Practical pathogenic bacteria LAB-3

Biochemical tests for the identification of Gram-positive bacterial species

1. **Catalase test: G+\G-**
2. **Oxidative/Fermentative: G+\G-**
3. **Oxidase test: G+\G-**
4. **Coagulase test: G+**
5. **Hippocrates hydrolysis test :G+**
6. **Bile Esculin test :G+**
7. **Bacitracin Susceptibility Test :G+**
8. **Optochin Susceptibility Test: G+**

9-Motility test: G+\G-

1- Catalase Test

- It determines the ability of bacteria to produce the catalase enzyme which forms gas bubbles when reacting with 3% H2O2.

- Catalase mediates the breakdown of hydrogen peroxide (H_2O_2) into oxygen and water.

Principle:

$2H_2O_2 \rightarrow 2H_2O + O_2$ (gas bubbles)

- A small inoculum of a bacterial isolate is mixed into hydrogen peroxide solution (3%). It is observed for the rapid elaboration of oxygen bubbles. The lack of catalase is evident by a lack of or weak bubble production.

- Catalase producing Gram-positive bacteria:

***Staphylococcus* spp**

- Catalase-positive bacteria include strict aerobes as well as facultative anaerobes.

-Catalase-negative bacteria may be anaerobes or facultative anaerobes (i.e. Streptococci).

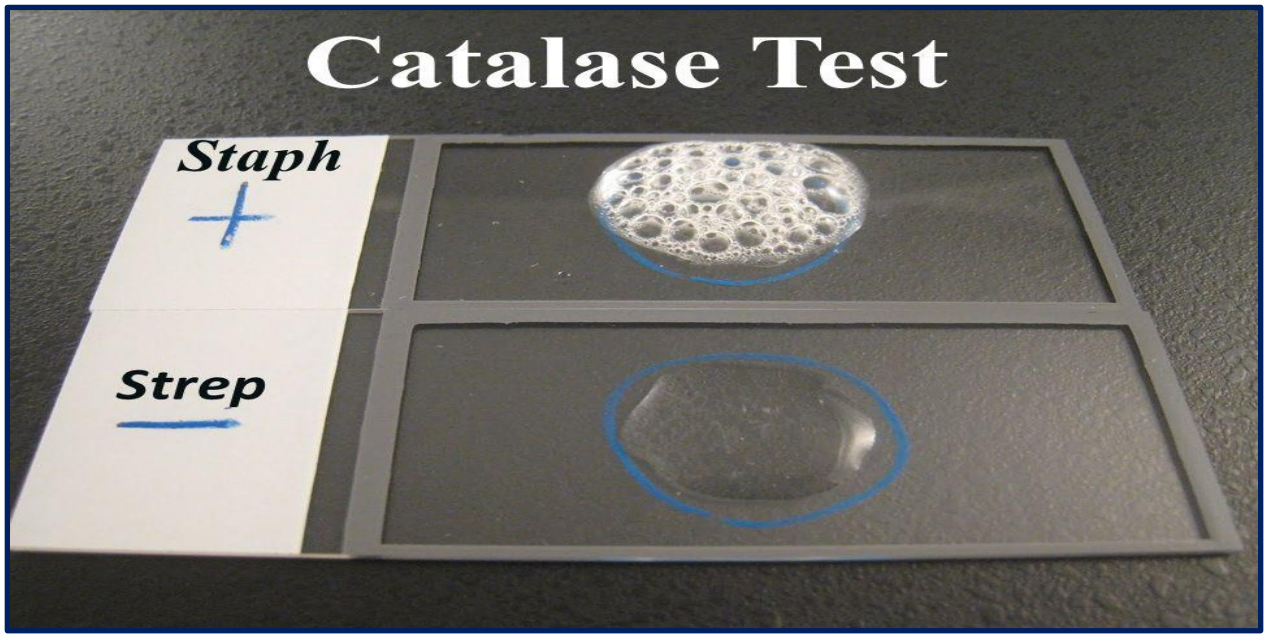
Percentage of H2O2 used in catalase test

Percentage	Purpose
3% H_2O_2	**Routine testing of aerobes**
15% H_2O_2	**Identification of anaerobic bacteria**
30% H_2O_2	***Neisseria* spp**

Procedure:

-Slide Test

1. **Transfer a small amount of bacterial colony to a surface of a clean, dry glass slide using a loop or sterile wooden stick (be sure the colony is visible to the naked eye on the slide).**
2. **Place a drop of 3% H_2O_2 onto the slide and mix.**
3. **A positive result is the rapid evolution of oxygen (within 5-10 seconds), as evidenced by bubbling.**
4. **A negative result is no bubbles or only a few scattered bubbles.**
5. **Dispose of your slide in the biohazard glass disposal container.**



-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 (O2 + water = bubbles) at the end of the wooden applicator stick.**

2- Oxidative/Fermentative Test

- During the metabolism process, bacteria either break down complex organic molecules aerobically or anaerobically.

- The aerobic metabolism process is referred to as the oxidation process whereas the anaerobic metabolism process is referred to as a fermentative process.

- Two tubes with Hugh and Leif son's medium are used where one tube is sealed with paraffin oil to create an anaerobic condition.

- Oxidative Gram-positive organism:

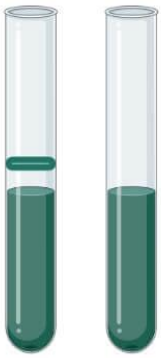
***Micrococcus* spp**

- Fermentative Gram-positive organism:

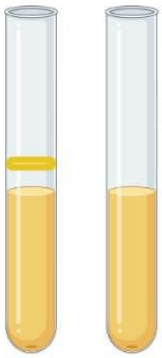
Staphylococcus aureus

- Gram negative : *Pseudomonas* spp

Oxidation/Oxidative-Fermentation (OF) Test

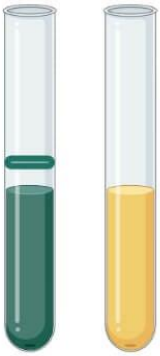


Control



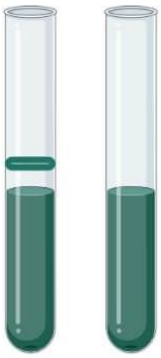
Fermentative

Eg. *E. coli*



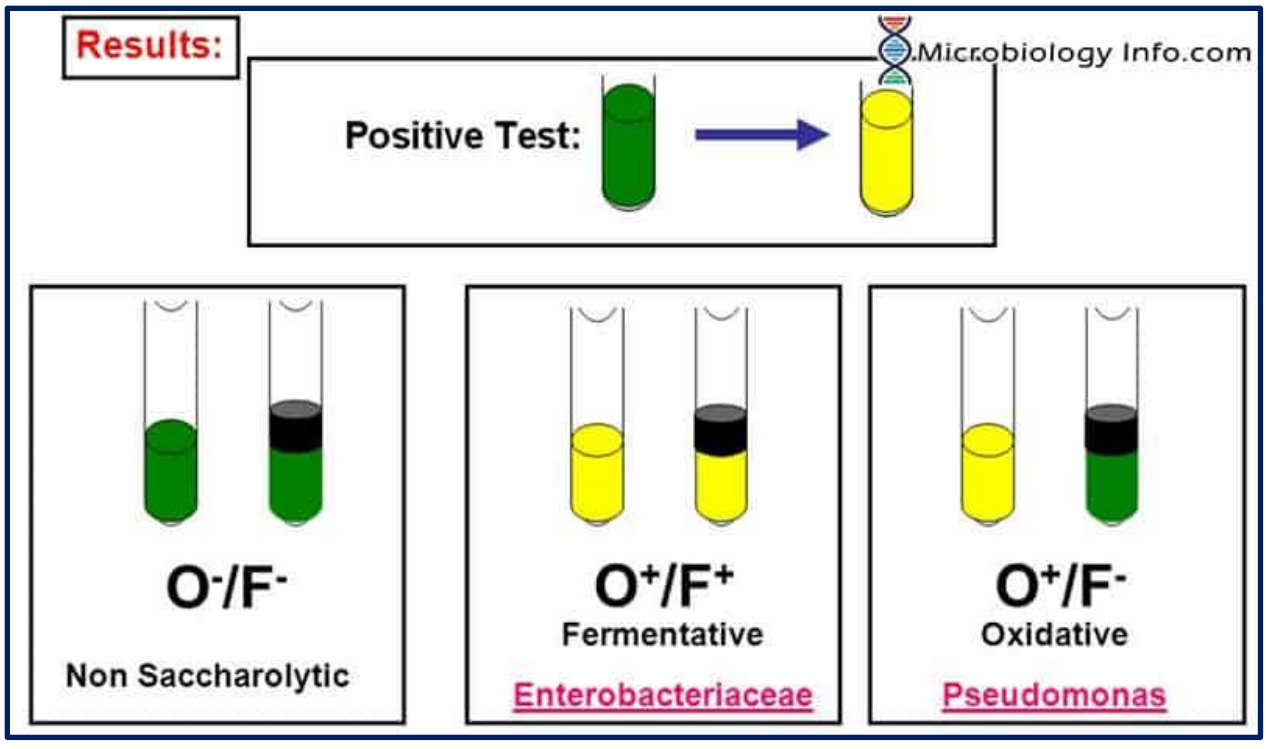
Oxidative

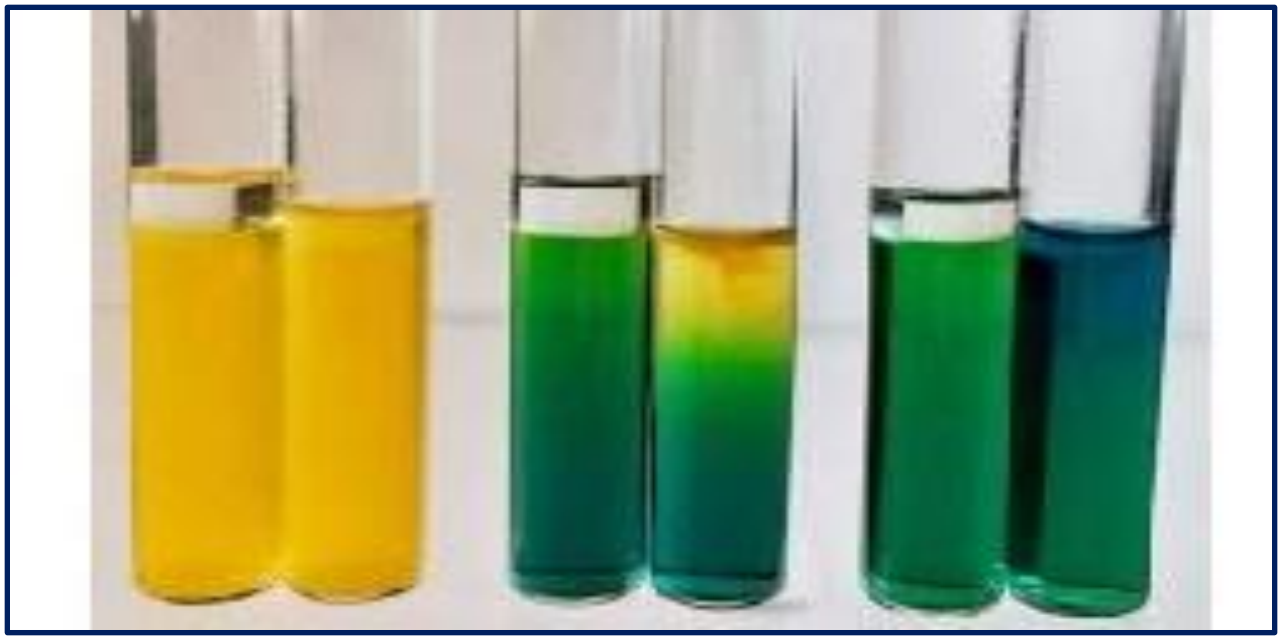
Eg. *Pseudomonas aeruginosa*



Non-Saccharolytic

Eg. *Alcaligenes faecalis*





3-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.

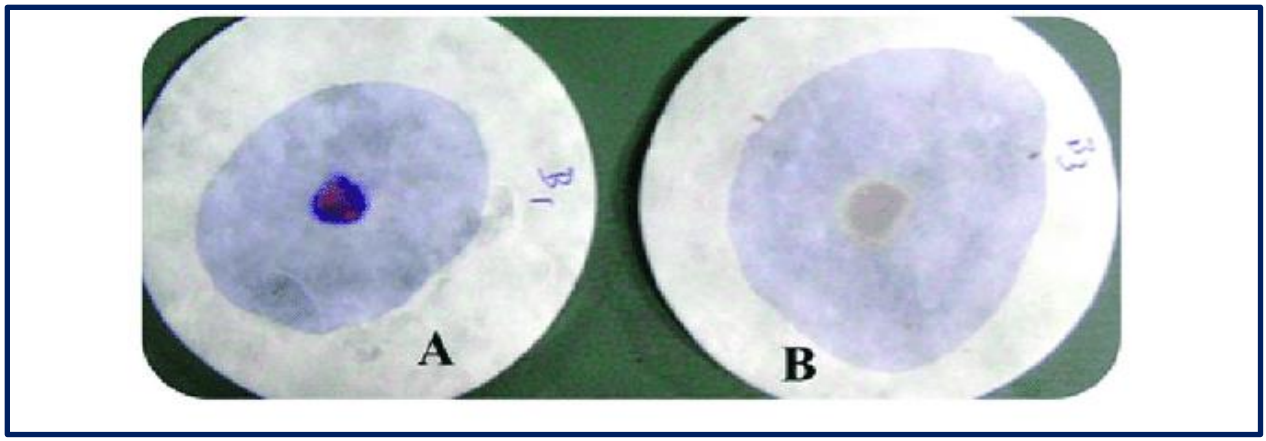
4- Observe the inoculated area of the paper of disks for the color change to deep blue or purple within 10 seconds because timing is very critical.

***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.



4- Coagulase Test

- The coagulase test is one way to differentiate the highly pathogenic *S. aureus* (coagulase-positive) from the other less pathogenic staphylococcal species on the human body (coagulase-negative Staphylococcus (CONS)).

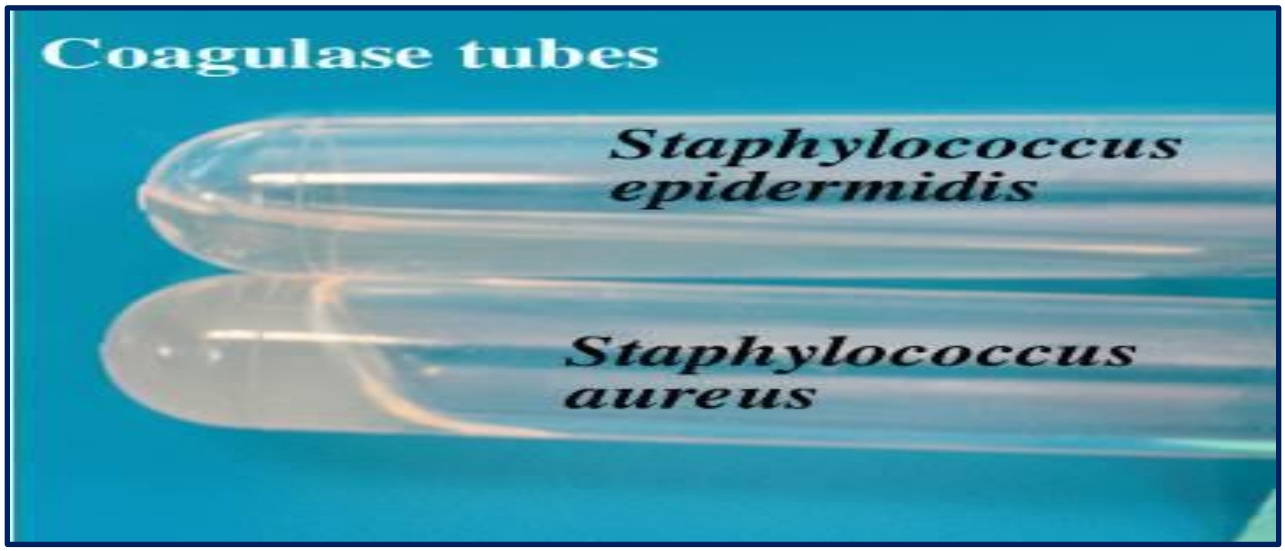
- *S. aureus* produces a bound and free form of coagulase that converts soluble fibrinogen into insoluble fibrin.

- Coagulase test is done either in a slide or in a tube which is determined by the form of coagulase produced.

- Cell bound coagulase is detected by the slide coagulase test which forms agglutination in case of positive results.

- Free coagulase is detected in a tube which forms a clot if the organism is tested positive.

- Coagulase producing Gram-positive organism: *Staphylococcus aureus*



5- Bile Esculin Test:

- The bile-esculin test is widely used to differentiate enterococci and group D streptococci (which are bile tolerant and can hydrolyze esculin to esculetin) from non-group D viridans group streptococci (which grow poorly on bile).

- Bile esculin agar medium is both selective as well as the differential medium in which its selective ingredient is bile that inhibits the growth

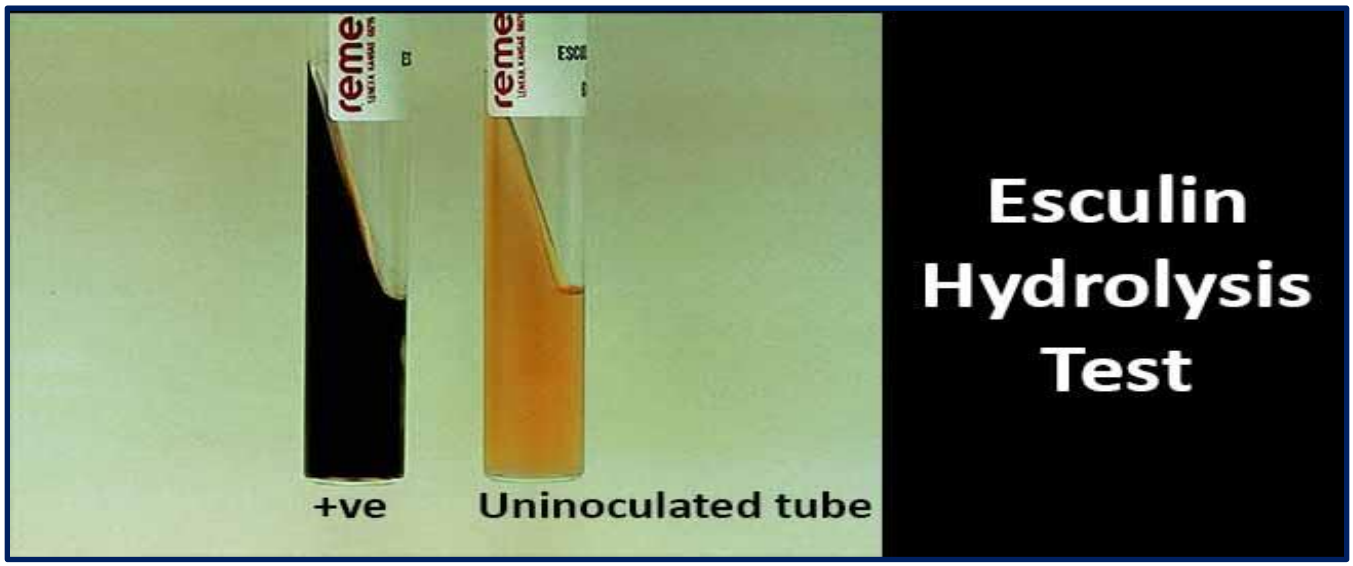
of other Gram-positive bacteria except enterococci and some streptococci species.

- Esculin is the differential ingredient that differentiates *Enterococcus* from *Streptococcus*.

- The bile esculin test determines the ability of bacteria to hydrolyze esculin when in the presence of bile salt, esculin is formed.

- Ferric citrate is present in the medium and when it reacts with esculin, it turns the entire medium dark brown to black due to the formation of the phenolic iron complex.

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



6- Hippurate hydrolysis test

Hippurate hydrolysis relies on the ability of the enzyme called hippurate hydrolase produced by microorganisms to hydrolyse sodium Hippurate to benzoic acid and glycine

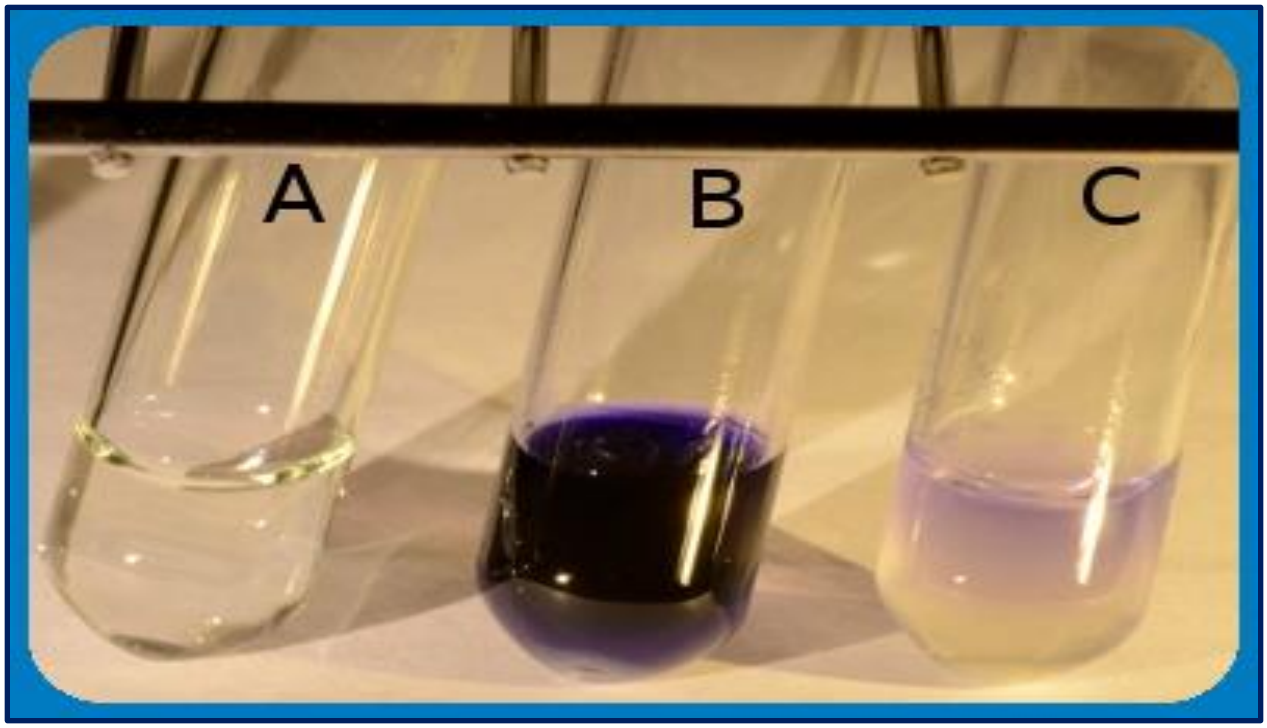
1- The Hippurate hydrolysis test detects the organism's ability to produce hippuricase enzyme that hydrolyzes hippurate substrate into glycine and benzoic acid.

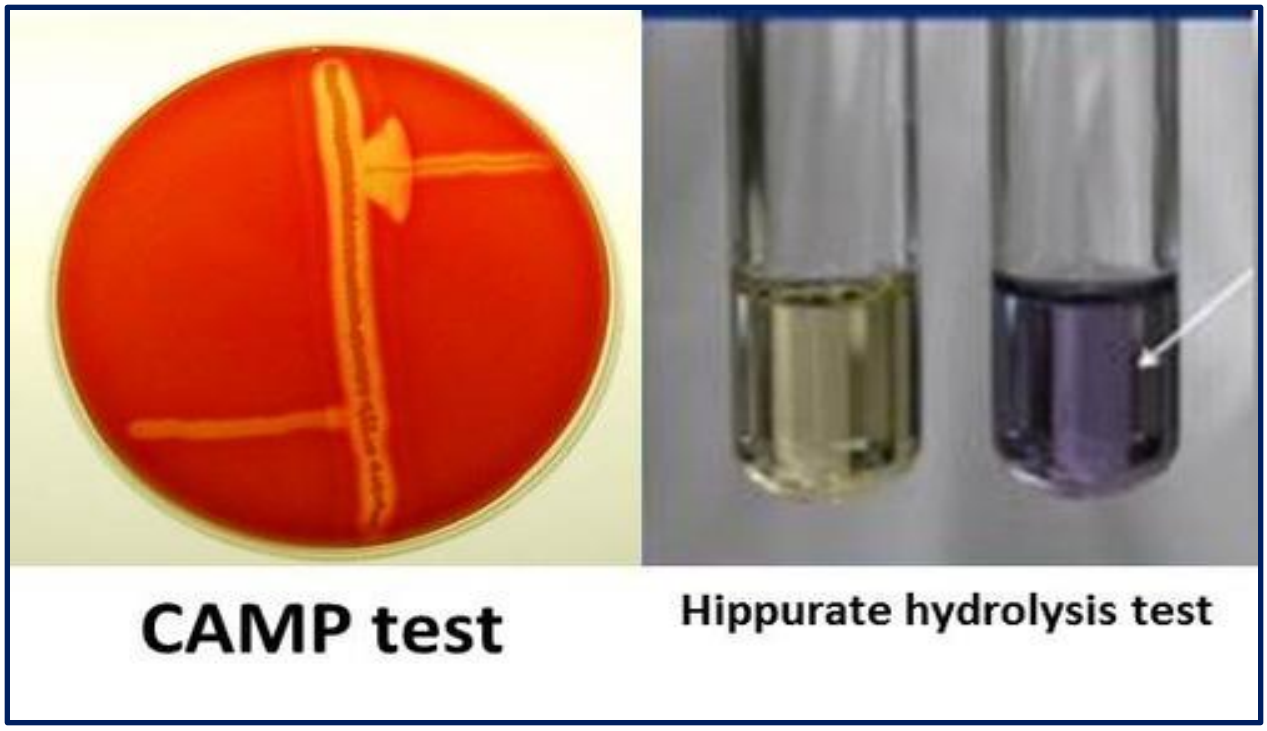
2- Glycine is detected by using the Ninhydrin reagent which forms a deep purple or violet color.

3-The Hippurate hydrolysis test differentiates β-hemolytic Streptococcus agalactiae from other β-hemolytic streptococci.

***Hippurate gram-positive organism:**

Streptococcus agalactiae





7- Bacitracin Susceptibility Test

- The Bacitracin test is used to distinguish β-hemolytic streptococci. It can distinguish between *Streptococcus pyogenes*, which forms a zone of inhibition around the bacitracin disc (Positive result), and *Streptococcus agalactiae*, which grows up to the disc (negative result).

- Bacitracin is antibiotic produced by group of *Bacillus subtilis*

- Bacitracin susceptibility test is a presumptive test for the differentiation between beta-hemolytic Group A streptococci and beta-hemolytic non-Group A streptococci.

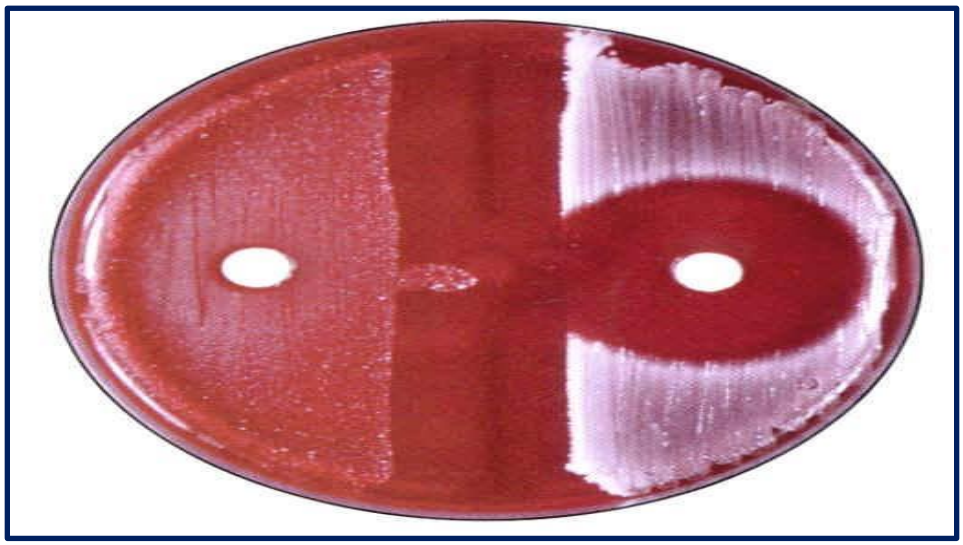
- This test is performed on the blood agar with a streaked culture of streptococci where the bacitracin disk is impregnated with sterile forceps before incubation.

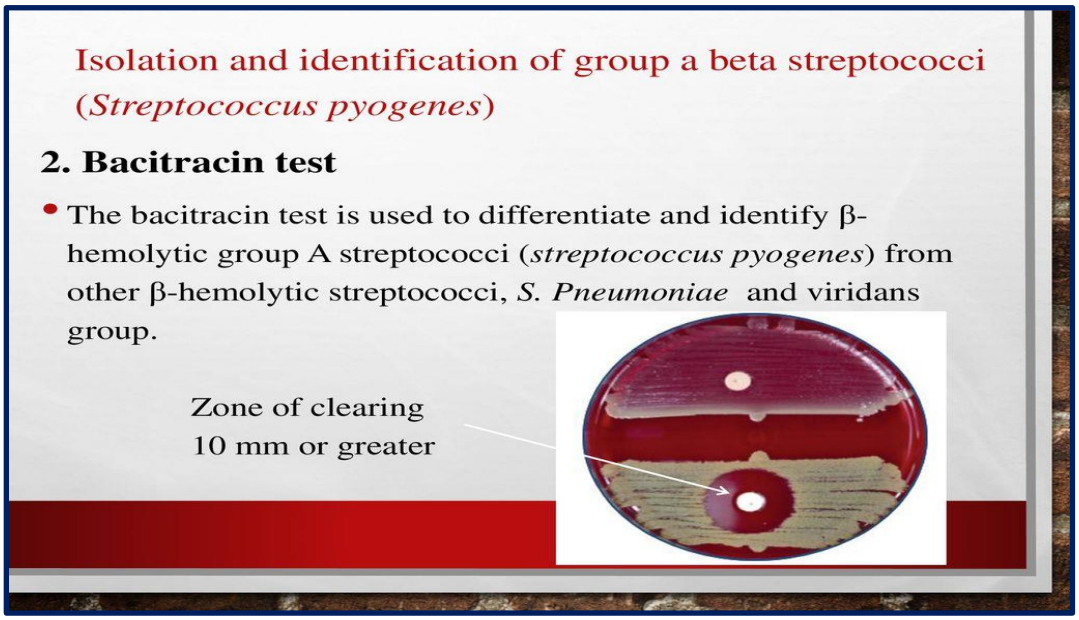
- A zone of inhibition will be observed if the isolated organism is beta-hemolytic Group A streptococci whereas beta-hemolytic non-Group A

streptococci will be resistant towards bacitracin showing no zone of hydrolysis and will grow all over the disk.

- Bacitracin sensitive Group A streptococci: *Streptococcus pyogenes*

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





8- Optochin Susceptibility Test:

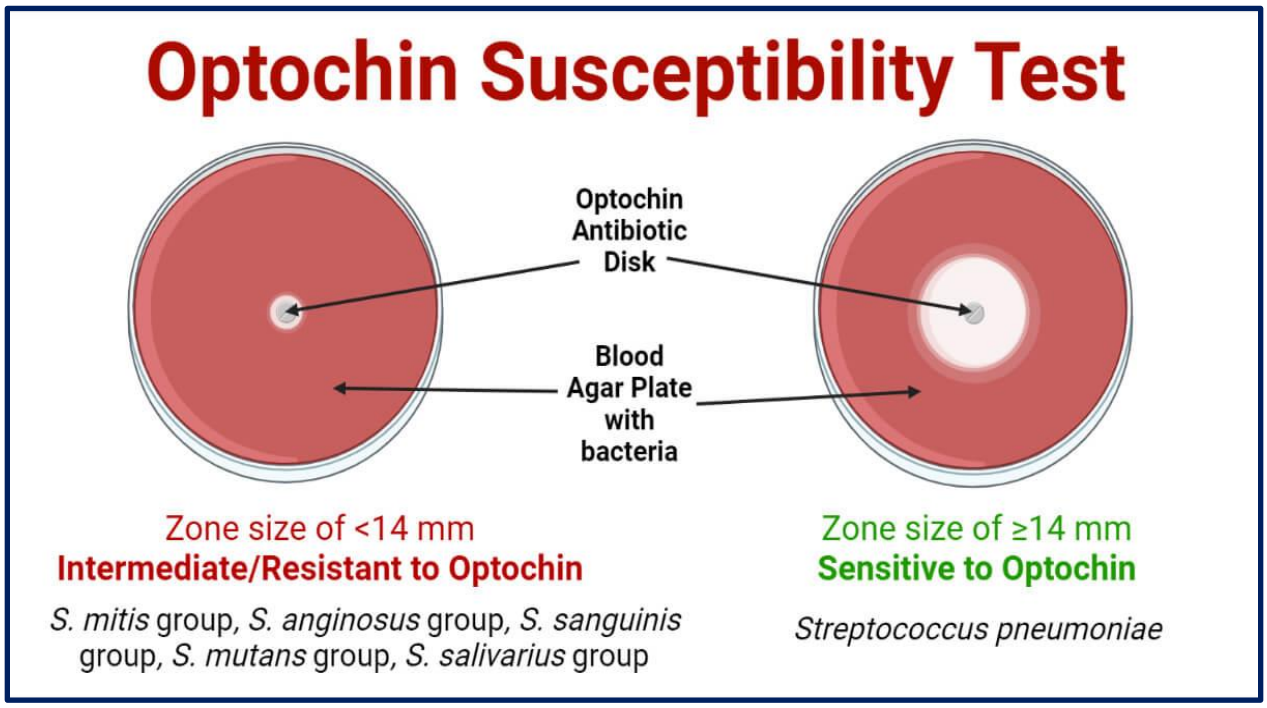
- It is a useful test for the identification of *Streptococcus pneumoniae*.

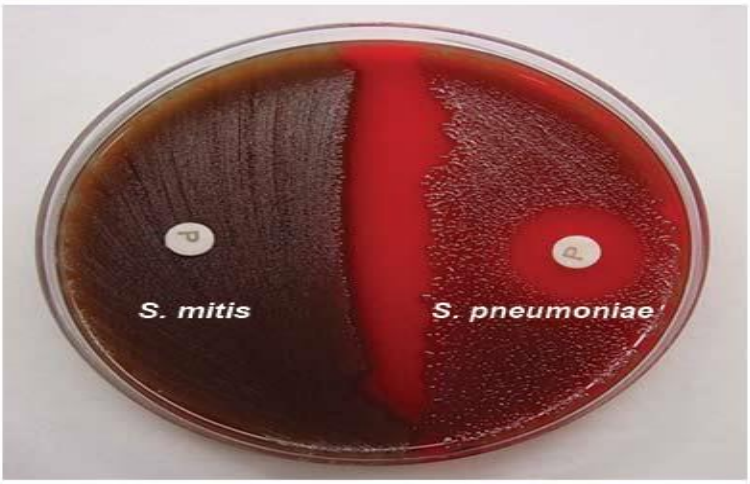
- Alpha-hemolytic *Streptococcus* is the most commonly susceptible bacteria for this test. Other alpha-hemolytic streptococcal species are optochin-resistant and do not display this clear zone of inhibition when in the presence of optochin.

- Differentiation of *Streptococcus pneumoniae* from other viridans streptococci depends on demonstrating optochin susceptibility, bile solubility, or detection of species-specific capsular polysaccharides.

- The optochin test is widely used in the form of filter paper discs, saturated with ethylhydrocupreine hydrochloride, which are applied directly to inoculated plates before incubation.

- A positive presumptive identification of *S. pneumoniae* is made when a well-defined zone of inhibition results around the saturated disk.





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.

9- 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.

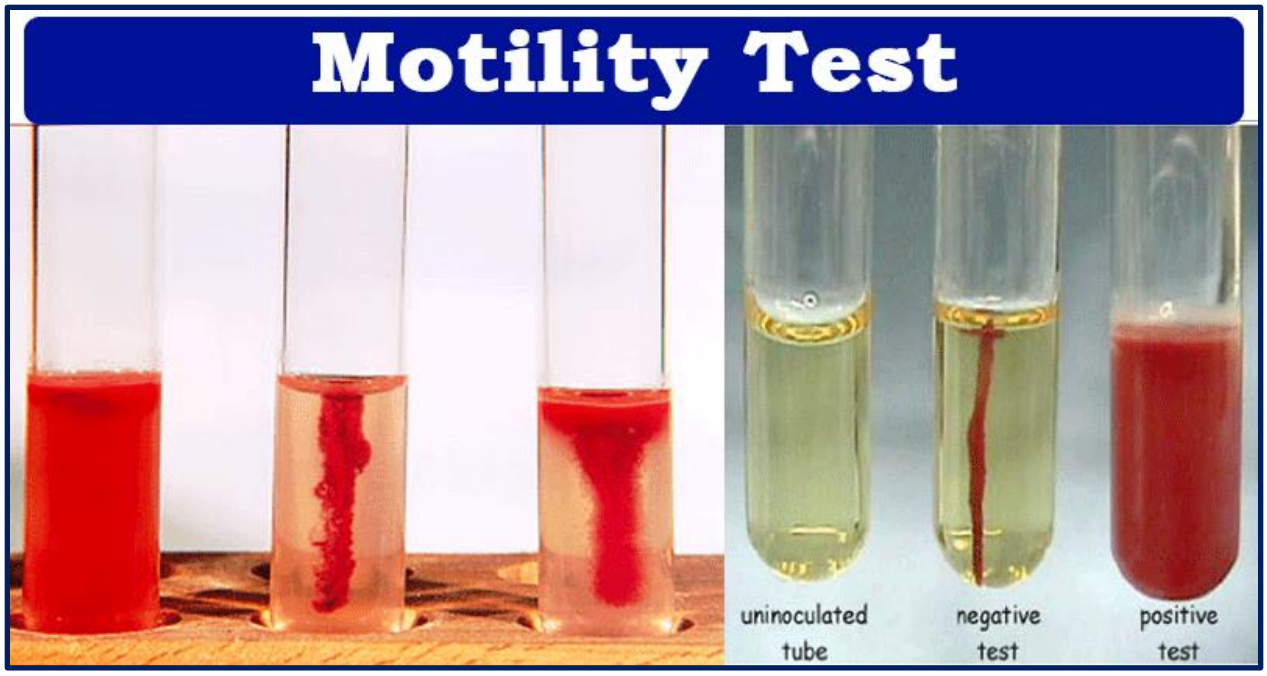
3- Incubate at 35°-37°C and examine daily for up to 7 days.

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*



10- 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.**

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).**

- **Positive result: Suspension clears in tube labelled test and remains turbid in control tube.**
- **Negative result: Suspension remains turbid.**

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