

Practical Pathogenic Bacteria

LAB: 1

Methods of Bacterial Identification

1-Microbial Causes of Infection

Infectious diseases are caused by different types of microorganisms, including:

- **Bacteria**
- **Viruses**
- **Fungi**
- **Parasites**

2- The source of the pathogen may be:

- **Exogenous:** acquired from the environment, animals, food, water, or other people.
- **Endogenous:** originating from the body's normal flora when host defenses are compromised

3- Specimen Selection, Collection, and Processing

Accurate laboratory diagnosis depends on proper specimen handling:

- The quantity of the specimen must be adequate and representative of the disease process.
- Specimens are selected based on clinical symptoms.
- Contamination must be avoided by using sterile equipment and aseptic techniques.
- Specimens should be transported to the laboratory as quickly as possible.
- Transport media may be required to preserve organisms.
- Specimens should be collected before antimicrobial therapy to ensure reliable results.

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4- Laboratory Specimens

Specimen	Associated Infection
Blood	Septicemia
Urine	Urinary tract infections
Stool	Gastrointestinal infections
Sputum	Respiratory infections
Vaginal swabs	Vaginal infections
Nose & ear swabs	ENT infections
Cerebrospinal fluid (CSF)	Central nervous system infections
Food & vomit	Food poisoning
Pus	Acne, burns, wound infections
Seminal fluid	Urethral discharge

5. Microscopic Examination

Gram Staining

Gram staining is a differential staining technique used to classify bacteria based on their ability to retain crystal violet dye.

- Gram-positive bacteria: appear **purple**: *Staphylococcus*, *Streptococcus*
- Gram-negative bacteria: appear **pink or red**: *Escherichia coli*, *Klebsiella*

Gram staining also reveals:

Cell shape (cocci or bacilli) Arrange

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What Is a Gram Stain?

A Gram stain is a laboratory test used to detect bacteria in clinical specimens or body fluids. It provides rapid preliminary information that helps healthcare providers select appropriate therapy.

Although Gram staining does not usually provide a definitive diagnosis, it is a fundamental technique in microbiology and an essential first step in bacterial identification. The method was developed by Hans Christian Gram in 1882.

Gram-positive organisms

Gram-positive bacteria have cell walls that contain thick layers of peptidoglycan, a substance that forms the cell walls of many bacteria. The peptidoglycan forms about 90% of the cell wall in gram-positive bacteria. This causes them to appear blue to purple under a Gram stain.

Gram-positive organisms include:

- *Staphylococcus* species.
- *Streptococcus* species.
- *Corynebacterium* species.
- *Clostridium* species.
- *Listeria* species.

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Gram-negative organisms

Gram-negative bacteria have cell walls with thin layers of peptidoglycan (10% of the cell wall) and high lipid (fatty acid) content. This causes them to appear red to pink under a Gram stain.

Gram-negative organisms include:

- *Neisseria gonorrhoeae* and *Neisseria meningitides*.
- *Moraxella* species.
- *Escherichia coli* (E. coli).
- *Pseudomonas* species.
- *Proteus* species.
- *Klebsiella* species.

Gram staining procedure

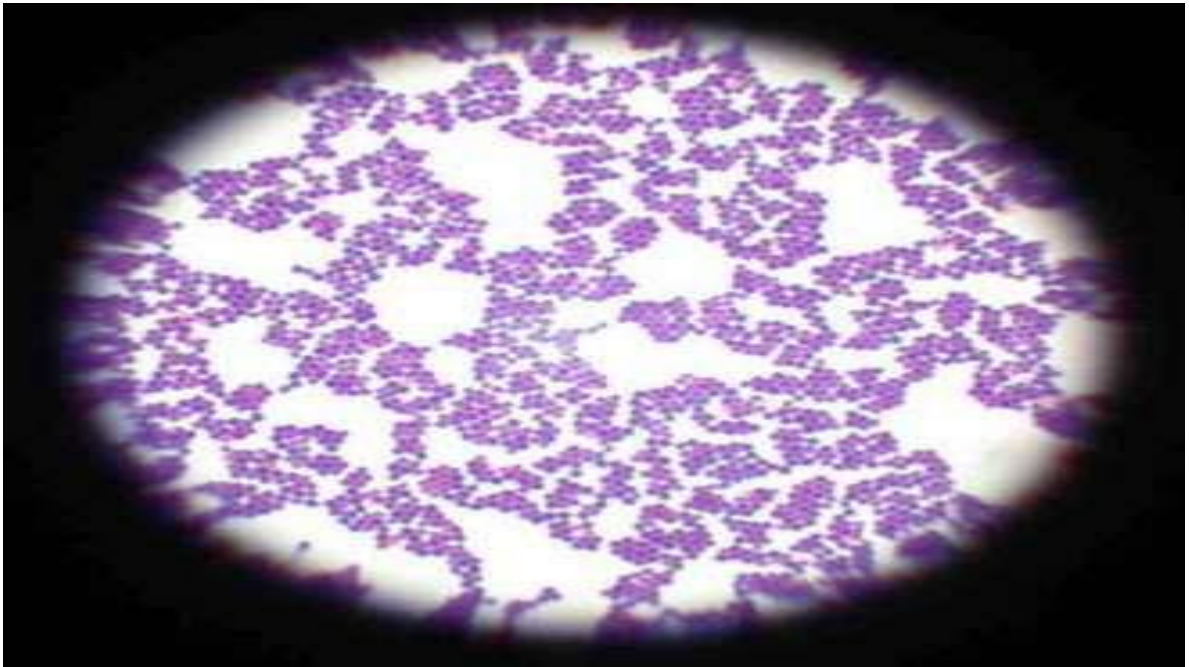
At the lab, a medical laboratory scientist smears or spreads the sample on glass microscope slides. These slides are known as smears. They then apply a series of stains to the smear to perform a Gram stain.

The Gram staining process includes four basic steps, including:

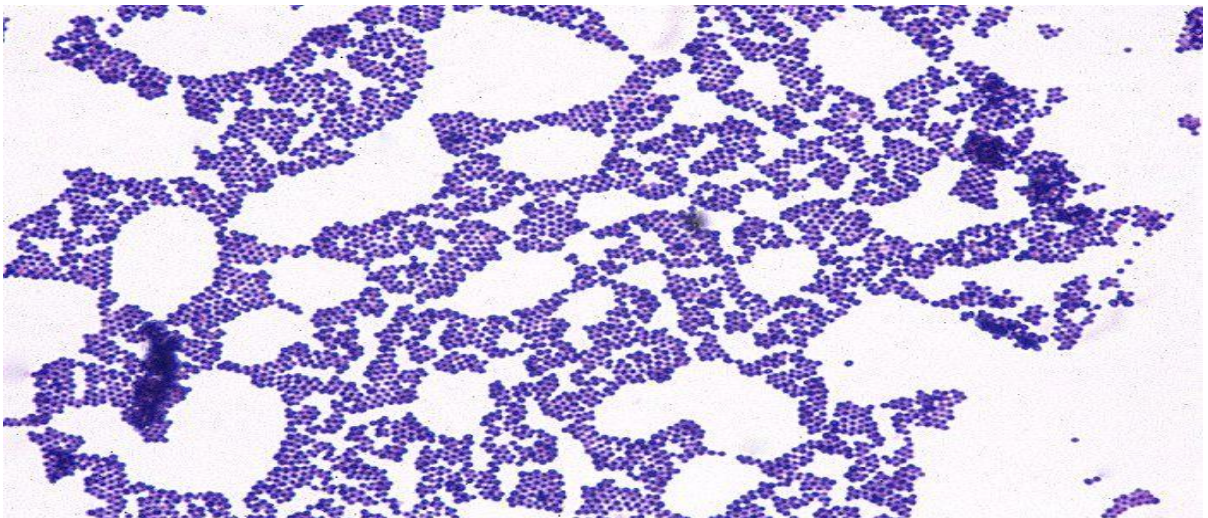
1. Applying a primary stain (crystal violet).1min
2. Adding a mordant (Gram's iodine).1min
3. Rapid decolorization with ethanol, acetone or a mixture of both.20 sec
4. Counterstaining with Safranin.0.5 min

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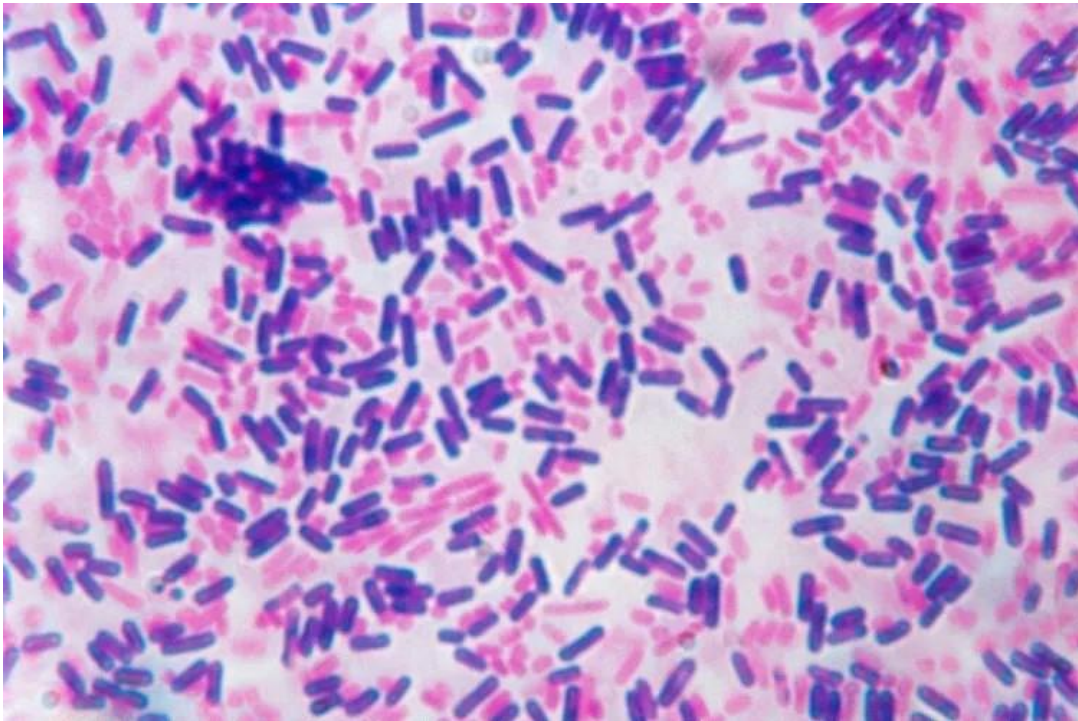
Staphylococcus spp = Cocci , Grape like clusters and Purple



Staphylococcus spp = Cocci, Grape like clusters and Purple

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Red: Gram negative Purple: Gram positive

6. Microbiological Culture Examination (Media Selection)

Culture examination allows isolation and identification of bacteria:

- **Media selection depends on the source of the specimen.**
- **Appropriate temperature and incubation conditions (aerobic or anaerobic) are required.**

Types of Media

- **Non-selective media: support the growth of many microorganisms.**
- **Selective media: contain inhibitory substances that allow growth of specific bacteria.**

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Colony Characteristics

- Size
- Color
- Odor
- Hemolysis on blood agar

7. Biochemical Identification

Biochemical tests identify bacteria based on their metabolic capabilities:

- Sugar fermentation
- Amino acid utilization
- Enzyme production

These tests may be:

- Single tests (e.g., coagulase test for *Staphylococcus aureus*)
- Multiple test systems for Gram-negative bacilli (clusters, chains, pairs)

Commercial biochemical identification kits are widely used in diagnostic laboratories.

8. Serologic Methods

Serologic tests detect specific antigens or antibodies:

- Known antigens are bound to latex particles.
- When mixed with corresponding antibodies, visible agglutination occurs.
- A positive reaction indicates the presence of the tested organism.

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9. Molecular Methods

Molecular techniques are the most sensitive and specific diagnostic methods:

- **Detect microorganisms based on DNA or RNA**
- **Allow rapid identification and characterization**

Polymerase Chain Reaction (PCR)

PCR is the most commonly used molecular method for bacterial identification.

10. Antibiotic Susceptibility Testing

- **Determines whether bacteria are susceptible or resistant to antimicrobial agents.**
- **Performed in vitro to guide appropriate treatment.**
- **Helps prevent misuse of antibiotics and reduces antimicrobial resistance.**

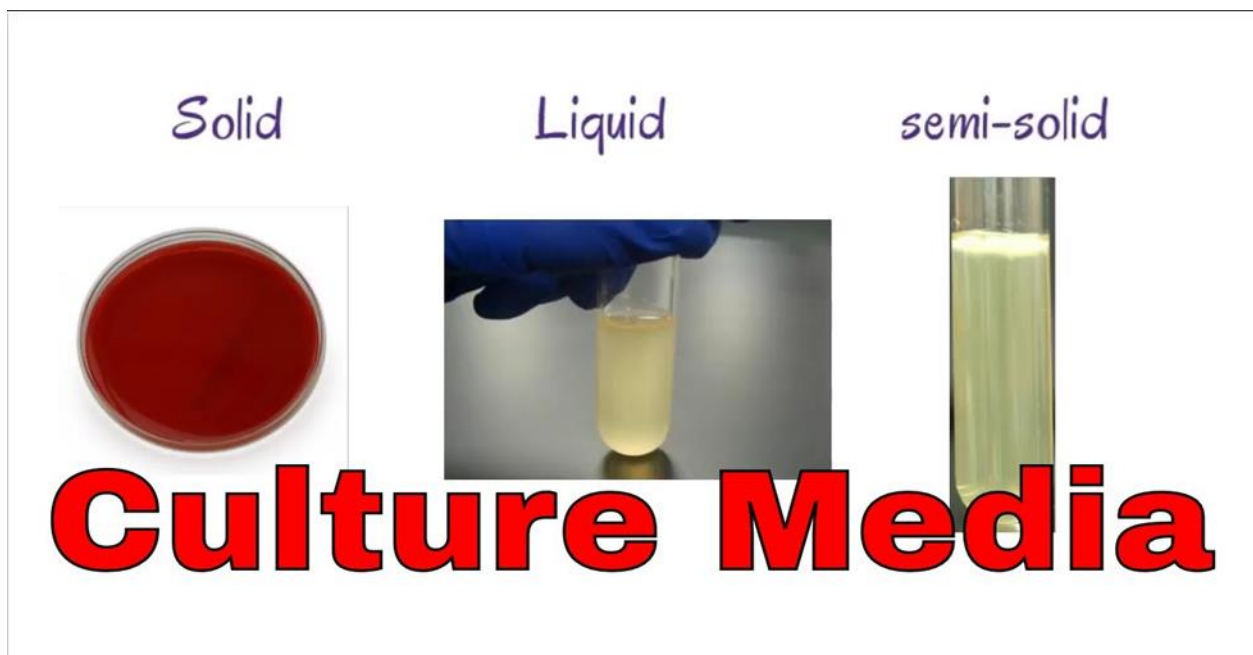
Culture Media used in Microbiology



Culture media

- Microorganisms, like all other living organisms, require essential nutrients to sustain life. Although all microorganisms share the same basic nutritional requirements, they differ in their specific needs for inorganic and organic compounds. By providing suitable environmental and nutritional conditions, microorganisms can be successfully cultivated under laboratory conditions.
- Based on this concept, culture media are defined as:
Artificial environments designed to simulate natural conditions and provide the necessary nutrients required for the growth and cultivation of microorganisms in the 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 is divided according to the purpose of its use

1- 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.

2-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 selective (suppressive) agents to the medium:

Certain chemical substances are added to culture media to inhibit the growth of unwanted microorganisms while allowing the growth of the desired ones.

These include:

- **Selective dyes** such as **crystal violet**, **methylene blue**, and **basic fuchsin**, which **inhibit the growth of Gram-positive bacteria without affecting Gram-negative bacteria**.
- **Selective antibiotics** such as **cycloheximide**, which suppresses the growth of **saprophytic fungi** and permits the growth of medically important fungi, such as dermatophytes, when added to Sabouraud dextrose agar.

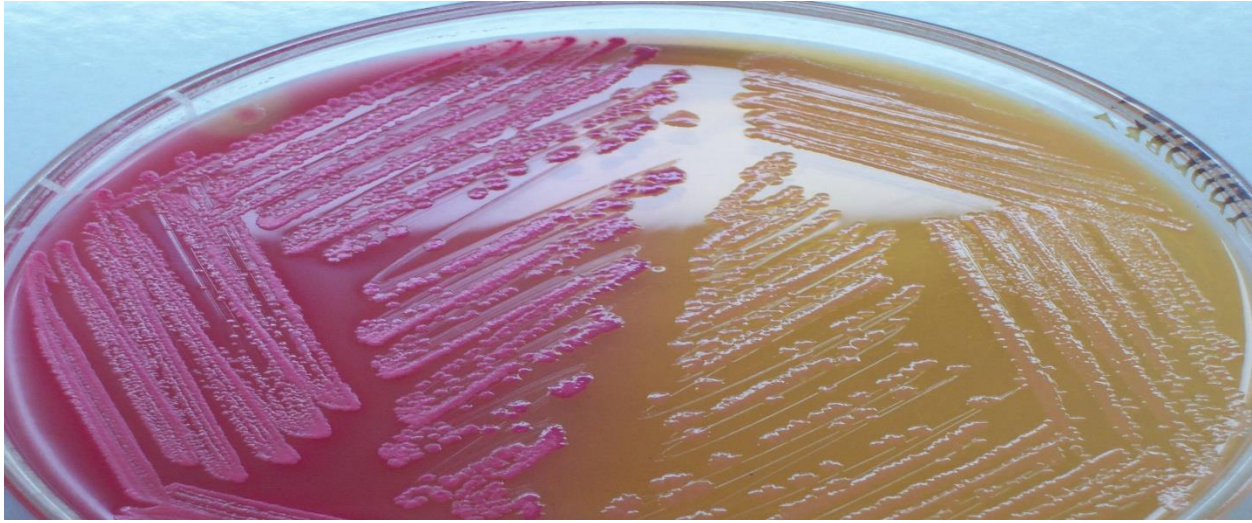
2. Manipulation of growth conditions:

Selective growth can also be achieved by adjusting environmental conditions to favor the growth of the desired microorganism, such as **temperature, aeration, and pH**, according to its specific growth requirements.

1-MacConkey agar (**selective differential media for gram negative bacteria**)

- **Sugar: Lactose**
- **PH-indicator: Neutral red**
- **The inhibitor for gram positive bacteria: crystal violet**
- **The inhibitor for non-enteric bacteria: bile salt**

- Lactose fermenter = pink colony
- Lactose non fermenter = pale colony



Principle of MacConkey Agar:

MacConkey agar is a selective and differential medium. It permits the growth of Gram-negative enteric bacteria while inhibiting the growth of Gram-positive and non-enteric bacteria.

The selective action of MacConkey agar is due to the presence of crystal violet and bile salts. Crystal violet inhibits the growth of Gram-positive bacteria, whereas bile salts suppress non-enteric bacteria. These inhibitory substances do not affect Gram-negative enteric bacteria because they are naturally adapted to survive in the presence of bile salts in the intestinal tract.

Differential property:

Many reagents or indicators are added to differential media to distinguish between bacterial species that grow on the same medium. These indicators are

usually dyes that function as pH indicators, detecting changes in acidity resulting from bacterial metabolic activity. Such changes are manifested by a visible color change in the medium.

3-XLD agar (Xylose Lysine Deoxycholate)

Selective differential media for Shigella and Salmonella

- Sugar: Xylose
- PH-indicator: phenol red
- H₂S indicator: Na - thiosulphate
- Gram positive & gram-negative inhibitor = Na- Deoxycholate
- 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 H₂S producing bacteria.



Shigella on XLD.

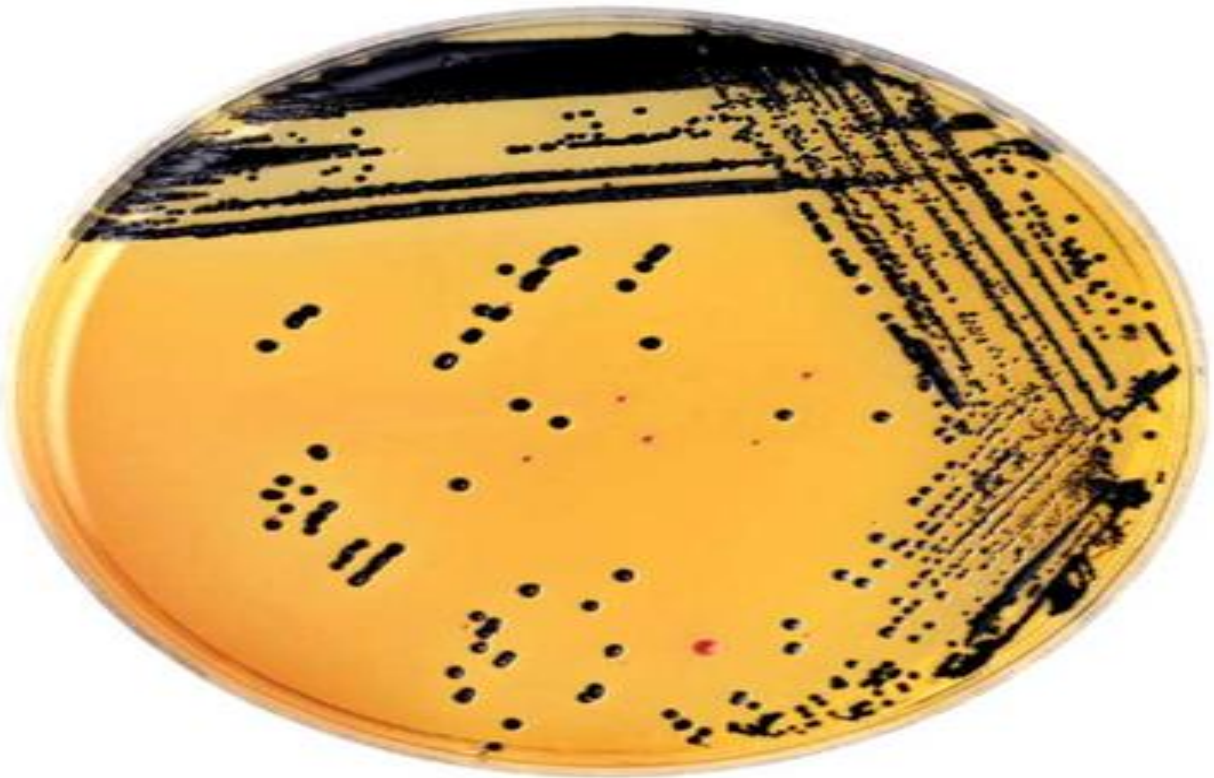


Salmonella on XLD.

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

4-S-S agar (Shigella Salmonella agar)

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



Salmonella on S-S agar pale colonies with black center

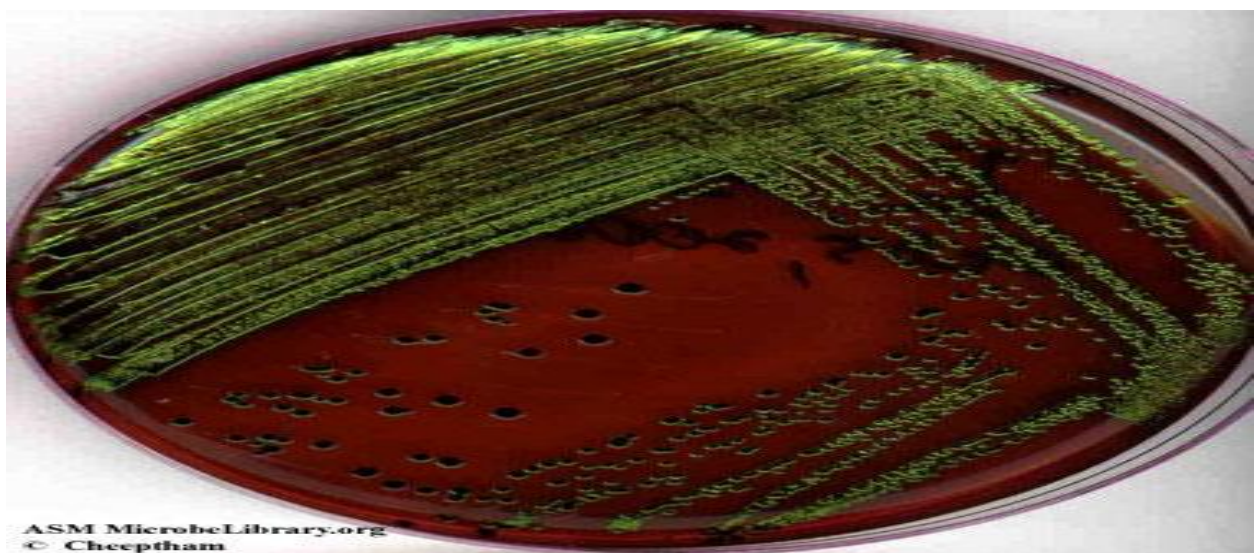


Shigella on S-S agar pale colonies

5-EMB agar (Eosin–Methylene Blue Agar) Selective differential media for Gram-negative enteric bacteria (Enterobacteriaceae)

- **Sugar: lactose**
- **pH indicator: Eosin and Methylene Blue dyes**
- **The inhibitors for Gram-positive bacteria = Eosin and Methylene Blue**
Shigella non fermenter lactose = pale colony
- **lactose fermenter = metallic green sheen colony (Escherichia coli) with a dark center**
- **Lactose fermenter = pink to purple colony (Enterobacter, Klebsiella)**
- **Non-lactose fermenter = light pink/colorless**

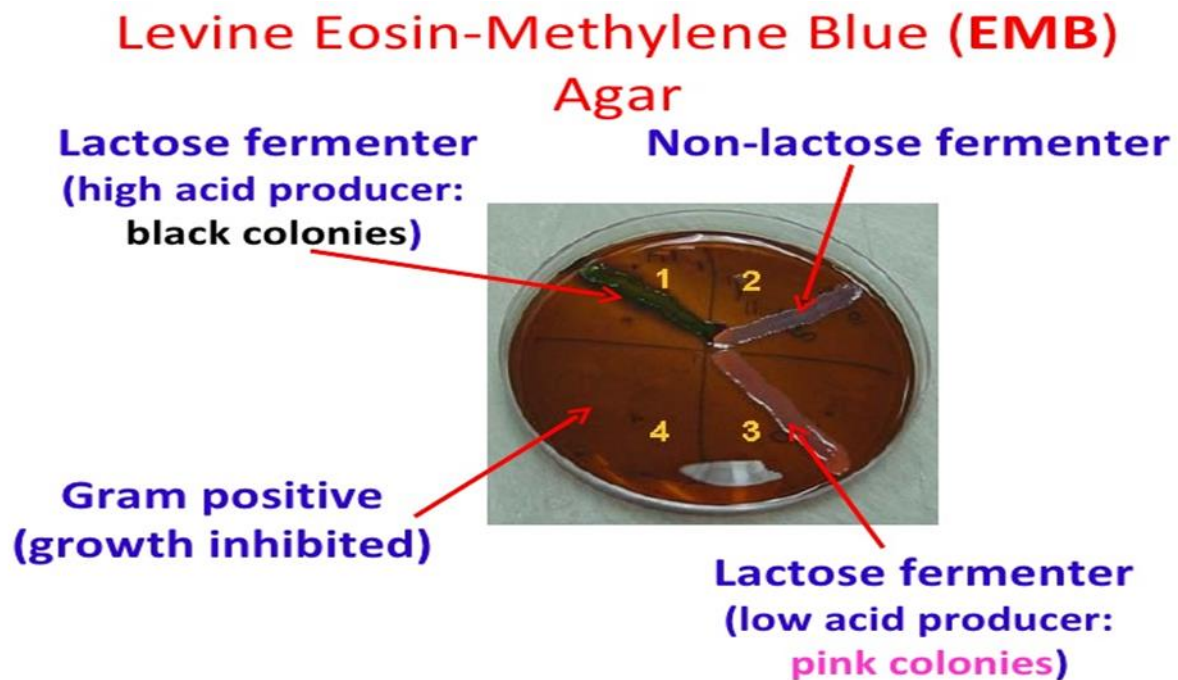
- Eosin Methylene Blue (EMB) agar is a **selective and differential medium**. It selectively supports the **growth of Gram-negative bacteria while inhibiting Gram-positive bacteria**.
- The medium is used for the isolation and differentiation of **Gram-negative enteric bacilli, especially coliforms and fecal coliforms**. Differentiation is based on **lactose fermentation**: lactose fermenters produce colored colonies, whereas **non-lactose fermenters form colorless colonies**.
- EMB agar is widely applied in water quality testing to detect coliforms and fecal coliforms, which indicate possible contamination by pathogenic microorganisms. It is also useful in differentiating organisms of the colon–typhoid–dysentery group ***Escherichia coli* colonies** grow with the **phenomenon of a green metallic sheen and a dark center**.



***Escherichia coli* on EMB agar (green metallic sheen)**



Klebsiella on EMB agar pink – purple mucoid colonies



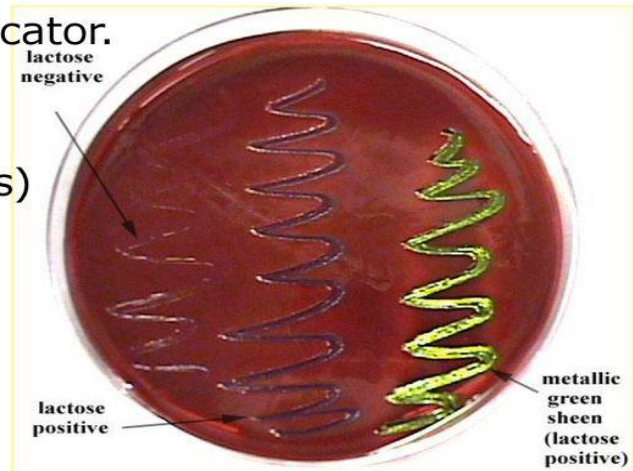
EMB (Eosin Methylene Blue) Agar

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

Eosin dye inhibits growth of Gram+ bacteria.

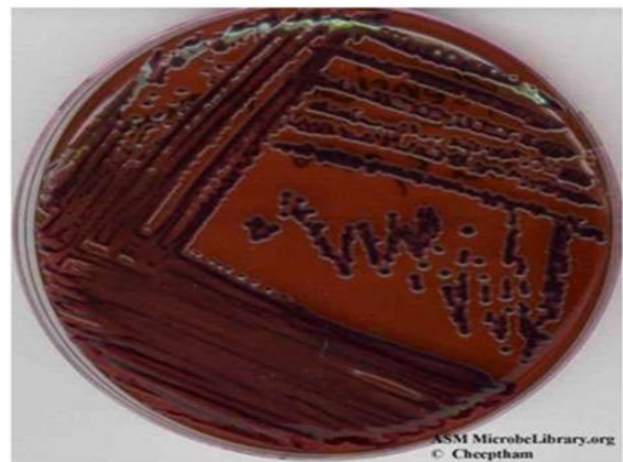
Methylene blue acts as indicator.

Lactose and sucrose
are nutrients
(fermentable carbohydrates)



On EMB

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



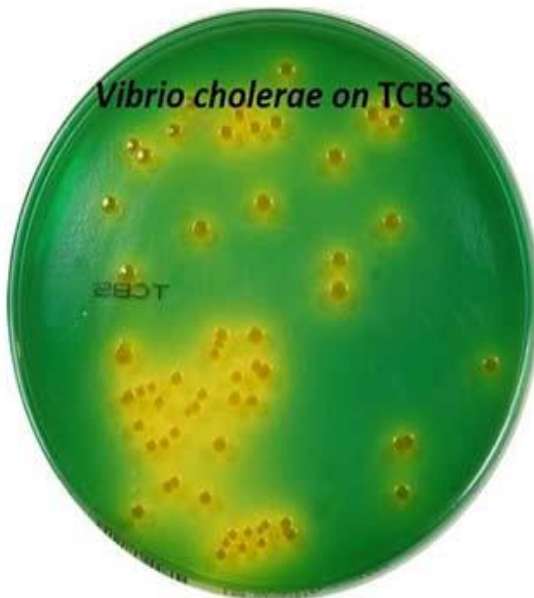
7-Thiosulfate–citrate–bile salts–sucrose agar, or TCBS agar

- **Selective** agar medium for isolation of *Vibrio* species
- **Highly selective** for *Vibrio cholerae* and *Vibrio parahaemolyticus*
- PH-indicator: Thymol blue and bromothymol blue
- It contains sodium thiosulfate and sodium citrate to inhibit Enterobacteriaceae, with sodium thiosulfate acts as a sulfur source
- Gram positive inhibitor: ox gall, which is a naturally occurring substance containing a mixture of bile salts (bile salts)
- Ferric citrate allows detection of hydrogen sulfide (H₂S) production
- Contains sucrose as a fermentable carbohydrate
- Alkaline pH favors growth of *Vibrio* species and inhibits other bacteria
- Sucrose fermenters form yellow colonies, non-fermenters form green or blue-green colonies

Typical colony morphology

- *V. cholerae*: Large yellow colonies.
- *V. parahaemolyticus*: Colonies with blue to green centers.

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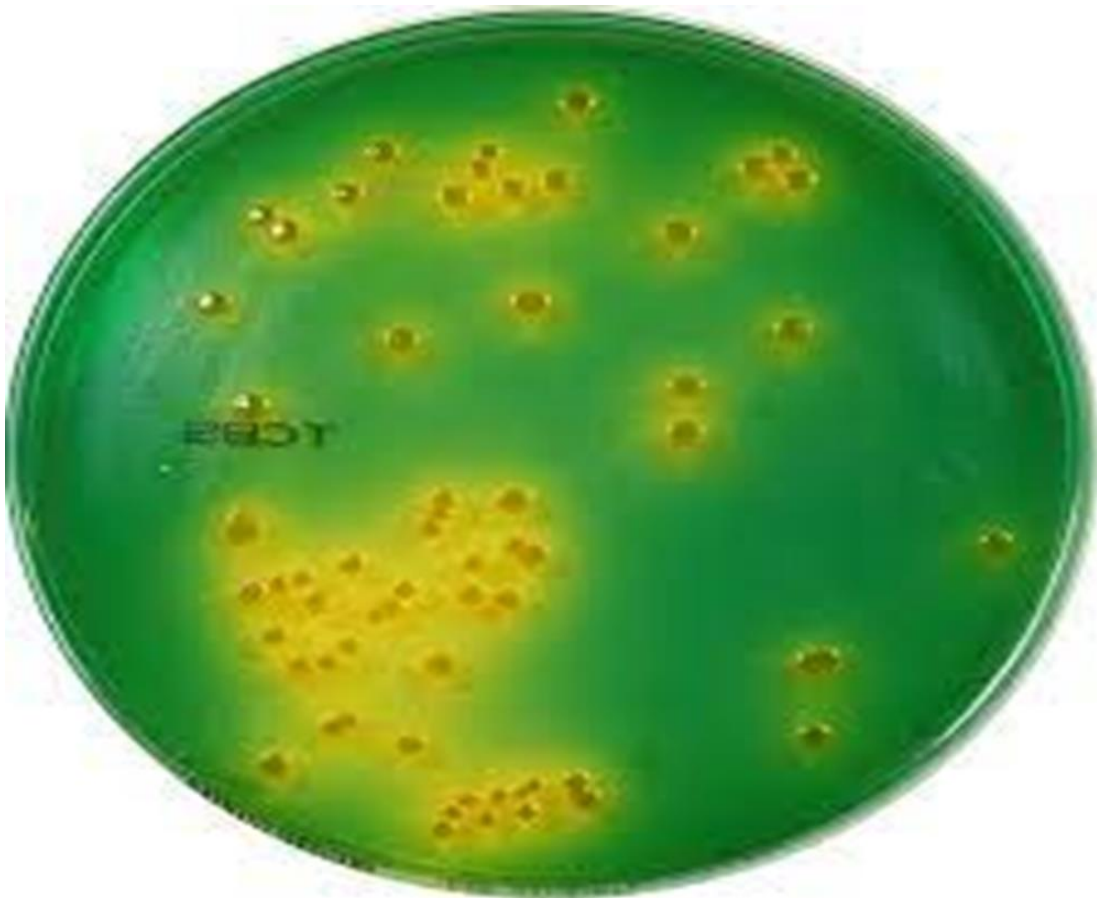


Vibrio cholerae on TCBS

Vibrio cholerae on TCBS Agar



Vibrio parahaemolyticus on TCBS Agar



Common Biochemical Tests Used

The following biochemical tests are routinely used for bacterial identification:

- Catalase Test (**Gram-positive / Gram-negative**)
- Oxidative–Fermentative (O/F) Test (**Gram-positive / Gram-negative**)
- Oxidase Test (**Gram-positive / Gram-negative**)
- Coagulase Test (**Gram-positive**)
- Hippurate Hydrolysis Test (**Gram-positive**)
- Bile Esculin Test (**Gram-positive**)
- Bacitracin Susceptibility Test (**Gram-positive**)
- Optochin Susceptibility Test (**Gram-positive**)
- Motility Test (**Gram-positive / Gram-negative**)

Motility Test

Procedure

1- Touch a straight needle to a colony of a recent culture (18- to 24 hour) growing on agar medium.

2- **Stab** once to a depth of only 1/3 to 1/2 inch in the middle of the tube.

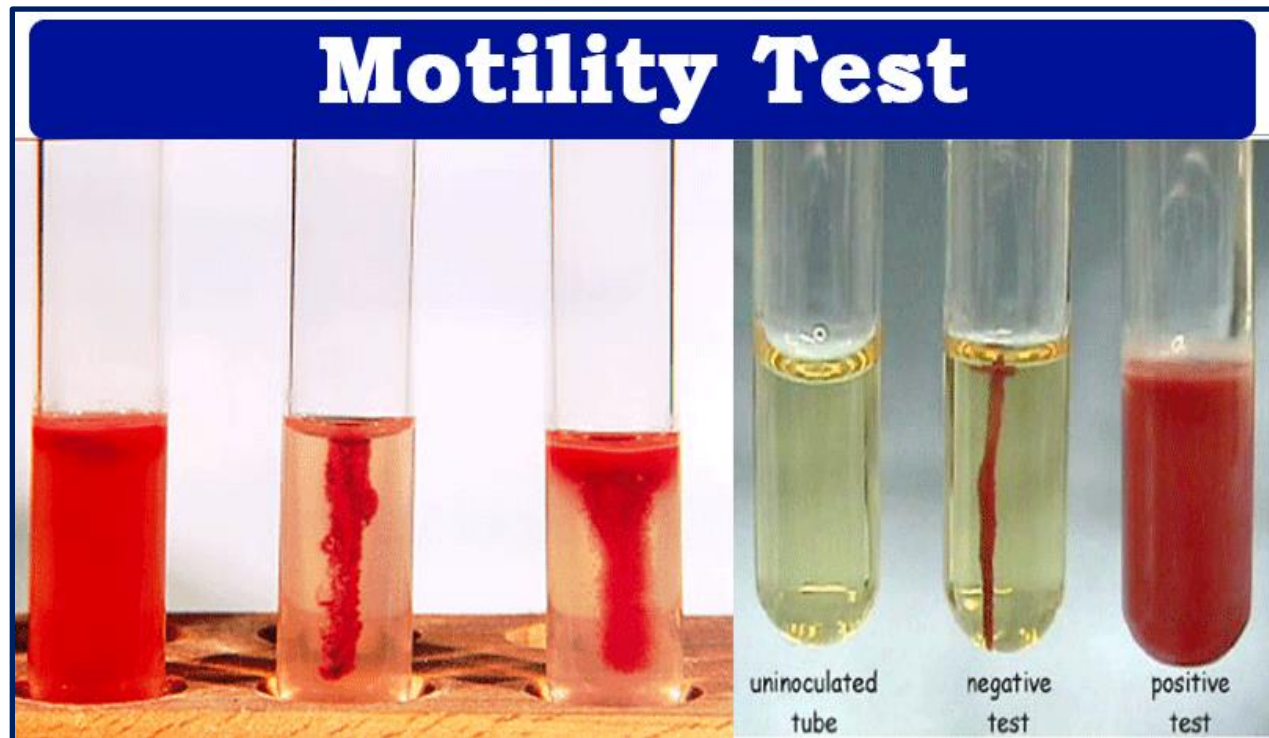
3- Incubate at 35°-37°C and examine daily for up to 7 days.

4- Observe for a diffuse zone of growth spreading out from the line of inoculation.

- **Semi solid medium 0.7-0.8% agar** is used to perform this test.

- Motile Bacteria: *Escherichia coli*, *Helicobacter pylori*, *Pseudomonas aeruginosa*.

- None Motile Bacteria: *Klebsiella*, *Shigella*, *Staphylococcus*



Staphylococcaceae Family

The Staphylococcaceae family consists of **Gram-positive cocci** that appear **spherical** under the microscope. They are commonly found as part of the **microbiota** of human skin and mucous membranes. Some species behave as **opportunistic pathogens**, while others are **highly virulent** and responsible for serious infections.

Morphology and General Characteristics

- Gram stain: Gram-positive
- Shape: Cocci
- Arrangement: Grape-like clusters
- Oxygen requirement: Facultative anaerobes (grow aerobically and anaerobically)
- Motility: Non-motile
- Spore formation: Non-spore forming
- Salt tolerance: Grow in 7.5% NaCl
- Bile salts: All species can grow in the presence of bile salts
- Catalase: Positive
- Oxidase: Negative
- Capsule: Present in some species/strains
- Coagulase: Positive or negative (species-dependent)
- DNase: Positive or negative (species-dependent)
- Mannitol fermentation: Positive or negative (species-dependent)

Genera and Important Species

- Genus: *Staphylococcus* Important species include: *Staphylococcus aureus*
Staphylococcus epidermidis, *Staphylococcus saprophyticus*

Culture Characteristics and Media

- Blood agar (**to observe hemolysis**)
- Mannitol Salt Agar (MSA) – **selective and differential**
- Baird-Parker agar

Growth on Baird-Parker Medium

- Most *Staphylococcus* species grow fermentatively
- *Staphylococcus saprophyticus* grows oxidatively

Mannitol Salt Agar (MSA)

Chapman Agar (Mannitol Salt Agar) is used for the isolation, **enumeration, and differentiation** of *Staphylococcus* species from clinical, food, antiseptic, and cosmetic samples.

Principle of Mannitol Salt Agar

- **Selectivity**: The medium contains a high salt concentration (**7.5% NaCl**), which **inhibits most Gram-negative bacteria and many Gram-positive bacteria**, allowing staphylococci to grow selectively.
- **Differentiation**: The medium contains **mannitol** as the **only fermentable sugar**. Fermentation of mannitol by the organism produces **acid, lowering the pH below 6.9**.
- **The phenol red pH indicator** then turns the medium **yellow**, **indicating a positive fermentation reaction**.
- **Non-mannitol-fermenting** staphylococci do **not change** the medium and it **remains red**.
- **Mannitol fermentation** → acid production → **yellow** color (pH < 6.9)
- **No fermentation** → peptone utilization → **red/pink** color

Interpretation

- ***Staphylococcus aureus***: Yellow colonies with a yellow halo
- **Non-pathogenic staphylococci**: Small red or pink colonies

Important note: The color change refers to the medium, not the colony itself. Some **pigmented bacteria** (e.g. micrococci) may produce colored colonies without fermenting mannitol.

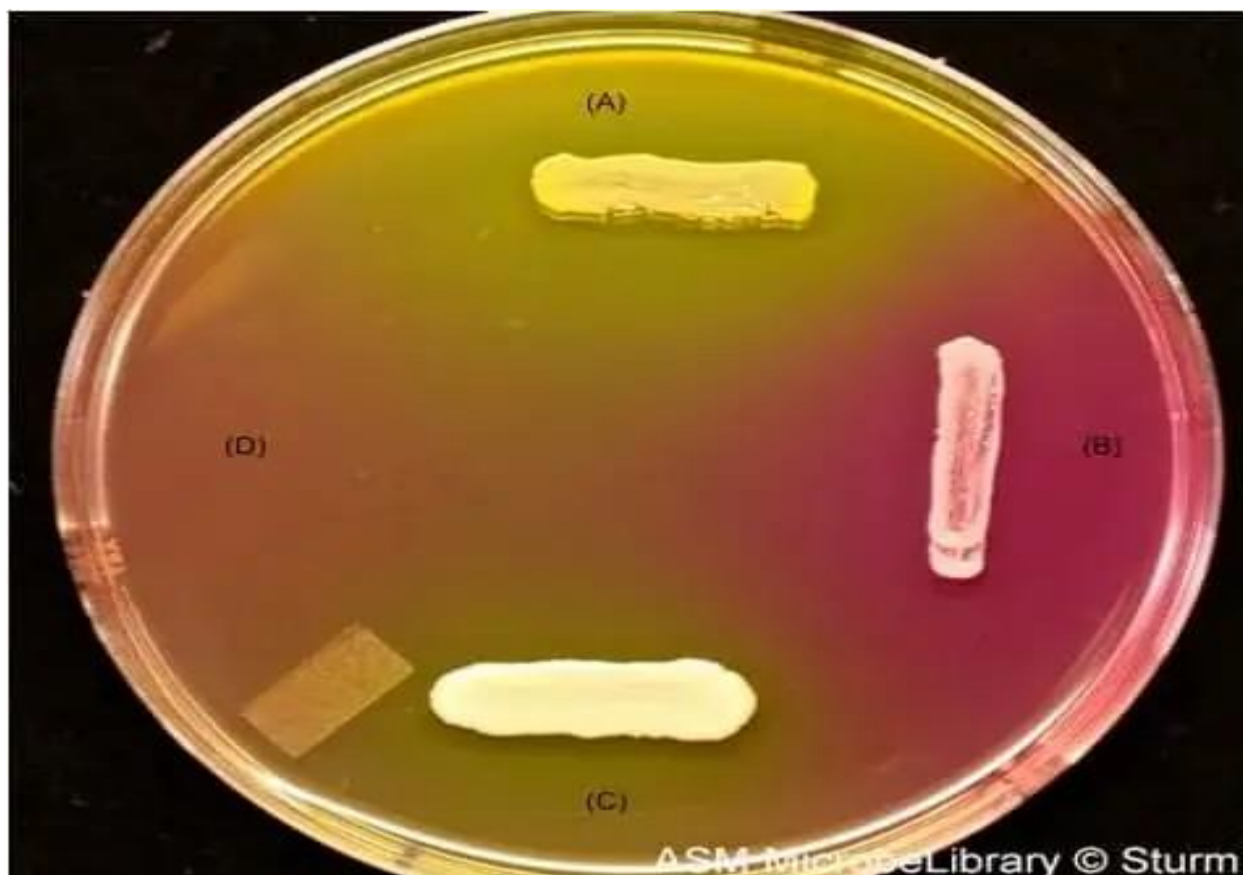


Figure : (A). *Staphylococcus aureus*: large yellow halo around growth indicates fermentation of mannitol.

(B). *Staphylococcus epidermidis*: Growth but not color change to the media indicating no fermentation of mannitol.

(C). *Staphylococcus saprophyticus*: small yellow halo around growth indicates fermentation of

mannitol. (10% of *S. saprophyticus* ferment mannitol)
(D). *E. coli*: no growth. Inhibited by the 7.5% NaCl



***Staphylococcus aureus* on mannitol salt agar**

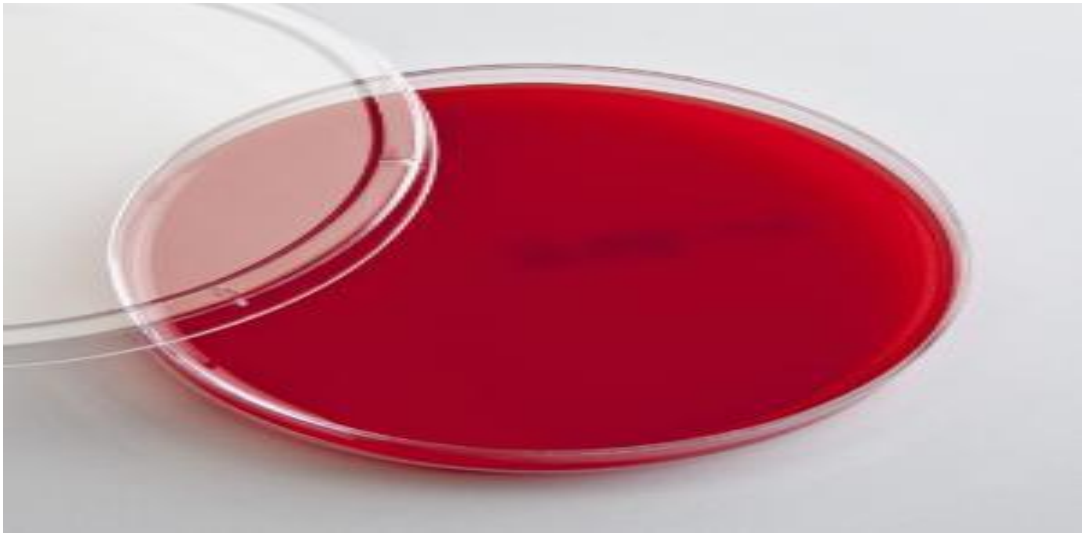


***Staphylococcus epidermidis* on mannitol salt agar**

Hemolysis on Blood Agar

▶ Blood agar (Enrichment differential media)

- ▶ Enrichment: for fastidious bacteria
- ▶ Differential: alpha hemolysis & beta hemolysis
- ▶ Add 5 ml blood \ 100 ml base of blood agar



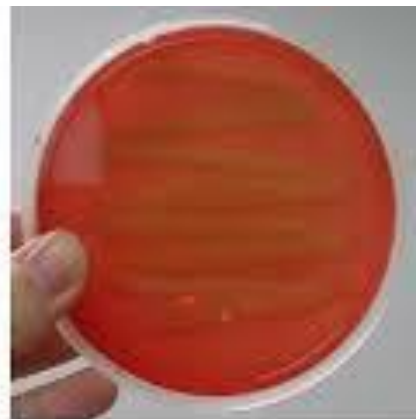
Blood agar



Beta Hemolysis



Alpha Hemolysis



Gamma Hemolysis

- *Staphylococcus aureus*: Beta-hemolysis
- Other staphylococci: Usually non-hemolytic or gamma hemolysis

Clinical Importance Blood agar

Blood agar is a general purpose, enriched medium often used to grow **fastidious organisms** and to **differentiate bacteria based on their hemolytic properties Beta, Alpha and Gamma hemolysis**. In the U.S., blood agar is usually prepared from tryptic soy agar or Columbia agar base with 5% sheep blood.



Biochemical Tests for the Identification of Gram-Positive Bacterial Species

Biochemical tests are essential tools used in clinical microbiology laboratories to identify Gram-positive bacteria based on their metabolic and enzymatic activities.

Catalase Test

Purpose The catalase test determines the ability of bacteria to **produce the enzyme catalase**, which **breaks down hydrogen peroxide (H₂O₂) into water and oxygen gas**.

Principle

$2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2 \uparrow$ The release of oxygen is observed as visible gas bubbles.

Test Method

A small amount of bacterial growth is mixed with hydrogen peroxide. The reaction is observed for immediate bubbling.

- **Positive result:** rapid formation of oxygen bubbles (within 5–10 seconds)
- **Negative result:** no bubbles or only a few weak bubbles

Interpretation

- **Catalase-positive:** Gram-positive bacteria: *Staphylococcus* spp.
- **Catalase-negative:** Gram-positive bacteria: *Streptococcus* spp. and *Enterococcus* spp.

Catalase-positive bacteria are usually strict aerobes or facultative anaerobes, while catalase-negative bacteria may be anaerobes.

Concentration of Hydrogen Peroxide Used

H₂O₂ Concentration	Purpose
3% H ₂ O ₂	Routine testing of aerobic bacteria
15% H ₂ O ₂	Identification of anaerobic bacteria
30% H ₂ O ₂	Identification of <i>Neisseria</i> spp.

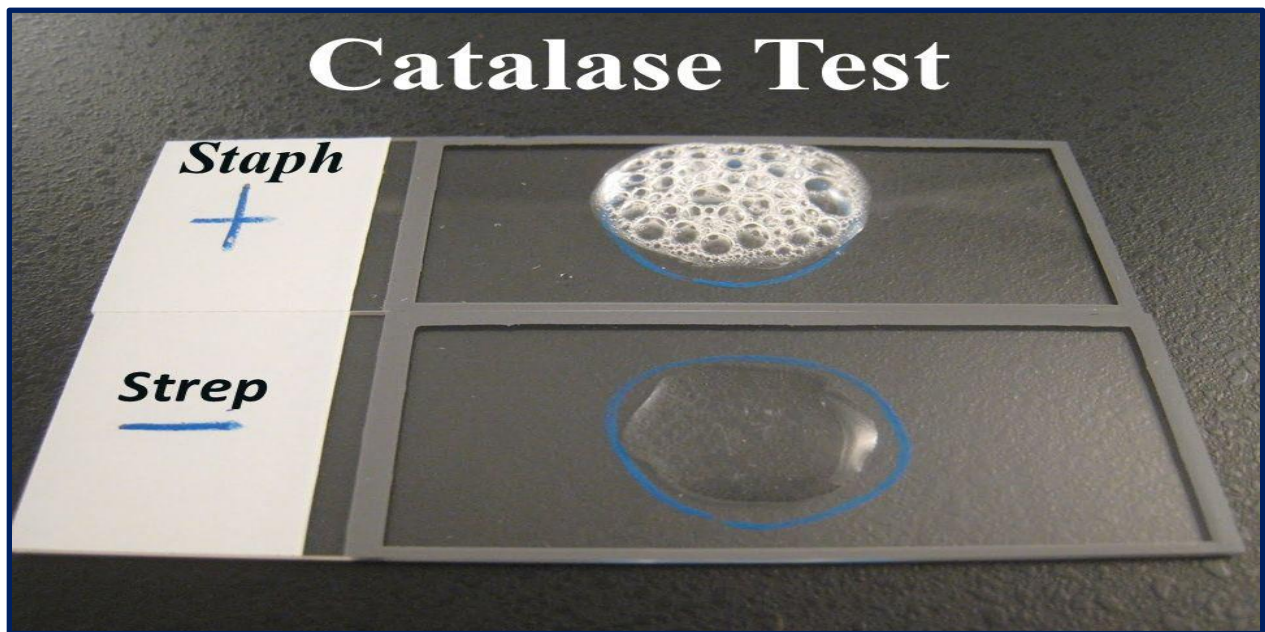
1-Procedure (Slide Method)

1. Using a sterile loop or wooden stick, transfer a small visible amount of a bacterial colony onto a clean, dry glass slide.
2. Add one drop of 3% hydrogen peroxide to the bacterial smear.
3. Mix gently and observe immediately.

4. Positive reaction: rapid bubbling within 5–10 seconds.
5. Negative reaction: absence of bubbles or only a few scattered bubbles.
6. Dispose of the slide in a biohazard glass disposal container.

Clinical Significance

- The catalase test is **one of the first and most important tests in Gram-positive bacterial identification.**
- It is particularly useful for **differentiating Staphylococci (catalase-positive) from Streptococci (catalase-negative).**
- It is often performed before more specific biochemical or serological tests.



2-Tube Test

1. Add 4 to 5 drops of 3% H_2O_2 to a test tube
2. Using a wooden applicator stick, collect a small amount of organism from a well-isolated 18 to 24-hour colony and place it into the test tube (*Note: Be careful not to pick up any agar (especially if using Blood Agar).*)

3. Place the tube against a dark background and observe for immediate bubble formation ($O_2 + \text{water} = \text{bubbles}$) at the end of the wooden applicator stick.



2-Oxidase Test

Principle:

This test is performed to determine or identify the presence of an **enzyme cytochrome oxidase** (of the electron transport chain) in bacterial cells. This enzyme sometimes called indophenol oxidase.

-The **reagent** used is (**tetramethyl-p-phenylene diamine dihydrochloride**) which is **oxidized** to a **purple-colored** end product called **indophenol** by the enzyme oxidase.

- The development of a **dark purple color** is a **positive** test that indicates the presence of oxidase, whereas **if the enzyme is not present**, the reagent remains reduced and is **colorless**.

Procedure:

- 1- Take a filter paper and moisturize it with the substrate i.e. 1% tetramethyl-p-phenylene diamine dihydrochloride or select a commercially available paper disk that has been saturated with the same substrate.

2- Remove a small portion of a bacterial colony (preferably not more than 24 hours old) from the agar surface with a sterile platinum wire or wooden stick.

3- Rub the sample on the filter paper or commercial disks.

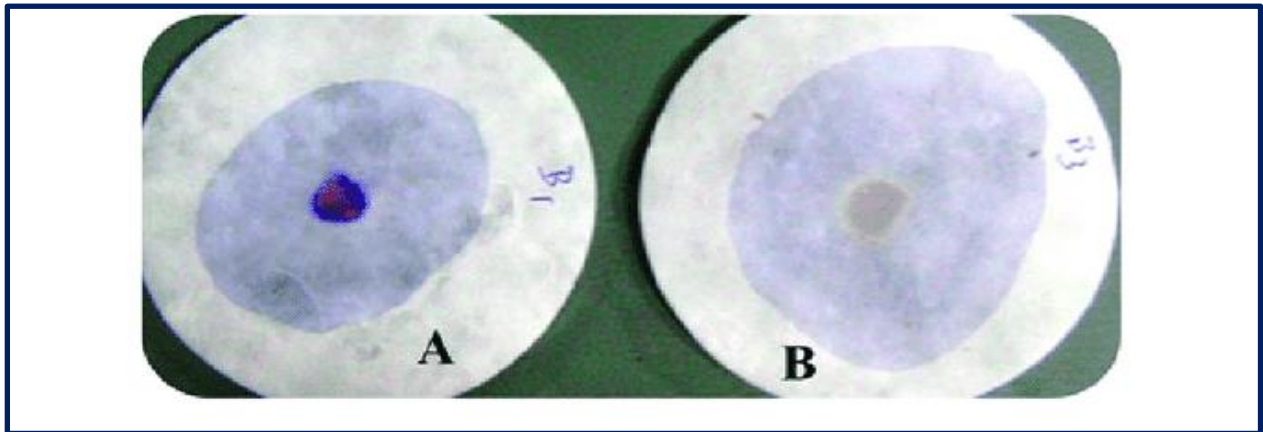
4- Observe the inoculated area of the paper or disks for the color change to **deep blue or purple** within 10 seconds because timing is very critical.

***Positive result:** Development of a **dark purple color** within 10 seconds of inoculation.

Neisseria gonorrhoeae, *Vibrio cholera*, *Pseudomonas* are oxidase positive.

***Negative result:** No change in color (no blue color seen)

Members of family Enterobacteriaceae like *E. coli* are oxidase negative.



3- Coagulase Test

The coagulase test is a biochemical laboratory test used to determine whether a bacterial isolate produces the enzyme coagulase, which converts soluble fibrinogen in blood plasma into insoluble fibrin, resulting in plasma clotting.

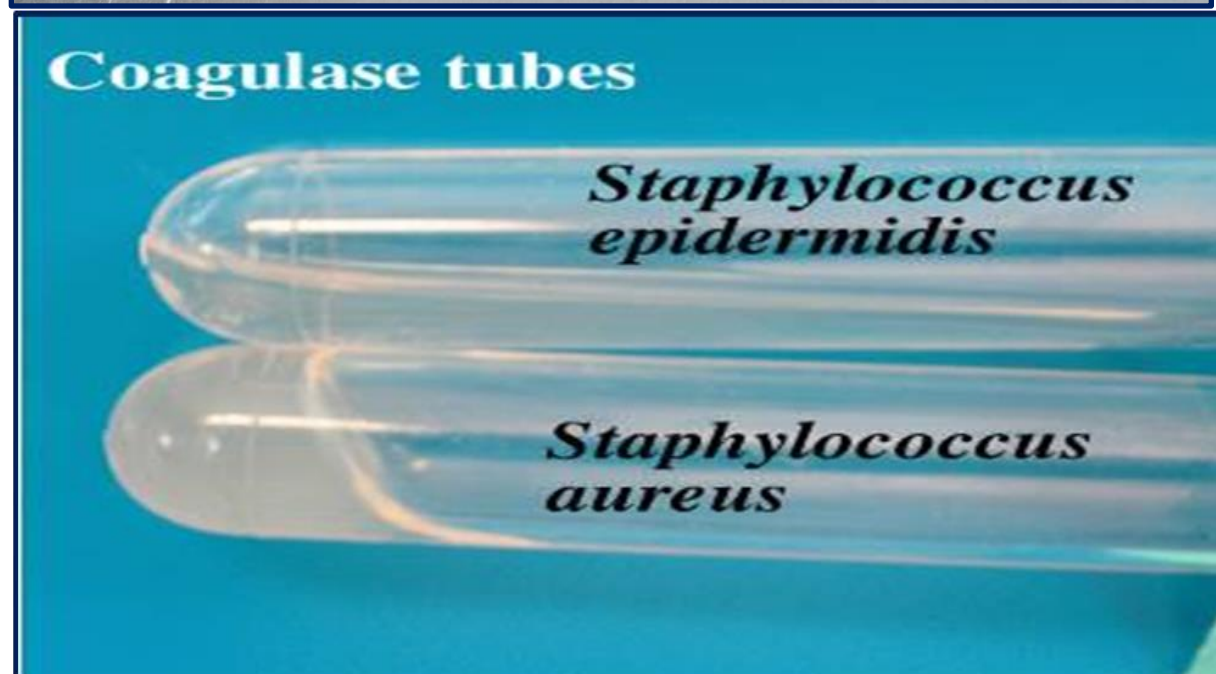
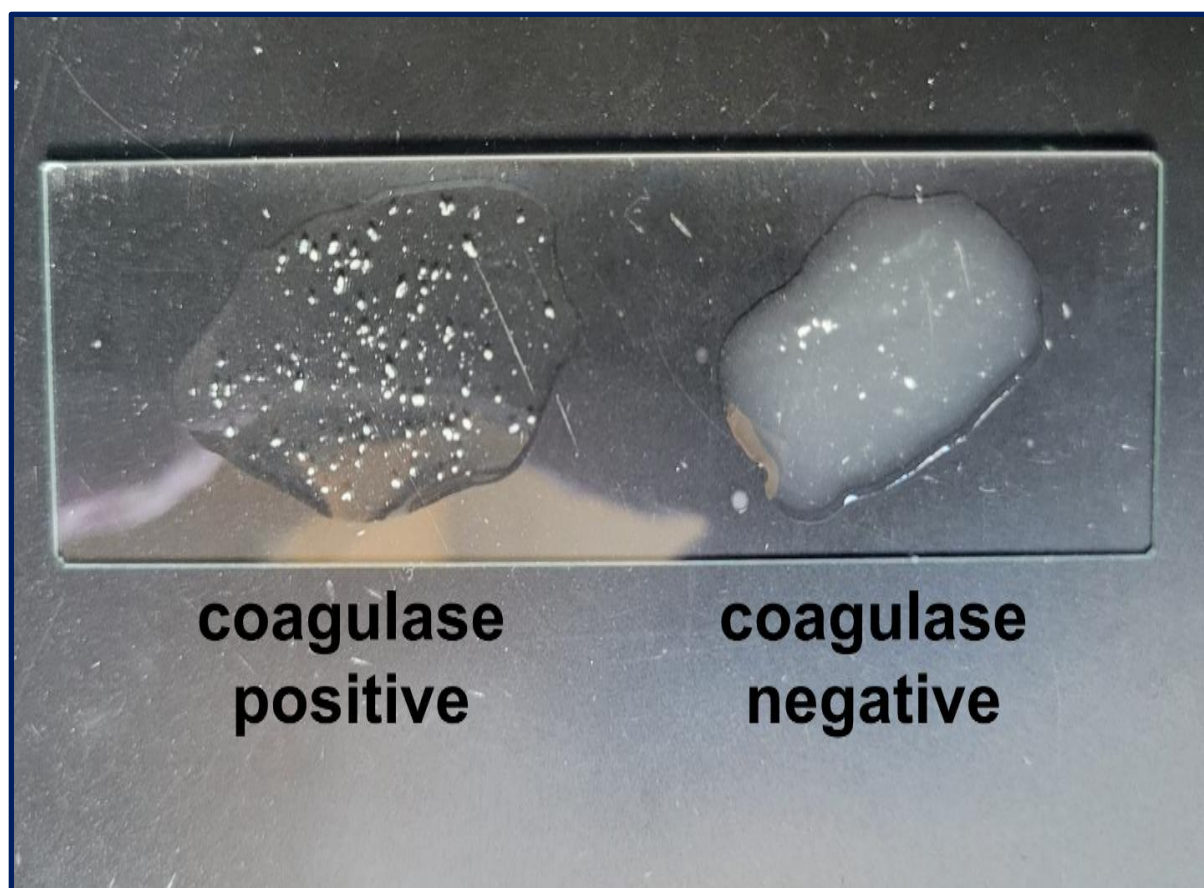
This test is of major importance in microbiology because it is used to differentiate the highly pathogenic *Staphylococcus aureus* (coagulase-positive) from (coagulase-negative) Staphylococci (CoNS), such as *Staphylococcus epidermidis* and *Staphylococcus saprophyticus*, which are generally less pathogenic and part of the normal human flora.

Staphylococcus aureus produces two forms of coagulase:

- Bound coagulase (clumping factor)
- Free coagulase

Accordingly, the coagulase test can be performed by two different methods, depending on the form of coagulase being detected:

- The slide coagulase test detects cell-bound coagulase and yields a positive result as visible agglutination.
- The tube coagulase test detects free coagulase and is considered positive when a stable clot forms in the plasma.



4-DNase Test

The DNase test is a presumptive biochemical test used to differentiate *Staphylococcus aureus*, which produces the enzyme deoxyribonuclease (DNase), from other *Staphylococcus* species that do not produce this enzyme.

- *S. aureus* produces a heat-stable thermonuclease. To detect this enzyme, the bacteria are first destroyed by heat, and the released DNase then reacts with the medium.
- Other bacteria that may give a positive DNase test include *Vibrio*, *Helicobacter*, *Moraxella*, *Serratia*, and *Aeromonas*.

Principle

- DNases are extracellular endonucleases that cleave DNA, releasing free nucleotides and phosphate.
- DNase agar is used to detect DNA hydrolysis. The medium may contain no indicator or indicators such as toluidine blue or methyl green.

Detection methods:

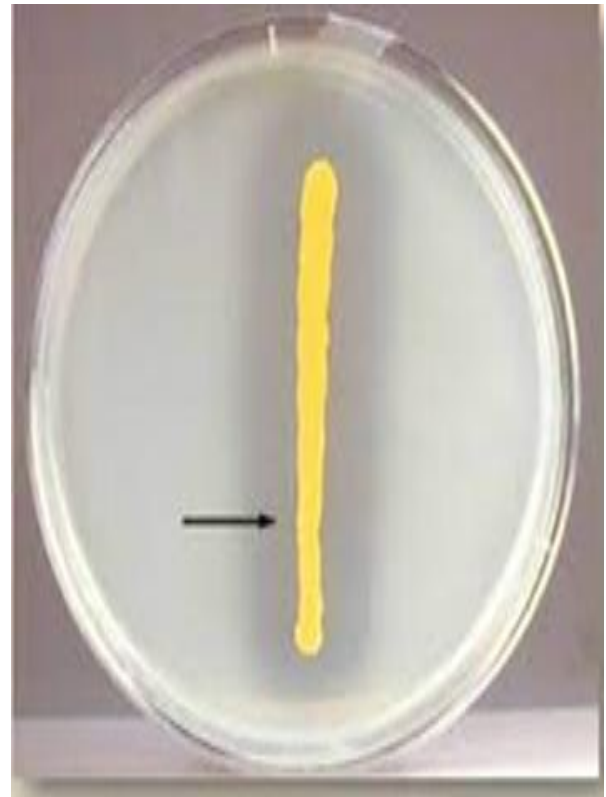
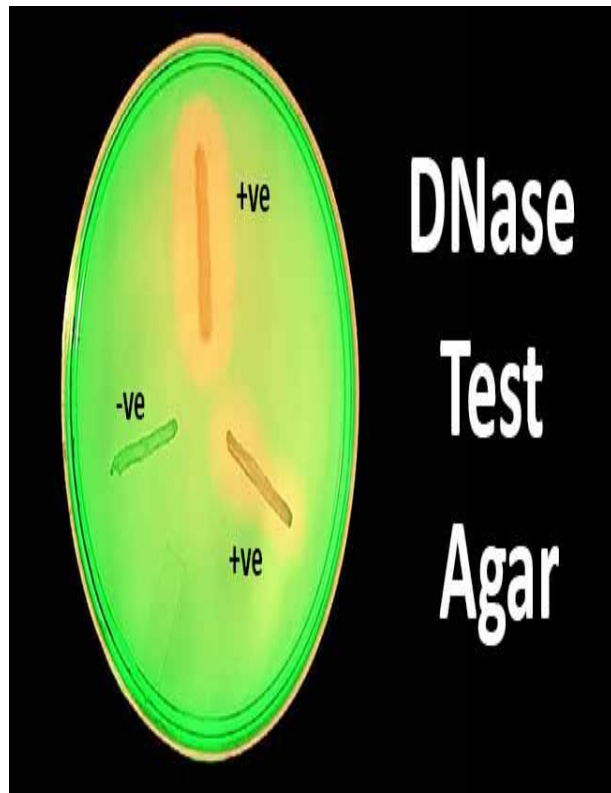
1. **DNase agar without indicator:** After incubation, the plate is flooded with 1N HCl. Unhydrolyzed DNA precipitates and makes the medium opaque, while DNA hydrolyzed by DNase-producing colonies forms a clear zone around the growth.
2. **DNase agar with methyl green:** DNA binds methyl green to produce a mint green color. Hydrolysis releases the DNA-methyl green complex, and free methyl green becomes colorless, forming a clear halo around DNase-positive colonies.
3. **DNase agar with toluidine blue O (TBO):** DNA forms a complex with TBO that changes structure upon hydrolysis, producing a bright pink color around positive colonies.

Uses

1. Detect the ability of an organism to hydrolyze DNA.
2. Differentiate *S. aureus* (DNase-positive) from other Staphylococci (DNase-negative).
3. Useful when plasma is unavailable for the coagulase test or when coagulase results are difficult to interpret.

Procedure

1. Using a sterile loop, pick several colonies from an 18–24 hour culture.
2. Inoculate the test and control organisms onto designated areas of the DNase agar.
3. Incubate the plate at 35–37°C for 24 hours.
4. Observe for color change: In DNase agar with methyl green, look for clear halos around colonies.
5. In DNase agar without indicators: Flood the agar surface with 1N HCl and remove excess acid.
6. Allow the reagent to absorb into the medium.
7. Look for a clear zone around colonies within 5 minutes, indicating DNA hydrolysis.



DNase test **positive**: *Staphylococcus aureus*, DNase test **negative**: *Staphylococcus epidermidis*

Novobiocin sensitivity test

To differentiate coagulase-negative staphylococci (CoNS) species, particularly *Staphylococcus saprophyticus* from other CoNS like *Staphylococcus epidermidis*.

Principle:

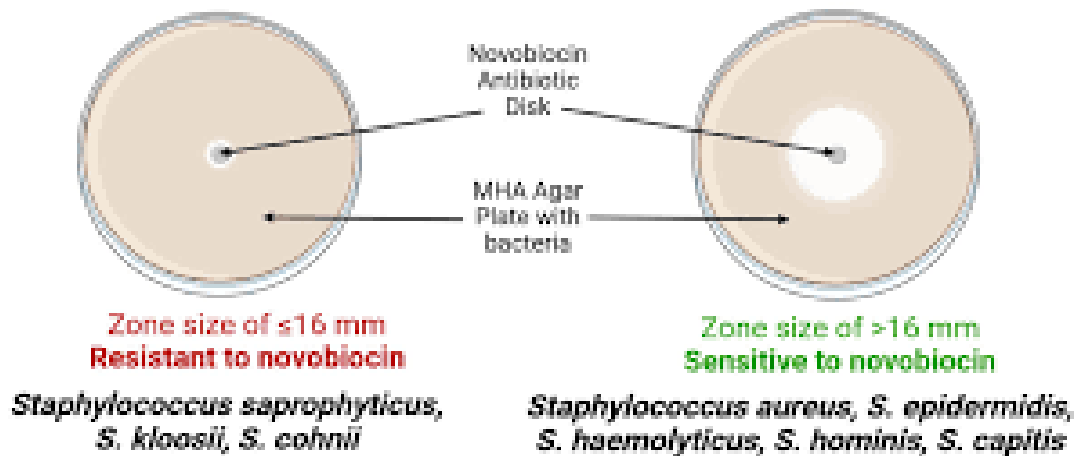
Novobiocin is an antibiotic that inhibits bacterial DNA gyrase, affecting bacterial DNA replication. The zone of inhibition around a novobiocin disc indicates sensitivity or resistance.

Species	Novobiocin Sensitivity	Clinical Relevance
<i>Staphylococcus saprophyticus</i>	Resistant	Common cause of urinary tract infections in young women. Resistance helps in identification.
<i>Staphylococcus epidermidis</i>	Sensitive	Normal skin flora; usually not resistant to novobiocin.
Other CoNS (most)	Sensitive	Used for identification in microbiology labs.

Procedure

1. Prepare a Mueller-Hinton agar plate.
2. Inoculate the plate with a pure culture of *Staphylococcus* (0.5 McFarland standard).
3. Place a 30 µg novobiocin disc on the agar.
4. Incubate at 35–37°C for 24 hours.
5. Measure zone of inhibition: ≥16 mm: Sensitive, ≤16 mm: Resistant

Novobiocin Susceptibility Test Results



Staphylococci can cause a wide range of infections, including:

- Skin and soft tissue infections
- Abscesses
- Urinary tract infections (UTIs)
- Septicemia
- Endocarditis

Clinical Specimens

Specimens commonly received in the laboratory include:

- Urine
- Blood
- Stool
- Swabs from ear, nose, eye
- Acne, burns, and wound swabs
- Catheter tips
- Seminal fluid

Differentiation between

Staphylococcus species

Tests	<i>S.aureus</i>	<i>S.epidermidis</i>	<i>S.saprophyticus</i>
Gram stain	Gram+ve cocci	Gram+ve cocci	Gram+ve cocci
Catalase	Positive	Positive	Positive
Oxidase	Negative	Negative	Negative
Blood agar	Beta	Gamma	Gamma
Coagulase	Positive	Negative	Negative
DNase	Positive	Negative	Negative
Mannitol fermentation	Ferment mannitol	Dose not ferment	Dose not ferment
Novobiocin sensitivity	S	S	R
Colony	Golden	White	Light yellow

S. epidermidis:

- Nutrient agar: White colonies
- Blood agar: Non haemolytic
- MSA: Non fermentative

S. saprophyticus:

- Nutrient agar: Yellow colonies
- Blood agar: Non haemolytic
- MSA: Non fermentative

Habitat:

- *S. aureus*
 - Nasal passages, skin, oral cavity and gastrointestinal tract.
- *S. epidermidis*
 - skin.
- *S. saprophyticus*
 - Rarely found in healthy humans.

Streptococcaceae Family

The Streptococcaceae family includes important human pathogens responsible for respiratory, skin, and systemic infections. Classification is based on hemolysis patterns and Lancefield grouping.

Morphology and General Characteristics

- Gram stain: Gram-positive
- Shape: Cocci
- Arrangement: Chains or pairs
- Oxygen requirement: Facultative anaerobes (some strict anaerobes)
- Motility: Non-motile
- Spore formation: Non-spore forming
- Capsule: Present in some species/strains
- Salt tolerance: Grow in 7.5% NaCl
- Bile tolerance: Grow in the presence of bile salts
- Catalase: Negative
- Oxidase: Negative
- Coagulase: Negative
- DNase: Negative

Genus: Streptococcus Important species:

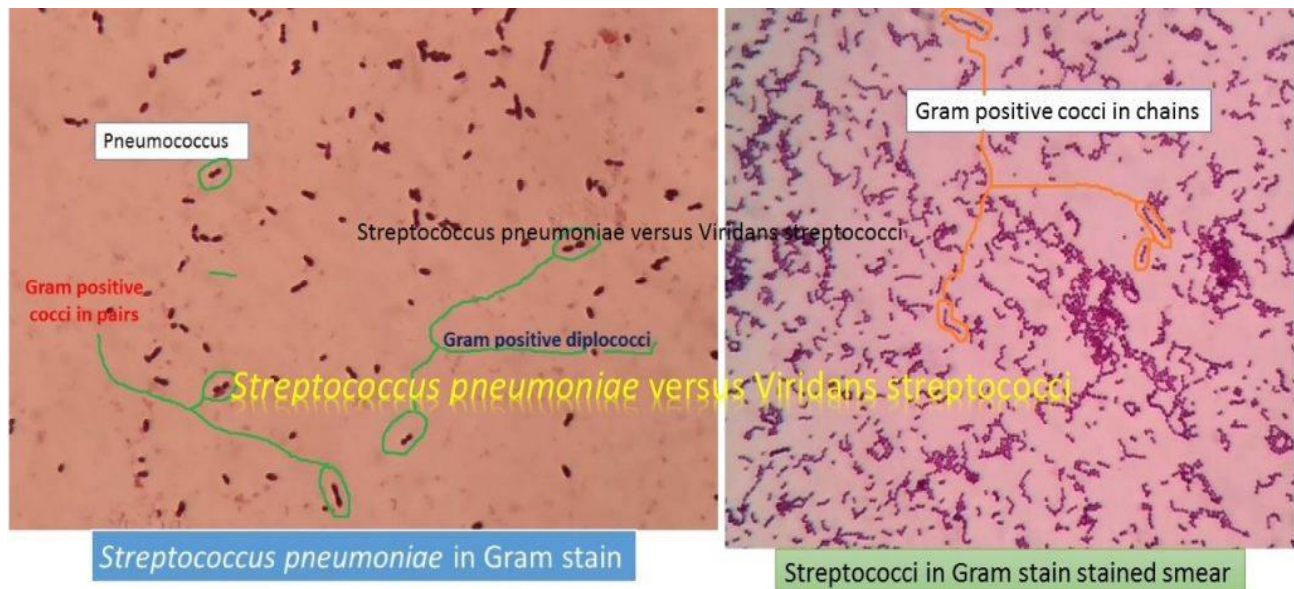
- *Streptococcus pyogenes* (Group A)
- *Streptococcus agalactiae* (Group B)
- *Streptococcus pneumoniae*
- Viridans streptococci (e.g., *S. salivarius*, *S. mitis*) (Group C)

Cultural and Biochemical Characteristics

- Grow best under facultative conditions; blood agar is preferred.
- Colony characteristics: small, 1–2 mm in diameter
- Hemolysis patterns on blood agar:
 1. **β -hemolysis**: clear lysis (e.g., *S. pyogenes*, *S. agalactiae*)
 2. **α -hemolysis**: greenish partial hemolysis (e.g., *S. pneumoniae*, viridans streptococci)
 3. **γ -hemolysis**: no hemolysis

Genus / Species	Morphology & Arrangement	Hemolysis	Key Biochemical Tests	Capsule	Preferred Media	Notes / Special Features
<i>S. pyogenes</i> (Group A)	Gram-positive cocci, chains of 4–10 cells	β -hemolysis	Bacitracin susceptible, Streptokinase +	Uncommon	Blood agar	Causes pharyngitis, skin infections, rheumatic fever
<i>S. agalactiae</i> (Group B)	Gram-positive cocci, chains	β -hemolysis	CAMP test +, Hippurate hydrolysis +	Uncommon	Blood agar	Neonatal sepsis & meningitis
<i>S. pneumoniae</i>	Gram-positive diplococci, "bullet-shaped"	α -hemolysis (green)	Optochin susceptible, Bile solubility +	Yes (polysaccharide)	Blood agar, Chocolate agar	Causes pneumonia, meningitis, otitis media
Viridans streptococci (e.g., <i>S. mitis</i> , <i>S. salivarius</i>) (Group C)	Gram-positive cocci, chains or pairs	α -hemolysis or γ -hemolysis	Optochin resistant, Bile solubility -	No	Blood agar	Normal oral flora; low virulence; can cause subacute bacterial endocarditis
Enterococci (formerly Group D Streptococcus) <i>Enterococcus faecalis</i>	Gram-positive cocci, pairs/chains	γ -hemolysis (usually)	Bile esculin +, Growth in 6.5% NaCl +	No	Blood agar, Bile esculin agar	Opportunistic pathogens, UTIs, nosocomial infections

Note: Most streptococci are facultative anaerobes, and some are obligate (strict) anaerobes.



Biochemical tests commonly used for identification:

1. Hemolysis pattern on blood agar
2. Bacitracin test (Group A identification)
3. CAMP test (Group B identification)
4. Optochin test (*S. pneumoniae* identification)
5. Streptokinase test
6. Bile solubility test (*S. pneumoniae*)
7. Bile esculin test
8. Hippurate hydrolysis test

Chocolate Agar and the Streptococcaceae Family

- Chocolate agar is an enriched culture medium.
- It is prepared by heating blood agar until the red blood cells are lysed.
- The name “chocolate” refers only to the brown color; it does not contain chocolate.

Relationship with Streptococcaceae

Members of the Streptococcaceae family are **fastidious organisms**, meaning some species require enriched media for optimal growth.

Streptococcus species that grow well on chocolate agar:

- *Streptococcus pneumoniae*
- *Streptococcus pyogenes*
- *Viridans streptococci*

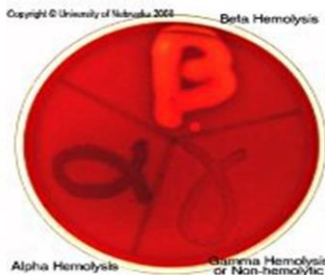
✓ **Note Hemolysis** cannot be determined on chocolate agar **because red blood cells are already destroyed.**

- Blood agar is the primary medium for identification and hemolysis classification of Streptococcus.
- Chocolate agar is used to support growth, not for hemolysis differentiation

1-Hemolysis pattern on blood agar

Hemolysis

- Beta (β) hemolysis
 - Complete lysis of RBC's surrounding colony causing a clear zone or clearing of blood from the agar
- Alpha (α) hemolysis
 - Partial lysis of RBC's surrounding colony causing a greenish discoloration in media
- Non-hemolytic
 - Also known as Gamma hemolysis (δ)
 - No lysing of RBC's and no color change of the medium surrounding colony



2- Bacitracin Susceptibility Test

The bacitracin test is a presumptive test used to **differentiate β -hemolytic streptococci**, particularly for distinguishing **Group A β -hemolytic streptococci** (*Streptococcus pyogenes*) from **β -hemolytic non-Group A streptococci**, such as *Streptococcus agalactiae*.

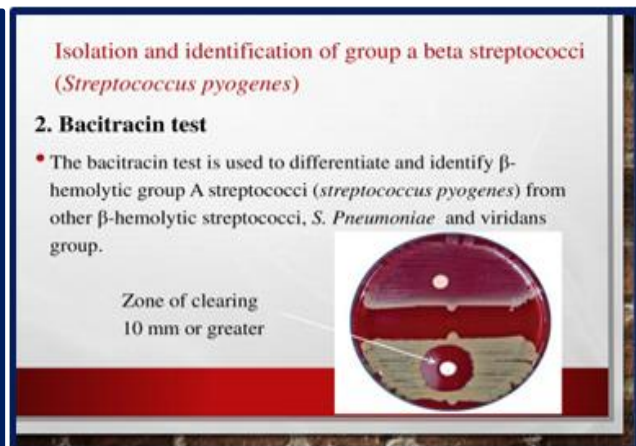
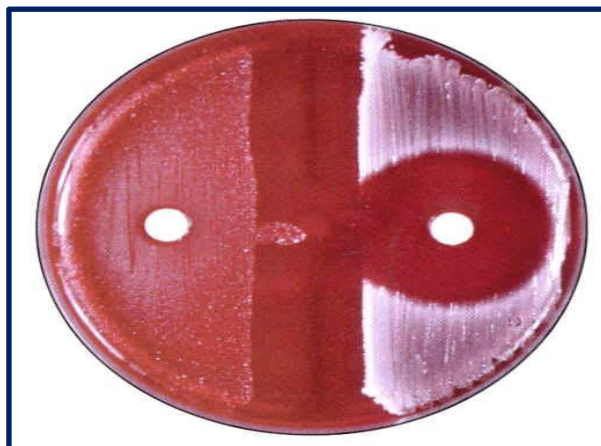
Bacitracin is an antibiotic produced by *Bacillus subtilis* and is used in this test to assess bacterial susceptibility.

The test is performed on blood agar by streaking the streptococcal isolate and placing a bacitracin disc onto the agar surface using sterile forceps, followed by incubation.

After incubation, **Group A β -hemolytic streptococci** (*S. pyogenes*) show a **clear zone of inhibition** around the bacitracin disc, indicating a **positive result**, whereas **β -hemolytic non-Group A streptococci** are **resistant to bacitracin** and grow up to the disc, showing **no zone of inhibition**, which represents a **negative result**.

- Bacitracin sensitive Group A streptococci: *Streptococcus pyogenes*

Bacitracin resistant non-Group A streptococci: *Streptococcus agalactiae*



3 -Christie–Atkins–Munch-Petersen (CAMP) test

The CAMP test is a biochemical test used in medical microbiology to identify Group B Streptococcus (*Streptococcus agalactiae*) based on its ability to produce the CAMP factor, an extracellular protein that enhances hemolysis.

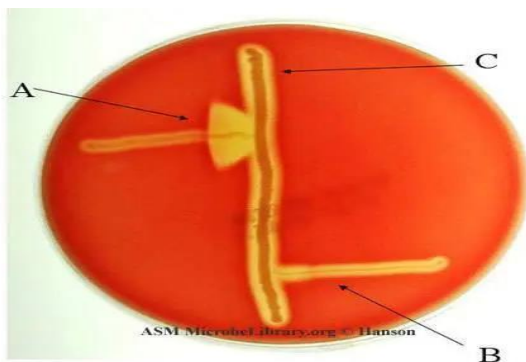
Principle

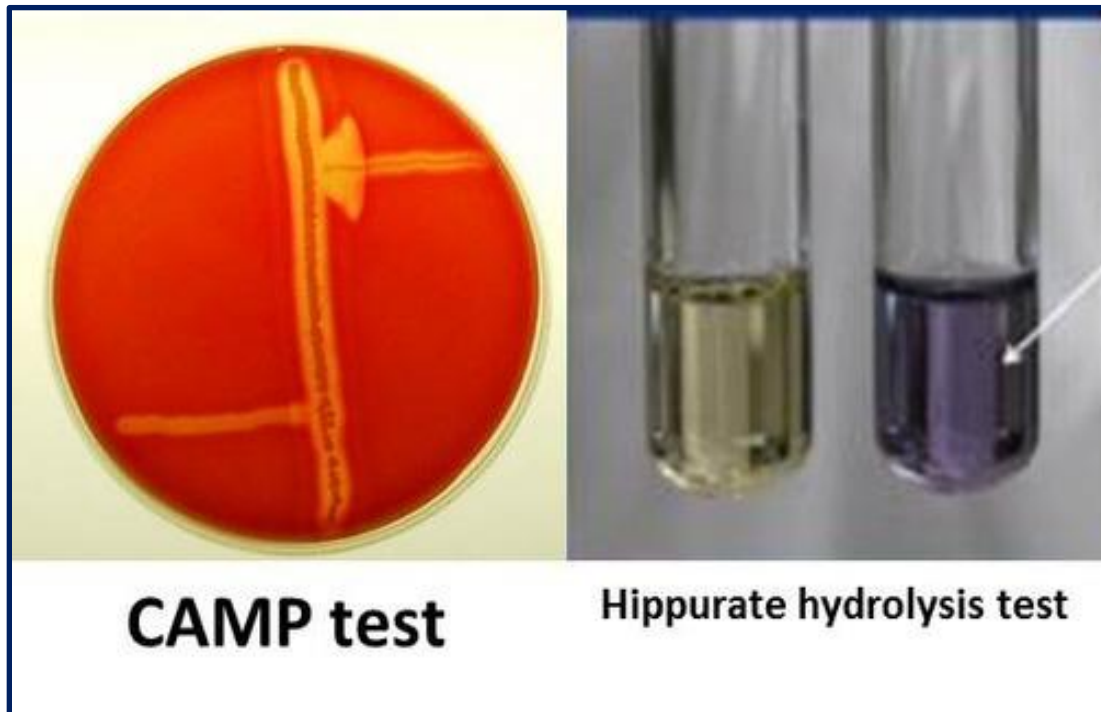
- *Streptococcus agalactiae* produces the **CAMP factor**, which acts synergistically with the β -hemolysin (sphingomyelinase C) produced by *Staphylococcus aureus*.
- When both organisms are cultured on sheep blood agar, the interaction between these two factors results in **enhanced β -hemolysis**, forming a characteristic **arrowhead-shaped zone** at the junction of the two organisms.

Procedure

- 1- A streak of β -hemolysin-producing *Staphylococcus aureus* is made down the center of a sheep blood agar plate.
- 2- The test organism (suspected streptococcus) is streaked perpendicular to the *S. aureus* streak, without touching it.
- 3- The plate is incubated at 35–37 °C for 18–24 hours.

A. *Streptococcus agalactiae*(positive), B. *Streptococcus pyogenes*(negative)





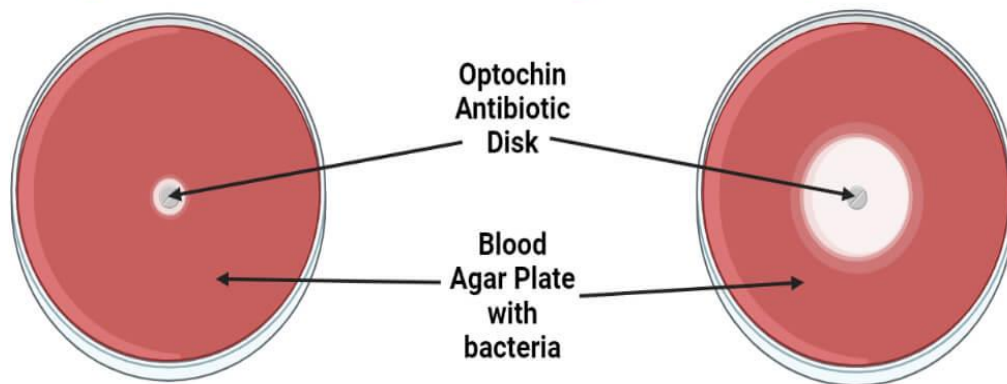
4- Optochin Susceptibility Test:

The optochin test is a useful and widely used method for the **identification of *Streptococcus pneumoniae***.

This test is commonly performed using filter paper discs impregnated with ethylhydrocupreine hydrochloride (optochin), which are placed directly onto inoculated blood agar plates prior to incubation.

Among **alpha-hemolytic streptococci**, *Streptococcus pneumoniae* is susceptible to optochin and therefore shows a clear zone of inhibition around the optochin disc. In contrast, other viridans group streptococci are optochin-resistant and do not exhibit a zone of inhibition.

Optochin Susceptibility Test

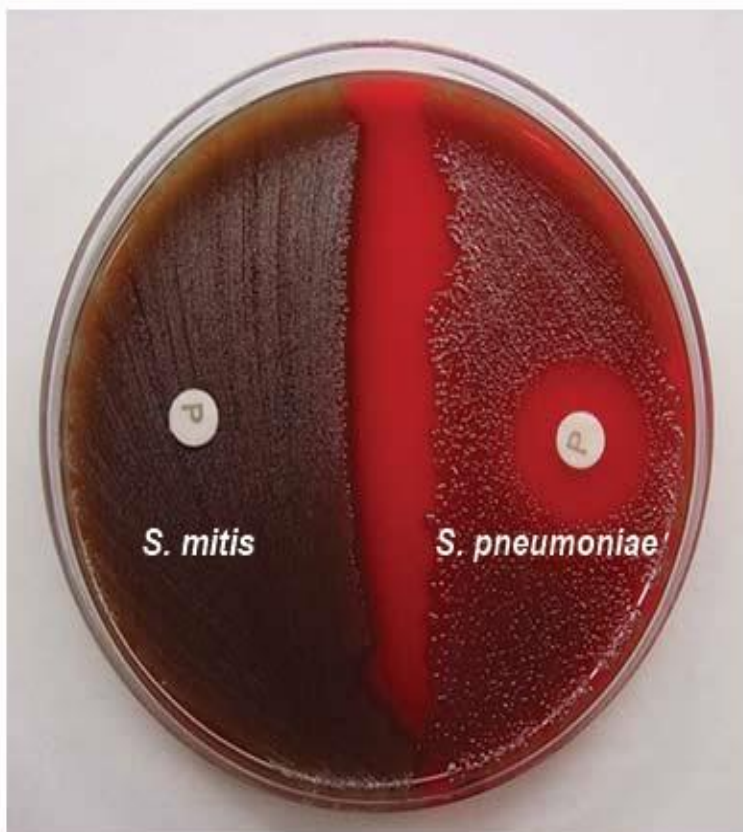


Zone size of <14 mm
Intermediate/Resistant to Optochin

S. mitis group, *S. anginosus* group, *S. sanguinis* group, *S. mutans* group, *S. salivarius* group

Zone size of ≥ 14 mm
Sensitive to Optochin

Streptococcus pneumoniae



Left Side

S. mitis

Resistant to optochin

Right Side

S. pneumoniae

Susceptible to optochin

Procedure:

1. Using an inoculating loop, select three to four well-isolated colonies of the alpha-hemolytic organism to be tested. An 18–24-hour culture of isolated organism can also be used for testing.
2. Streak the isolate onto one-half of a TSA-5% sheep blood agar plate so as to obtain confluent growth.
Note: Use of media other than TSA-5% sheep blood agar is not recommended, as false identification may result.
3. Using sterile forceps, place an optochin disk onto the inoculated surface of the agar.
4. Press disk gently with the sterile forceps or loop so that the disk adheres firmly to the agar surface.
5. Incubate the plate at 35 +/- 2.0 degrees C. for 18-24 hours in 5-10% CO₂ enriched environment.
6. If zone of inhibition is present, measure the diameter with a millimeter ruler or caliper

Positive result: Zone of inhibition is **14 mm or greater** in diameter with 6 mm disk.

Negative result: **No zone of inhibition** or a zone of inhibition of **<14mm** diameter.

4. Streptokinase test

Streptokinase is a **fibrinolytic enzyme**. Its primary function is to **dissolve blood clots**, which facilitates the spread of bacteria within body tissues.

Mechanism of action:

1. Streptokinase **binds to plasminogen present in the blood.**
2. It **converts plasminogen into plasmin**, the active enzyme.
3. Plasmin degrades fibrin, the main structural component of blood clots.
4. This results in clot dissolution, allowing bacteria to spread more easily through tissues.

Clinical significance:

Streptokinase enhances tissue invasion and dissemination of bacteria, **particularly *Streptococcus pyogenes***, thereby increasing the severity of infection.

5- Bile Salt Solubility Test

- It is the test which differentiates *Streptococcus pneumoniae* (positive- soluble) from **alpha-hemolytic streptococci (negative- insoluble)**. *Streptococcus pneumoniae* is a bile soluble species whereas all other alpha-hemolytic streptococci are bile resistant.

Principle:

S. pneumoniae has an **autolytic enzyme** which can be demonstrated by allowing a broth culture to age in the incubator; at 24 hours the broth is **turbid**; after a few days the medium will **become clear**.

Bile or a solution of a bile salt (e.g., **sodium desoxycholate**) rapidly lyses pneumococcal colonies. **Lysis depends on the presence of an intracellular autolytic enzyme, amidase.** Bile salts lower the surface tension between the bacterial cell membrane and the medium, thus accelerating the organism's natural autolytic process. Bile salts activate the autolytic enzyme which induces clearing of the culture.

Reagents:

2% sodium deoxycholate (bile salt) solution

- Dissolve 2 gram of sodium deoxycholate into 100 ml sterile distilled water.

10% sodium deoxycholate (bile salt) solution

- Dissolve 10 gram of sodium deoxycholate into 100 ml sterile distilled water.

Procedure:

Tube Method

1. Prepare a heavy suspension of a pure culture in 2 ml of 0.85% saline.
 2. Divide the organism suspension into two tubes.
 3. Adjust the turbidity to that of 0.5-1 McFarland standard.
 4. To one tube (test tube), **add 2 drops of 2% sodium deoxycholate** and mix.
 5. To the other tube (control tube), add 2 drops of sterile water distilled water and mix.
 6. Leave both tubes for 10-15 minutes at 35-37°C.
 7. Observe for a clearing of turbidity in the tube containing **2% sodium deoxycholate**.
 8. If negative, continue to incubate up to 3 hours. Observe again for clearing.
- **Positive result:** Suspension clears in tube labelled test and remains **turbid in control tube**. - **Negative result:** **Suspension remains turbid.**

Plate Method

1. Incubate the sample on 5% sheep blood agar for 12 to 24 hours.
2. Place one to two drops of 10% sodium deoxycholate to the side of a freshly isolated colony (18 -24 hours) on 5% sheep blood agar.
3. Gently wash the solution over the colony with dislodging the colony from the medium.
4. Incubate the culture plate at 35-37°C for 30 minutes.
5. Examine for lysis of colony (Disappearance of the colony).

Note: Partial clearing (partial solubility) is not considered positive for *S. pneumoniae* identification.



6- Bile Esculin Test:

- The bile-esculin test is widely used to **differentiate Enterococcus species** and **group D streptococci**, which are **bile tolerant** and **capable of hydrolyzing esculin**, from non-group **D viridans streptococci**, which grow poorly in the presence of bile and do not hydrolyze esculin.

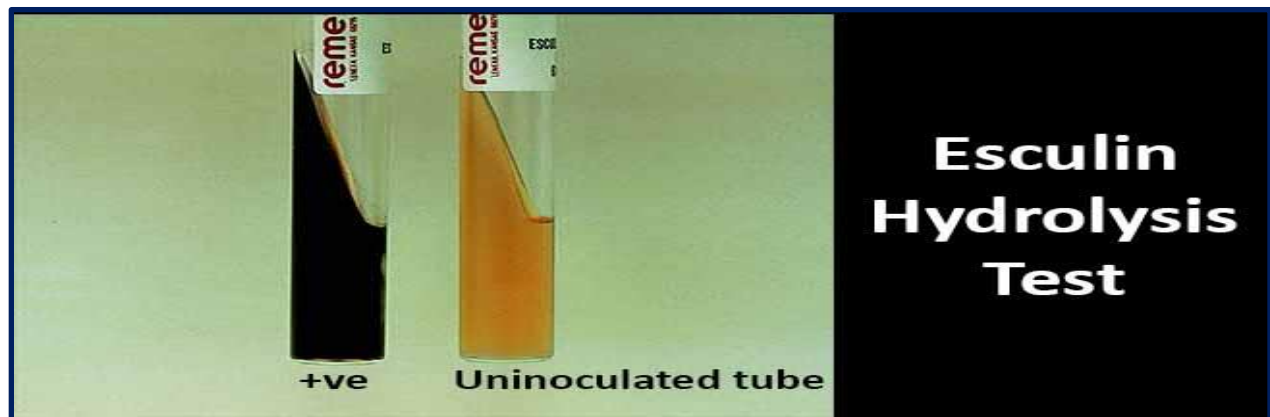
Bile esculin agar is both a selective and differential medium.

- The **selective** agent is bile salts, which **inhibit the growth of most Gram-positive bacteria** except **enterococci and some streptococci**.
- Esculin acts as the **differential component**, allowing differentiation between **Enterococcus** and **Streptococcus species** based on their **ability to hydrolyze esculin**.

The bile esculin test determines the ability of bacteria to **hydrolyze esculin** in the presence of **bile salts**, producing **esculetin as a reaction product**.

The medium also contains **ferric citrate**, which **reacts with esculetin** to **form a dark brown to black phenolic iron complex**, resulting in blackening of the medium, which indicates a **positive test result**.

- Bile esculin **Gram-positive organism: *Enterococcus faecalis***



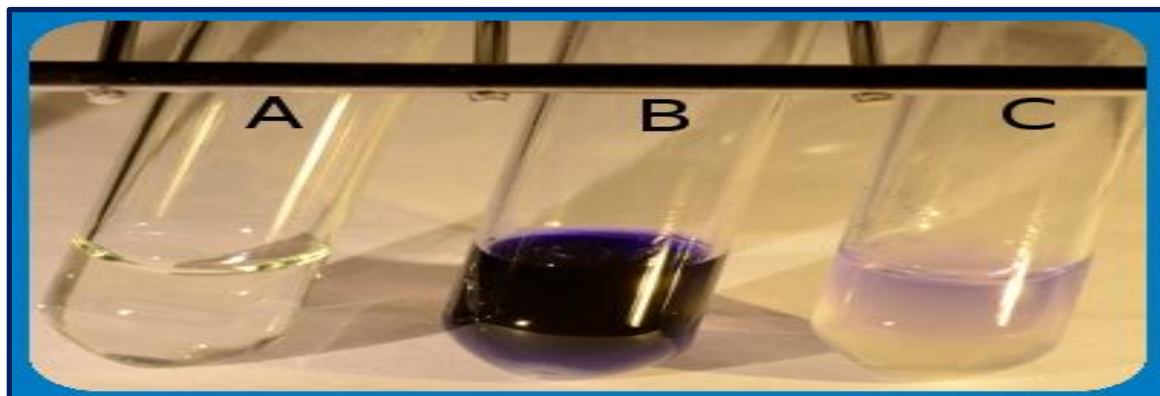
7- Hippurate hydrolysis test

The hippurate hydrolysis test is based on the **ability of certain microorganisms** to produce the **enzyme hippurate** hydrolase (**hippuricase**), which **hydrolyzes sodium hippurate** into **benzoic acid and glycine**.

The presence of **glycine** is detected by the addition of **ninhydrin reagent**, which **reacts with glycine** to produce a **deep purple or violet color**, indicating a **positive result**.

This test is particularly useful for **differentiating β -hemolytic *Streptococcus agalactiae* (Group B Streptococcus)** from other **β -hemolytic streptococci**, which are typically hippurate-negative.

*Hippurate gram-positive organism: *Streptococcus agalactiae*



Streptococcus pneumoniae

Characteristics

- ❖ **Gram** - Positive
- ❖ **Shape** – Diplococcus, “lancet shaped”
- ❖ **Motility** – None
- ❖ **Oxygen Requirements** -
Facultative anaerobe
- ❖ **Oxidase** – Negative
- ❖ **Catalase** – Negative
- ❖ **Hemolysis** – Alpha
- ❖ **Optochin** - Sensitive
- ❖ **Capsule** - Polysaccharide
- ❖ **Bile** – Soluble
- ❖ **Virulence Factors**
 - ❖ Pneumolysin
 - ❖ Toxic to the bacteria, once the bacteria are dead, it is released into the environment
 - ❖ Kills endothelial cells, paralyzes respiratory cilia, reduces phagocytic ability, increases anti-inflammatory cytokines, causes platelet activation
 - ❖ IgA protease
 - ❖ Choline Binding Protein
 - ❖ Attachment to epithelial cells

Epidemiology and Risk Groups

- Worldwide
- Part of normal flora of skin and nose
- Patients with Sickle Cell Disease
- Patients without a spleen

Symptoms

- Pneumococcal meningitis
 - Fever, headache, stiff neck, nausea
 - Most common bacterial cause
- Otitis Media
- Pneumoniae
 - Causes a unilobular pneumonia with rust-colored sputum
 - Most common cause of bacterial pneumonia in the world
 - Shaking chills, high fever, hemoptysis, chest pain
- Acute Rhinosinusitis

Transmission

- ☐ Contaminated air droplets
- ☐ Part of the normal flora

Treatment

- ✓ Children get the protein-conjugated vaccine to activate T-cells
- ✓ Adults over 65 can get the polysaccharide vaccine to activate B-cells
- ✓ Treat with Azithromycin or cephtriaxone

Diagnosis

- Culture and biochemical tests

Differences between *Str. pneumoniae* & Viridans Streptococci

Property	Pneumococci	Viridans Streptococci
Morphology	Lanceolate, diplococci	Spherical / oval cocci In long chains
Capsule	Present	Absent / Slime
Colony	Draughtsman	Dome
Bile solubility	Soluble	Insoluble
Optochin sensitivity	Sensitive	Resistant
Animal pathogenicity (mouse)	Virulent	Avirulent

Viridans Streptococci

Characteristics

- ❖ **Gram** - Positive
- ❖ **Shape** – Cocci
- ❖ **Motility** – None
- ❖ **Oxygen Requirements** - Facultative anaerobe
- ❖ **Oxidase** – Negative
- ❖ **Catalase** – Negative
- ❖ **Hemolysis** – Alpha
- ❖ **Optochin** - Resistant
- ❖ **Bile** – Insoluble

Epidemiology and Risk Groups

- Worldwide
- Part of normal flora of mouth

Transmission

- ☐ Contaminated air droplets
- ☐ Part of the normal flora

Symptoms

- Dental cavities
 - *Streptococcus mutans* associated with this
- Subacute infective endocarditis
 - Grabs onto previously damaged heart valves and creates a fibrin-platelet aggregates to form a vegetation
 - *Streptococcus sanguinis* is associated with this

Diagnosis

- Culture and biochemical tests

Treatment

- ✓ Good dental hygiene
- ✓ Amoxicillin for dental procedures

Streptococcus species

Tests	<i>S.pyogenes</i>	<i>S.pneumoniae</i>
Gram stain	Gram+ve &cocci	Gram+ve &cocci
Arrangement	chain	pair
Catalase	Negative	Negative
Oxidase	Negative	Negative
Streptokinase	Positive	Negative
Capsule	Negative	Positive
Inulin	None ferment	Ferment
Bile solubility	Negative	Positive soluble
CAMP reaction	Negative	Negative
Blood hemolysis	Beta	Alpha
Bile esculin	Negative	Negative
Nacl 6.5%	Negative	Negative
Motility	Negative	Negative
Spore forming	Negative	Negative
Optochin	R	S
Bacitracin	S	R

Biochemical Tests for Identification of Gram-Negative Bacteria

IMViC Tests is an acronym for four biochemical tests used in bacterial identification:

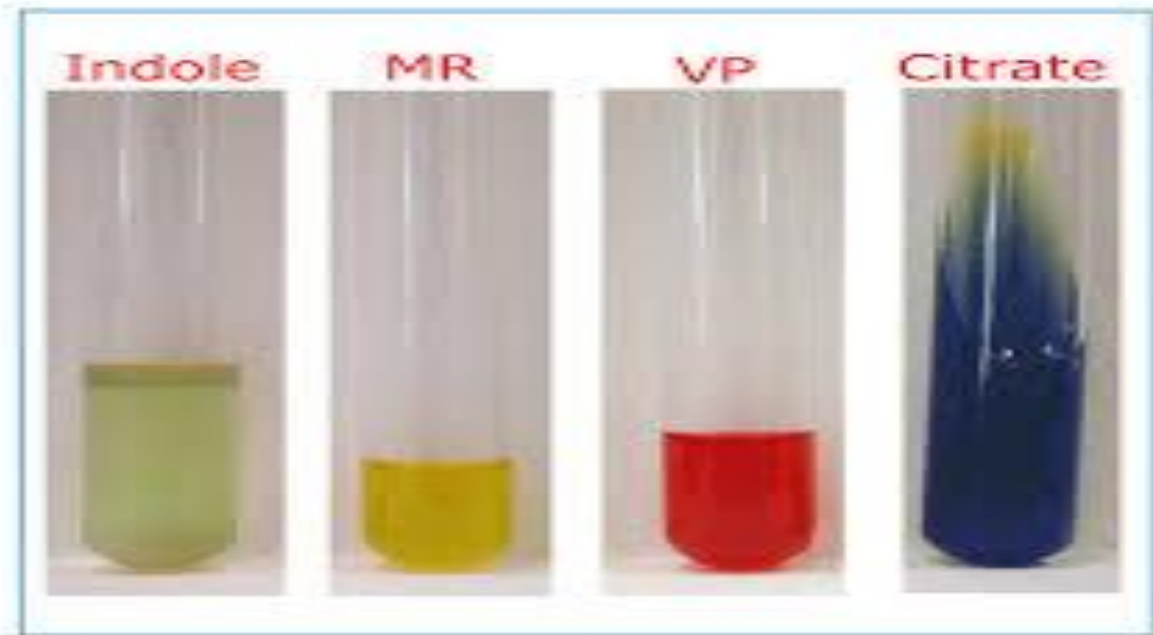
- **I** – Indole production
- **M** – Methyl Red test
- **V** – Voges–Proskauer test
- **C** – Citrate utilization test

Purpose

- Especially useful for differentiating members of the **Enterobacteriaceae family**.
- Each test evaluates a specific metabolic characteristic:
 - **Indole:** Ability to produce indole from tryptophan
 - **Methyl Red:** Mixed acid fermentation of glucose
 - **Voges–Proskauer:** 2,3-butanediol fermentation (acetoin production)
 - **Citrate:** Ability to use citrate as the sole carbon source

The **IMViC tests** are a set of biochemical tests used in microbiology laboratories to identify coliform bacteria. Coliforms are **Gram-negative rods** that are **aerobic or facultative anaerobic** and **can ferment lactose with gas production** within 48 hours. Their presence is commonly used as an indicator of **fecal contamination**.





IMViC series = Klebsiella & Enterobacter

Indole Test

- The organism is inoculated and grown in **peptone water broth**. The medium contains tryptophan as a substrate.
- If the organism produces the **enzyme tryptophanase**, it breaks down tryptophan into:

Indole, Pyruvate, Ammonium

- The broth is sterilized at 121°C for 15 minutes before use.
- After incubation, indole (if produced) is extracted from the broth using xylene.
- To detect indole production, Kovac's reagent is added after incubation.
- **Composition of Kovac's reagent:**
 - Amyl alcohol
 - Para-dimethylaminobenzaldehyde (p-DMAB)
 - Concentrated hydrochloric acid (HCl)

- **Kovac's reagent** is **used to** determine the ability of an organism to produce indole from the amino acid tryptophan.
- **Positive result:** Formation of a **pink to red layer** on the surface (top) of the liquid.

Indole Test – Method

- Inoculate tryptone water with the tested microorganism.
- Incubate at 37°C for 24 hours.
- After incubation, add 1 mL of Kovac's reagent.
- Shake the tube gently.
- Read the result immediately.

Kovac's Reagent – Preparation

- Dissolve 10 g of p-dimethylaminobenzaldehyde in 150 mL of amyl, isoamyl, or butyl alcohol.
- Heat in a 56°C water bath until completely dissolved.
- Allow to cool.
- Slowly add 50 mL of concentrated HCl.
- Store in a glass-stoppered brown bottle in the refrigerator.
- The reagent should be light yellow in color.

Ehrlich's Reagent – Preparation

- Dissolve 1 g of p-dimethylaminobenzaldehyde in 95 mL of 95% ethyl alcohol.
- Slowly add 20 mL of concentrated HCl.
- Store in a glass-stoppered brown bottle in the refrigerator.
- The reagent should be light yellow in color.

Indole test

- Media culture: peptone water
- Reagent: kovacs
- Enzymes: Tryptophanase
- Substrate: Tryptophan

Results Interpretation

- Positive test (e.g., *Escherichia coli*): Formation of a bright pink/red color in the top layer. Indicates the presence of indole. Indole positive (+)
- Negative test (e.g., *Klebsiella* spp.): No color change (no pink/red layer). Indicates indole was not produced. Indole negative (–). Medium remains yellow or turns orange

Summary of the Indole Test

- Culture Medium: Peptone water (Tryptone broth)
- Substrate: Tryptophan
- Reagent: Kovac's reagent (or Ehrlich's reagent)
- Enzymatic pathway:
 - Indole test → Tryptophan metabolism by the enzyme tryptophanase

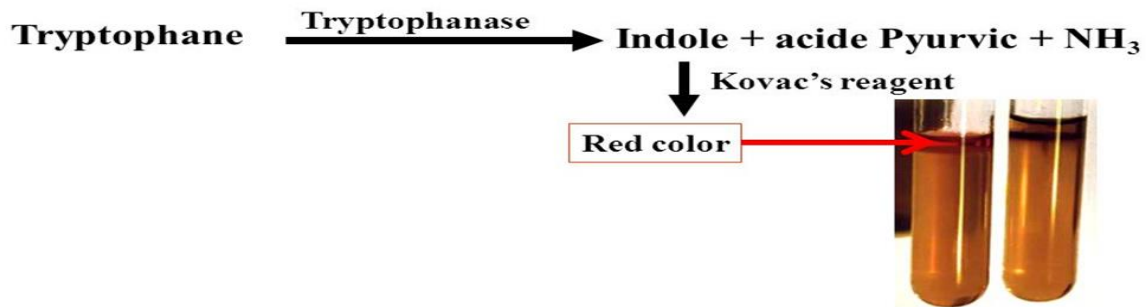
Results

- Indole Positive: Formation of a bright red or pink layer on the surface of the broth.
Example: *Escherichia coli*
- Indole Negative: No red or pink color; the medium remains yellow or turns orange.
Example: *Klebsiella*

IMViC: Indole Test

- Principal

- Some microorganisms can metabolize tryptophane by the tryptophanase



Methyl Red (MR) and Voges–Proskauer (VP) Tests

- Both tests use the same culture medium called MR-VP broth.
- After incubation, the broth is divided into two separate tubes:
 - One tube for the Methyl Red (MR) test.
 - One tube for the Voges–Proskauer (VP) test.

Methyl Red (MR) Test

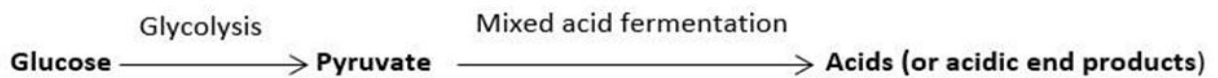
Purpose

- Detects production of stable organic acids from glucose fermentation.
- Identifies organisms that use the mixed acid fermentation pathway (using pyruvate as a substrate).

Principle

- Some bacteria ferment glucose and produce large amounts of organic acids.
- These acids significantly lower the pH of the medium.
- The pH indicator methyl red is added after incubation:

- **Red color** → pH below 4.4 (**positive result**). Indicates use of mixed acid fermentation.
- **Yellow color** → pH above 6.0–6.2 (**negative result**). Indicates use of 2,3-butanediol fermentation pathway instead of mixed acid fermentation.



Methyl Red (MR) Test

1. Sterile Glucose Phosphate Broth (GPB) medium (0.5 mL in each tube).
2. Test culture suspension.
3. **Methyl red indicator.**

Preparation of Methyl Red Indicator

- Dissolve 0.1 g methyl red in 300 mL of 95% ethanol.
- Add distilled water to make the final volume 500 mL.

Procedure

1. Inoculate GPB medium with the culture suspension.
2. Incubate at 37°C for 24 hours.
3. Add 5–6 drops of methyl red indicator.
4. Observe color change immediately.

Voges–Proskauer (VP) Test

Purpose

- Detects production of **acetylmethylcarbinol (acetoin)**.
- Identifies organisms that use the 2,3-butanediol fermentation pathway.

Reagents Used

- **Alpha-naphthol**
- **Potassium hydroxide (KOH)**

Method

- Add **alpha-naphthol** and **KOH** to the second tube.
- Shake vigorously to **allow oxygen exposure**.
- Allow the tube to stand for **5–10 minutes**.

Positive VP test: Development of **a pinkish-red color**. Indicates production of **acetoin** and **use of the 2,3-butanediol** pathway.

Negative VP test: No red color develops.

Voges–Proskauer (VP) Test

Principle

- In the 2,3-butanediol fermentation pathway, **pyruvate** is converted **into acetoin** (acetyl methyl carbinol).
- In the presence of **oxygen and KOH**, **acetoin** is oxidized to **diacetyl**.
- **Diacetyl** reacts with the **guanidine component** of peptone in the presence of **α-naphthol**, forming **a pink-red color**. (especially at the top of the broth).
- After **aerobic incubation** in MR-VP broth, reagents are added.
- A positive reaction develops **within 30 minutes** (not more than 1 hour).

Requirements

1. Sterile Glucose Phosphate Broth (GPB) (0.5–1.0 mL per tube).
2. Test culture suspension (e.g., *Enterobacter*, *Klebsiella*).
3. 5% α-naphthol in absolute ethanol
4. 40% KOH solution May contain 0.5% creatinine (optional).

Procedure

1. Inoculate GPB medium with culture suspension.
 2. Incubate at 37°C for 24–48 hours.
 3. Add 0.6 mL of 5% α-naphthol and mix well.
 4. Add 0.2 mL of 40% KOH, shake vigorously.
 5. Observe for color change within 5–30 minutes.
- **Results** **Positive VP test:** Development of pink to red color
 - **Negative VP test:** No red color (medium may remain brown)

VP Positive Bacteria

- *Klebsiella* spp.
- *Enterobacter* spp.
- *Proteus mirabilis*
- *Serratia* spp.
- *Staphylococcus aureus*
- Viridans streptococci (except *S. mitis* and *S. vestibularis*)

VP Negative Bacteria

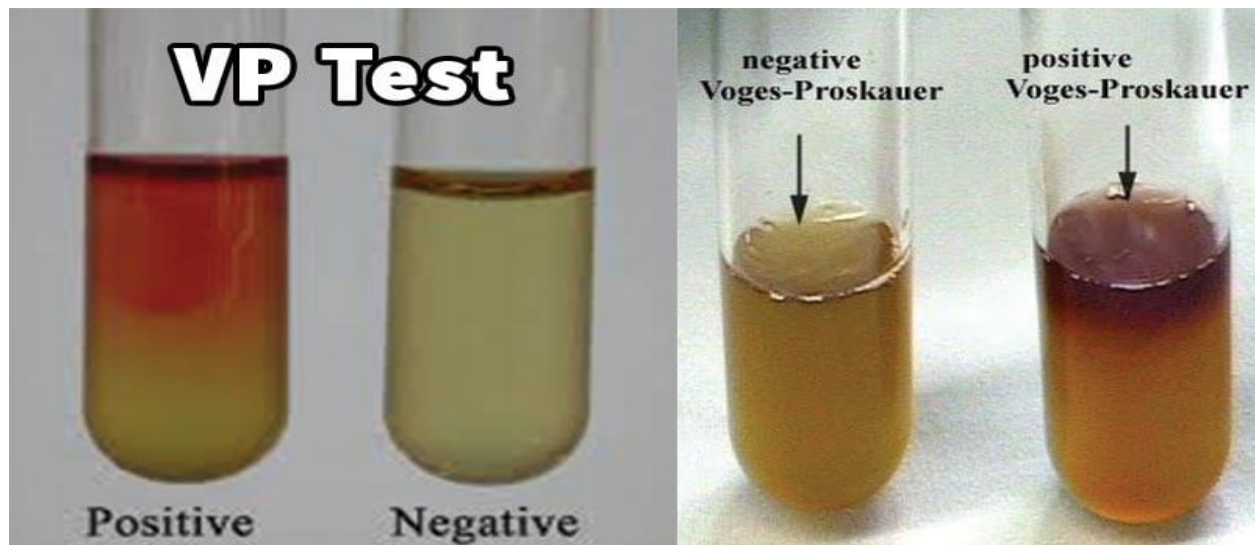
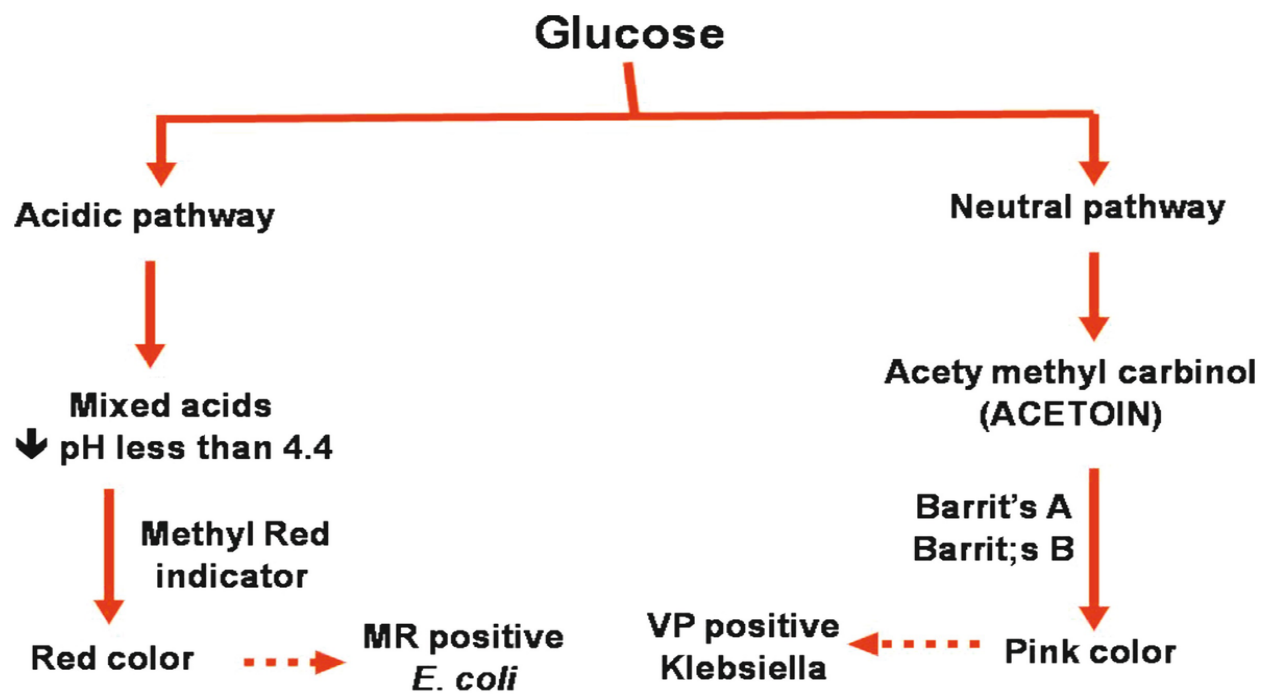
- *Escherichia* spp.
- *Proteus vulgaris*
- *Citrobacter freundii*

Summary of MR–VP Test

- Culture Medium: Glucose Phosphate Broth (MR-VP broth)
- Substrate: Glucose
- Reagents:
 - MR test → Methyl red
 - VP test → Barritt's reagents (α -naphthol + KOH)
- Enzymatic pathways:
 - MR → Mixed acid fermentation
 - VP → 2,3-butanediol fermentation

Results

- MR Positive: Red
- MR Negative: Yellow
- VP Positive: Red / Pink
- VP Negative: No red color



Citrate Test (Simmons' Citrate Test)

- In the 1930s, (Stewart A. Koser) studied bacterial catabolism of organic acids.
- He demonstrated that citrate utilization could differentiate bacteria in natural environments.
- Citrate metabolism helped distinguish between:
 - Soil and aquatic coliforms (non-fecal contamination) → able to grow.
 - Fecal coliforms → unable to grow on citrate.

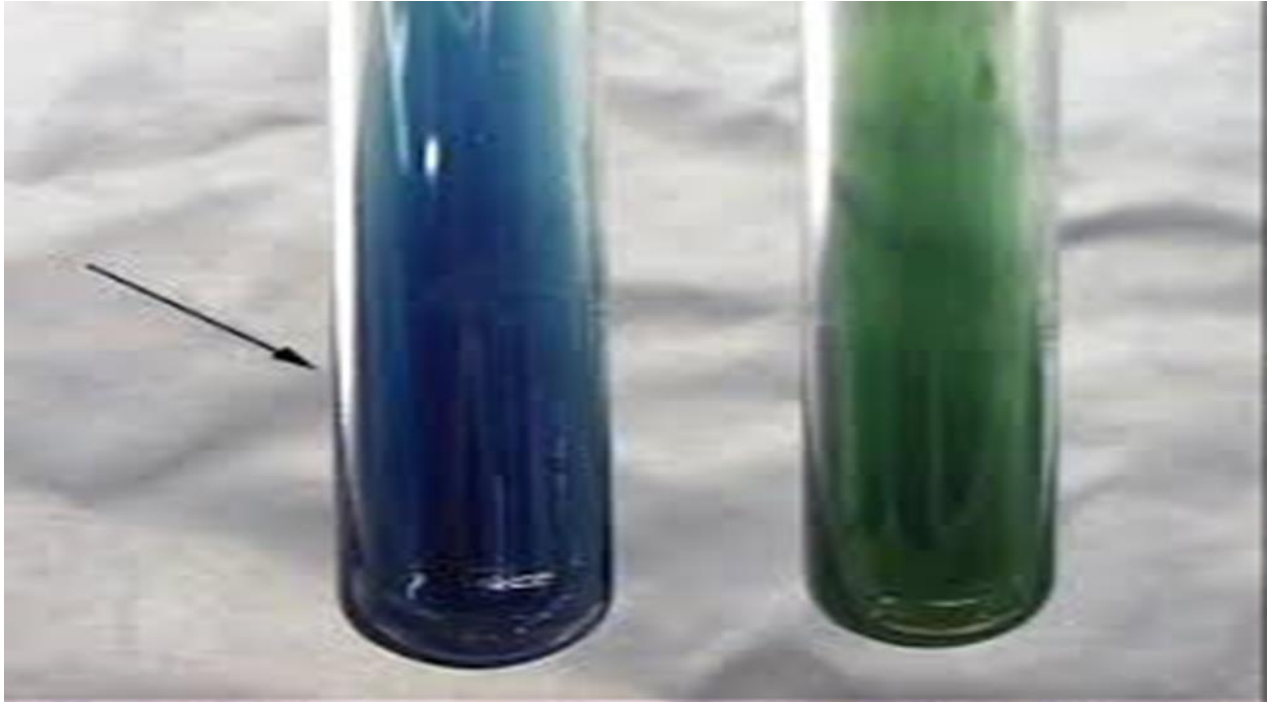
Purpose of the Test

- The ability of a microorganism to use citrate as the sole carbon and energy source.
- Commonly used to differentiate members of the Enterobacteriaceae family.

Medium Used

Simmons' Citrate Agar, which contains:

- Sodium citrate → sole carbon source.
- Ammonium ions (ammonium phosphate) → sole nitrogen source.
- pH indicator → Bromothymol blue (BTB)
- The medium is prepared as a slant to increase the surface area and enhance bacterial growth.
- If the organism can utilize citrate, it converts ammonium salts into ammonia.
- Ammonia production increases the pH (alkaline reaction).
- The pH change is detected by Bromothymol blue:
 - Green at neutral pH. Medium remains green Negative result.
 - Blue in alkaline conditions. Indicates citrate utilization Positive result Prussian blue.
 - BTB helps reduce false-positive results by clearly indicating pH change.



Citrate Utilization Test

Principle

- Some bacteria can use citrate as the sole carbon source.
- These bacteria produce the enzyme citrase, which breaks citrate into:
 - Oxaloacetic acid
 - Acetic acid
- Oxaloacetic acid is then decarboxylated to form:
 - Pyruvate
 - Carbon dioxide (CO₂)

Alkaline Products Formation

1. Formation of Sodium Carbonate Released CO₂ reacts with water and sodium from sodium citrate: $\text{CO}_2 + \text{H}_2\text{O} + \text{Na}^+ \rightarrow \text{Na}_2\text{CO}_3$ Sodium carbonate (alkaline) increases the pH of the medium.

2. Formation of **Ammonium Hydroxide** Utilization of ammonium salts (nitrogen source) produces **ammonia**. Ammonia **forms ammonium hydroxide** (NH_4OH), which is alkaline.

Ammonium salt → Ammonium hydroxide (alkaline) **Final Effect on pH**

The combined effect of: Sodium carbonate and Ammonium hydroxide Raises the **pH above 7.6**.

Requirements

1. Test culture suspension.
2. Sterile Koser's citrate broth (1.0 mL per tube) or Simmons' citrate agar slant.

Procedure

1. Inoculate Koser's broth or Simmons' citrate agar with the culture suspension.
2. Incubate at 37°C for 24 hours.
3. Observe results:
 - In Koser's broth → Check for turbidity (growth).
 - In Simmons' agar → Check for growth and color change to blue.

Test Summary

- Culture medium: Simmons' citrate agar
- pH indicator: Bromothymol blue
- Enzyme: Citrase
- Substrate: Sodium citrate (Na-citrate)

Results

- Positive test:
 - Growth present
 - Medium turns blue
- Negative test:
 - No growth Medium remains green

Fecal Coliforms



Indole positive



MR positive



VP negative



Citrate
negative

Nonfecal Coliforms



Indole negative



MR negative



VP positive



Citrate
positive

These **IMViC** tests are useful for differentiating the family *Enterobacteriaceae*, especially when used alongside the **Urease test**.

The IMViC results of some important species are shown below.

Species	Indole	Methyl Red	Voges-Proskauer	Citrate
<i>Escherichia coli</i>	Positive	Positive	Negative	Negative
<i>Staphylococcus aureus</i>	Negative	Positive	Positive	Negative
<i>Shigella spp.</i>	Negative	Positive	Negative	Negative
<i>Salmonella spp.</i>	Negative	Positive	Negative	Positive
<i>Klebsiella spp.</i>	Negative	Negative	Positive	Positive
<i>Proteus vulgaris</i>	Positive	Positive	Negative	Negative
<i>Proteus mirabilis</i>	Negative	Positive	Negative	Positive
<i>Citrobacter freundii</i>	Negative	Positive	Negative	Positive
<i>Enterobacter aerogenes</i>	Negative	Negative	Positive	Positive

TSI agar (Triple Sugar Iron Agar) Test, Sugar fermentation, CO₂ & H₂S Production Test.

T= Triple, S= Sugar, I= Iron, A=Agar

Sugar = Glucose 0.1%, Lactose 1%, Sucrose 1%

PH indicator = Phenol red

Iron= Ferric Ammonium Citrate

H₂S indicator = Sodium Thiosulfate

TSI agar

Triple Sugar Iron Agar

0.1%
dextrose

1.0%
sucrose

1.0%
lactose

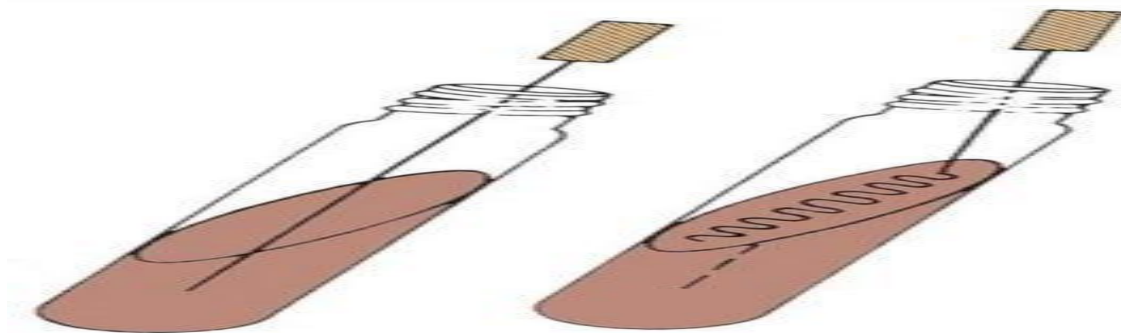
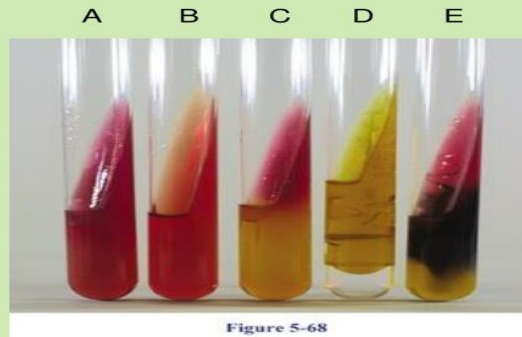
(a) Red/red (no sugar fermentation)

(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 H₂S

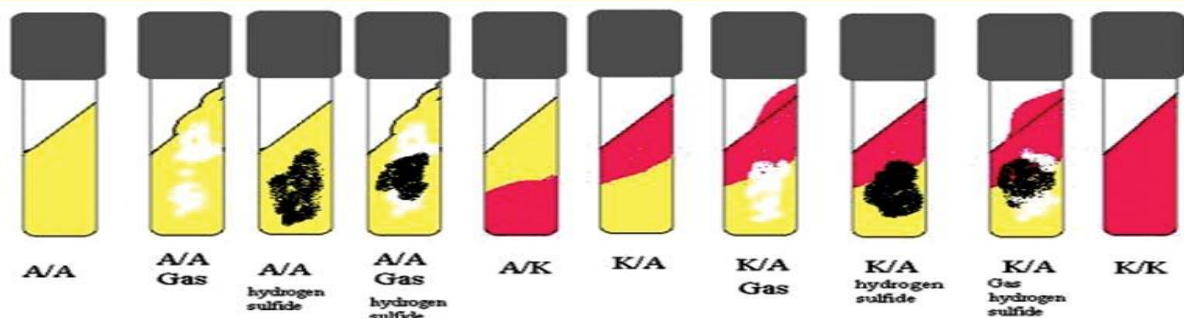


Objectives of TSIA (Triple Sugar Iron Agar) Test

1- To determine whether a **gram-negative bacilli** ferments **glucose and lactose or sucrose** and forms **hydrogen sulfide (H₂S)**.

2-To **differentiate members** of the **Enterobacteriaceae family** from other **gram-negative bacilli**.

Triple Sugar Iron Agar (TSIA) Test



Principle TSIA (Triple Sugar Iron Agar) Test

Purpose

- Designed to differentiate among members of the Enterobacteriaceae.
- Distinguishes:
 - Gram-negative bacilli that ferment glucose
 - Other gram-negative intestinal bacilli (non-fermenters).
- Differentiation is based on:
 - Fermentation of glucose, lactose, and/or sucrose
 - Production of hydrogen sulfide (H₂S)
 - Gas production

Composition of TSIA Medium

- Carbohydrates ratio:
 - 10 parts lactose
 - 10 parts sucrose
 - 1 part glucose
- Peptone
- Phenol red → Acid-base indicator
- Sodium thiosulfate → Sulfur source
- Ferrous ammonium sulfate (or ferrous sulfate) → H₂S indicator

Indicators and Their Roles

- Phenol red:
 - Orange-red at neutral pH
 - Yellow in acidic conditions (carbohydrate fermentation)
 - Deep red in alkaline conditions (peptone utilization)

Ferric ammonium citrate

- Detect H₂S production
- Black precipitate forms in the butt

1-Carbohydrate Fermentation; Glucose is fermented first Within **8–12 hours**, the entire medium turns yellow (acidic).

After **18–24 hours**: **Butt** (anaerobic area): **Remains yellow** Due to **continued acid production** from glucose fermentation.

Slant (aerobic area): **Reverts to red** (**alkaline**) Due to oxidation of **acids and peptone** metabolism
Formation of **CO₂, H₂O, and alkaline amines**

2- Lactose and/or Sucrose Fermentation If **lactose and/or sucrose** are fermented (in addition to glucose). **Large amounts of acid** are produced. **Both slant and butt** remain **yellow** (**acidic**) after **18–24 hours**.

3- non-fermenters: If the organism **does not ferment** glucose **Both slant and butt** remain **red** (**alkaline**). The organism **utilizes peptones only**.

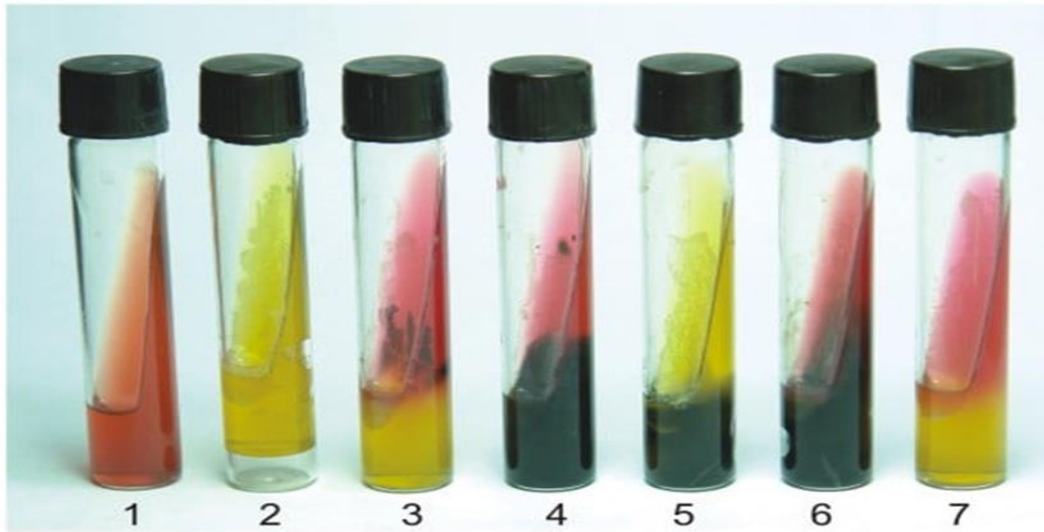
4-Gas Production (CO₂ and H₂) **Bubbles** in agar **Cracks** in the medium Agar **lifted** from bottom or **sides of the tube**

5-Hydrogen Sulfide (H₂S) Production

- The production of **H₂S** occurs by the reduction of **sodium thiosulfate** to H₂S.
- An acidic environment is required for this reaction.
- H₂S reacts with ferric ammonium citrate.
- This reaction produces blackening of the agar butt in the tube.

Sodium thiosulfate → (by bacterial reduction) H₂S

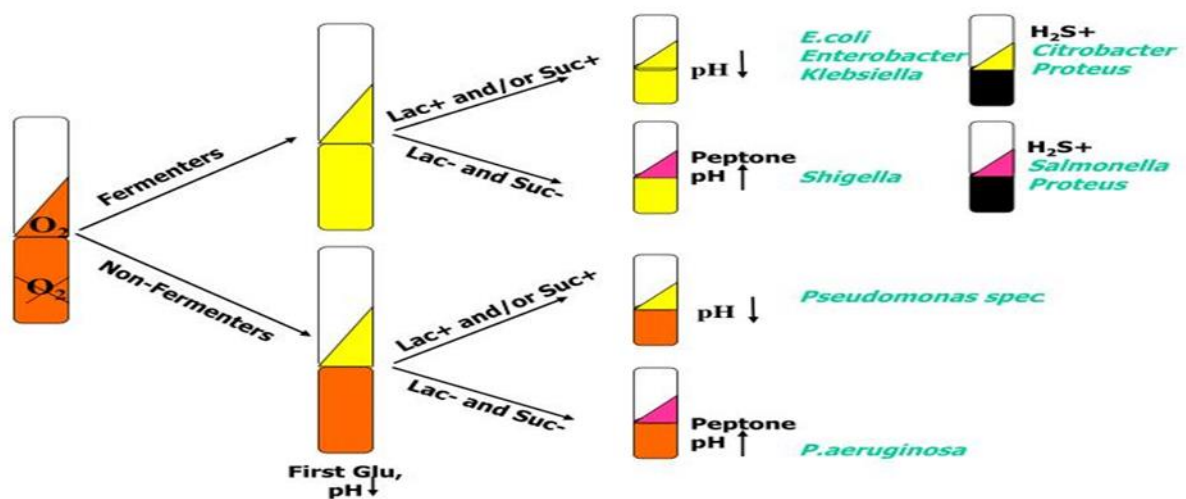
H₂S + Ferric ammonium citrate → FeS (black precipitate)



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

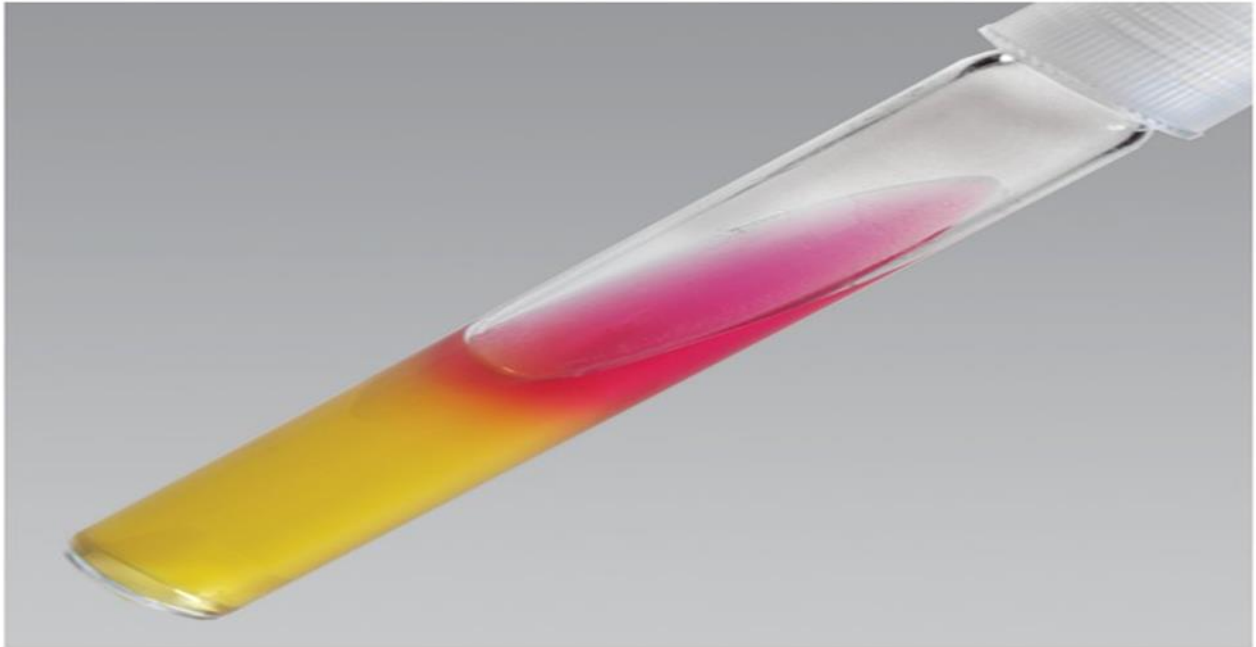
Understanding the Reactions in TSI-Agar



Procedure of TSIA (Triple Sugar Iron Agar) Test

1. With a straight inoculating needle, touch the top of a well-isolated colony.
2. Inoculate the TSI agar by:
 - First stabbing through the center of the medium down to the bottom of the tube.
 - Then streaking the surface of the agar slant.
3. Leave the cap loosely on the tube and incubate at 35–37°C in ambient air for 18–24 hours.
4. After incubation, examine the medium for:
 - **Color change in the slant and butt.**
 - **Blackening of the medium (H₂S production).**
 - **Cracks or splits in the agar (gas production).**

S.N.	Result (slant/butt)	Symbol	Interpretation
1	Red/Yellow	K/A	Glucose fermentation only, peptone catabolized.
2	Yellow/Yellow	A/A	Glucose and lactose and/or sucrose fermentation.
3	Red/Red	K/K	No fermentation, Peptone catabolized under aerobic and/or anaerobic conditions.
4	Yellow/Yellow with bubbles	A/A, G	Glucose and lactose and/or sucrose fermentation, Gas produced.
5	Red/Yellow with bubbles	K/A, G	Glucose fermentation only, Gas produced.
6	Red/Yellow with bubbles and black precipitate	K/A, G, H ₂ S	Glucose fermentation only, Gas produced, H ₂ S produced.
7	Yellow/Yellow with bubbles and black precipitate	A/A, G, H ₂ S	Glucose and lactose and/or sucrose fermentation, Gas produced, H ₂ S produced.
8	Red/Yellow with black precipitate	K/A, H ₂ S	Glucose fermentation only, H ₂ S produced.
9	Yellow/Yellow with black precipitate	A/A, H ₂ S	Glucose and lactose and/or sucrose fermentation, H ₂ S produced.



Result = Alkaline\ Acid, no CO₂, no H₂S \ *Shigella*. spp

Quality Control of TSIA Test

Test organism	Slant	Butt	Gas production	H ₂ S production
<i>Escherichia coli</i> ATCC25922	Yellow	Yellow	+	–
<i>Pseudomonas aeruginosa</i> ATCC27853	Red	Red	–	–
<i>Salmonella enterica</i> ATCC14028	Red	Yellow	+	+
<i>Shigella sonnei</i> ATCC9290	Red	Yellow	–	–

Urease Test

Principle

- Urease is a constitutive enzyme that hydrolyzes urea into:



- Ammonia reacts with CO₂ and water to form ammonium carbonate.
- Ammonium carbonate increases the alkalinity (pH) of the medium.
- The pH indicator phenol red changes:
 - Orange-yellow (acidic/neutral) → original color
 - Bright pink (alkaline) → positive result

Media Used

- Christensen's urea agar (solid medium – slant)
- Stuart's urea broth (liquid medium)
- May also be part of combined media such as MIU (Motility–Indole–Urease) test.

Microorganisms Tested

- Gram-negative enteric pathogens (e.g., *Yersinia* spp.)
 - Fastidious Gram-negative rods: *Brucella*, *Helicobacter pylori*, *Pasteurella*
- Gram-positive rods: *Corynebacterium*, *Rhodococcus*,
- Yeasts: *Cryptococcus* spp

Procedure

A. Christensen's Urea Agar

1. Streak the entire slant surface with a heavy inoculum from an 18–24-hour pure culture.
Do not stab the butt (it serves as a color control).
2. Incubate at 35°C with loosened caps observe for color change at: 6 hours
3. 24 hours (or longer if required).

B. Stuart's Urea Broth

1. Inoculate broth with a heavy inoculum from an 18–24-hour pure culture.
2. Shake gently to suspend bacteria.
3. Incubate at 35°C with loosened caps.
4. Observe for color change at: 8 hours, 12 hours, 24 hours

Results rapid Urease Positive Organisms

- e.g., *Proteus* spp., *Morganella morganii*, some *Providencia stuartii*.
- Strong positive reaction within 1–6 hours (agar) or 8–24 hours (broth).
- Medium turns bright pink (magenta).

Delayed Urease Positive Organisms

- e.g., *Klebsiella* spp., *Enterobacter* spp.
- Weak pink color at 6 hours.
- Stronger reaction after extended incubation.
- Note (Stuart's broth):
 - Delayed positives may not show reaction due to high buffering capacity of the medium.

Urease Negative Organisms

- e.g., *Escherichia coli*. Medium remains yellowish/light orange (no color change).
- Results are usually read within 24 hours.

Color Interpretation Summary

Result	Color Change	Interpretation
Positive	Light orange → Bright pink (magenta)	Urease produced
Negative	Remains light orange/yellow	No urease production

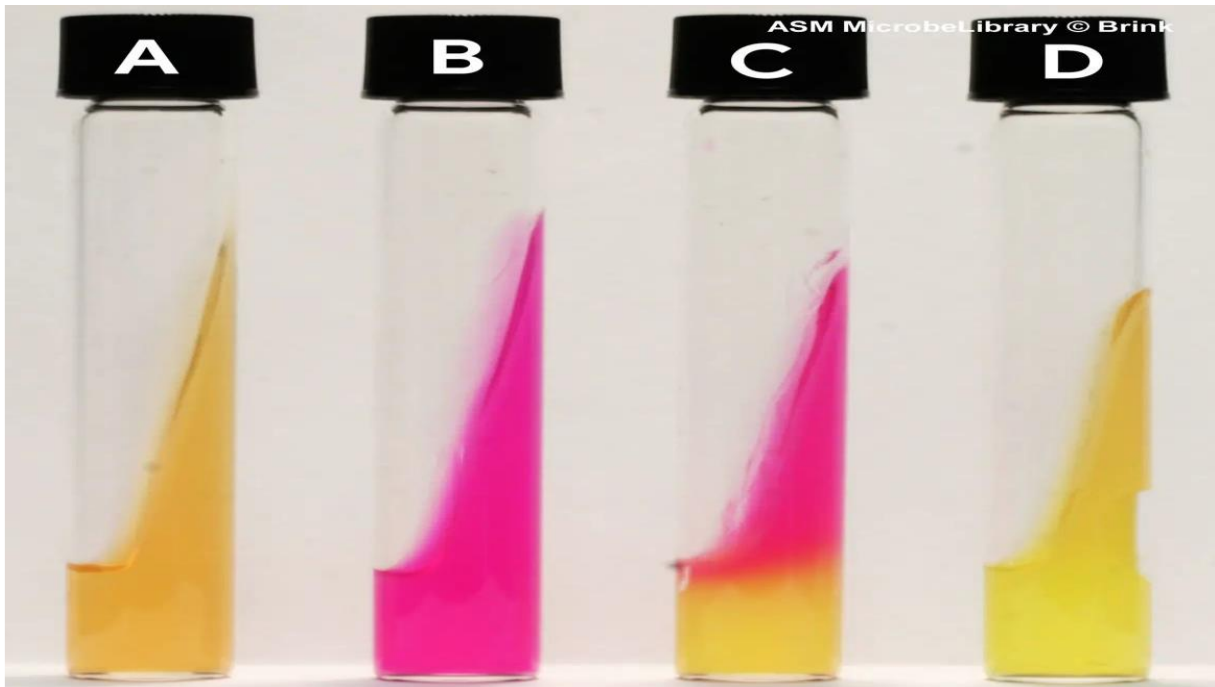


Figure: Urea agar test (a) uninoculated, (b) *Proteus mirabilis* (rapidly urease positive), (c) *Klebsiella pneumoniae* (delayed urease positive), (d) *Escherichia coli* urease negative).



Figure: positive result pink color (Urease positive)

Diagnostic Utility of Urease Test

1. Identification of Bacteria and Yeasts

- Part of identification of several **Enterobacteriaceae**: *Proteus* spp., *Klebsiella* spp.
- Also useful for identifying: *Cryptococcus* spp., *Brucella* spp., *Helicobacter pylori*

2. Differentiation of *Proteus* spp.

- Urease-positive organisms like *Proteus* can be distinguished from other non-lactose fermenting **Enterobacteriaceae**.

3. Presumptive Detection of *H. pylori*

- Biopsy tissue is placed directly in urease broth.
- Positive urease reaction indicates the presence of *H. pylori*.
- Commercial urease agar kits are available for clinical use.

4. Differentiation of Yeasts

- *Cryptococcus neoformans* → urease-positive
- *Candida albicans* → urease-negative

5. Urea Breath Test (Non-invasive)

- Detects *H. pylori* infection based on urease activity.
- Highly sensitive and specific.

Common Urease-Positive Organisms

1. *Proteus* spp.

2. *Klebsiella* spp.

Urease Test Medium (Urea Agar)

- Substrate: Urea
- pH Indicator: Phenol red
- Enzyme Tested: Urease

Urease Test Summary

Culture media:

- Christensen's urea agar (solid slant)
- Stuart's urea broth (liquid medium)

pH indicator: Phenol red

Enzyme: Urease

Substrate: Urea

Principle:

- Urease hydrolyzes urea into ammonia (NH_3) and carbon dioxide (CO_2).
- Ammonia increases the pH of the medium, producing an alkaline reaction.

Results

- Positive test:
 - o medium changes from light orange to bright pink (magenta)
 - o Indicates urease production
 - o Seen in organisms such as *Proteus* and *Morganella morganii*
- Negative test: No color change Medium remains light orange or yellow Indicates absence of urease Seen in organisms such as *Escherichia coli*

Oxidative/Fermentative Test

The Oxidative/Fermentative (O/F) test is used to determine whether bacteria metabolize carbohydrates by oxidation (aerobic metabolism) or by fermentation (anaerobic metabolism).

Principle

The O/F test uses Hugh and Leifson's medium, which contains:

- A specific carbohydrate (**usually glucose**)
- pH indicator (**bromothymol blue**)

Two tubes of the medium are inoculated with the test organism:

- One tube is left open to allow **aerobic conditions**.
- The second tube is sealed with sterile paraffin oil to create **anaerobic conditions**.

Acid production from **carbohydrate metabolism** causes **a color change in the medium**.

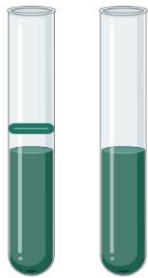
Interpretation of Results

Reaction Pattern	Interpretation
Yellow color in open tube only	Oxidative metabolism
Yellow color in both tubes	Fermentative metabolism
No color change in either tube	Non-saccharolytic organism

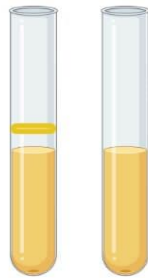
Examples of Bacterial Reactions

- Oxidative Gram-positive organisms: ***Micrococcus* spp.**
- Fermentative Gram-positive organisms: ***Staphylococcus aureus***
- Gram-negative oxidative organism: ***Pseudomonas* spp.**
- The O/F test helps **differentiate** *Micrococcus* spp. (oxidative) from *Staphylococcus* spp. (fermentative).
- It is also valuable in identifying **non-fermenting Gram-negative bacilli**, such as *Pseudomonas* spp.
- The test is commonly used as an initial step in **bacterial identification**.

Oxidation/Oxidative-Fermentation (OF) Test

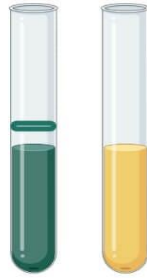


Control



Fermentative

Eg. *E. coli*



Oxidative

Eg. *Pseudomonas aeruginosa*



Non-Saccharolytic

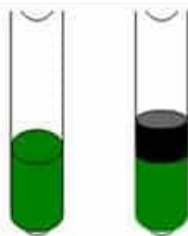
Eg. *Alcaligenes faecalis*

Results:

Positive Test:

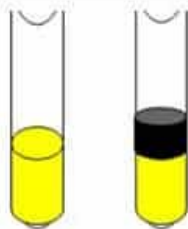


Microbiology Info.com



O⁻/F⁻

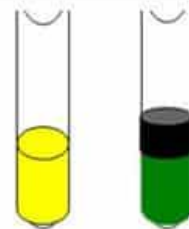
Non Saccharolytic



O⁺/F⁺

Fermentative

Enterobacteriaceae



O⁺/F⁻

Oxidative

Pseudomonas



Enterobacteriaceae

Enterobacteriaceae is a large family of Gram-negative bacteria that includes more than 30 genera and over 120 species.

This family contains many harmless symbionts as well as several well-known pathogens such as *Salmonella*, *Escherichia coli*, *Klebsiella*, and *Shigella*. Other medically important genera include *Enterobacter* and *Citrobacter*.

Members of this family are commonly referred to as enterobacteria or enteric bacteria.

Morphology and General Description

- Members are bacilli (rod-shaped), typically 1–5 μm in length.
- On blood agar, they usually form medium to large gray colonies, although some species may produce pigments.
- Most species are motile by means of peritrichous flagella, while some genera are non-motile.
- Most possess type I fimbriae, which are involved in adhesion to host cells.
- They are non-spore forming bacteria.

Some Important Genera

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

General Characteristics of Enterobacteriaceae

1. Gram-negative
2. Rod-shaped / coccobacilli
3. Facultative anaerobes
4. Non-spore forming
5. Capsule: present or absent (+/-)
6. Motility: present or absent (+/-)
7. Catalase positive
8. Oxidase negative

Laboratory Identification

To identify different genera of **Enterobacteriaceae**, microbiologists commonly use the following tests:

- **Phenol red** carbohydrate fermentation tests
- **Tryptone broth** (for indole production)
- **Phenylalanine agar**: detects phenylalanine deaminase, which converts phenylalanine to phenylpyruvic acid
- **Methyl Red (MR) test**: detects stable acid end products of glucose fermentation
- **Voges-Proskauer (VP) test**: detects acetylmethylcarbinol production
- **Catalase test**: detects the enzyme catalase, which breaks down hydrogen peroxide into water and oxygen
- **Oxidase test**: detects the presence of cytochrome oxidase (negative in Enterobacteriaceae)
- **Gelatin hydrolysis test**: detects gelatinase activity

Lactose-Fermenting Genera

- **Escherichia**
- **Klebsiella**
- **Enterobacter**

Escherichia coli

***Escherichia coli* (*E. coli*)** is a Gram-negative, oxidase-negative, catalase-positive, facultative anaerobic, non-spore forming coliform bacterium.

Cells are typically rod-shaped, measuring approximately 2.0 μm in length and 0.25–1.0 μm in diameter, with a cell volume of about 0.6–0.7 μm^3 .

- Motility is due to peritrichous flagella.
- Optimum growth temperature is 37 °C, although some laboratory strains can grow at temperatures up to 49 °C.

Medical Importance

E. coli normally inhabits the intestinal tract of healthy humans and animals.

Most strains are harmless or cause mild, self-limiting diarrhea. However, some pathogenic strains, such as *E. coli* O157:H7, can cause:

- Severe abdominal cramps
- Bloody diarrhea
- Vomiting



Figure : *Escherichia coli* on MacConkey agar (Dry pink colonies)

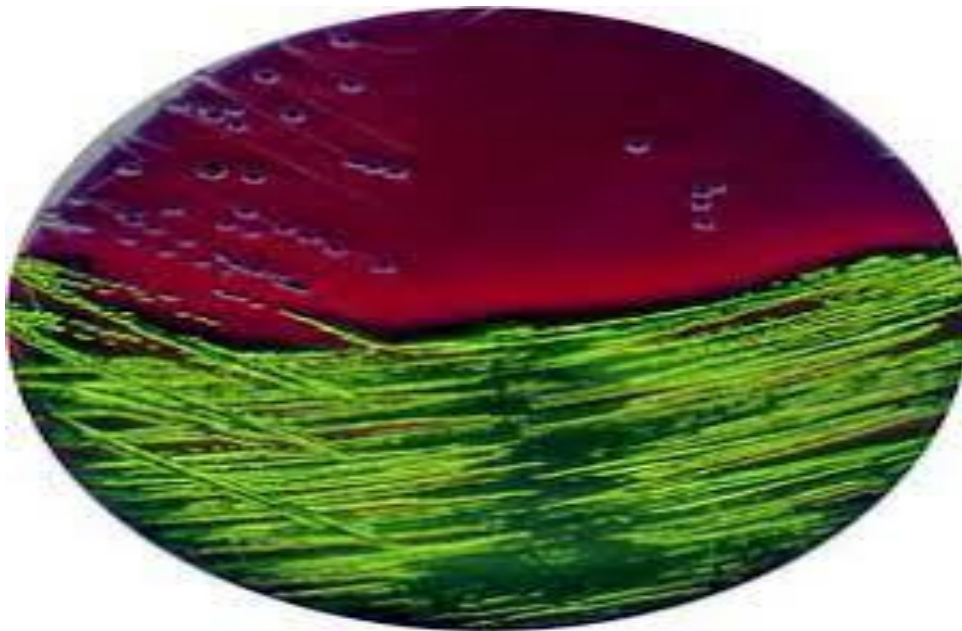


Figure : *Escherichia coli* on EMB agar (Dark colony and Green metallic sheen).

General characteristics

Escherichia coli – Characteristics

- Gram negative
- Coccobacilli / short rods
- Facultative anaerobes
- Non-spore forming
- Capsule: present (microcapsule or prominent capsule depending on genus)
- Motility: variable 80% motile ,20% non-motile
- Catalase: positive
- Oxidase: negative
- Urease: negative
- IMViC: +, +, -, -
- TSI agar: Acid/Acid, CO₂ +, H₂S -
- Lactose fermenting on MacConkey agar (**Dry pink colonies**), green metallic sheen on EMB agar

Genus: *Klebsiella spp*

General Description

Klebsiella is a genus of Gram-negative, oxidase-negative, catalase-positive, rod-shaped bacteria characterized by a prominent polysaccharide capsule.

They are widely distributed in nature (water, soil, plants, animals, humans) and are part of the normal flora of the nose, mouth, and intestines.

- Usually, non-motile
- Cells are short and thick rods
- Size: 0.3–1.5 µm wide × 0.5–5.0 µm long
- Grow well on routine laboratory media
- Facultative anaerobes
- Optimal growth: 35–37 °C, pH ≈ 7.2

- Capsule is important for virulence and serologic identification

Important Species

- *Klebsiella pneumoniae*
- *K. oxytoca*
- *K. granulomatis*
- *K. variicola*
- *K. quasipneumoniae*
- *K. michiganensis*
- *K. grimontii*

Biochemical Characteristics (Klebsiella spp)

- Gram negative
- Non-spore forming
- Capsule: positive
- Motility: non-motile
- Catalase: positive
- Oxidase: negative
- Urease: positive
- IMViC: -, -, +, +
- TSI: A/A, CO₂ +, H₂S -
- Lactose fermentation: mucoid pink colonies



Figure: *Klebsiella* on EMB agar (mucoid purple colonies)

Genus: *Enterobacter* spp

General Description

Enterobacter is a genus of Gram-negative, facultative anaerobic, non-spore-forming rods belonging to the family Enterobacteriaceae.

They are opportunistic pathogens, commonly causing urinary and respiratory tract infections, especially in hospitalized or immunocompromised patients.

- **Part of the coliform group**
- **Not fecal coliforms (do not grow at 44.5 °C with bile salts)**
- **Lactose fermenters with gas production**
- **Oxidase negative, catalase positive**
- **Indole negative**
- **Urease: variable**

General Characteristics (*Enterobacter spp*)

- **Gram negative**
- **Coccobacilli / short rods**
- **Facultative anaerobes**
- **Non-spore forming**
- **Capsule: variable**
- **Motility: motile**
- **Catalase: positive**
- **Oxidase: negative**
- **Urease: variable**
- **IMViC: -, -, +, +**
- **TSI: A/A, CO₂ +, H₂S -**
- **Lactose fermentation: pink to mucoid colonies**

Culture Appearance

- **MacConkey agar: pink colonies**
- **Tryptic Soy agar: rough or smooth colonies**
- **Encapsulated strains may appear mucoid**



Rough and smooth colony growth of *Enterobacter cloacae* bacteria on **Tryptic Soy agar**



The encapsulated strain of *Enterobacter aerogenes* on MacConkey medium

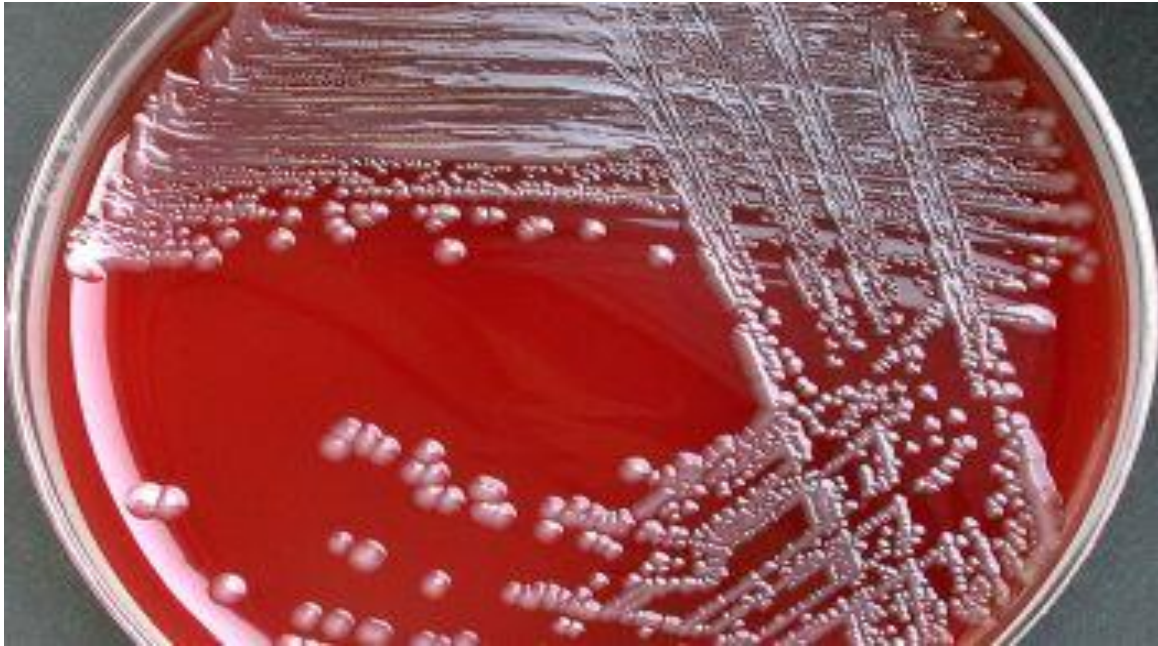


Enterobacter aerogenes on MacConkey medium

EMB Agar (Summary)

- **Selective for Gram-negative bacteria**
- **Differential for lactose fermentation**
- **Results:**
 - **Strong acid:** metallic green sheen (*E. coli*)
 - **Moderate acid:** pink/purple (*Enterobacter, Klebsiella*)
 - **No fermentation:** colorless (*Pseudomonas*)

Tests	E.coli	Klebsiella	Enterobacter
Catalase	+	+	+
Oxidase	-	-	-
Lactose	F	F	V
Urease	-	+	V
IMViC	+,+,-,-	-, -, +, +	-, -, +, +
TSI	A/A CO ₂ +, H ₂ S -	A/A CO ₂ +, H ₂ S -	A/A CO ₂ +, H ₂ S -
Motility	+	-	+
Spore	-	-	-
Capsule	microcapsule	have	V
H ₂ S	-	-	-
EMB agar	Green metallic sheen	Mucoid pink to Purple	Pink to Purple
MacConkey agar	Pink	Mucoid pink	Pink
Glucose	F	F	F



Enterobacter cloacae

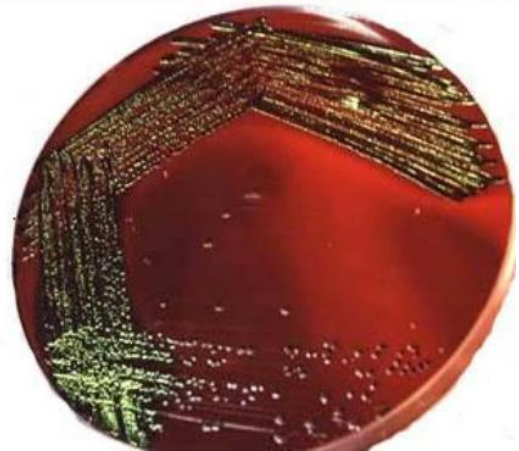


Enterobacter aerogenes

EMB plate



*Enterobacter
aerogenes* on EMB



Family: Enterobacteriaceae

Lactose non fermenting

Genus: *Proteus. spp*

Genus: *Shigella. spp*

Genus: *Salmonella. spp*

1- Genus: *Proteus. spp*

A) Important Species and Associated Infections

***Proteus vulgaris*: Urinary tract infections (UTIs), Wound infections.**

***Proteus mirabilis*: UTIs, Wound infections, Nosocomial (hospital-acquired) infections.**

***Proteus penneri*: UTIs, Wound infections, Nosocomial infections.**

B) General Characteristics

- Gram-negative bacteria.
- Pleomorphic: appear as bacilli or coccobacilli.
- Actively motile with peritrichous flagella.
- Non-lactose fermenters.
- Facultative anaerobes.
- Non-capsulated.
- Non-spore forming.
- Show swarming growth on agar media.
- Grow at temperatures between 25–37 °C.

C) Natural Habitat

Some species are free-living in: Water, Sewage, Soil, Vegetables

- Others are part of the normal intestinal flora.
- Infection occurs when bacteria leave the intestinal tract.

D) Diseases Caused by *Proteus* species may cause:

- Common causes of Urinary tract infections (UTIs).
- Bacteremia.
- Pneumonia.
- Focal infections in debilitated patients.
- Infections in patients receiving contaminated intravenous infusions.
- Occasionally infect normal individuals.
- Very common in patients with:
 - Indwelling urinary catheters.
 - Anatomical or functional abnormalities of the urinary tract.
- UTIs caused by *Proteus* tend to be more severe than those caused by *Escherichia coli*.

E) Pathogenesis and Virulence Factors

- **Produce urease enzyme.** Urease causes rapid hydrolysis of urea → release of ammonia. This leads to: **Alkaline urine** (Formation of urinary stones in the bladder and ureters)
- **Ammonia also:** Inactivates the complement system (especially C4).
- **Motility:** Helps bacterial entry into the bladder.
- **Endotoxin:** Causes fever and shock in septicemia.

F) Serological Classification

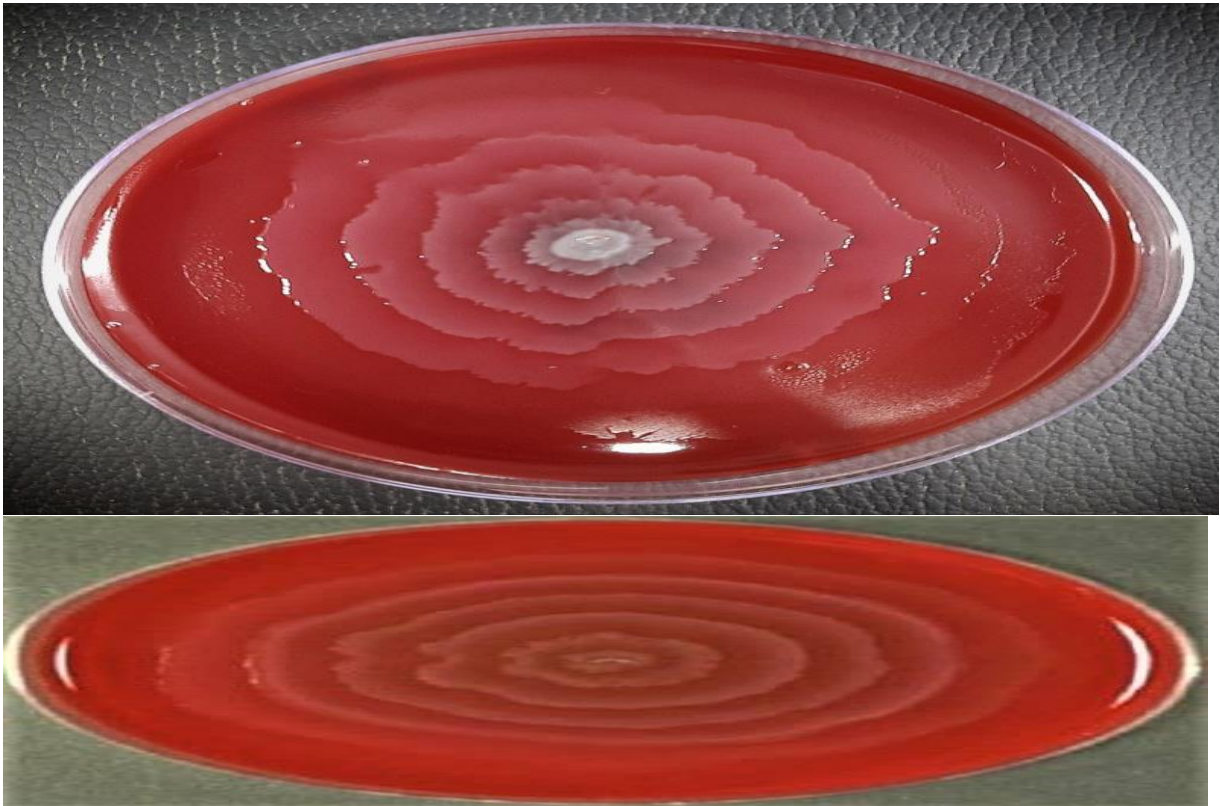
- Not reliable due to cross-reactivity with *Typhus*.
- Differentiation between biotypes is done by: Carbohydrate fermentation tests.

G) Enzymes Produced by *Proteus* spp. several important enzymes:

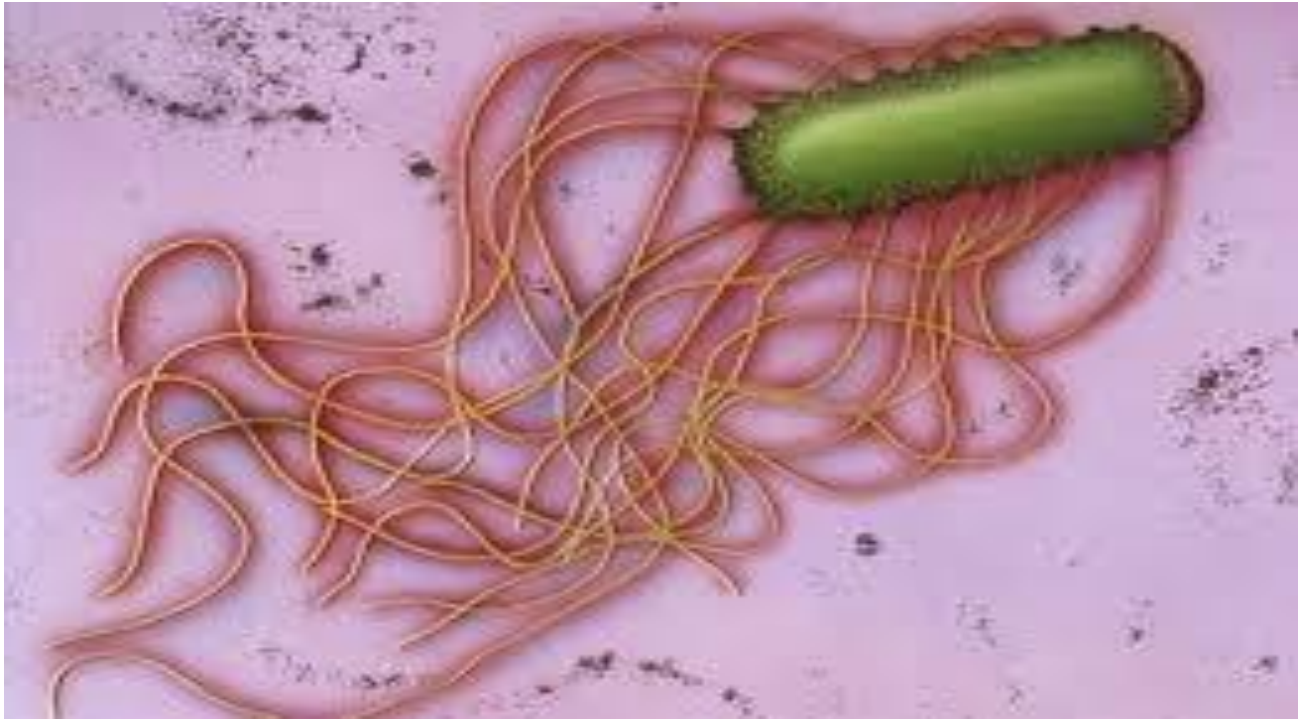
- **Protease.**
- **Gelatinase.**
- **Phenylalanine deaminase.**
- **Urease.**
- **Hemolysin.**

H) Antibiotic Sensitivity

- **Highly sensitive to: Piperacillin. Cefotaxime. Gentamycin.**
- **Drug of choice: Piperacillin.**

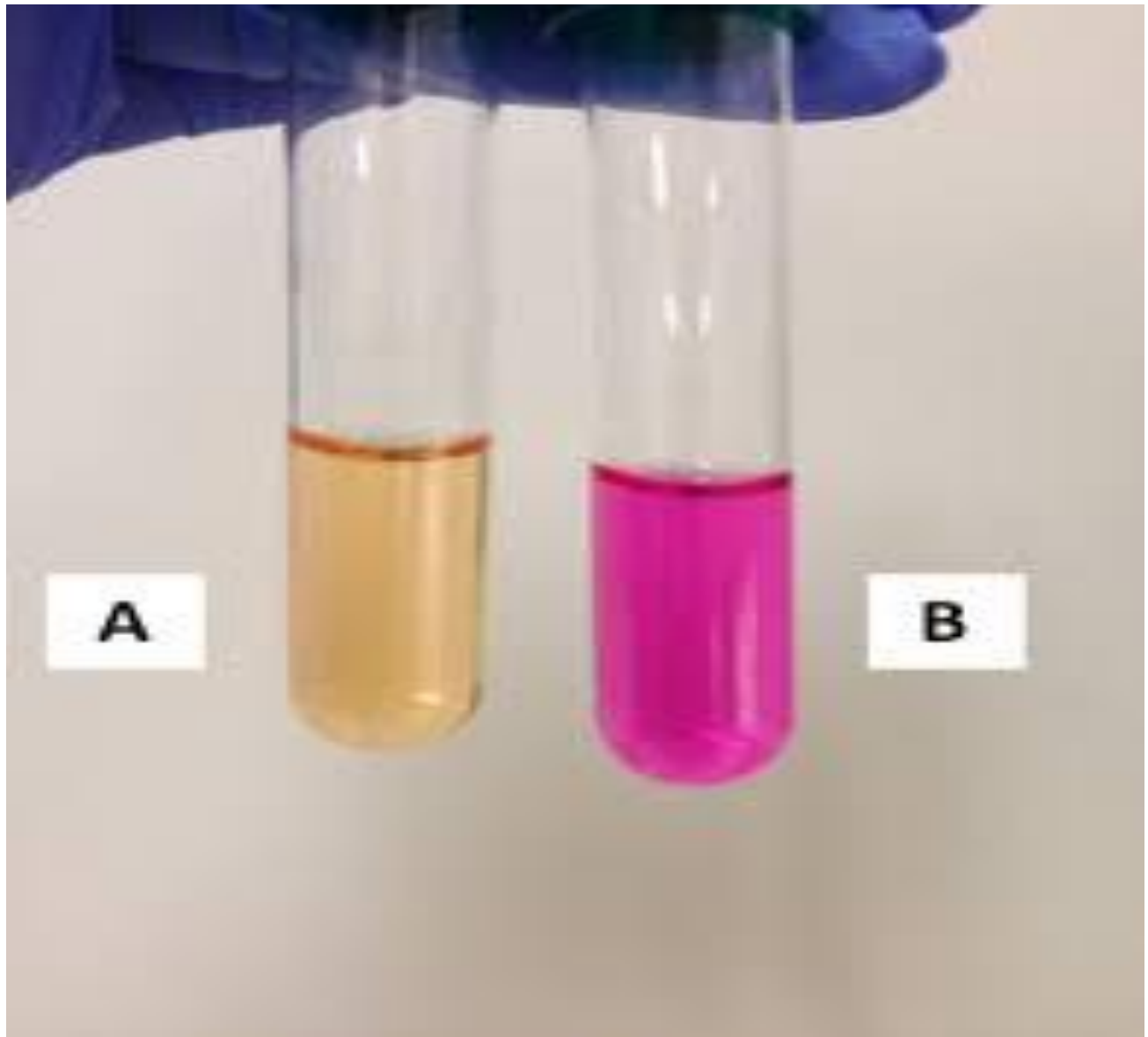


Proteus swarming on blood agar



Urease producing bacteria

1. Proteus
2. Klebsiella
3. Staphylococcus
4. Psuedomonas
5. Providentia
6. Ureaplasma



Proteus urease test positive (pink color).

Some factors inhibit the swarming phenomena:

- 1- Adding 4% agar to media.**
- 2- Presence of bile salts (MacConkey agar).**
- 3- Anaerobic conditions.**

Diagnostic tests for proteus spp.:

1- Gram stain: Gram negative bacilli or coccobacilli (pleomorphic).

2- Inoculation MacConkey agar.

3- Blood agar (swarming and hemolysis)

4-TSI agar test.

5- Urease test.

6- IMViC test.

7- Gelatin liquefaction.

8- Phenylalanine deaminase test:

- **Also known as:** Phenyl pyruvic acid (PPA) test.
- **Purpose:** Tests the ability of an organism to produce the enzyme phenylalanine deaminase.
- **Enzyme Function:** Removes the amine group from the amino acid phenylalanine

Produces phenyl pyruvic acid (PPA) and ammonia.

- **Detection:** Phenyl pyruvic acid reacts with ferric salts to give a green color.
- **Useful for initial differentiation of:** Proteus sp, Morganella spp, Providencia spp.
- **Differentiates them from other Enterobacteriaceae.**

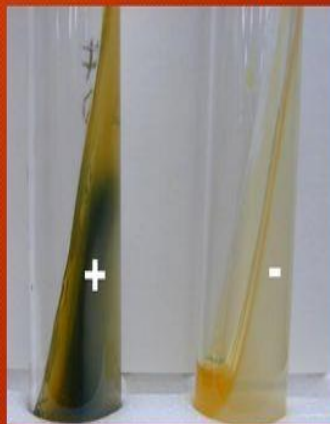
Procedure:

1. Inoculate the long slant of phenylalanine agar with the tested organism.
2. Incubate the tube at 37 °C for 18–24 hours.
3. Add 4–5 drops of 10% ferric chloride solution along the slope.
4. **Positive test:** Green color develops in the fluid and on the slope. **Negative test:** No green color appears.

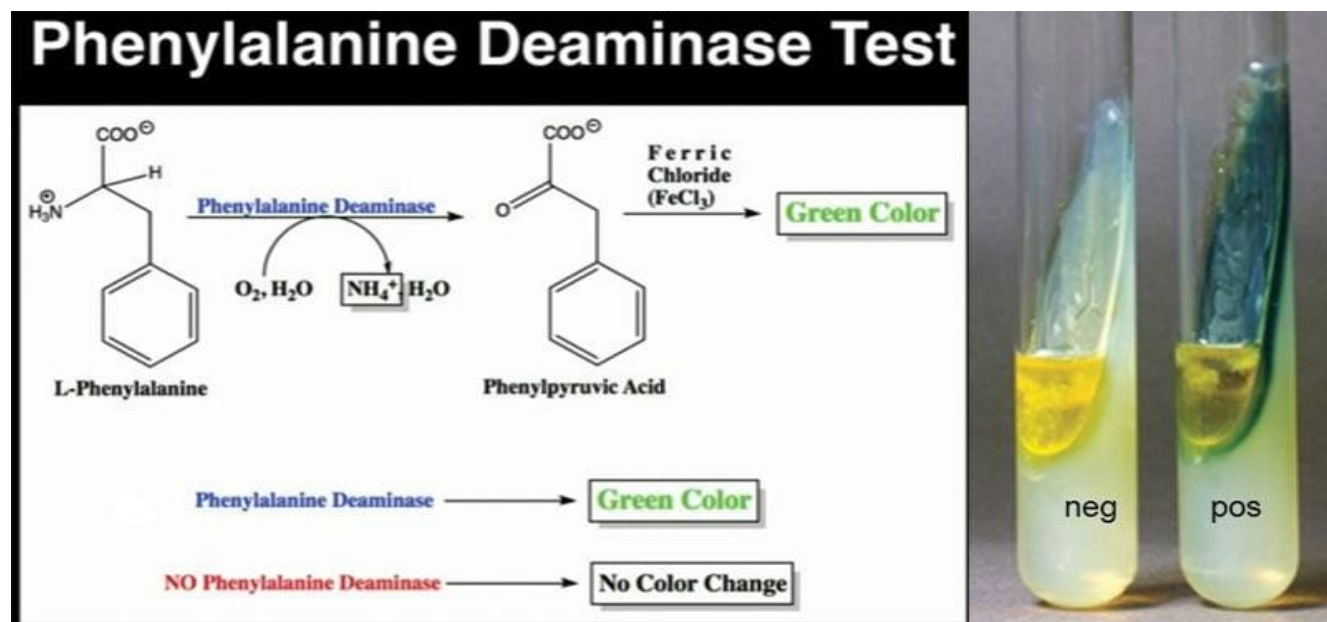
Phenylalanine Deaminase Test

13

- Used to identify bacteria possessing the enzyme phenylalanine deaminase
 - Phenylalanine deaminase converts phenylalanine to phenylpyruvic acid + NH_3
 - Ferric chloride (FeCl_3) reacts with the phenylpyruvic acid and changes color from yellow to green



Reagent = FeCl_3 , positive result = green color



Some biochemical and culture characteristics of *Proteus spp.*

tests	<i>P.mirabilis</i>	<i>P.vulgaris</i>	<i>P. penneri</i>
IMViC	-, +, -, V	+, +, -, V	-, +, -, -
TSI	A/K CO ₂ +, H ₂ S +	A/KCO ₂ +, H ₂ S +	A/KCO ₂ +, H ₂ S -
Catalase	+	+	+
Oxidase	-	-	-
Urease	+	+	+
Capsule	+	-	-
Swarming	+	+	+
H ₂ S	+	+	-
Motility	+	+	+
MacConkey agar	L.N. F	L.N. F	L.N. F
Phenylalanine	+	+	+
Glucose	+readily	+ readily	+
Maltose	-	+readily	+
Sucrose	Ferment slowly	Ferment readily	+
Blood hemolysis	Non hemolysis	Non hemolysis	Beta
Gelatinase	+	+	+

Biochemical reactions of Proteus, Morganella, and Providencia species

Species	Urea	Cit	Ind	Suc	H ₂ S	PDA	GG
<i>P. mirabilis</i>	+	D	-	-	+	+	+
<i>P. vulgaris</i>	+	D	+	+	+	+	D
<i>M. morganii</i>	+	-	+	-	-	+	D
<i>P. rettgeri</i>	+	+	+	+	-	D	D
<i>P. stuartii</i>	-	+	+	+	-	+	-

Key: Urea = Urease test, Cit = Citrate test, Ind = Indole test,
 Suc = Sucrose fermentation, H₂S = Hydrogen sulphide
 production, PDA = Phenylalanine deaminase test, GG = Gas
 from glucose fermentation, D = Different strains give
 different results

Genera: Shigella and Salmonella

A) **Shigella: Serogroups and Species**

- Serogroup A: *Shigella dysenteriae* (12 serotypes).
- Serogroup B: *Shigella flexneri* (6 serotypes).
- Serogroup C: *Shigella boydii* (23 serotypes).
- Serogroup D: *Shigella sonnei* (1 serotype).

General Characteristics

- Gram-negative rods, cylindrical shape.
- Non-motile.
- Non-spore forming.
- Encapsulated.
- Non-lactose fermenters (except *S. sonnei*, which is lactose fermenter).
- Colonies appear pale on MacConkey agar.
- Facultative anaerobes.
- Part of human intestinal flora if present in small numbers.
- Highly virulent: as few as ~200 cells can cause infection.
- Transmission: via contaminated food with fecal matter.
- Growth temperature: 10–42 °C; optimum: 37 °C.
- Pathogenesis: Causes dysentery → destruction of intestinal epithelial cells (cecum and rectum).
- All Shigella spp. ferment glucose without gas, except *S. flexneri*.

Specimens for Detection

- Stool (4–5 days post-infection).
- Mucous or blood from intestines.
- Rectal swab.

B) Salmonella

Important Species

- *Salmonella typhi*
- *Salmonella paratyphi A*
- *Salmonella paratyphi B*
- *Salmonella typhimurium*

- *Salmonella enteritidis*
- *Salmonella ariwuna*
- *Salmonella choleraesuis*
- *Salmonella gallinarium*
- *Salmonella schottmuelleri* para A

General Characteristics

- Gram-negative bacilli.
- Non-spore forming.
- Motile (except *S. gallinarium*).
- Non-lactose fermenters (N.L.F.).
- Urease-negative.
- Citrate-utilizers.
- H₂S producers.
- Growth temperature: 4–40 °C.
- Biochemical tests are not always dependable; serotyping is used for identification.
- Resistant to some chemicals: brilliant green, Na-tetracholate, Na-deoxycholate (used in selective media).
- Transmission: human feces, animals, birds, reptiles; through contact, food, or water.
- Causes gastroenteritis and food poisoning.

Pathogenicity

- Acute gastroenteritis: 10⁵–10⁸ cells of *S. typhi* or *S. typhimurium*.
- Septicemia and local infections: by all *Salmonella* spp.
- Enteric fever: 10⁴–10⁶ cells of *S. typhi* or *S. paratyphi* A/B.

Specimens : Stool, urine, blood, serum (for serology).

Serological Diagnosis

- Widal test: detects somatic (O) and flagellar (H) antigens.
- Phage typing.

Laboratory Diagnostic Tests

1. Gram stain.
2. MacConkey agar – pale colonies.
3. **SS agar**: selective and differential; **Shigella** → pale colonies, **Salmonella** → black center (H_2S).
4. IMViC tests.
5. Motility.
6. Glucose fermentation.
7. TSI test.
8. Mannitol fermentation.
9. Gelatin hydrolysis.
10. Phenylalanine test.
11. **XLD agar (Xylose Lysine Deoxycholate)**:
 - Selective for Salmonella & Shigella.
 - pH ~7.4 → bright pink/red.
 - Sugar fermentation → yellow color.
 - Salmonella decarboxylates lysine → alkaline → **red colonies**; produces **H_2S → black center**.
 - Contains lactose & sucrose.
12. Urease test.
13. CDA (Citrate Deoxycholate Agar) – selective for Salmonella & Shigella.
14. **Brilliant Green Agar** – selective/differential; Salmonella **grows**, ***S. typhi* may not**.

15. **Bismuth Sulfite Agar – selective for Salmonella; *S. typhi* → black colonies; inhibits Gram-positive bacteria.**



Figure : Salmonella on S-S agar (black colonies)

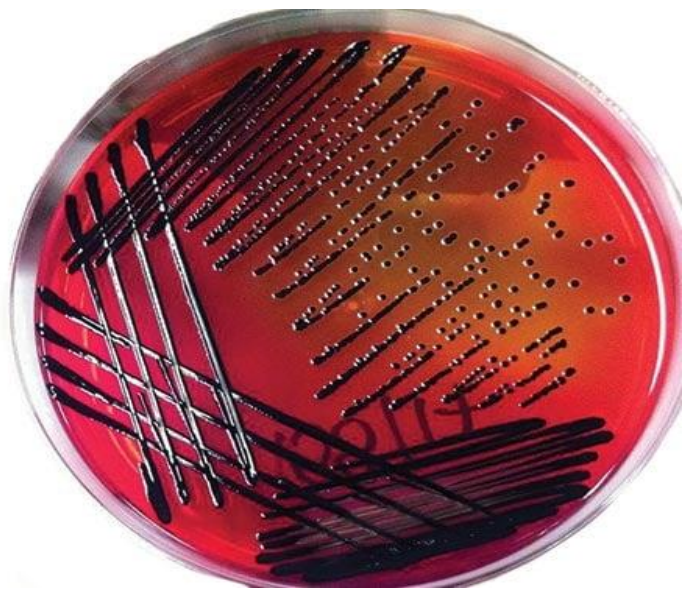


Figure: Shigella on S-S agar (pale colonies)

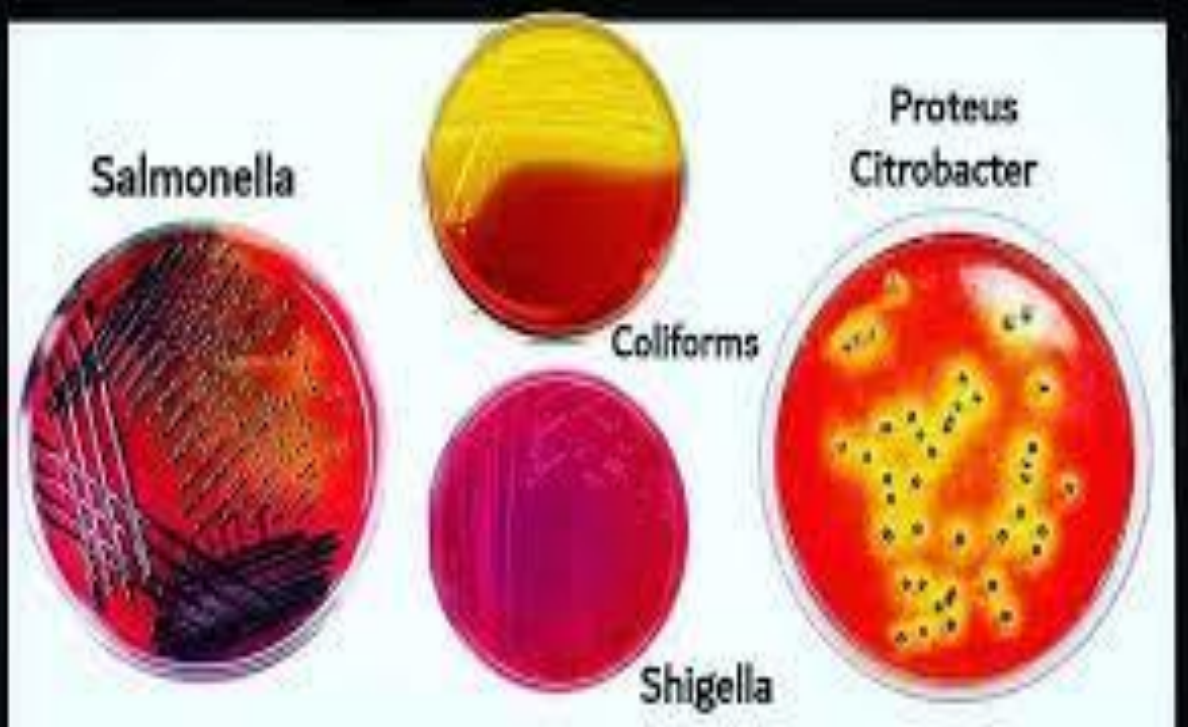


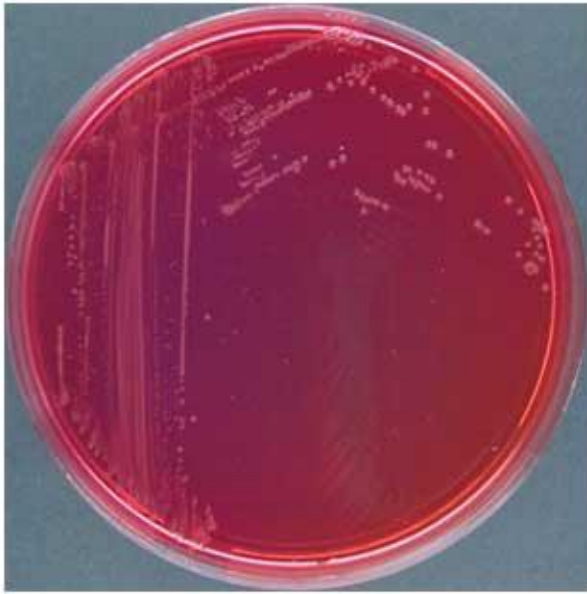
Figure : Salmonella on XLD agar (yellow colonies with black center)

Salmonella
Typhimurium
on
XLD agar.

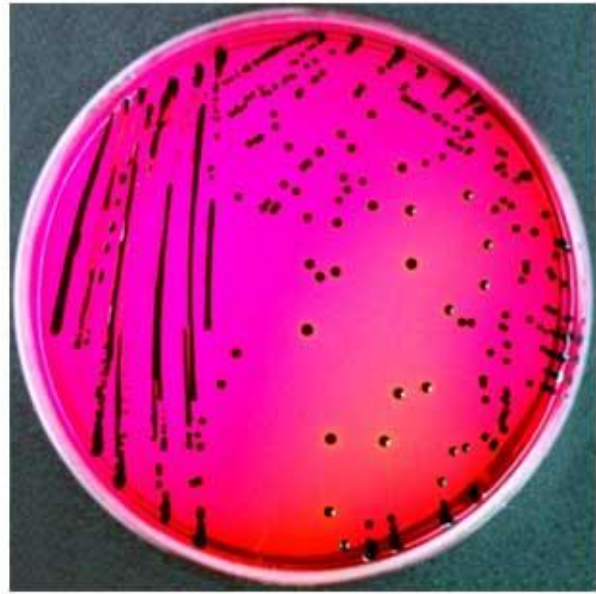


XLD Agar media



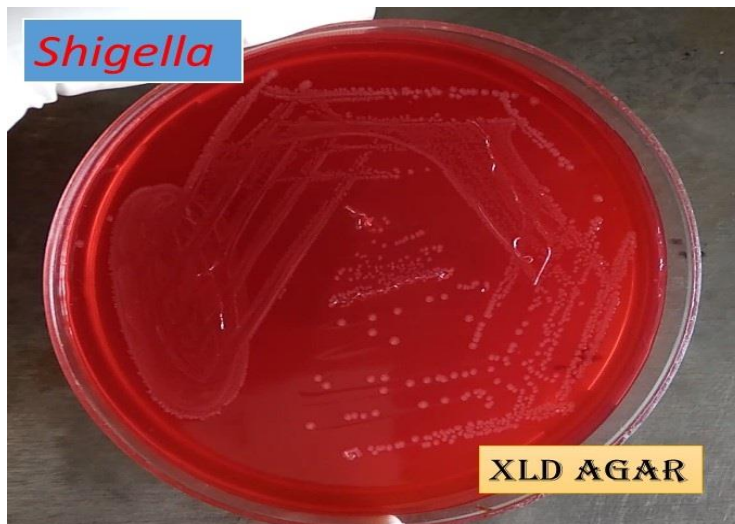


***Shigella* on XLD.**



***Salmonella* on XLD.**

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



Colony characteristics on XLD agar

Organisms	Colony characteristics
<i>Salmonella</i> H2S positive	Red colonies with black centers
<i>Shigella</i> spp. and <i>Salmonella</i> H2S negative	Red colonies
<i>E. coli</i>	Large, flat, yellow colonies
<i>Proteus</i> spp.	Red to Yellow colonies
<i>Enterobacter</i> / <i>Klebsiella</i>	Mucoid, yellow colonies

Some biochemical and culture characteristics of *Shigella* spp.

tests	<i>Sh.dysenteriae</i>	<i>Sh.flexneri</i>	<i>Sh.boydii</i>	<i>Sh.sonnei</i>
IMViC	V , + , - , -	V , + , - , -	V , + , - , -	- , + , - , -
TSI	A/K CO2- ,H2S -	A/KCO2-,H2S -	A/KCO2 -,H2S -	A/KCO2 -,H2S -
Catalase	+	+	+	+
Oxidase	-	-	-	-
Urease	N	N	N	N
H2S	N	N	N	N
Motility	N	N	N	N
MacConkey agar	L.N.F	L.N.F	L.N.F	
Phenylalanine	N	N	N	N
Glucose	+ NO gas	-	+ NO gas	+ NO gas
Mannitol	N	P	P	P
Gelatinase	N	N	N	N
S-S agar	Pale colony	Pale colony	Pale colony	Pale colony

P=positive, N=negative

Some biochemical and culture characteristics of *Salmonella* spp.

tests	<i>S.typhi</i>	<i>S.thyphimurium</i>
IMViC	- , + , - , -	- , + , - , +
TSI	A/K CO2 +, H2S -	A/K CO2+, H2S +
Catalase	+	+
Oxidase	-	-
Urease	-	-
H2S	-	+
Motility	+	+
MacConkey agar	L.N. F	L.N. F
Phenylalanine	-	-
Glucose	+	+
Maltose	+ gas	+ gas
S-S agar	Pale colony	Pale colony with black center