Practical Pathogenic Bacteria

LAB:4

Bacterial Identification (Gram Negative Bacteria)

1- IMViC Test

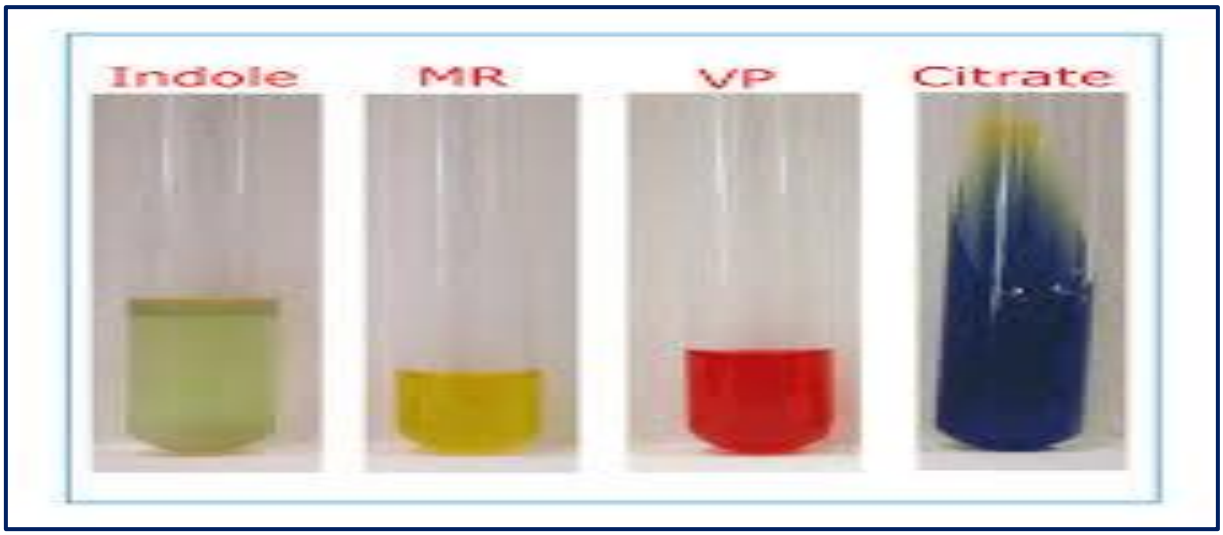
I= Indole ring production

M= Methyl red

V= Voges Proskauer

C= Citrate utilization

- **IMViC test is a group of individual tests used for differentiating the members of Enterobacteriaceae family (the coliform group), especially when used alongside the Urease test.**
- **Coliforms are Gram-negative, aerobic, or facultative anaerobic rods.**
- **They produce gas from lactose within 48 hours.**
- **The presence of some coliforms indicates a fecal contamination.**



IMViC series = *Klebsiella* and *Enterobacter*

Indole Production Test

Principle of Indole Production Test

- The bacterium is grown in peptone water (tryptone water) broth (medium).

- The medium contains tryptophan (substrate), which under the action of enzyme tryptophanase (enzyme) is converted to an Indole molecule, pyruvate and ammonium.

- To test the broth for indole production, Kovac's reagent is used.

- **Kovac's reagent consist of amyl alcohol and para-dimethylaminobenzaldehyde and concentrated hydrochloric acid.**

- Kovac's reagent is actually used to determine ability of an organism to separate indole from amino acid tryptophan and it is added after incubation.

- A positive result is indicated by forming a red ring on the top of the medium (*E. coli*).
- A negative result is indicated by forming a yellow ring on the top of the medium (*Klebsiella*).

Procedure of Indole Test

1- Inoculate the tryptone water medium with the tested bacterium.

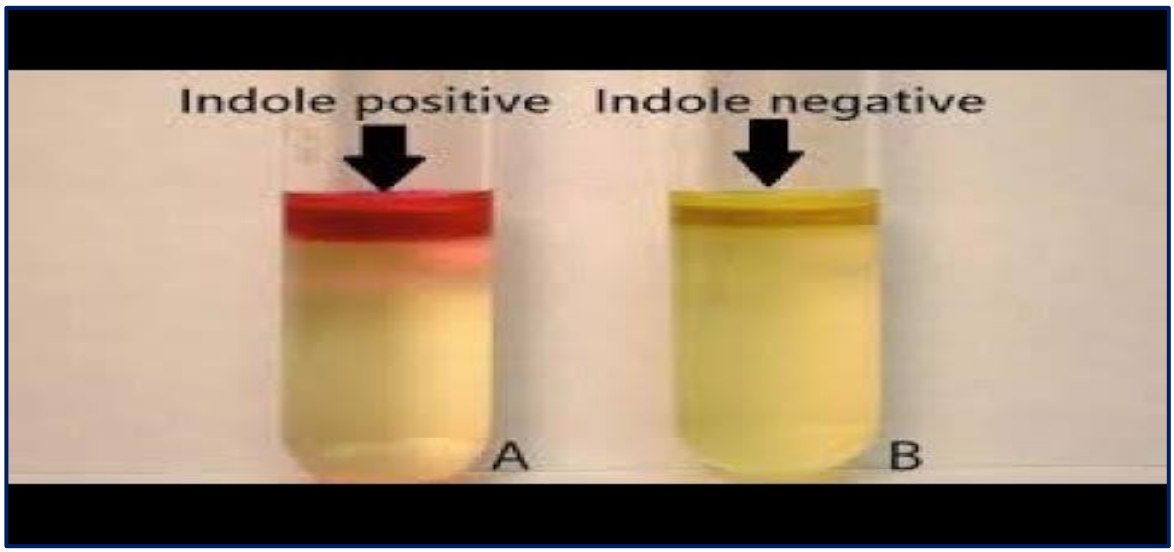
2- Incubate at 37°C for 24 hrs. After the incubation interval, add 1 ml of Kovac's reagent, shake the tube gently and read immediately.

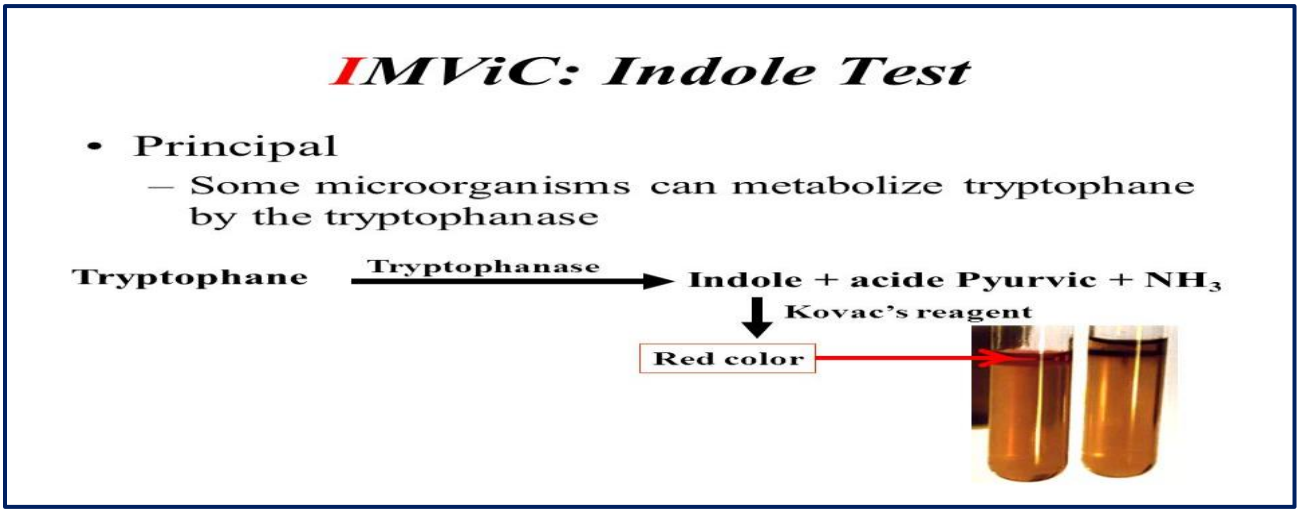
3- The Indole-positive result (red ring).

4- The Indole-negative result (yellow ring).

Kovac's Reagent Preparation:

Dissolve 10 g of p-dimethylaminobenzalaldehyde in 150 ml of amyl, isoamyl or butyl alcohol. Heat it in a 56°C water bath until dissolved. Cool. Slowly add 50 ml of conc. HCL. Store it in a glass-stoppered brown bottle in the refrigerator. This reagent should be light yellow in color.





Methyl Red and Voges–Proskauer Test

- These two tests use the same medium which is called MR-VP broth.

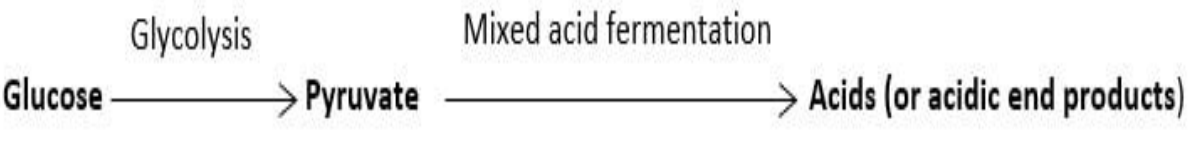
- After growth, the broth is separated into two different tubes, one for the methyl red (MR) test and one for the Voges-Proskauer (VP) test.

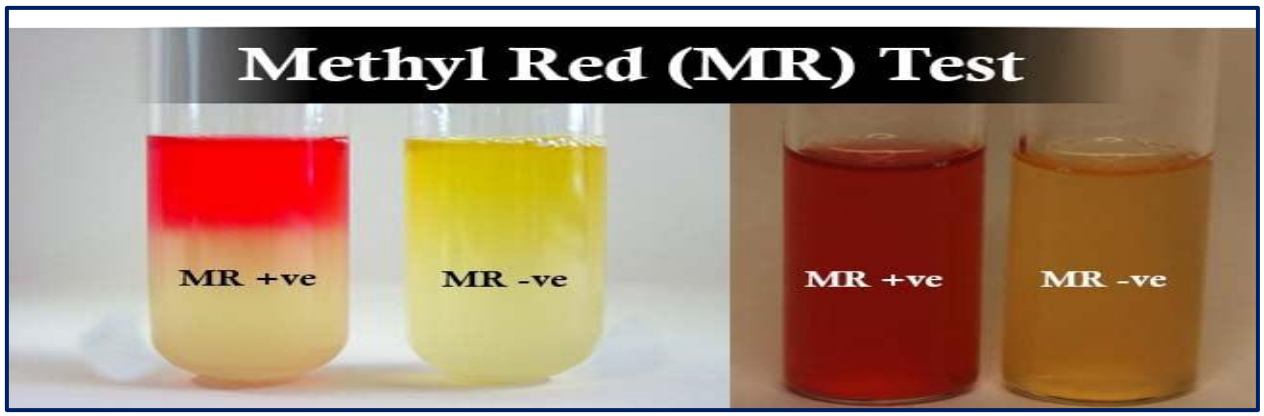
Principle of Methyl Red Test

- The methyl red test detects production of mixed acids from glucose fermentation using (mixed acid fermentation pathway) using pyruvate as a substrate.

- The pH indicator methyl red is added and a red color appears at a pH lower than 4.2, indicating a positive result.

- If the solution remains yellow (pH = 6.2 or above), this indicates a negative result (butanediol fermentation). Methyl red is yellow at a pH above 6.0, but it turns red at a pH below 4.4.





Procedure of MR Test

1. **Inoculate the MR-VP medium with the tested bacterium.**
2. **Incubate at 37°C for 24 hrs.**
3. **Add 5-6 drops of methyl red indicator.**
4. **The positive result is indicated by a red color of the medium.**
5. **The negative result is indicated by the medium remaining yellow or turning orange.**

Uses of MR Test

The methyl red test is used to differentiate between different bacterial species based on their ability to produce stable acid end products during glucose fermentation. It is commonly used for the identification of enteric bacteria, such as *Escherichia coli* and *Enterobacter aerogenes*.

Principle of VP Test

- The VP test is performed using two indicators alpha-naphthol (Barrit's A) and potassium hydroxide (Barrit's B) to test for the presence of acetyl methyl carbinol (acetoin), an intermediate of the (butanediol fermentation pathway).

- After 48 hrs of incubation, both reagents are added, the tube is shaken vigorously then allowed to sit for 5-10 minutes.

- A pink-red color at the surface of the medium indicates a positive result (*Klebsiella spp., Enterobacter spp., Viridans Streptococci* except *S. mitis, Proteus mirabilis, Serratia spp., Staphylococcus aureus*).

- No pink-red color indicates a negative result (*Escherichia spp., Proteus vulgaris, Citrobacter freundii*).

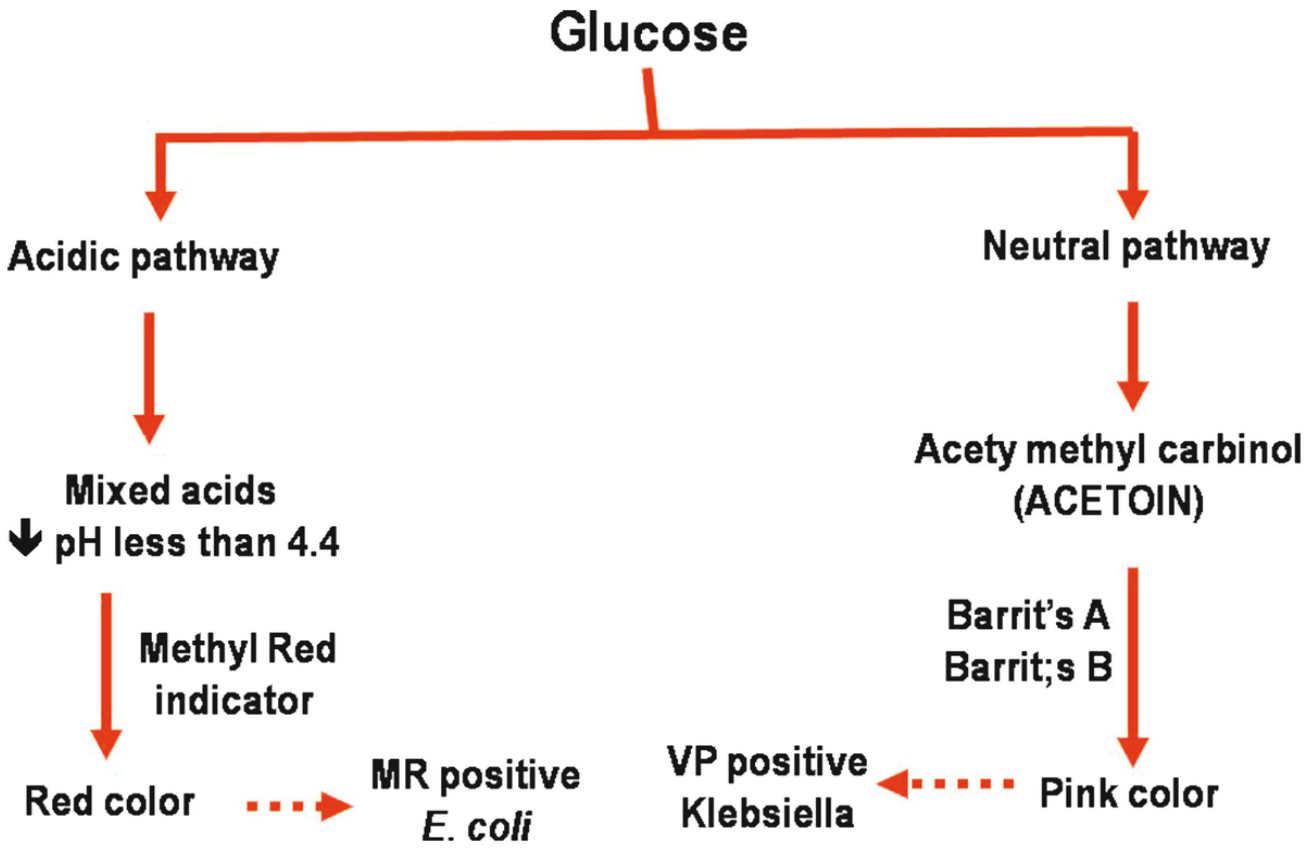
Procedure of VP Test

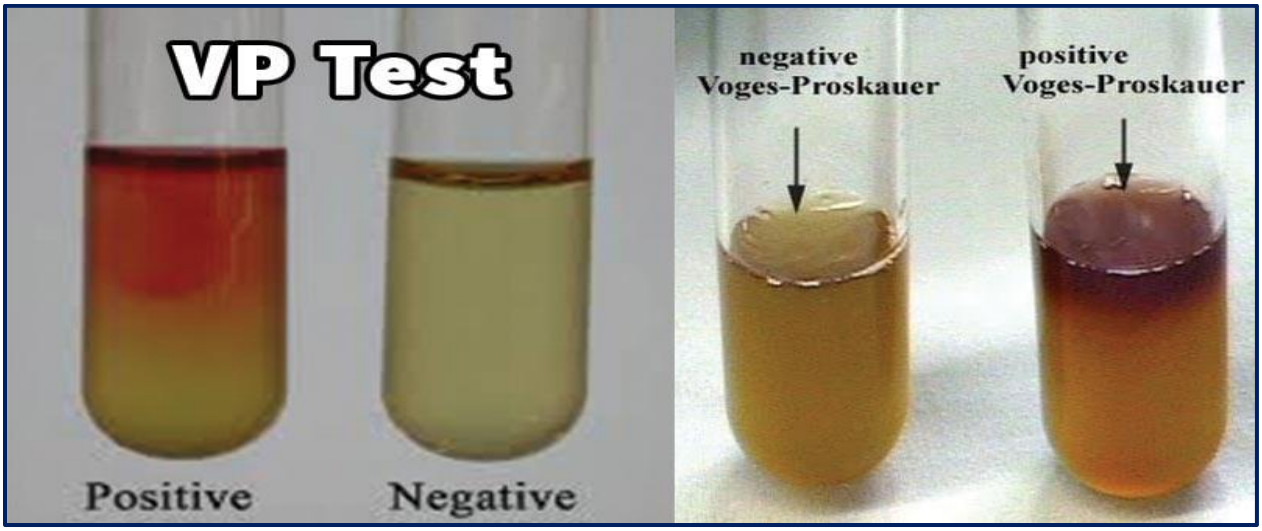
1. **Inoculate The MR-VP medium with the tested bacterium.**
2. **Incubate at 37°C for 24 hrs.**
3. **Add six drops of reagent A (5% α-naphthol solution) and mix well by shaking.**
4. **Add two drops of reagent B (40% KOH solution) and mix well by shaking.**

5. **Positive VP test is indicated by a red color of the medium, within 5 minutes. A negative VP test is indicated by the medium remaining brown.**

Uses of VP Test

The VP test is often used to differentiate between members of the Enterobacteriaceae family, which includes important human pathogens such as *Escherichia coli*, *Salmonella*, and *Shigella*.





Citrate Utilization test

Principle of Citrate Utilization Test

- Simmon's citrate agar is the medium used in this test to determine the ability of a microorganism to use citrate as its sole carbon and energy source.

- This agar contains citrate and ammonium ions (nitrogen source) and Bromothymol blue (BTB) as a pH- indicator.

- **The bacterial species that produce citrase enzyme can utilize citrate (substrate) as their sole source of carbon.**
- **The produced enzyme will break citrate into oxaloacetic acid and acetic acid. The oxaloacetic acid will then be decarboxylated to produce pyruvate and CO_2.**
- **Released CO_2 will combine with H_2O and excess sodium from sodium citrate to produce alkaline sodium carbonate. The sodium carbonate will increase the medium pH and turn it into alkaline.**

$CO_2 + H_2O$ + excess sodium from sodium citrate → Na_2CO_3 (alkaline)

- **Additionally, the released CO_2 will stimulate the metabolism of ammonium salts. Utilization of the ammonium salts as a source of nitrogen will cause the production of ammonia (or ammonium hydroxide) which will increase the medium pH and turn it into alkaline.**

  Ammonium salt → Ammonium hydroxide (alkaline)

- **The combined effect of ammonium hydroxide and sodium carbonate will increase the pH of the medium above 7.6 which will turn the pH-indicator BTB from deep forest green (at neutral pH) to Prussian blue (royal blue).**

- A blue color indicates a positive result (*Salmonella typhimurium*).
- A green color indicates a negative result (*Escherichia coli*).

Procedure of Citrate Utilization Test

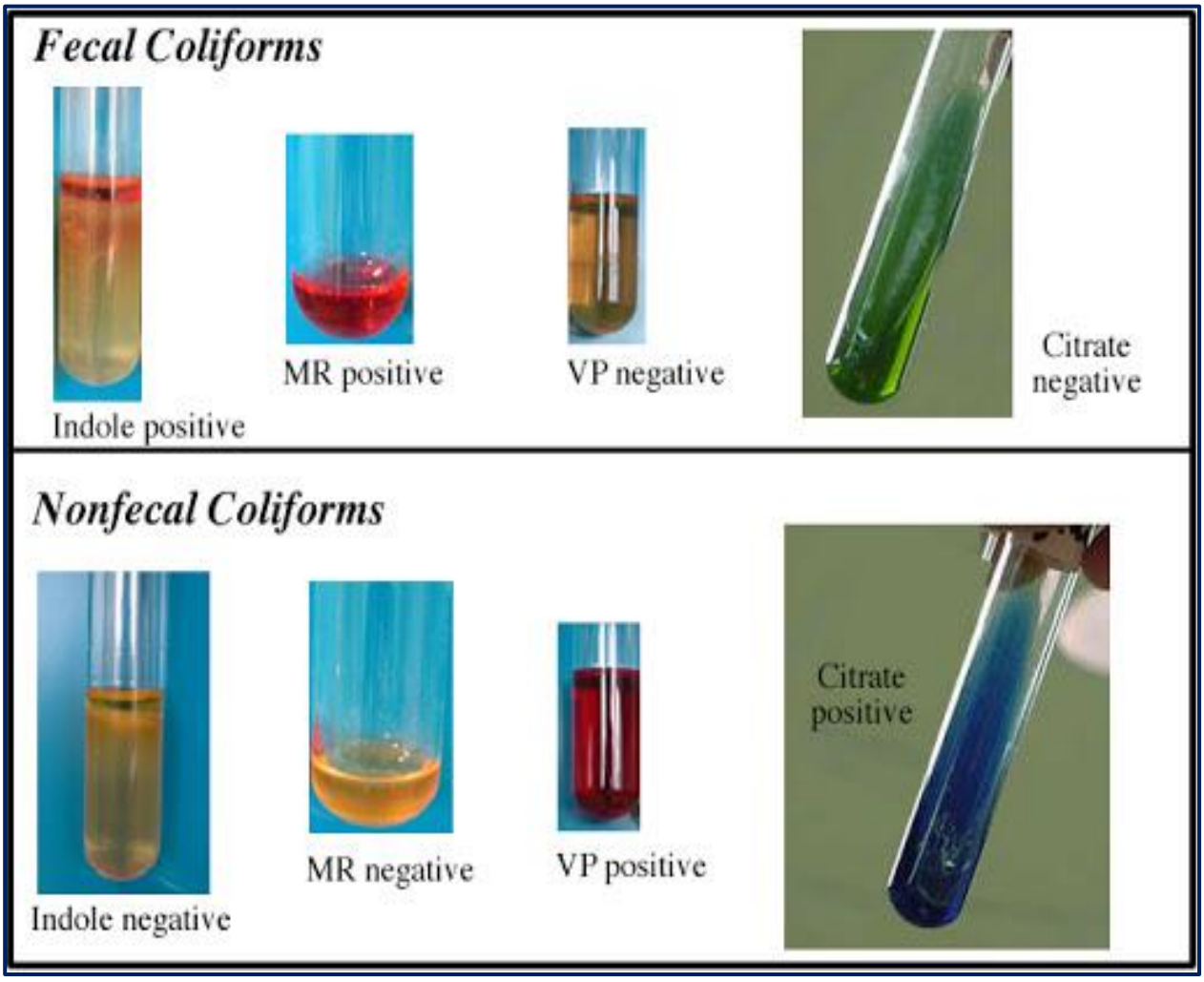
1. **Inoculate Simmon's citrate agar with the tested bacterium.**
2. **Incubate at 37°C for 24 hrs.**
3. **Check for growth and change in color of from green to blue.**

Uses of Citrate Utilization Test

- Citrate metabolism could be an indicator for bacteria found in natural environments.

- Citrate could be used to distinguish bacterial coliforms found in soil, and aquatic environments, such as *Enterobacteriaceae*, and coliforms with fecal contamination.

- It was found that coliforms without fecal contamination grow, while the coliforms with fecal contamination did not grow.


Fecal Coliforms
Indole positive
MR positive
VP negative
Citrate negative
Nonfecal Coliforms
Indole negative
MR negative
VP positive
Citrate positive

IMViC tests of bacterial species

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

2- TSI agar (Triple Sugar Iron Agar) Test

Principle of TSIA (Triple Sugar Iron Agar) Test

- Sugars are Glucose 0.1%, Lactose 1%, Sucrose 1% .

- Phenol red is the pH-indicator used to indicate the acidification, while both sodium thiosulfate and ferrous ammonium sulfate are indicators for H_2S formation.
- The pH-indicator phenol red is used for detecting carbohydrate fermentation which is indicated by the change in medium color from orange-red to yellow in the presence of acids.
- In the case of oxidative decarboxylation of peptone, alkaline products are built and the pH increases. This is indicated by the change in color of the medium from orange-red to deep red.
- Sodium thiosulfate and ferrous ammonium sulfate present in the medium detect the production of hydrogen sulfide and are indicated by the black color in the butt of the tube.

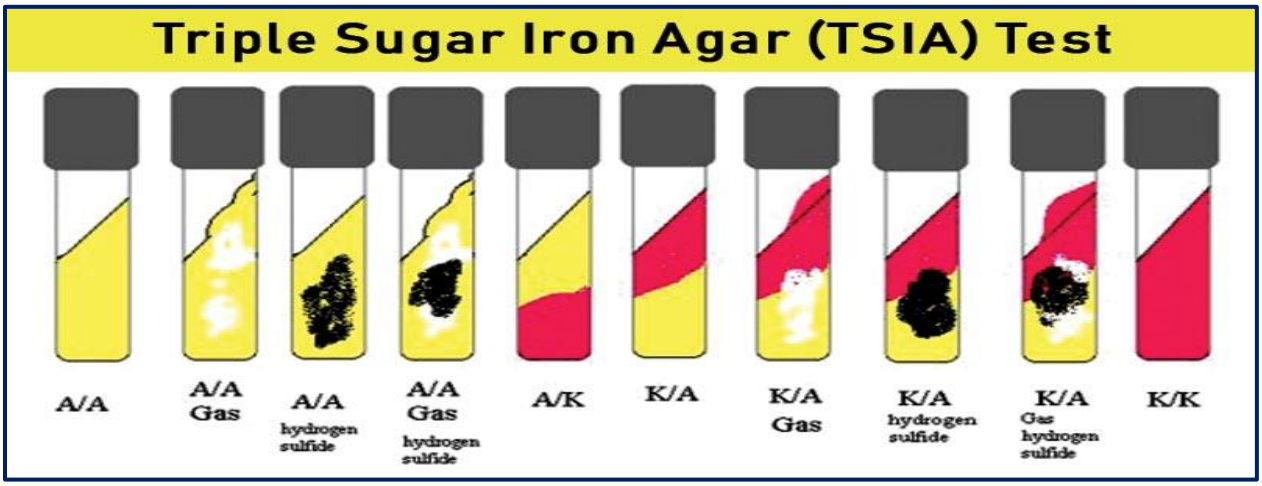
- Glucose is utilized first by a fermentative bacterium and the entire medium becomes acidic (yellow) in 8 to 12 hours. The butt remains acidic even after an 18 to 24 hours incubation period because of the presence of organic acids resulting from the fermentation of glucose under anaerobic conditions in the butt of the tube.
- The slant, however, reverts to the alkaline (red) state because of oxidation of the fermentation products under aerobic conditions on the slant. This change is a result of the formation of CO_2 and H_2O and the oxidation of peptones in the medium to alkaline amines.

- When, in addition to glucose, lactose, and/or sucrose are fermented, the large amount of fermentation products formed on the slant neutralizes the alkaline amines and renders the slant acidic (yellow), provided the reaction is read in 18 to 24 hours.
- If the slant and butt become alkaline, glucose has not been fermented. Organisms showing this reaction are defined as non-fermenters and derive their nutrients from the peptones present in the medium.
- The formation of CO2 and hydrogen gas (H2) is indicated by the presence of bubbles or cracks in the agar or by the separation of the agar from the sides or bottom of the tube.
- The production of H2S (sodium thiosulfate reduced to H2S) requires an acidic environment, and reaction with the ferric ammonium sulfate produces a blackening of the agar butt in the tube.

Uses of TSIA Test

1- To determine whether a Gram-negative bacillus ferments glucose and lactose or sucrose and forms hydrogen sulfide (H2S) and CO2.

2- To differentiate members of the Enterobacteriaceae family and to distinguish them from other Gram-negative intestinal bacilli.



TSI agar

Triple Sugar Iron Agar

0.1% dextrose

1.0% sucrose

1.0% lactose

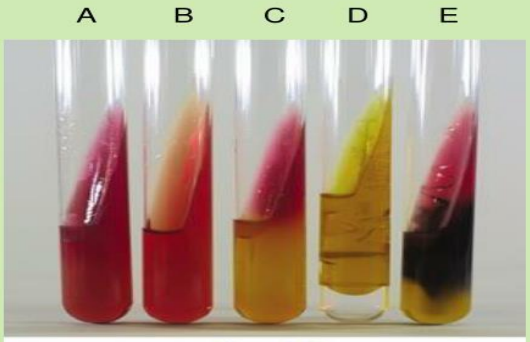
(a) Red/red (no sugar fermentation)

(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 H_2S



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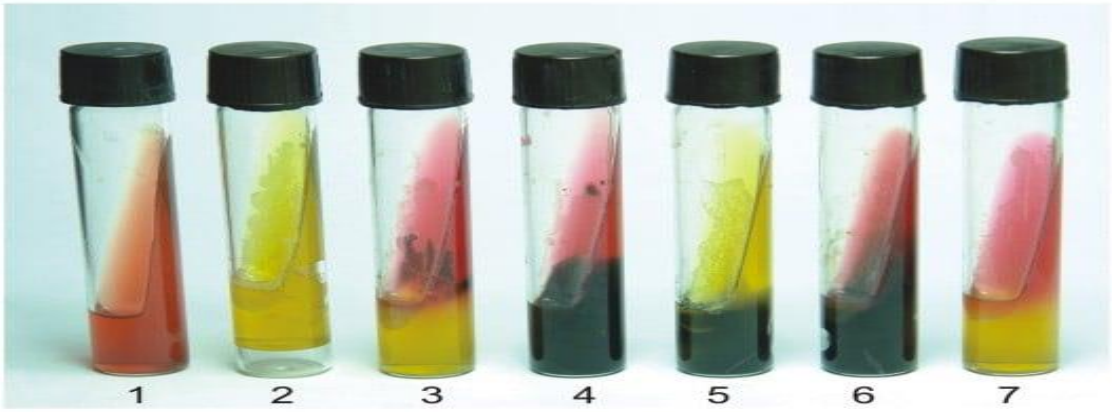


Triple Sugar Iron Agar (M021)

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

Procedure of TSIA Test

- **With a straight inoculating needle, touch the top of a well-isolated colony.**
- **Inoculate TSI agar by first stabbing through the center of the medium to the bottom of the tube and then streaking the surface of the agar slant.**
- **Leave the cap on loosely and incubate the tube at 35°-37°C for 18 to 24 hours.**
- **Following incubation, examine for color change in slant and butt, blackening and cracks in the medium.**

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_2S	Glucose fermentation only, Gas produced, H_2S produced.
7	Yellow/Yellow with bubbles and black precipitate	A/A,G,H_2S	Glucose and lactose and/or sucrose fermentation, Gas produced, H_2S produced.
8	Red/Yellow with black precipitate	K/A,H_2S	Glucose fermentation only, H_2S produced.
9	Yellow/Yellow with black precipitate	A/A,H_2S	Glucose and lactose and/or sucrose fermentation, H_2S produced.

3- Urease Test

- **Media used in urease test is either an agar (Christensen's urea agar) or a broth (Stuart's urea broth).**

- **Urease is an enzyme that hydrolyzes urea to carbon dioxide and ammonia in the presence of water.**

- **Many bacterial species have a urease enzyme, especially that infect the urinary tract.**

- **Ammonia combines with carbon dioxide and water to form ammonium carbonate which changes the medium into alkaline, turning the pH-indicator phenol red from yellow color to bright pink.**

- **Urease positive bacteria include: *Proteus*, *Pseudomonas*, *Klebsiella*, *Helicobacter pylori*, *Staphylococcus aureus*, *S. epidermidis* and *S. saprophyticus*.**

- **Urease negative bacteria include: *Escherichia coli* and *Enterobacter*.**

Procedure of Urease Test

- **For Christensen's urea agar**

1. **Streak the entire slant surface with a bacteria inoculum from an 18–24 hour pure culture.**
2. **Incubate tubes with loosened caps at 35°C.**
3. **Observe the slant for a color change at 6 hours and 24 hrs unless specified for longer incubation**

- **For Stuart's Urea Broth**

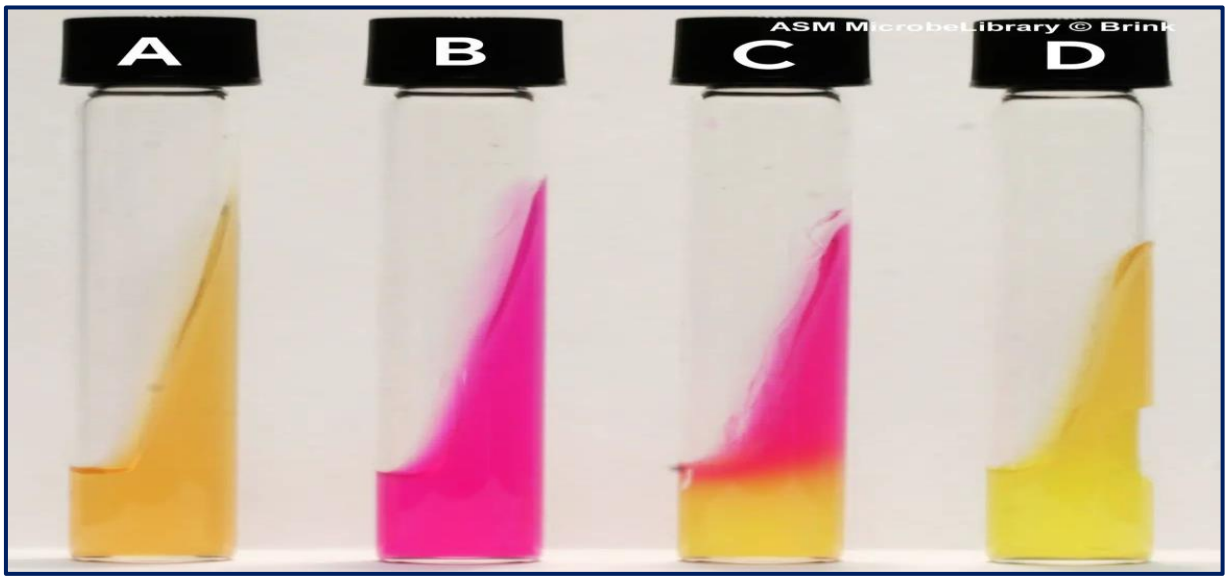
1. **Inoculate the broth with a bacterial inoculum from an 18-24 hour pure culture**
2. **Shake the tube gently to suspend the bacteria.**
3. **Incubate the tubes with loosened caps at 35°C.**
4. **Observe the broth for a color change at 8, 12, 24 hrs.**

Results

- Rapid urease positive for Christensen's urea agar: the bacterial species that hydrolyze urea rapidly (*Proteus vulgaris*) produce a positive result (pink color) within 1-2 hrs of incubation. In Stuart's urea broth, the rapid positive result appears after 24 hrs.

- Delayed urease positive for Christensen's urea agar: the bacterial species that hydrolyze urea slowly (*Klebsiella pneumoniae*) produce pink color after 6 hrs of incubation. In this test, the slant (upper part of the medium) changes to pink, while the butt (lower part of the medium) requires 3-5 days to change entire butt to pink. In Stuart's urea broth, the rapid positive result appears after 24 hrs. In Stuart's urea broth, *Klebsiella pneumoniae* acts as urease negative.

- In both agar and broth, the medium remains yellow if the organism is urease negative (*Escherichia coli*).



Urea agar test (A) uninoculated, (B) *Proteus mirabilis* (rapidly urease positive), (C) *Klebsiella pneumoniae* (delayed urease positive), (D) *Escherichia coli* (urease negative).

Left: urease positive (pink), Right: urease negative (yellow)