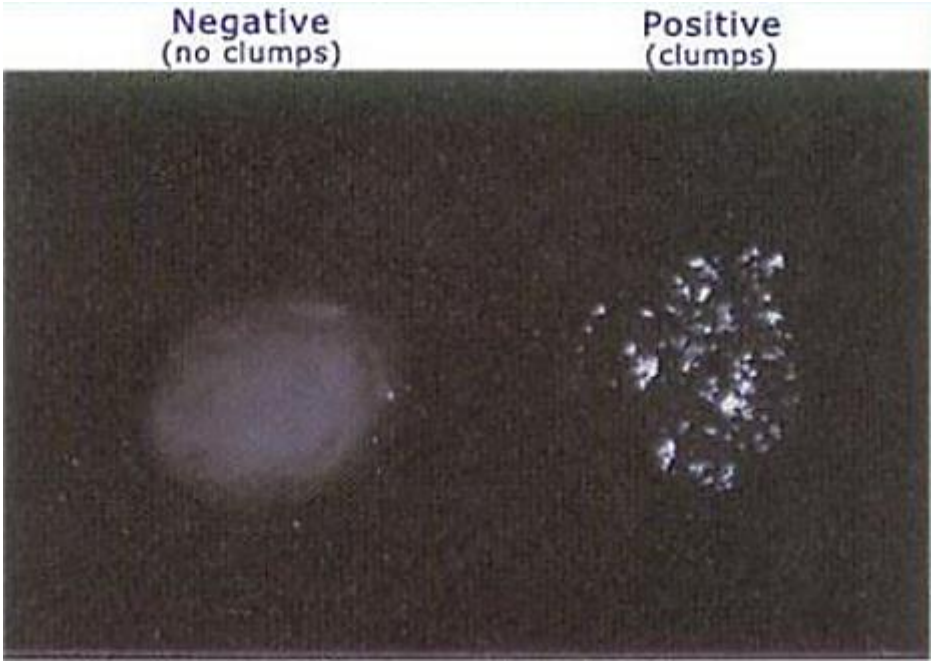
Staphylococcus epidermidis: causes infection of native heart valves and intravascular protheses.

Staphylococcus saprophyticus: causes urinary tract infections, mainly in sexually active women.

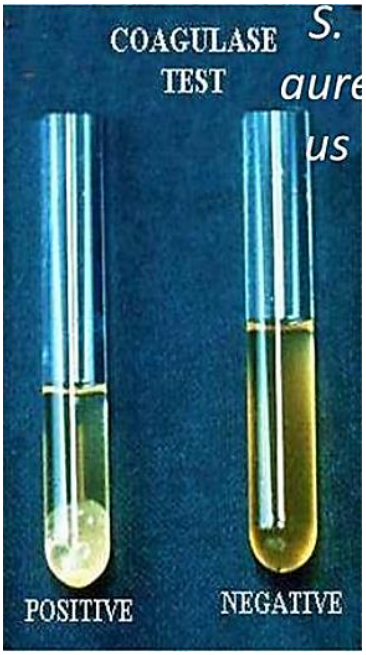
CONS that are less commonly implicated as pathogens include: *S. hominis, S. haemolyticus, S. cohnii, s. lugdunensis, S. saccharolyticus, S. schleiferi, S. simulans* and *S. warneri* .

Coagulase test

The coagulase test has traditionally been used to differentiate *Staphylococcus auerus* from coagulase-negative staphylococci. *S. aureus* produces two forms of coagulase (i.e., bound coagulase and free coagulase). Bound coagulase, otherwise known as "clumping factor", can be detected by carrying out a slide coagulase test, and free coagulase can be detected using a tube coagulase test.



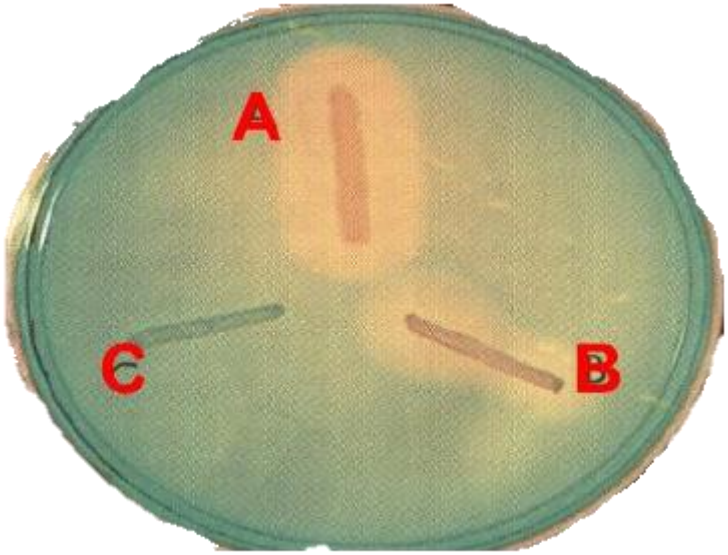
Slide coagulase (Bound) causes bacterial cells to agglutinate in the plasma



Free coagulase (tube) active enzyme produced by *S. aureus*
Fibrinogen Fibrin

DNase test:

DNase agar is a differential medium that tests the ability of organism to produce an exoenzyme, called deoxyribonuclease or DNase, that hydrolyzes DNA. DNase agar contains nutrients for the bacteria, DNA, and methyl green as an indicator. Methyl green is a cation which binds to the negatively-charged DNA Deoxyribonuclease allows the organisms that produce it to break down DNA into smaller fragments. When the DNA is broken down, it no longer binds to the methyl green, and a clear halo will appear around the areas where the DNase producing organism has grown. If we using DNase agar without indicator, Flood the plate with IN Hydrochloric Acid. Leave the plate to stand for a few minutes to allow the reagent to absorb into the plate. Decant excess hydrochloric acid and then examine the plate within 5 minutes against a dark background.



Deoxyribonuclease test

A. Positive - Staphylococcus aureus

B. Positive -Serratia marcescens

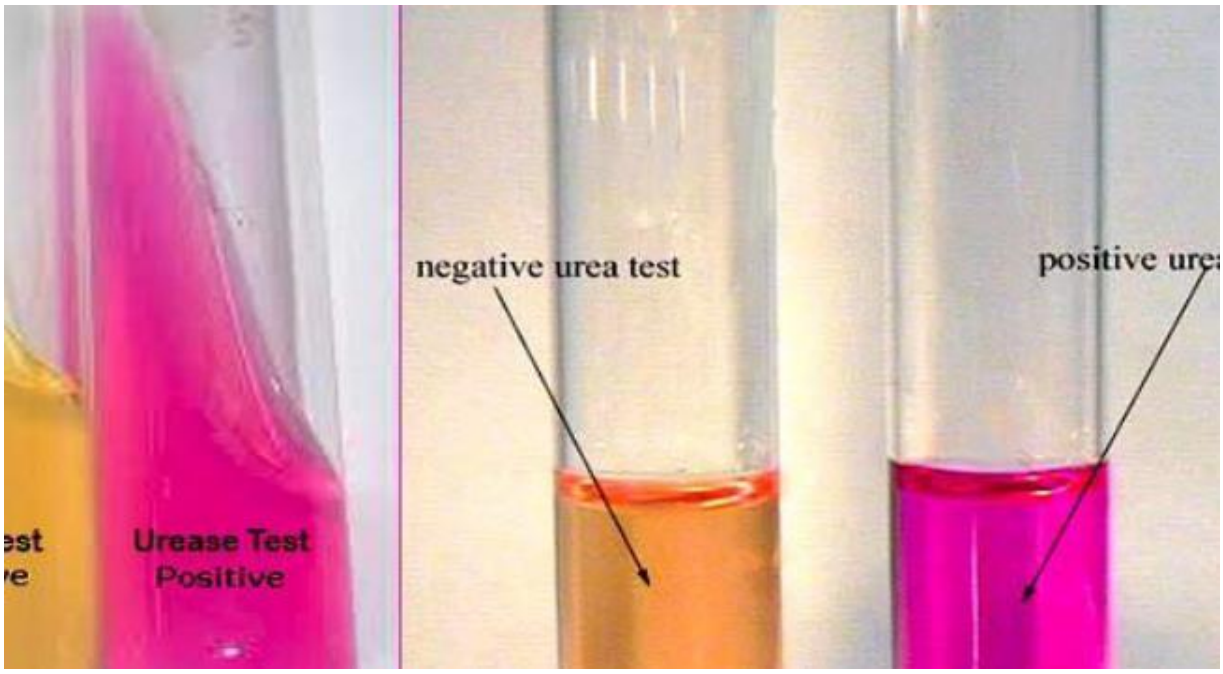
C. Negative -Staphylococcus epidermidis

UREASE TEST

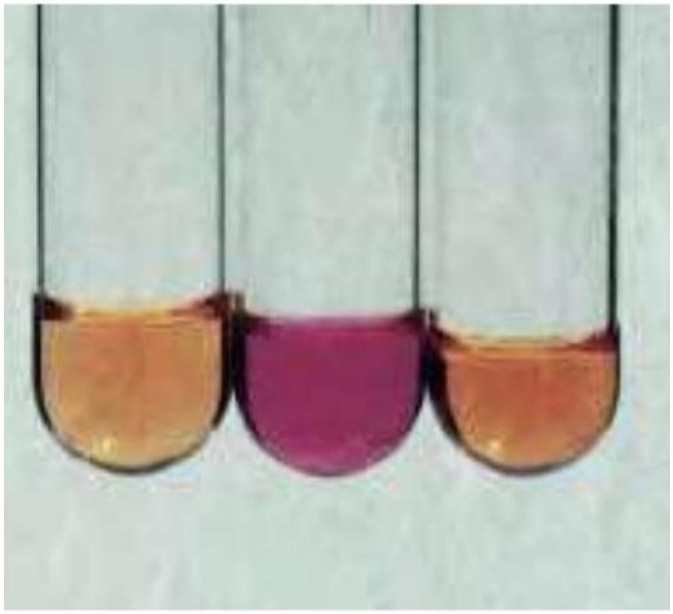
Aim: To determine the ability of microbes to degrade urea by urease.

Principle:

- Urea is diamide carbonic acid often referred as carbamide.
- The hydrolysis of urea is catalyzed by specific enzyme urease to yield 2 moles of ammonia.
- Urease attacks the nitrogen and carbon bond in urea and forms ammonia.
- The presence of urease is detected, when the organisms are grown in urea broth.
- Medium containing the pH indicator phenol red.
- Splitting of urea creates the alkaline condition which turns phenol red to deep pink in color.
- Mainly used for identification of Proteus spp. from other genus of lactose nonfermenting enteric organisms.



UREASE TEST



UREA BROTH
From left to right—uninoculated, positive (*Proteus*) *and negative*

Properties of Staphylococcus species				
Species	Catalase	Coagulase	Mannitol	Novobiocin
S. aureus	positive	positive	positive	sensitive
S. epidermidis	positive	negative	negative	sensitive
S. saprophyticus	positive	negative	negative	resistant

Lab 5 streptococcus

TAXONOMY

Rank	Scientific name
Family	• Streptococcaceae
Genus	• *Streptococcus*
Species (medically important spp.)	• *S. pyogenes* • *S. agalactiae* • *S. dysgalactiae* • *S. bovis* • *S. anginosus* • *S. sanguinis* • *S. mitis* • *S. mutans* • *S. pneumoniae*

OVERVIEW

- Streptococcus is a genus of coccus (spherical) Gram-positive bacteria.
- Cell division in this genus occurs along a single axis in these bacteria, thus they grow chains or pairs.
- Currently, over 50 species are recognized in this genus.
- This genus has been found to be part of the salivary microbiome.

GENERAL CHARACTERISTICS

- Gram +ve bacteria arranged in chains or pairs.
- Facultative anaerobes.
- Fastidious growth requirements.
- Catalase negative.
- Have beta, alpha or gamma hemolytic colonies on blood agar.
- In humans, it is major normal flora in oral cavity.
- In 1984, many bacteria formerly considered Streptococcus were separated out into the genera Enterococcus and Lactococcus.

Diseases caused by Streptococcus			
Group	**Hemolysis type**	**Streptococcus spp**	**Diseases**
A	β	*S. pyogenes*	Tonsillitis, Bronchopneumonia, Scarlet fever, Cellulites, Glomerulonephritis, Rheumatic fever.
B	β	*S. Agalactia* (CAMP +ve)	Neonatal endocarditis and meningitis.
C	β	*S. equisimilis*	Throat infection, Puerperal fever
D	α	*S. viridans*	Subacute endocarditis to heart failure.
	α	*S. Pneumoniae* (Optochin +ve)	Pneumonia.

Streptococcus spp.

• Samples

Pus (*S. pyogenes, Enterococcus spp. anaerobic streptococci*)

Urine (beta-hemolytic *streptococci & Enterococcus spp.*)

Sputum (*S. pyogenes, S. pneumoniae*)

Synovial fluid (*S. pyogenes, S. pneumoniae* and anaerobic streptococci)

Pleural fluid (*S. pyogenes, S. pneumoniae*)

Ascitic fluid (*S. pyogenes, S. pneumoniae, S. agalactiae & viridans streptococci*)

Blood (*S. pyogenes, S. pneumoniae & viridans streptococci*)

Throat swab (*S. pneumoniae, non-hemolytic streptococci & lactobacilli*)

CSF (*S. agalactiae & S. pneumoniae*)

Cervical swab (*S. pyogenes*)

Lab. Diagnostic tests:

1- Gram stain: G +ve cocci

2- Blood agar for hemolysis detection.

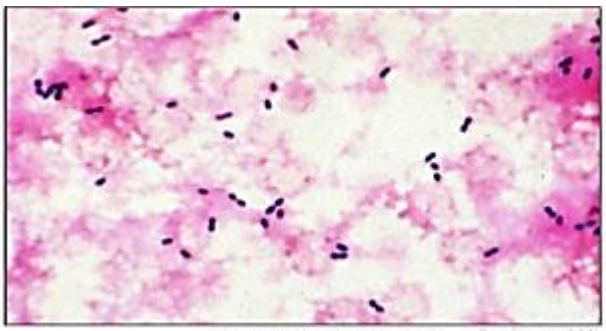
3- Bile solubility test:

The principle of the bile (sodium deoxycholate or sodium taurocholate) solubility test is to distinguishes Strepto. pneumoniae from all other alpha- hemolytic streptococci. Strepto. pneumoniae is bile soluble whereas all other alpha-hemolytic streptococci are bile resistant. Sodium deoxycholate (2% in water) will lyse the pneumococcal cell wall. Strepto. pneumoniae produces autolytic enzymes (autolysins) that cause lysis of older cells. These autolysins are responsible for the sunken centers that are observed in older Strepto. pneumoniae colonies on agar media. The bile solubility test uses bile salts to accelerate the lytic process.

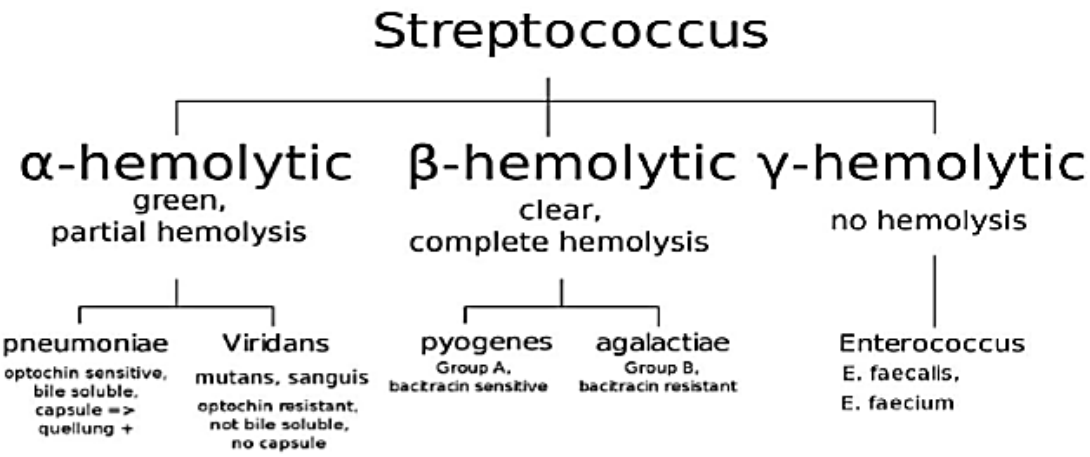
Microscopic feature

All streptococci are Gram +ve in chains or pairs except *S. pneumoniae* it is Gram +ve diplococci.

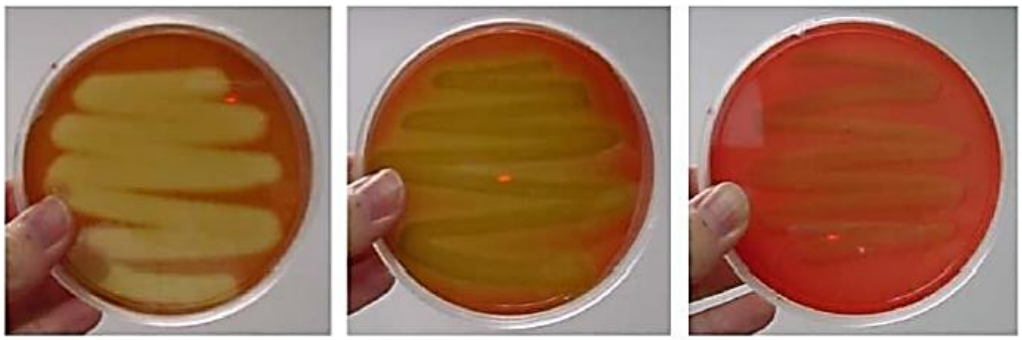
S. pyogenes



S. pneumoniae

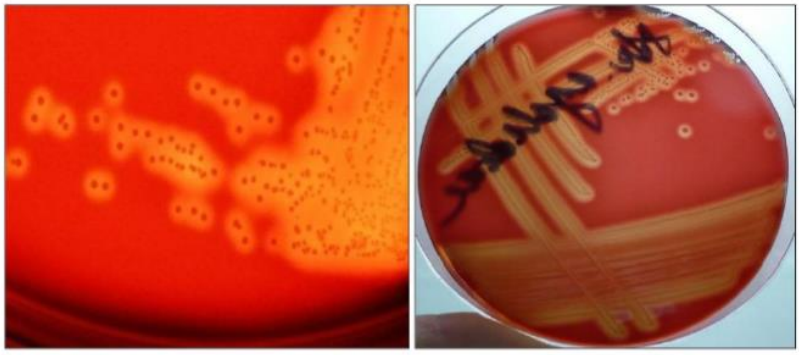


HEMOLYSIS ON BLOOD AGAR



Beta Hemolysis | Alpha Hemolysis | Gamma Hemolysis

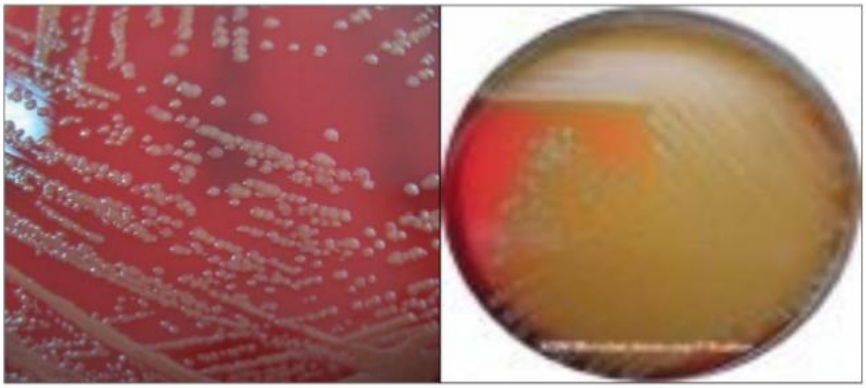
Streptococcus spp.



S. pyogenes
0.5 – 1 mm in diameter, colorless, dry, shiny or mucoid, beta-hemolytic colonies

S. pneumoniae
2 mm in diameter, grey mucoid colonies, small zone of beta-hemolysis.

Streptococcus spp.



S. pneumoniae
smooth, glistening, wet looking, mucoid colonies

S. pneumoniae
alpha-hemolytic colonies

4- Streptokinase test:

Kinases (also known as fibrinolysins) have the opposite effect of coagulase. Streptokinase is the name of a kinase produced by streptococci. Thus, the positive indication of this test is the lysis of the plasma clot.

In addition to the Coagulase test the Streptokinase teat help to differentiate Streptococcus from Staphylococcus.

* All species of Streptococcus are producing streptokinase except S. pneumoniae (-) To make the test prepper a clot from plasma by adding Cacl2 then add bacterial growth to see streptokinase production.

* **Clot test** = 0.2 ml human plasma + 0.25 (Cacl2) + 0.8 saline + 0.5 ml culture

* **Control** = 0.2 ml human plasma + 0.25 (Cacl2) + 0.8 saline + 0.5 ml saline.

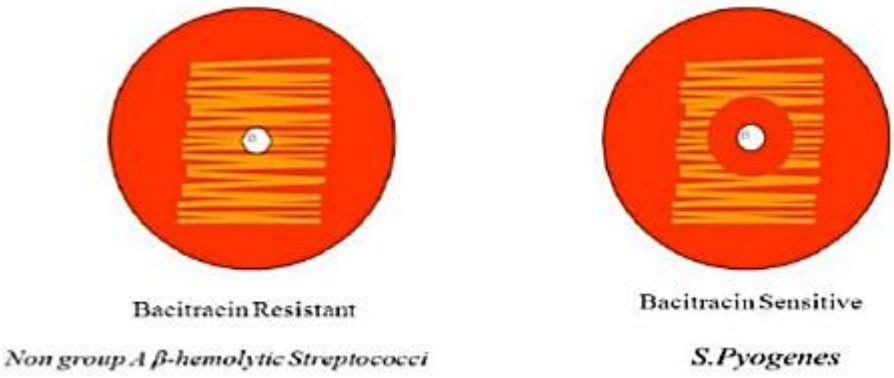
5- Carbohydrate fermentation (To differentiate between Strept. spp.)

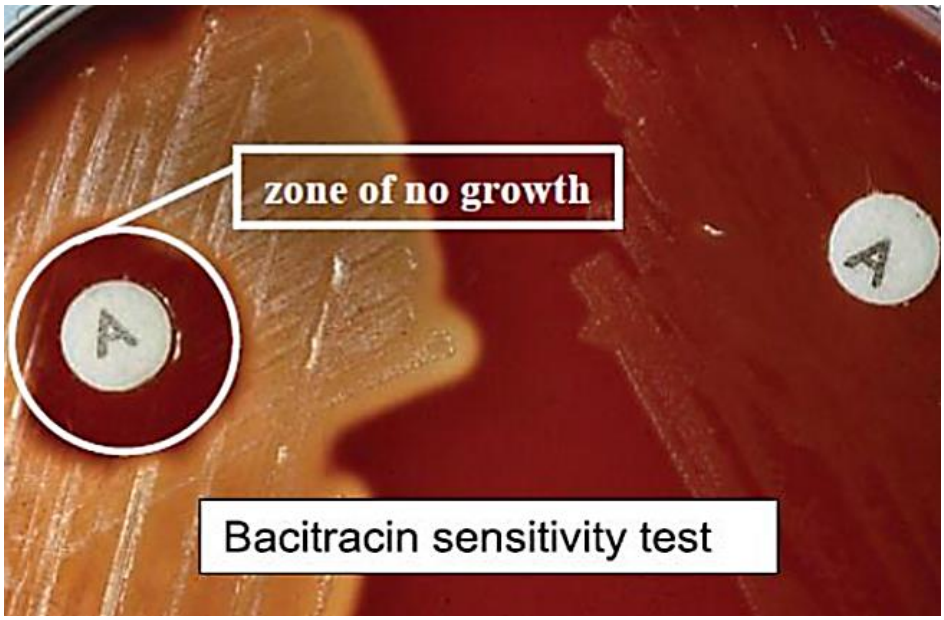
Because Strept. are fastidious a Muller-Hinton medium with CHO is used for fermentation, the indicator is bromothymol blue and the sugars that used will be glucose, inulin, mannitol and lactose.

BIOCHEMICAL TESTS

1. Catalase test: All Streptococcus spp. are catalase negative.

2. Bacitracin test: used to determine the effect of a small amount of bacitracin (0.05 IU not higher) on an organism. Streptococcus pyogenes (Group A Streptococci) is inhibited by the small amount of bacitracin in the disk; other beta- hemolytic streptococci usually are not.





3. Optochin test: *S. pneumoniae* strains are sensitive to optochin so that it is used to differentiate S. pneumoniae from other alpha-hemolytic streptococci.

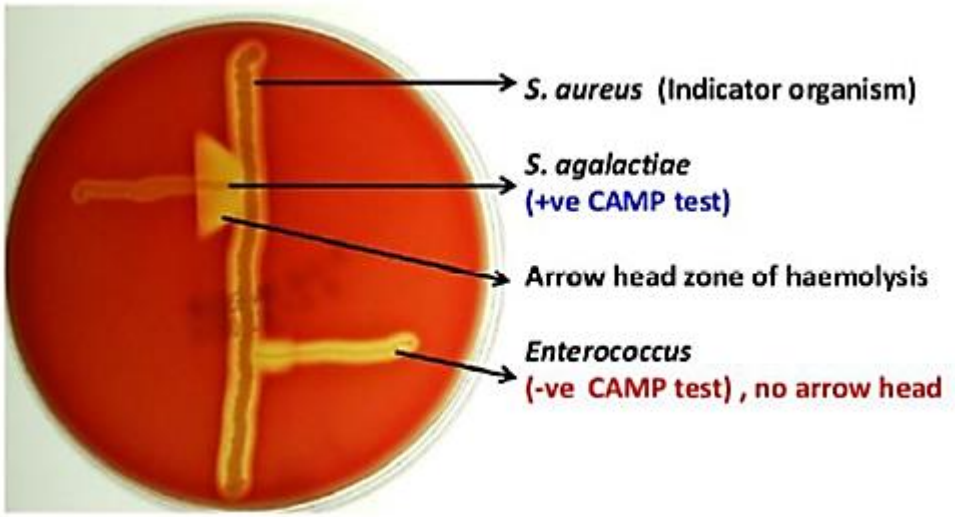
***Streptococcus* spp.**

Bacitracin test
Streptococcus pyogenes

Optochin test
Streptococcus pneumoniae

4. Christie Atkins Munch-Petersen (CAMP) test:

is used for the presumptive identification of Group B beta-hemolytic streptococci, *(Streptococcus agalactiae).*

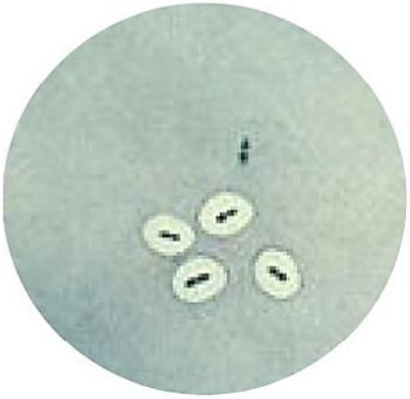


8- Growth in 6.5 % Nacl:

The salt tolerance test is performed using Tryptic Soy Broth with added sodium chloride (regular table salt) to create an overall salt concentration of 6.5%. It is a selective medium which tests the ability of an organism to survive in a salt-rich environment. (+ve) turbidity and (-ve) where there is no growth.

9- Quelling reaction:

also called the Neufeld reaction, is a biochemical reaction in which antibodies bind to the bacterial capsule of *Streptococcus pneumoniae*. The antibody reaction allows these species to be visualized under a microscope. If the reaction is positive, the capsule becomes opaque and appears to enlarge (see figure below) The test is performed by mixing bacterial growth w standard specific anti-capsular Ag. swelling of the cap indicates the (+ve) result.



LAB:6
Enterobacteriaceae

Enterobacteriaceae is a large family of Gram-negative bacteria. It was first proposed by Rahn in 1936, and now includes over 30 genera and more than 120 species.

Enterobacteriaceae includes, along with many harmless symbionts, many of the more familiar pathogens, such as *Salmonella*, *Escherichia coli*, *Klebsiella*, and *Shigella*. Other disease-causing bacteria in this family include *Enterobacter* and *Citrobacter*. Members of the Enterobacteriaceae can be referred to as enterobacteria or "enteric bacteria",

Members of the Enterobacteriaceae are bacilli (rod-shaped), and are typically 1–5 μm in length. They typically appear as medium to large-sized grey colonies on blood agar, although some can express pigments.

Most have many flagella used to move about, but a few genera are nonmotile. Most members of Enterobacteriaceae have peritrichous, type *I* fimbriae involved in the adhesion of the bacterial cells to their hosts.

They are not spore-forming

Citrobacter, Enterobacter, Escherichia, Hafnia, Morganella, Providencia, Klebsiella, Proteus, Salmonella, Shigella and Serratia.

General characteristics

1- Gram negative

2- Coccobacilli

3- facultative anaerobes

4- Non spore forming

5- capsule + or –

6- motile + or –

7- Catalase +

8- Oxidase –

Identification

To identify different genera of Enterobacteriaceae, a microbiologist may run a series of tests in the lab. These include:

- **Phenol red**
- **Tryptone broth**
- **Phenylalanine agar for detection of production of deaminase, which converts phenylalanine to phenyl pyruvic acid**
- **Methyl red or Voges-Proskauer tests depend on the digestion of glucose. The methyl red tests for acid end products. The Voges Proskauer tests for the production of acetylmethylcarbinol.**
- **Catalase test on nutrient agar tests for the production of enzyme catalase, which splits hydrogen peroxide and releases oxygen gas.**
- **Oxidase test on nutrient agar tests for the production of the enzyme oxidase, which reacts with an aromatic amine to produce a purple color.**
- **Nutrient gelatin tests to detect activity of the enzyme gelatinase.**

IMVIC test

IMVIC is a series of tests that are useful in the identification of enteric bacteria.

Tests include:

- 1.1= Indole test
- 2. M = Methyl red test (MR)
- 3. VI = Voges-Proskauer test (VP)
- 4. C = Citrate test

Indole test

Media culture :peptone water

Reagent ;kovacs

Enzymes: Tryptophanase

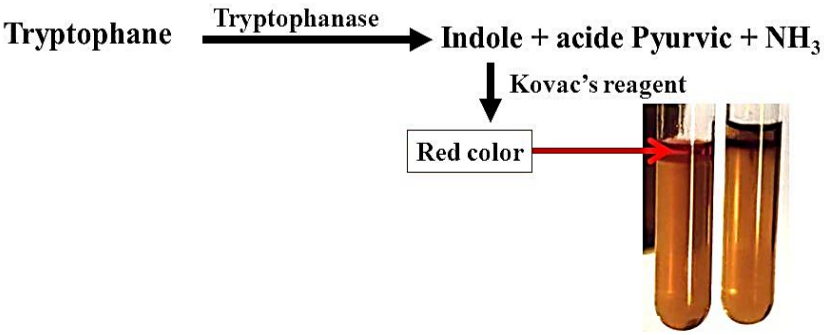
Substrate :Tryptophan

Positive result: red ring

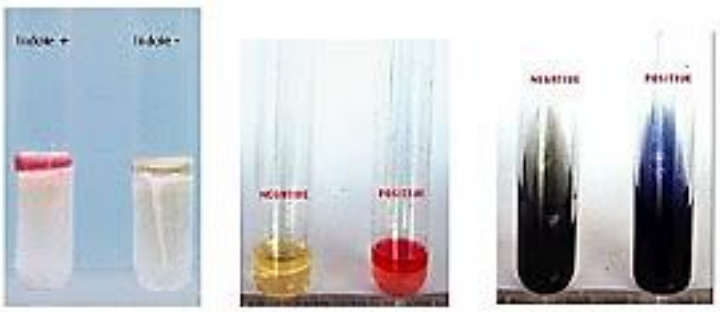
Negative result :no change

IMViC: Indole Test

- Principal
  - Some microorganisms can metabolize tryptophane by the tryptophanase



IMViC Test



Indole MR- VP Citrate

Methyl red – Voges proskauer test

Media culture :Glucose phosphate

Reagent : Methyl red& Barrits

Enzymes:

Substrate : Glucose

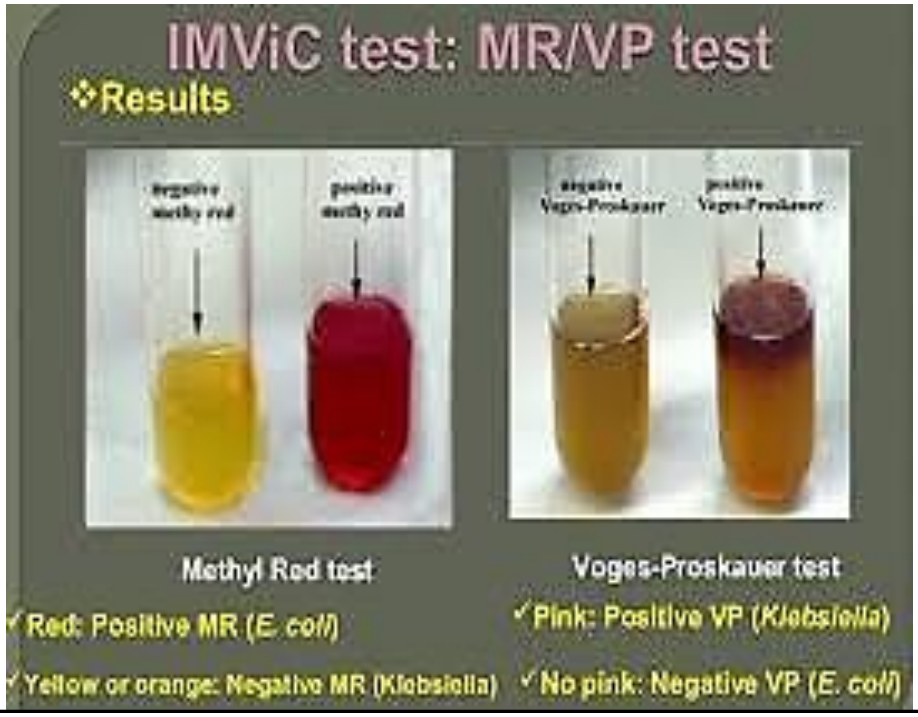
Positive result :M=red color ..V=pink color

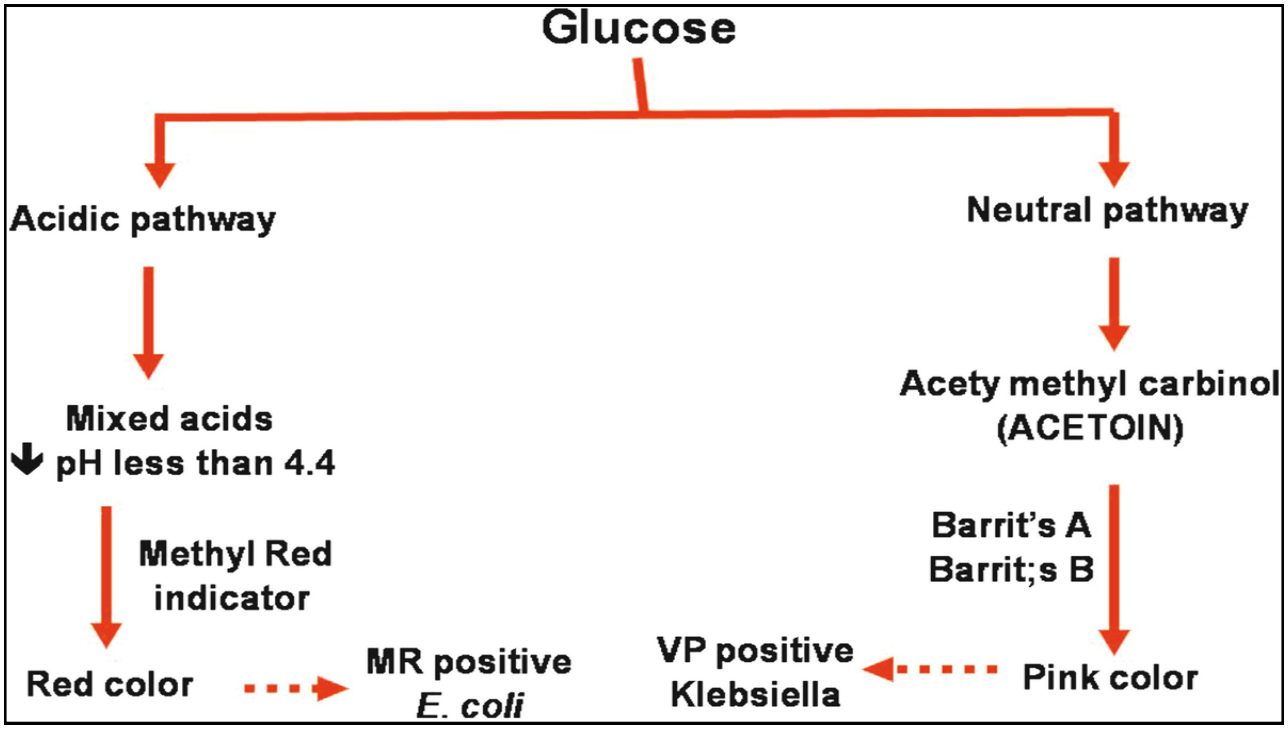
Negative result :yellow color

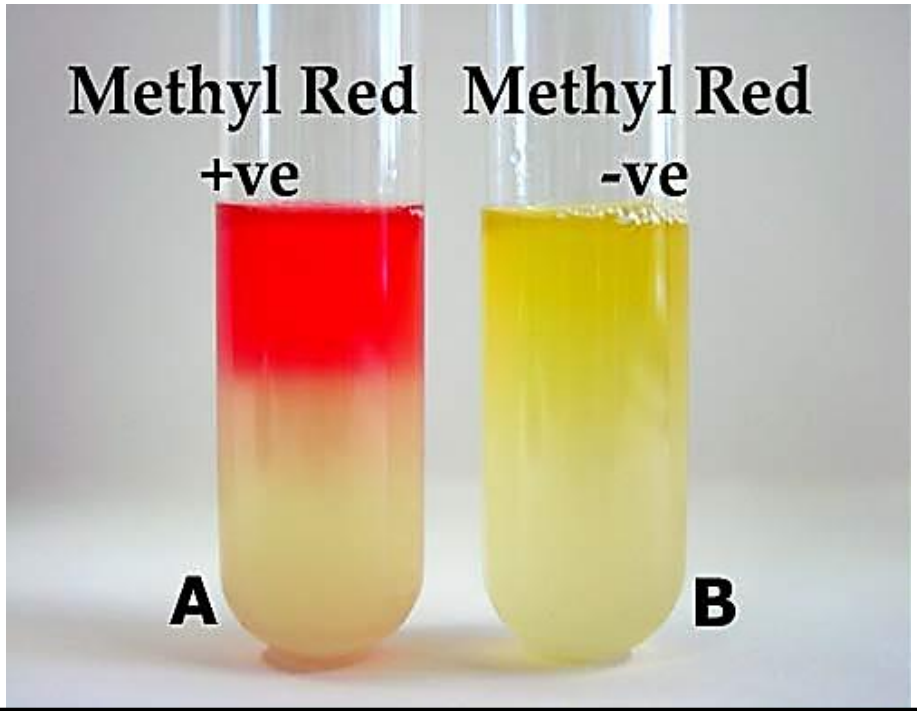
Methyl Red Test

- Used to determine the ability of a bacteria to oxidize glucose and produce stable acid end products
- Methyl red is a pH indicator (red at pH less than 4.4 and yellow at a pH greater than 6)
- The combination medium used for this test is the MR-VP (methyl red/Voges-Proskauer) broth

Acid production: positive methyl red
End products of neutral pH : positive Voges-Proskauer


IMViC test: MR/VP test
Results
Methyl Red test
Voges-Proskauer test
Red: Positive MR (E. coli)
Yellow or orange: Negative MR (Klebsiella)
Pink: Positive VP (Klebsiella)
No pink: Negative VP (E. coli)


Glucose
Acidic pathway
Neutral pathway
Mixed acids
pH less than 4.4
Methyl Red
indicator
Red color
MR positive
E. coli
Acety methyl carbinol
(ACETOIN)
Barrit's A
Barrit;s B
Pink color
VP positive
Klebsiella



Citrate utilization test

Media culture :Simmon citrate agar

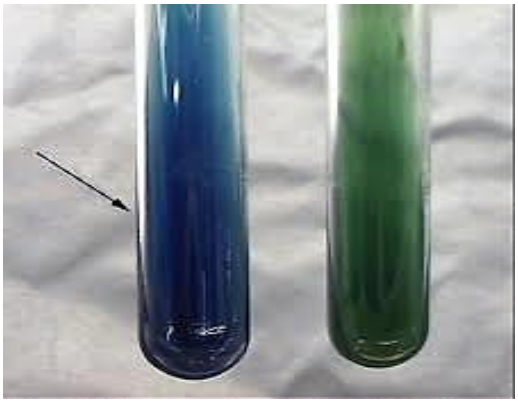
PH indicator :Bromothymol blue

Enzymes: Citrase

Substrate :Na-citrate

Positive result : Blue color

Negative result :NO growth



Fecal Coliforms

Indole positive

MR positive

VP negative

Citrate negative

Nonfecal Coliforms

Indole negative

MR negative

VP positive

Differentiation of enteric bacteria by IMViC tests:

Genus	Indole	M.R.	V.P.	Citrate
Escherichia	+	+	-	-
Enterobacter	-	-	+	+
Klebsiella	-	-	+	+
Salmonella	-	+	-	+
Proteus	+	+	-	+

Triple sugar iron agar (TSI test)

Triple Sugar Iron (TSI) Agar

Major ingredients of TSI:

Carbohydrates: Glucose - 0.1%
Lactose - 1%
Sucrose - 1%

Peptone (Nitrogen source)

Sodium thiosulphate (Sulfur source)

Ferrous sulfate (H2S indicator)

Phenol red (pH indictor) turn into yellow at acidic pH

It is pored into a test tube to produce a slant and a deep butt

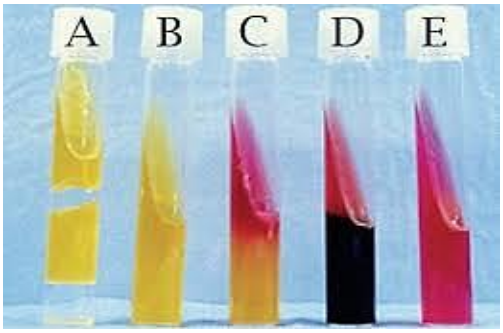
If only glucose is fermented, the slant and butt initially turn yellow from the small amount of acid produced; as the fermented products are subsequently oxidized the slant turns Alkaline (RED).

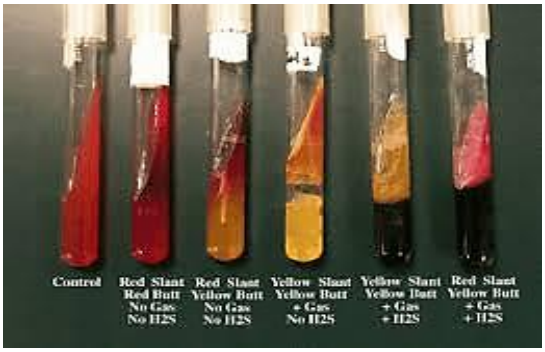
If lactose or sucrose is fermented so much acid is produced that slant and butt remain Acid (YELLOW).

The result read

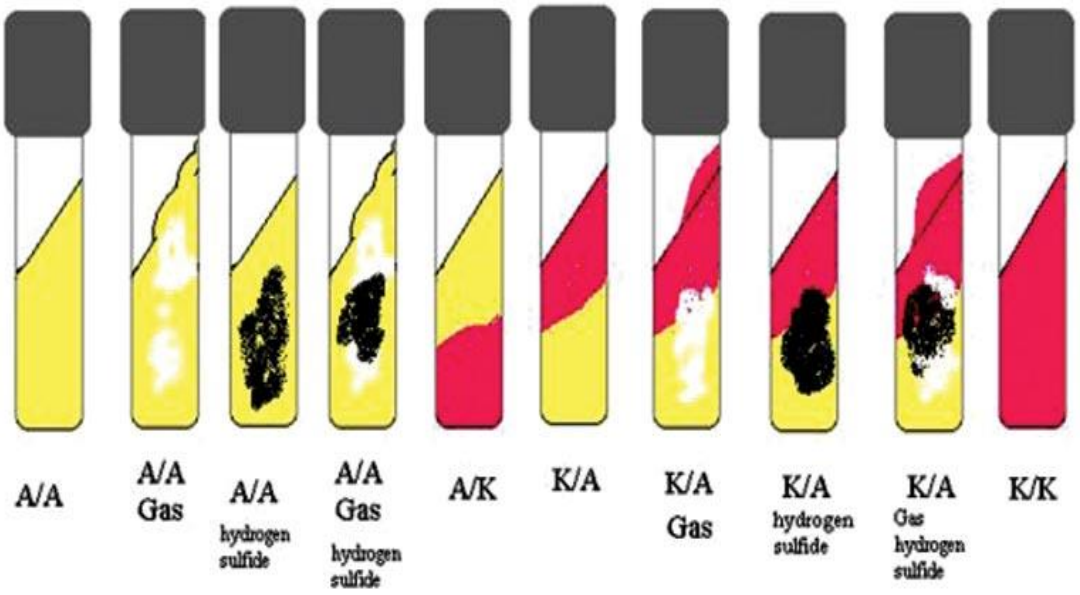
- Slant , butt , CO2 production ,H2S production
- Four result
- If lactose fermenter : Acid (yellow slant) \ Acid (yellow butt) .
- If lactose non fermenter : Alkaline (red slant)\ Acid (yellow butt) .
- CO2 production :Gas break the solid media
- H2S production : black sediment

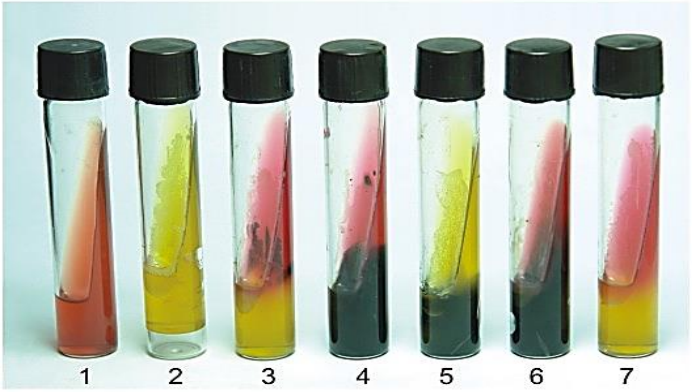
Escherichia coli
ATCC 25922


A
B
C
D
E


Control
Red Slant
Red Butt
No Gas
No H2S
Red Slant
Yellow Butt
No Gas
No H2S
Yellow Slant
Yellow Butt
+ Gas
No H2S
Yellow Slant
Yellow Butt
+ Gas
+ H2S
Red Slant
Yellow Butt
+ Gas
+ H2S

Triple Sugar Iron Agar (TSIA) Test

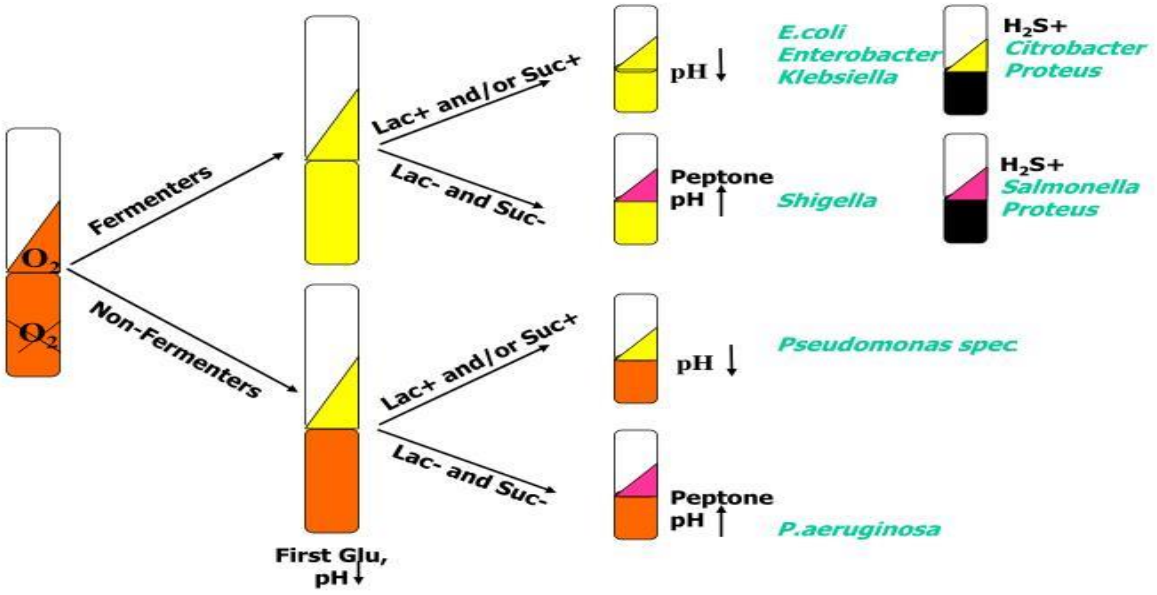


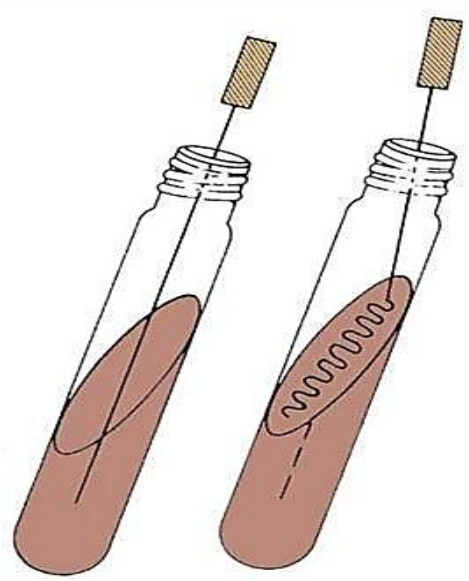


Triple Sugar Iron Agar (M021)

1. Control
2. Escherichia coli ATCC 25922
3. Salmonella Typhi ATCC 6539
4. Proteus vulgaris ATCC 13315
5. Citrobacter freundii ATCC 8090
6. Salmonella Typhimurium ATCC 14028
7. Shigella flexneri ATCC 12022

Understanding the Reactions in TSI-Agar





TSI agar

Triple Sugar Iron Agar

0.1% dextrose

1.0% sucrose

1.0% lactose

A B C D E

Figure 5-68

(a) Red/red (no sugar fermentation)

(b) Control

(c) Red/yellow (Glucose fermented but lactose and sucrose not fermented)

(d) Yellow/yellow (Glucose fermented. Lactose and/or sucrose fermented)

(e) Red/yellow with H_2S

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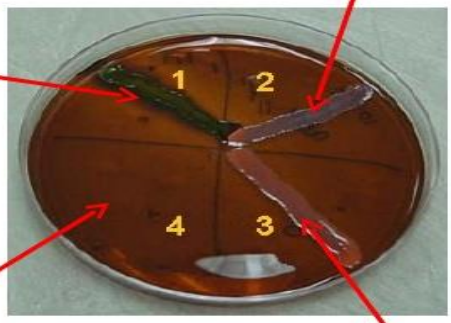
LAB: 6 PART 2
Enterobacteriaceae

- *Escherichia coli*
  General characteristics
- 1-Gram negative bacteria (red)
- 2- Coccobacilli
- 3- Facultative anaerobes
- 4-Motile
- 5-Non spore forming
- 6- Capsule -ve (microcapsule)
- 7-Catalase positive ,,,Oxidase negative ,,,
- 8- Urease negative
- 9-Lactose fermenter (pink colonies)(on MacConkey agar)

Levine Eosin-Methylene Blue (EMB) Agar

Lactose fermenter (high acid producer: black colonies)

Non-lactose fermenter



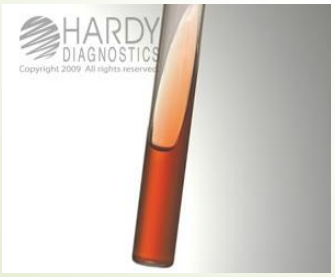
Gram positive (growth inhibited)

Lactose fermenter (low acid producer: pink colonies)

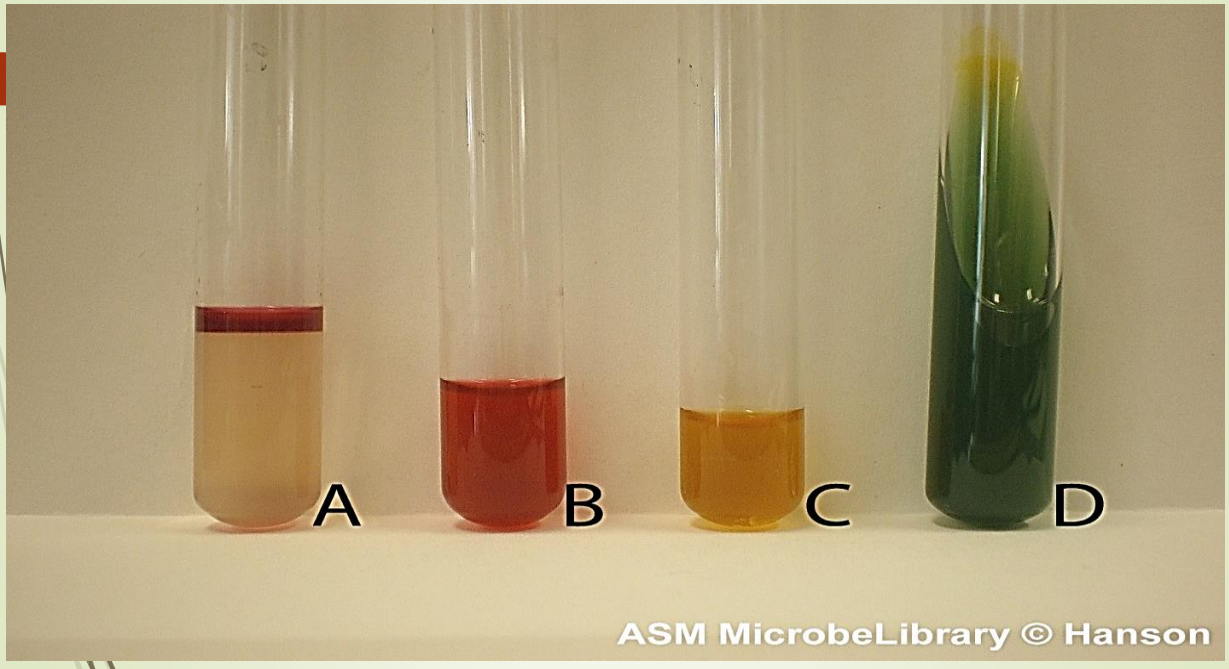
10-IMViC test: ++--

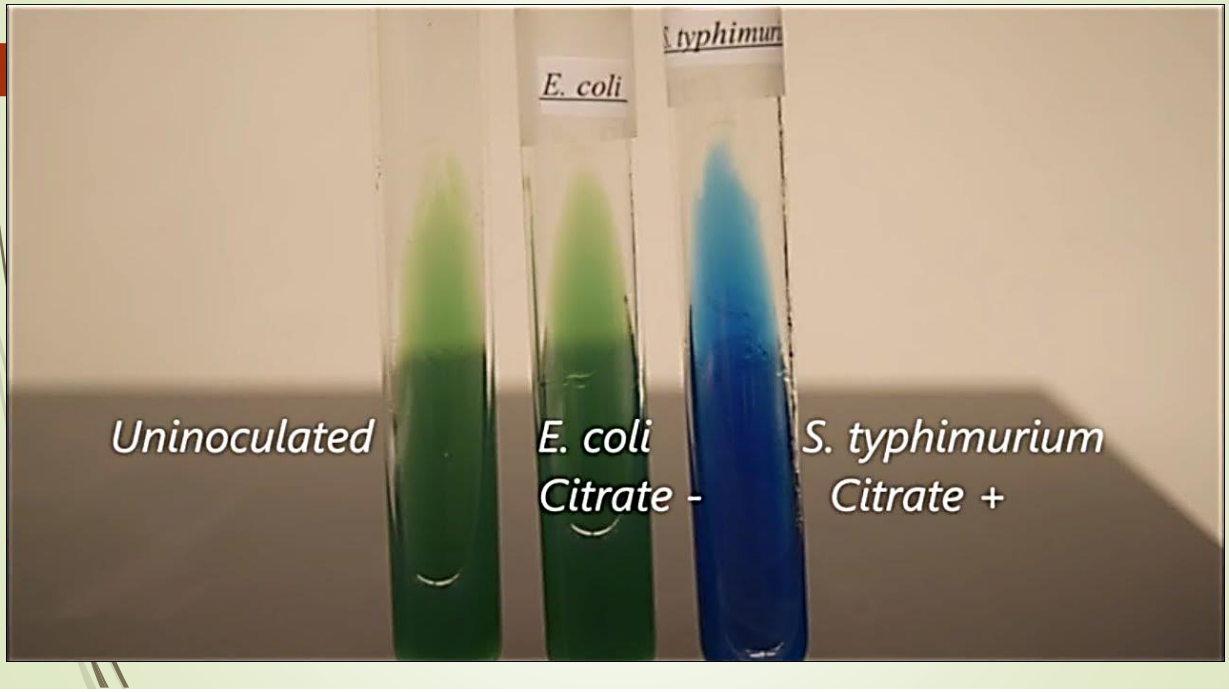
11-TSI test: Acid \Acid\ CO2+\H2S –

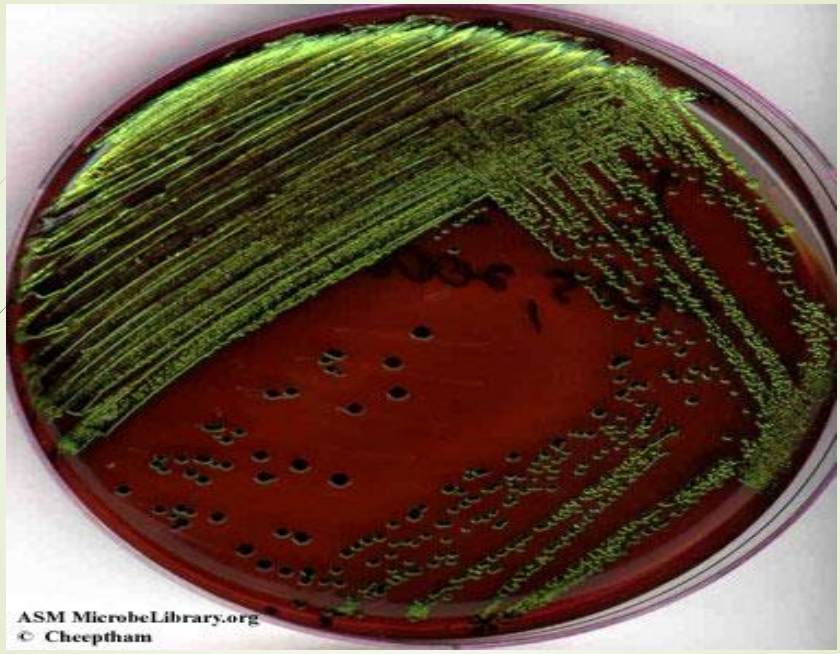
12- black colonies on (EMB agar) with green metallic sheen because high acid product



Escherichia coli
ATCC 25922


A
B
C
D
ASM MicrobeLibrary © Hanson


E. coli
Uninoculated
E. coli
Citrate -
S. typhimurium
Citrate +


ASM MicrobeLibrary.org
© Cheeptham

- *Klebsiella spp*

General characteristics

- 1-Gram negative bacteria (red)
- 2- Coccobacilli
- 5-Facultative anaerobes
- 4- Non Motile
- 5-Non spore forming
- Capsule +ve
- 7-Catalase positive ,,,Oxidase negative
- 8-Lactose fermenter (mucoid pink colonies)(on MacConkey agar)
- 9-Urease positive

10-IMViC test: -,-,+,+

11-TSI test: Acid \Acid\ CO2+\H2S –

12- purple or pink mucoid colonies on (EMB agar) because low acid product

RESULTS OF IMViC TESTS FOR COLIFORMS

	Indole	MR	VP	Citrate
Klebsiella and Enterobacter	-	-	+	+

IMViC Reactions

	I	M	Vi	C
Escherichia coli	+	+	–	–
Edwardsiella tarda	+	+	–	–
Proteus vulgaris	+	+	–	–
Klebsiella pneumoniae	–	–	+	+
Klebsiella oxytoca	+	–	+	+
Enterobacter spp.	–	–	+	+
Serratia marcescens	–	–	+	+
Citrobacter freundii	–	+	–	+
Citrobacter koseri	+	+	–	+

Dr.T.V.Rao MD

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Pathogenic Enterobacteriaceae

Escherichia coli:

تحتوي عدة أنماط ممرضة pathotypes أهمها:

1. **(UPEC) Uropathogenic *E. coli*:** مسؤولة عن نسبة عالية من **التهابات الجهاز البولي**.
2. **(MAEC) Meningitis-associated *E. coli*:** مسؤولة عن مرض **التهاب السحايا**.
3. **(IPEC) Intestinal pathogenic *E. coli*:** مسؤولة عن مجموعة من **الأمراض الاسهالية** التي يمكن أن تنتقل بالغذاء والماء الملوثين.

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- *Enterobacter spp*

General characteristics

- 1-Gram negative bacteria (red)
- 2- Coccobacilli
- 5-Facultative anaerobes
- 4- Motile +ve
- 5-Non spore forming
- Capsule -ve
- 7-Catalase positive ,,,Oxidase negative
- 8-Lactose fermenter (pink colonies)(on MacConkey agar)
- 9-Urease –ve

10-IMViC test: -,-,+,+

11-TSI test: Acid \Acid\ CO2+\H2S –

12- pink colonies on (EMB agar) because low acid product

Enterobacter (Motile +ve ,,, Urease –ve)

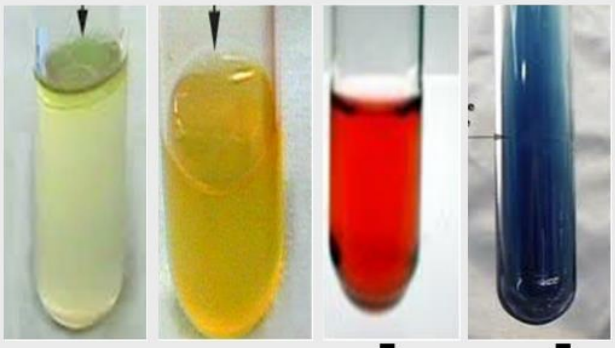
Klebsiella (Motile -ve ,,, Urease +ve)

مستعمرات *Enterobacter Sp*. على الوسط الغذائي EMB

مستعمرات *Enterobacter aerogenes* على الوسط الغذائي EMB agar

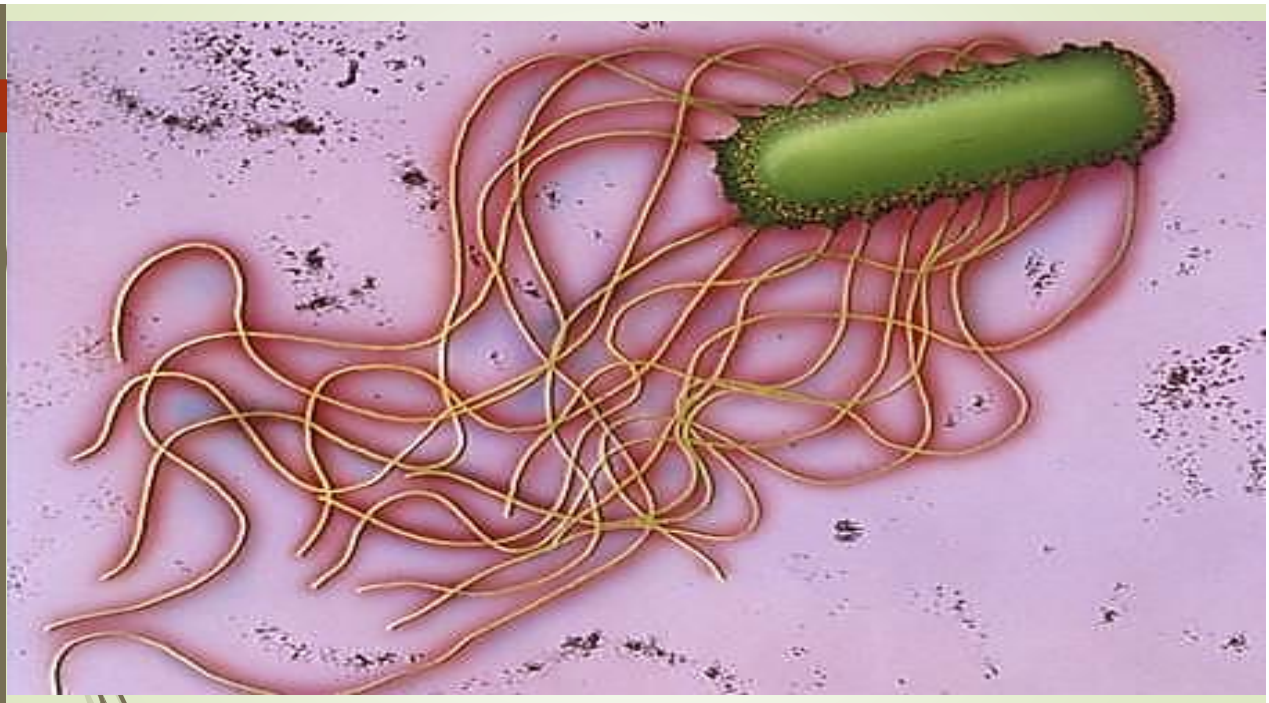
RESULTS OF IMViC TESTS FOR COLIFORMS

	Indole	MR	VP	Citrate
Klebsiella and Enterobacter	-	-	+	+



- *Proteus spp*
- *1- Gram negative*
- *2-Rod shape*
- *3- Facultative anaerobes*
- *4- pleomorphic*
- *5- Motile +(peritrichous)*
- *6-Capsule –ve*
- *7- nonspore forming*
- *8-Catalase +ve ,,Oxidase –ve ,,Urease +ve (very active)*
- *9-Swarming +ve*
- *10- H2S +ve (black sediment).*
- *11- non lactose fermenter (pale colonies).*

- 12-IMViC test v,+,-,v
- 13 - TSI test K\ A H2S + , CO2 +
- 14-Phenylalanine +ve
- Proteus (swarming ,,H2S producer)
- *P.vulgaris* (UTI,wound infection) ,(Indole +ve ,maltose +ve)
- *P. mirabilis* (UTI,wound infection , nosocomial infections) ,(Indole -ve ,maltose -ve)
- *P. rettgeri* (gastroenteritis)
- *P. penneri* (UTI,wound infection , nosocomial infections)

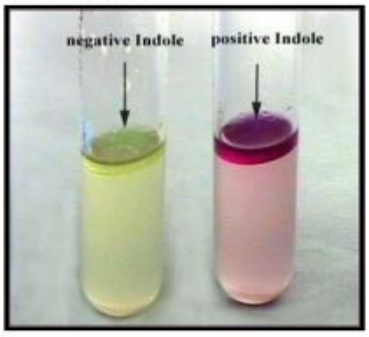


IMViC test

1-indole test:

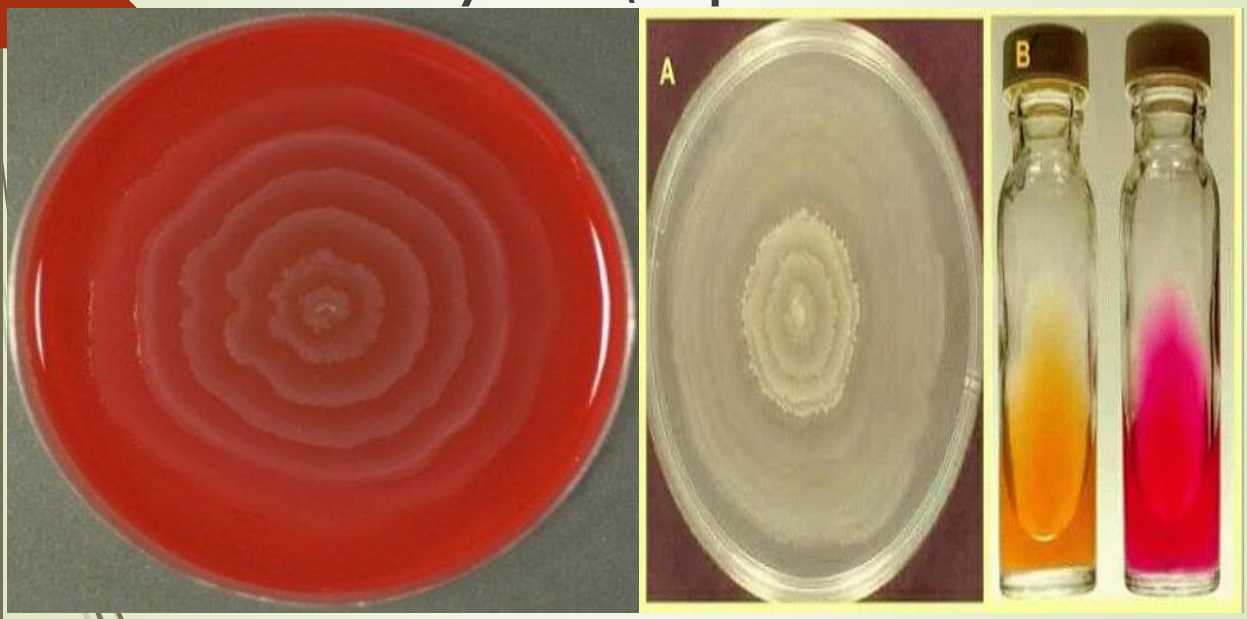
is used to determine the ability of bacteria to convert tryptophan into indole.

P. mirabilis can be differentiated from *p.vulgaris* by indole test.



P. mirabilis → **negative**
P. vulgaris → **positive**

15-Blood hemolysis v \ Alpha & Beta



TSI agar

Triple Sugar Iron Agar

0.1% dextrose

1.0% sucrose

1.0% lactose

A B C D E

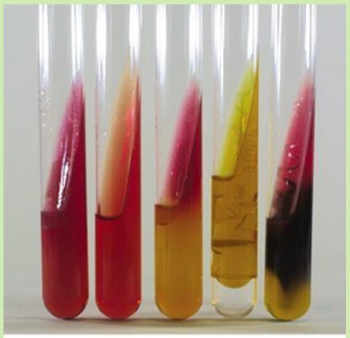


Figure 5-68

(a) Red/red (no sugar fermentation)

(b) Control

(c) Red/yellow (Glucose fermented but lactose and sucrose not fermented)

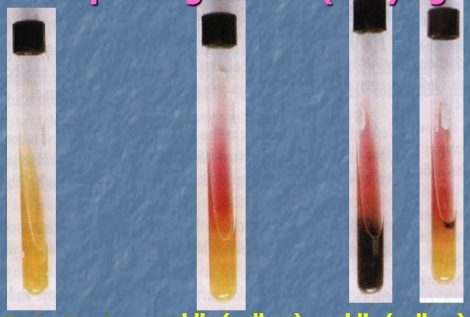
(d) Yellow/yellow (Glucose fermented. Lactose and/or sucrose fermented)

(e) Red/yellow with H_2S

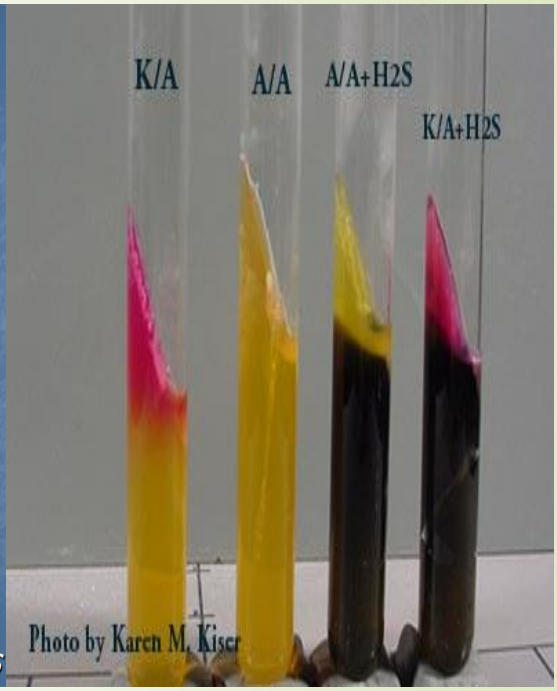
Definitive tests to differentiate between Lactose fermenters & Lactose Nonfermenters

- **Growth on Triple Sugar Iron (TSI) agar:**

Results:



	E.Coli & Klebsiella	Shigella	Proteus & Salmonells
Butt:	acidic (yellow)	acidic (yellow)	acidic (yellow)
Slant:	acidic (yellow)	alkaline (red)	alkaline (red)
H_2S Production:	-ve	-ve	+ve



LAB:8
Shigella & Salmonella

Shigella – species and serogroups

SPECIES	SEROGROUP	SEROTYPE
S. dysenteriae	A	1 – 15
S. flexneri	B	1 – 6 (15 subtypes)
S. boydii	C	1 - 18
S. sonnei	D	1

- **General characteristics**

1-Gram negative

2-Rod shape

3- Facultative anaerobes

4-Non spore forming

5- Non motile

6-Uncapsulated

7- Non lactose fermenter(Pale colonies or Colorless colonies).except *Sh. Sonnei*(*Late lactose ferm*)

8-H2S –ve

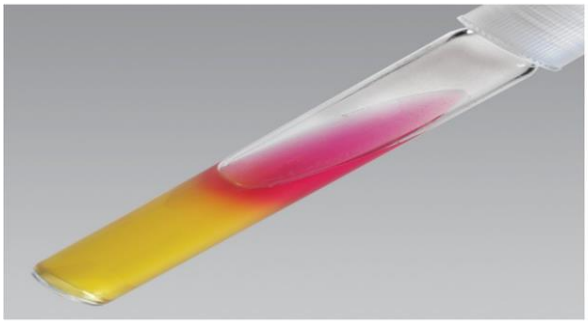
9-Catalase +ve ,,,Oxidase –ve

10-IMViC test : V,+,-, - except *Sh. sonnei* indol –ve

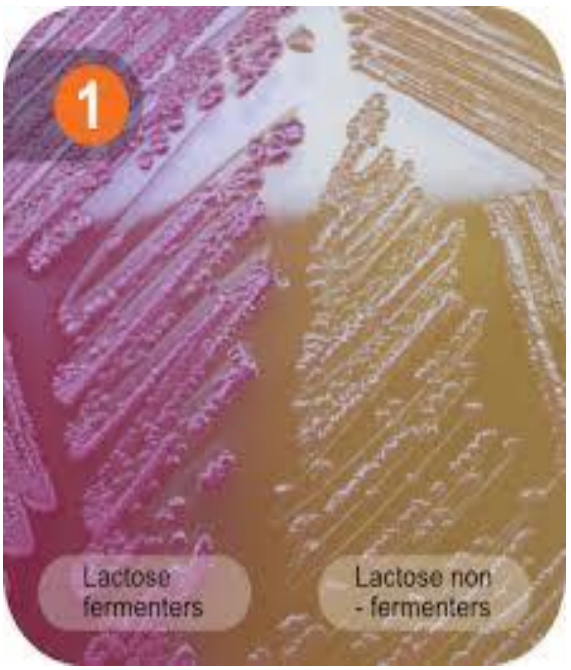
11-TSI test : K\A , CO2 –ve, H2S –ve

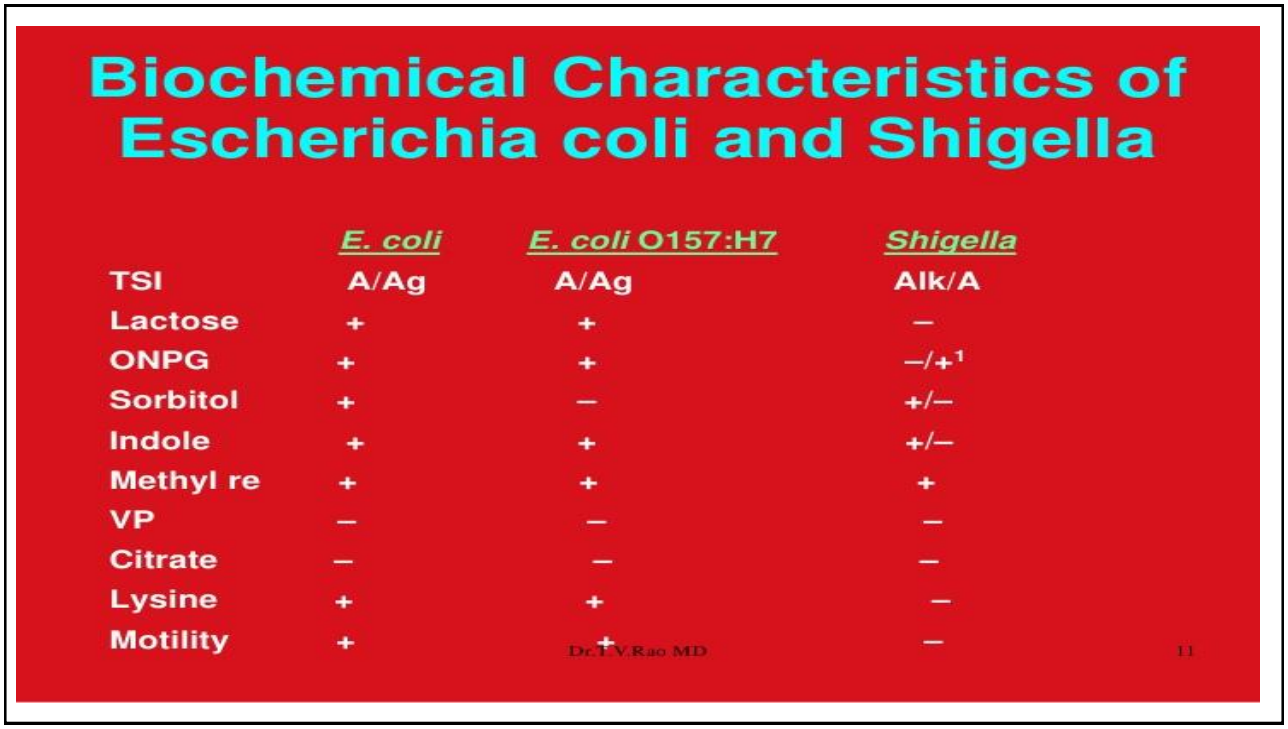
12-Glucose ferm except *Sh. Flexneri*

13-Mannitol ferm except *Sh. dysenteriae*



On **MacConkey agar** *Shigella* species producing pale non-lactose fermenting colonies.





Test	S. dysenteriae	S. flexneri	S. boydii	S .sonnei
Indole	V	V	V	-
M	+	+	+	+
V	-	-	-	-
C	-	-	-	-
TSI	K\A,-,-	K\A,-,-	K\A,-,-	A\A,-,-
Motility	-	-	-	-
Glucose	+	-	+	+
Lactose	LNF	LNF	LNF	LF
Mannitol	-	+	+	+
Urease	-	-	-	-
Gelatinase	-	-	-	-
MacConkey agar	Pale colonies	Pale colonies	Pale colonies	Pink colonies
S-S agar	Pale colonies	Pale colonies	Pale colonies	Pink colonies
Xylose	-	-	-	-

Genus : Salmonella

General characteristics

1-Gram negative

2-Rod shape

3- Facultative anaerobes

4-Non spore forming

5- Motile +ve except *S.gallinarium*

6-Uncapsulated except S.typhi

7- Non lactose fermenter(Pale colonies or Colorless colonies) on MacConkey agar .

8-H2S +ve except S.typhi

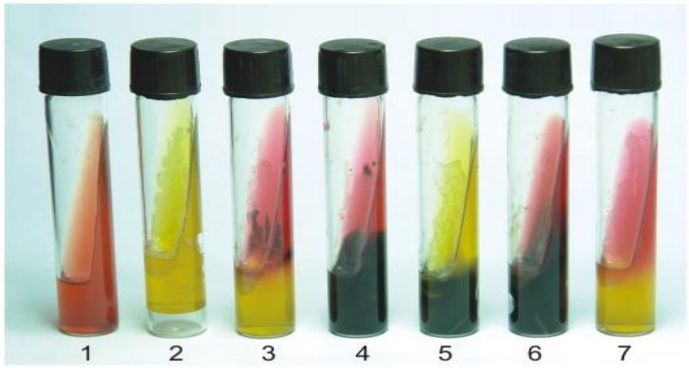
9-Catalase +ve ,,,Oxidase –ve

10-IMViC test :-,+,-, + except *S. typhi citrate* –ve

11-TSI test : K\A , CO2 +ve, H2S +ve , except *S. typhi H2S-ve*

12-Glucose ferm

13-Mannitol ferm+ve

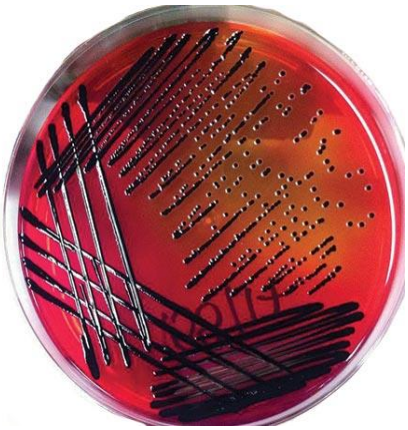


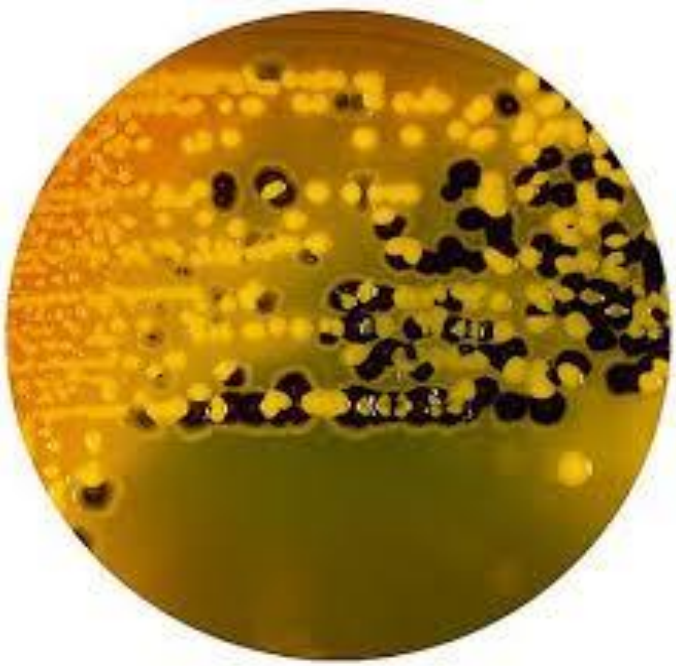
Triple Sugar Iron Agar (M021)

1. Control
2. Escherichia coli ATCC 25922
3. Salmonella Typhi ATCC 6539
4. Proteus vulgaris ATCC 13315
5. Citrobacter freundii ATCC 8090
6. Salmonella Typhimurium ATCC 14028
7. Shigella flexneri ATCC 12022

Test	S. typhi	S. Typhimurium
Indole	-	-
M	+	+
V	-	-
C	-	+
TSI	K\A,co2 +, H2S -	K\A, C02 +, H2S +
Motility	+	+
Glucose	+	+
Lactose	LNF	LNF
Mannitol	+with gas	+with gas
Xylose	+	+
Urease	-	-
XLD agar	Yellow colonies	Yellow colonies With black center
MacConkey agar	Pale colonies	Pale colonies
S-S agar	Pale colonies	Pale colonies with black center

Salmonella Typhimurium on XLD agar.





Lab 9 Family: Pseudomonadaceae

Pseudomonas known to cause disease in humans are associated with opportunistic infections

Genus: Pseudomonas Spp.

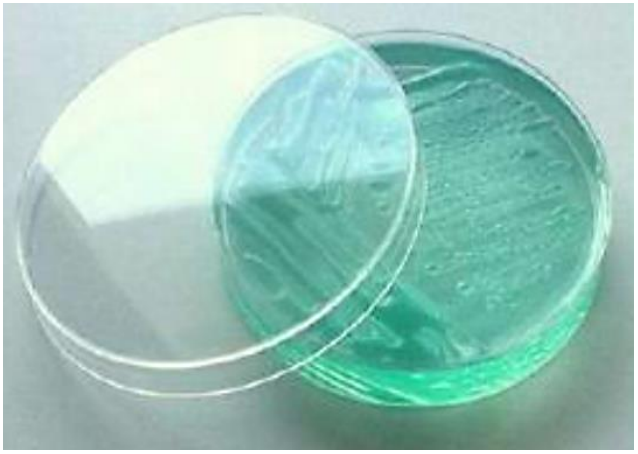
- *Pseudomonas aeruginosa*
- *Pseudomonas fluorescence*
- *Pseudomonas putida*

(Fluorescent group)

- *Pseudomonas pseudoalcaligene* (opportunistic)
- *Pseudomonas mallei*
- *Pseudomonas pseudomallei*
- *Pseudomonas cepatia*

Pseudomonas

A large group of rods, aerobic, non-sporing, gram-negative bacteria motile by polar flagella, Opportunistic pathogens, Found In nature; water, soil, other moist environments Some of them are pathogenic to plants



P. aeruginosa

- Forms round colonies with a fluorescent greenish color, sweet odor, and ß-hemolysis.

- Pyocyanin (nonfluorescent) → **blue green.** *(P. aeruginosa)*
- Pyoverdin (fluorescen) → **yellow green.** *(P. fluorescence)*
- Pyorubin → **red**
- Pyomelanin → **black**

The pigments test is done o MacConkey and Milk agar Because of their light color

اختبار الصبغات يجرى على الـ Milk والـ MacConkey لان لونهم فاتح ويبين عليهم أي صبغة 😁

Note: Pyocin is bacteriocin produced by ***P. aeruginosa***

While **Pyocyanin** is pigment produced by ***P. aeruginosa***

- Some strains have a prominent capsule (alginate).

Identification of *P. aeruginosa* is usually based on oxidase test and its colonial morphology: b-hemolysis, the presence of characteristic pigments and sweet odor, and growth at 42 C.

P. acruginosa → produce **blue green** pigment (Pyocyanin).

P. fluorescence → produces **yellow green** pigment (Pyoverdin).

Pyoveridin (composed from 2 pigments fluorescen A and fluorescen B). Other produces red pigment - Pyorubin) Some produce black pigment-Pyomelanin.

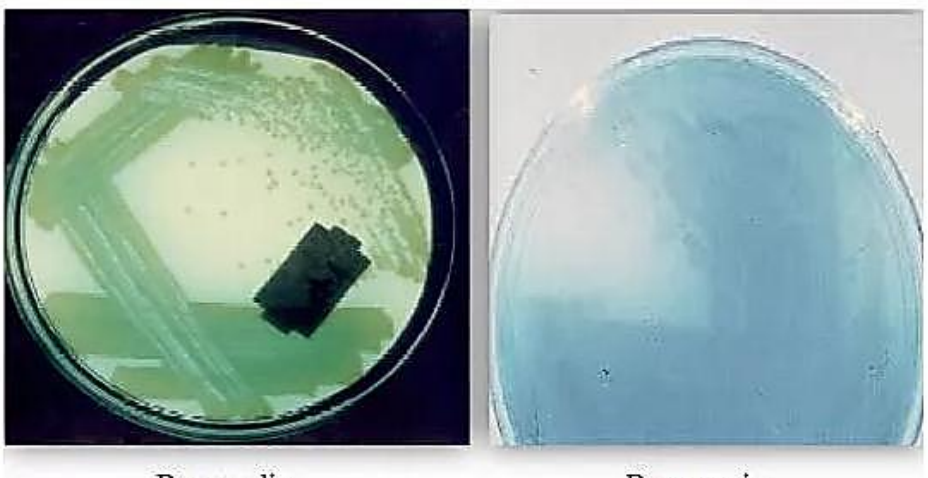
Pigment production

- Some strains produce diffusible pigments:

  Pyocyanin (blue); fluorescein (yellow); pyorubin (red).

- *P. aeruginosa* produces characteristic grape-like odor (العنب المتخمر او المتعفن) and blue-green pus & colonies.

- Broad antibiotic resistance.



Pyoverdin Pyocyanin

Pathogenicity:

P. aeruginosa is the most important species, it is invasive and toxigenice produce infection in patients with abnormal host defense and is an important nosocomial pathogen, they cause UTI, otitis media and septic shock, and the main infection of Pseudomonas is bum infection and wound infection.

They may found in antiseptic solution, eve drops, grows well in dettol. heating 55 C kill Pseudomonas so it could survive in detergents, it shows also resistant to different and multiple antibiotics.

Pseudomonas aeruginosa:

On Blood agar

B-hemolysis

On MacConkey agar

Nan-lactose fermenting

On Nutrient agar

Large, opaque irregular colonies with butyrous consistency & fruity odor or earthy smell

Enzymes and toxins:

They are extracellular include hemolysin, lipase, collagenase, protease, the most important toxin is **exotoxin A** which cause blockage of protein synthesis which leads to tissue necrosis.

Classification:

1. **1-** Biochemical test
2. **2-** Serological (H-Ag, O-Ag, 110 serotype).
3. **3-** Pyocin typing: Pseudomonas produce pyocin which is an antimicrobial agent.
4. **4-** Phage typing
5. **5-** Sensitivity pattern antibiotics.

Drug of choice: Carbenicillin (Pyopen)

Specimens: skin lesion, pus, spinal fluid, sputum and urine

Laboratory Diagnostic tests:

1- Gram stain: G -ve bacilli

2- Milk agar (for pigmentation).

3- Blood agar (for hemolysis).

4- Kling A & King B (selective and differential)

5- MacConkey agar.

6- TSI 7- IMVIC

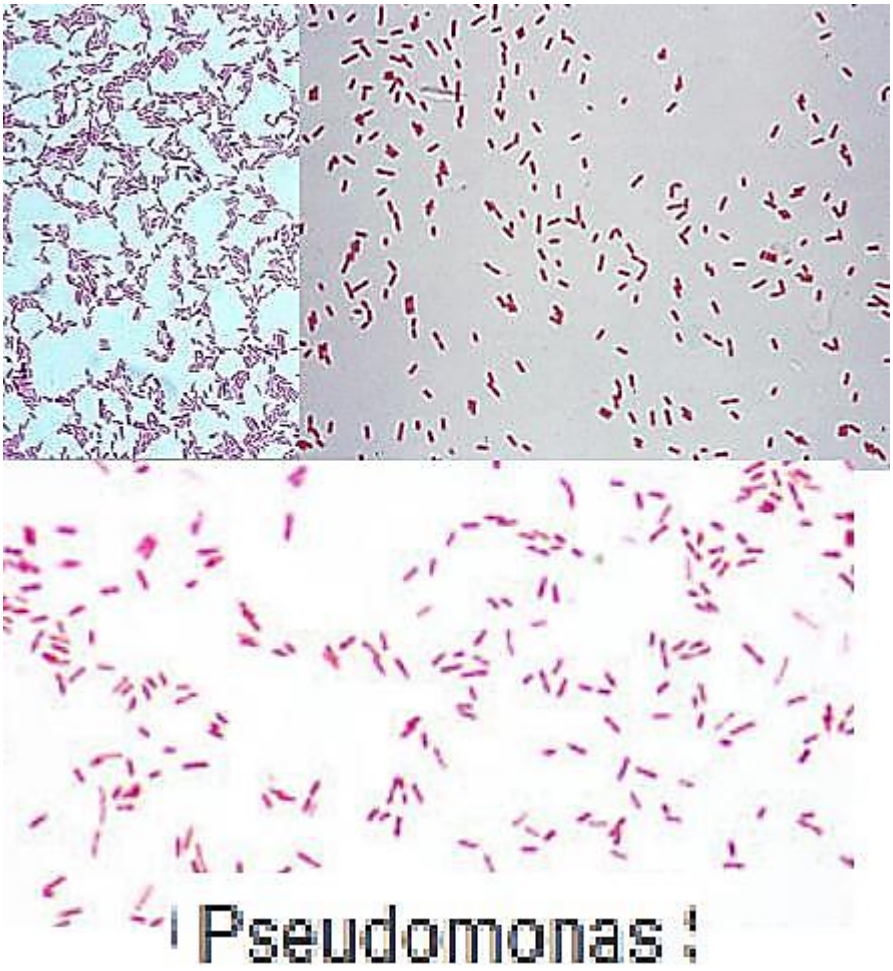
7- IMVIC

8- Motility

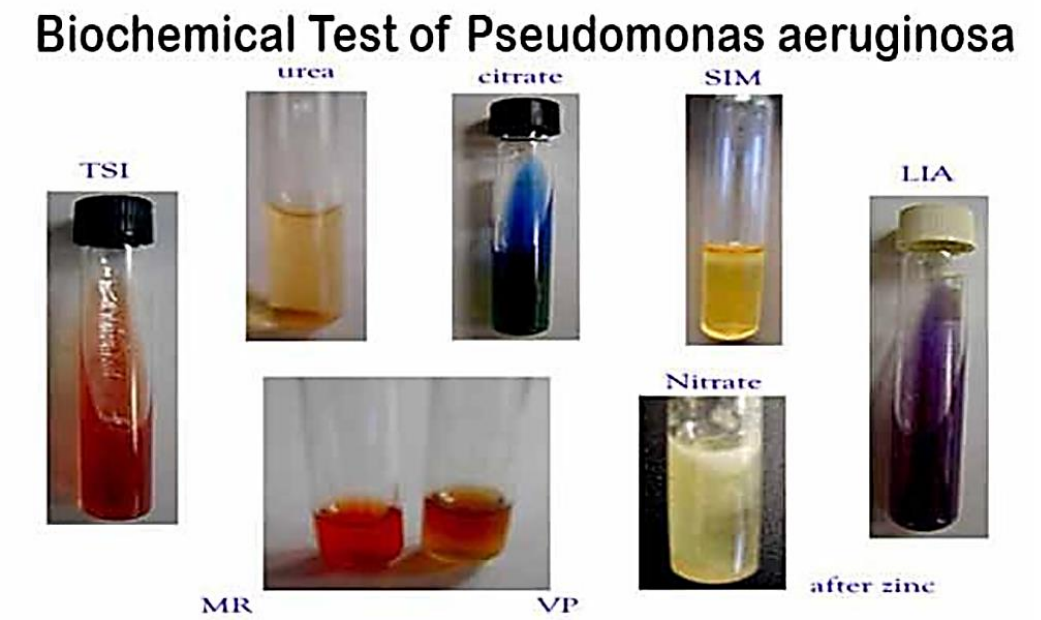
9- O/F (oxidation-fermentation) contain 1% glucose, bromothymol blue. K_2HPO_4 buffering, add paraffin on the slant to produce anaerobic condition, inoculation by stabing, the color change to yellow.

10-Nitrate broth.

11-oxidase and catalase.


Pseudomonas !

Test	*P. aeruginosa*	*P. fluorescence*
Indole	-	-
MR	-	-
VP	-	-
SC	+	+
TSI	K/K - -	K/K - -
Nitrate	+	+
Motility	+	+
Growth at 42 C°	+	-
Growth at 4 C°	-	+
King A	+ pyocianin	(- +) - pigment
King B	+ fluorescen	+ fluorescen
MacConkey	NL.F. transparent, irregular	N.L.F. transparent, irregular
oxidase	+	+
catalase	+	+
OF medium	O (+) / F (-)	O (+) / F (-)



Acinetobacter

The genus *Acinetobacter* comprises 38 validly named species.

Domain: Bacteria

Phylum: Proteobacteria

Class: Gammaproteobacteria

Order: Pscudomonadales

Family: Moraxellaceae

Genus: Acinetobacter

Species:

A. albensis
A. apis
A. bohemicus
A. boissieri
A. bouvetii
A. equi
A. gandensis

الاجناس

The *Acinetobacter* has more than 50 species, most of which are nonpathogenic environmental organisms. The most common infection- causing species is ***A. baumannii***, followed by ***Acinetobacter calcoaceticus*** and ***Acinetobacter lwoffii***.

Acinetobacter is often cultured from hospitalized patients' sputum or respiratory secretions, wounds, and urine. In a hospital setting, Acinetobacter commonly colonizes irrigating solutions and intravenous solutions.

Introduction

- The name, Acinetobacter, comes from the Latin word for "**motionless**," because they lack cilia or flagella with which to move.
- Have 32 species, *A. baumanii* and *A. lwoffii* have greatest clinical importance.
- Most species are not significant sources of infection. However, one opportunistic species, ***Acinetobacter baumannii*** is found primarily in hospitals and poses a risk to people who have suppressed immunity.
- >2/3 of *Acinetobacter* infections are due to *A. baumannii*.

Acinetobacter

- Gram-Negative
- Coccobacilli
- Strictly aerobic
- Nonmotile
- Catalase positive
- Oxidase negative

Epidemiology

Environmental reservoirs:

- Soil
- Fresh water
- Vegetables
- Animals Body lice, fleas, ticks

In the hospital

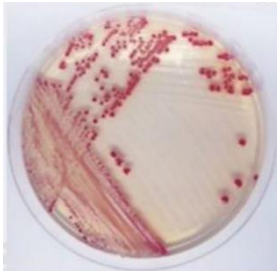
- Environmental surface.
- Ventilators, dialysis machines, air ventilation systems, water sources.
- Hands Contaminated suction equipment.
- Respiratory, urinary, GI tracts & wounds of patients.

Growth Requirement

- ✓ Aerobic
- ✓ Grow at 44° C
- ✓ **Differential Media:** MacConkey Agar
- ✓ **Selective Media:** Chrom Agar and Leeds Acinetobacter Agar

Leeds Acinetobacter Agar


MacConky Agar


CHROM Agar

Biochemical Reactions

- Oxidase negative (opposite to *Neisseria spp.* or *Moraxella spp.*)
- Haemolytic
- Indole negative
- Catalase positive

Biochemical Profile

- Both *A. baumannii* and *A. lwoffii* are Catalase positive and Oxidase Negative.
- ***baumannii*** ferment glucose, xylose and lactose but ***A. lwoffii*** cannot ferment.

Molecular Detection

- *baumannii* and *A. lwoffii* can be detected by PCR.
- **RecA** specific primers are used to detect recA gene in *A. baumannii*, giving a 382 bp fragment.
- **est** specific primers are used to detect est gene in A. lwoffii, giving a 309 bp product.

Pathogenesis

- Opportunistic pathogen.
- Survive under dry conditions.

Virulence Factors

- Polysaccharide capsule, prevent complement activation, delay phagocytosis.
- Fimbriae (adhere to human bronchial epithelium).
- Pilli colonization of environmental surface to form biofilms).

Transmission

Acinetobacter can be spread from person to person (infected or colonized patients), contact with contaminated surfaces of exposure to the environment.

Antibiotic Resistance

Acinetobacter species are capable of accumulating multiple antibiotic resistance genes, leading to the development of multidrug-resistant or even panresistant strains.