LAB:5 **Practical Pathogenic bacteria**

Bacterial Identification (Gram negative Bacteria)

Biochemical tests for identification of Gram-negative bacterial species

1- IMViC Test

I= Indole ring production

M= Methyl red

V=Voges Proskauer C=Citrate utilization

C=Citrate utilization

IMViC tests are a group of individual tests used inmicrobiology lab for identify an organism in the coliform group. A coliform is a gram negative, aerobic, or facultative anaerobic rod, which producesgas from lactose within 48 hours. The presence of some coliforms indicate fecal contamination.

The term "IMViC" is an acronym for each of these tests. "I" is for indole test; "M" is for methyl red test; "V" is for Voges-Proskauer test, and "C" is for citrate test. The lower case "i" is merely for "in" as the Citrate test requires coliform samples to be placed "in Citrate".

These tests are useful in distinguishing membersof Enterobacteriaceae.

IMViC series = Klebsiella & Enterobacter

Indole test

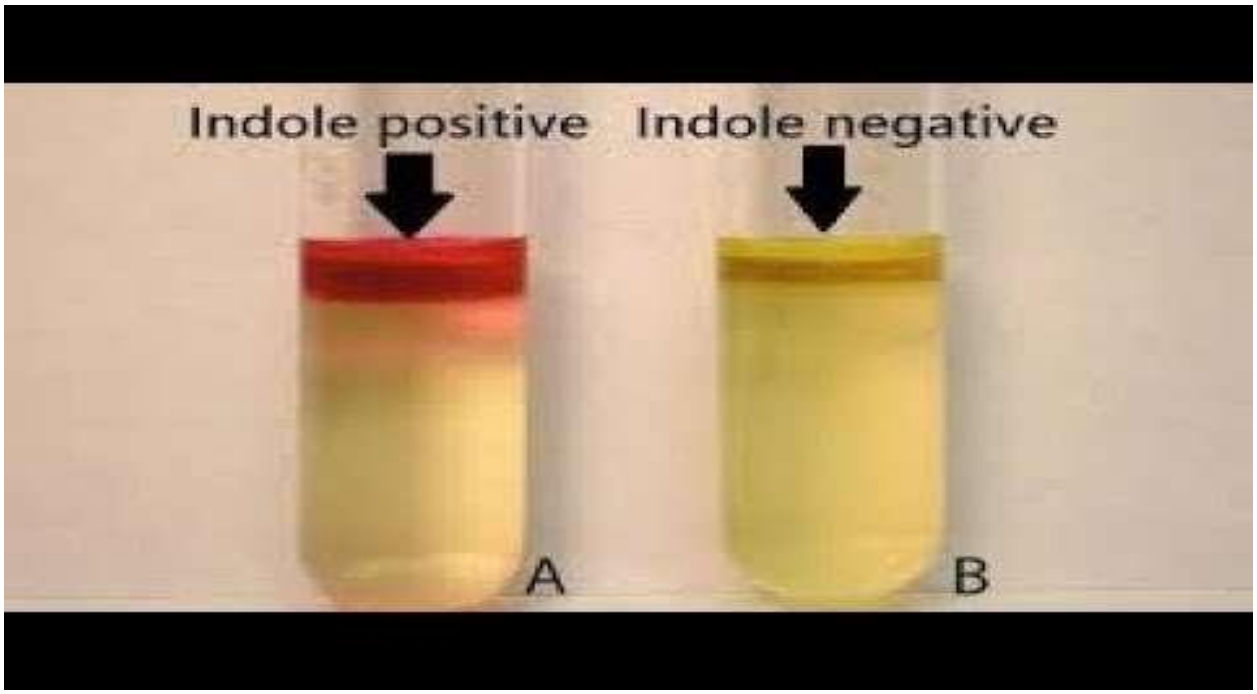
In this test, the organism is grown in peptone water broth. It contains tryptophan, which under the action of enzyme Tryptophanase is converted to an Indole molecule, , Kovac's reagent it is added after incubation . Kovac's reagent consist of amyl alcohol and para-dimethylaminobenzaldehyde and concentrated hydrochloric acid. Kovac's reagent is actually used to determine ability of an organism to separate indole from amino acid tryptophan. A positive result is indicated by a pink/red layer forming on top of the liquid.

Indole test Method:

Inoculate Tryptone water with the tested microorganism Incubate at 37°C for 24 hours After incubation interval, add 1 ml Kovacs reagent, shake the tube gently and read immediately

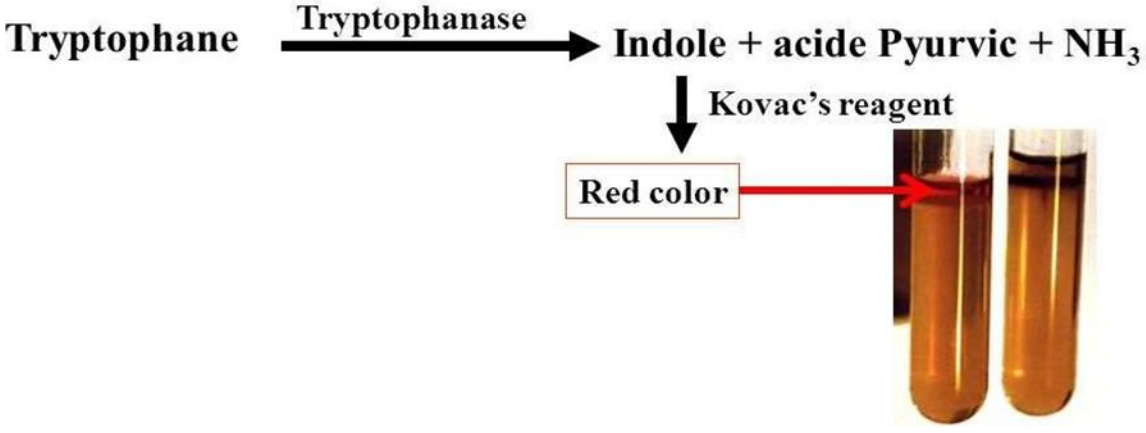
Result: A bright pink color in the top layer indicates the presence of indole and the absence of color means that indole was not produced. e.g. E. coli (+) , Klebsiella (-).

- **Indole test**
- **Media culture :peptone water**
- **Reagent ;kovacs**
- **Enzymes: Tryptophanase**
- **Substrate :Tryptophan**
- **Positive result: red ring**
- **Negative result :no change (yellow ring)**



IMViC: Indole Test

- Principal
  - Some microorganisms can metabolize tryptophane by the tryptophanase



Methyl red and Voges–Proskauer test

These tests both use the same broth for bacterial growth. Thebroth 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.

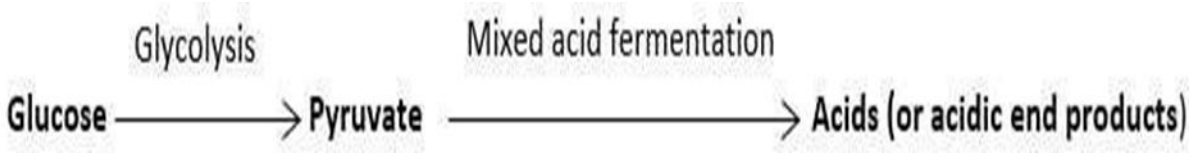
The methyl red test detects production of acids formed during metabolism using pyruvate as a substrate. a red color appears at pH lower than 4.2, indicating a positive test and

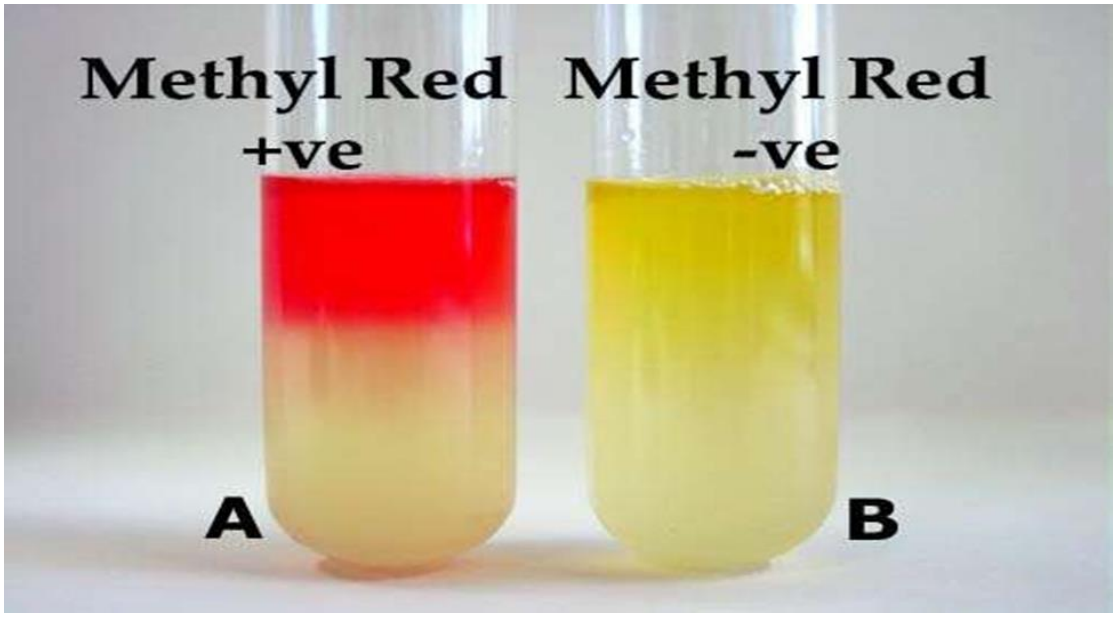
yellow color (pH = 6.2 or above) indicates a negative test.

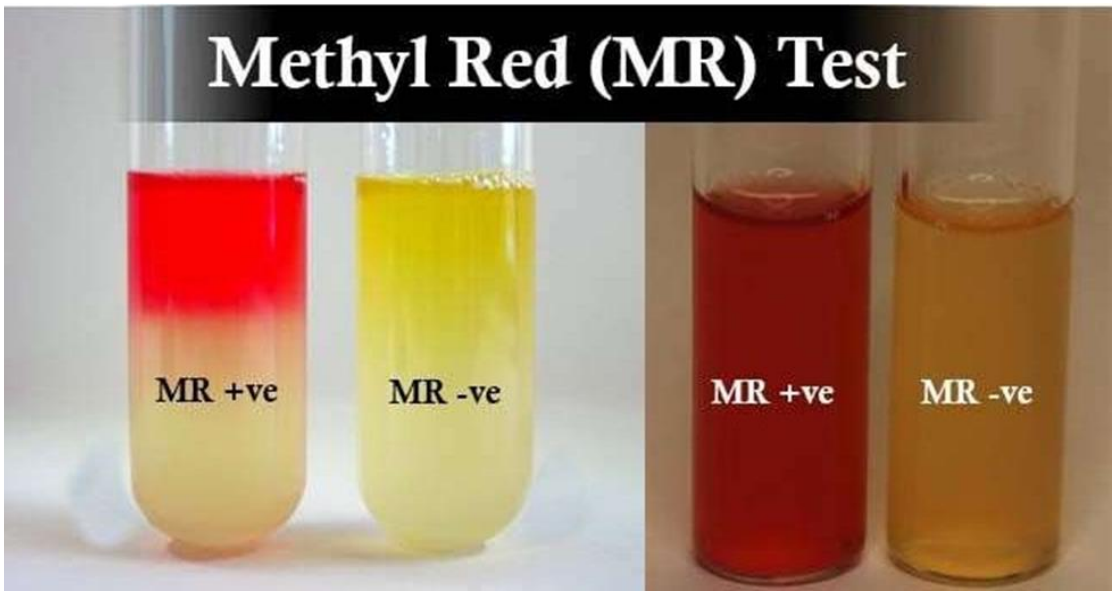
A positive result for VP test is pink – red color at the top of the broth. No change in color represents a negative VP test.

Principle of Methyl Red Test

The principle of the test is based on the ability of bacteria to produce organic acids during glucose fermentation, which lowers the pH of the medium.
In the methyl red test, a pH indicator called methyl red. the bacteria ferment glucose and produce organic acids. If the bacteria produce enough acid to lower the pH of the medium below 4.4, the methyl red indicator will turn red.







Requirements of MR Test

1. Sterile glucose phosphate broth **(GPB) medium** (0.5ml in eachtube)
2. Test culture suspension
3. Methyl red indicator
4. Dissolve 0.1 g methyl red in **300 ml 95% ethanol**. Add distilledwater to make up the volume to 500ml.

Procedure of MR Test

1. Inoculate the GPB medium with culture suspension.
2. Incubate at 37°C for 24 hours.
3. Add 5-6 drops of methyl red indicator.
4. Positive test is indicated by a bright red color of the medium.
   A negative test indicated by the medium remaining yellow orturning orange

Principle of VP Test

Pyruvate can be metabolized into a neutral intermediate product called 'acetoin'.

A positive result is indicated by the development of pink – red color at the top of the broth. No change in color represents a negative VP test.

VP Positive Bacteria:

Klebsiella spp., Enterobacter spp., Viridans Streptococci(except S. mitis, and S. vestibularis), Proteus mirabilis, Hafnia spp., Serratia spp., Staphylococcus aureus,

VP Negative Bacteria:

Escherichia spp., Proteus vulgaris, Citrobacter freundii.

Requirements VP Test

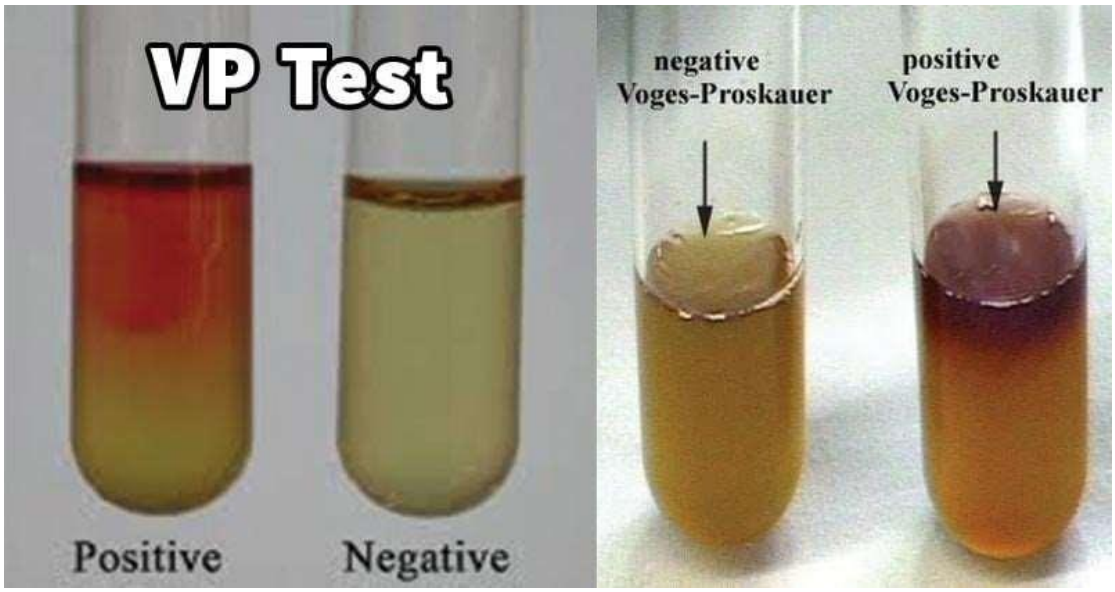
1. **Sterile glucose phosphate broth (GPB) medium (0.5-1.0ml in each tube).**
2. **Test culture suspension (Enterobacter / Klebsiella).**
3. **5%- α-naphthol in absolute ethanol.**
4. **40% KOH solution (containing 0.5% creatinine).**

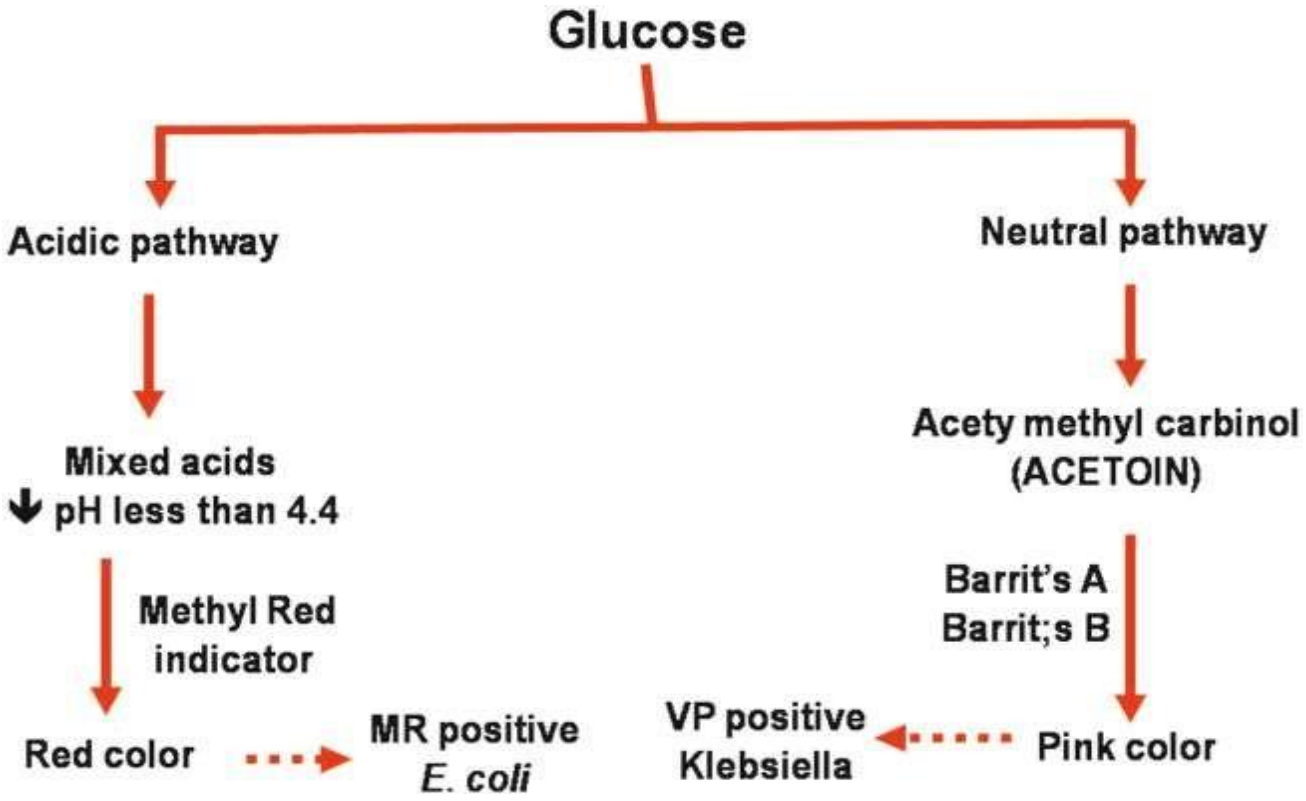
Procedure of VP Test

1. **Inoculate GPB medium with the culture suspension.**
2. **Incubate at 37°C for 24 hours.**
3. **Add 0.6 ml of 5% α-naphthol and mix well.**
4. **Add 0.2 ml of 40% KOH solution, shake well.**
5. **Positive VP test is a red color of the medium, within 5 minutes. Negative VP test is brown.**

Methyl red – Voges proskauer test

- **Media culture :Glucose phosphate**
- **Reagent : Methyl red & Barrits**
- **Substrate : Glucose**
- **Positive result :M=red color ..V= Red – pinkish color**
- **Negative result :yellow color**





Citrate test

to use citrate as its sole carbon and energy source. The This test uses Simmon's citrate agar to determine the ability of a microorganism agar contains citrate and ammonium ions (nitrogen source) and Bromothymol blue (BTB) as a pH indicator. Bromothymol blue was added in order to reduce false positives. The citrate agar is green before inoculation, and turns blue, because of BTB as a positive test indicator, meaning citrate is utilized. The test is also prepared on a slant to maximize bacterial growth for an even better indication of the use of citrate.

Principle of Citrate Utilization Test

Some bacteria can utilize 'citrate' as their sole source of carbon. Such bacteria produce citrase enzymes which will break the citrate. the pH indicator bromothymol blue will turn in the medium from deep forest green (at neutral pH) to Prussianblue.

Requirements of citrate utilization Test

1. **Test culture suspension.**
2. **Sterile Koser's citrate medium (1.0 ml in each tube) /Simmon's citrate medium (agar slant).**
3. **pH indicator bromothymol blue.**

Citrate Utilization Test Procedure

1. **Inoculate the medium (Koser's broth or Simmon's agar)with the culture suspension.**
2. **Incubate at 37^{o}C for 24 hours.**
3. **Check for turbidity (indicating positive test) in Koser'smedium; growth and change in colour of indicator to blue on Simmon's citrate agar (positive test).**

Citrate utilization test

Media culture: Simmon citrate agar

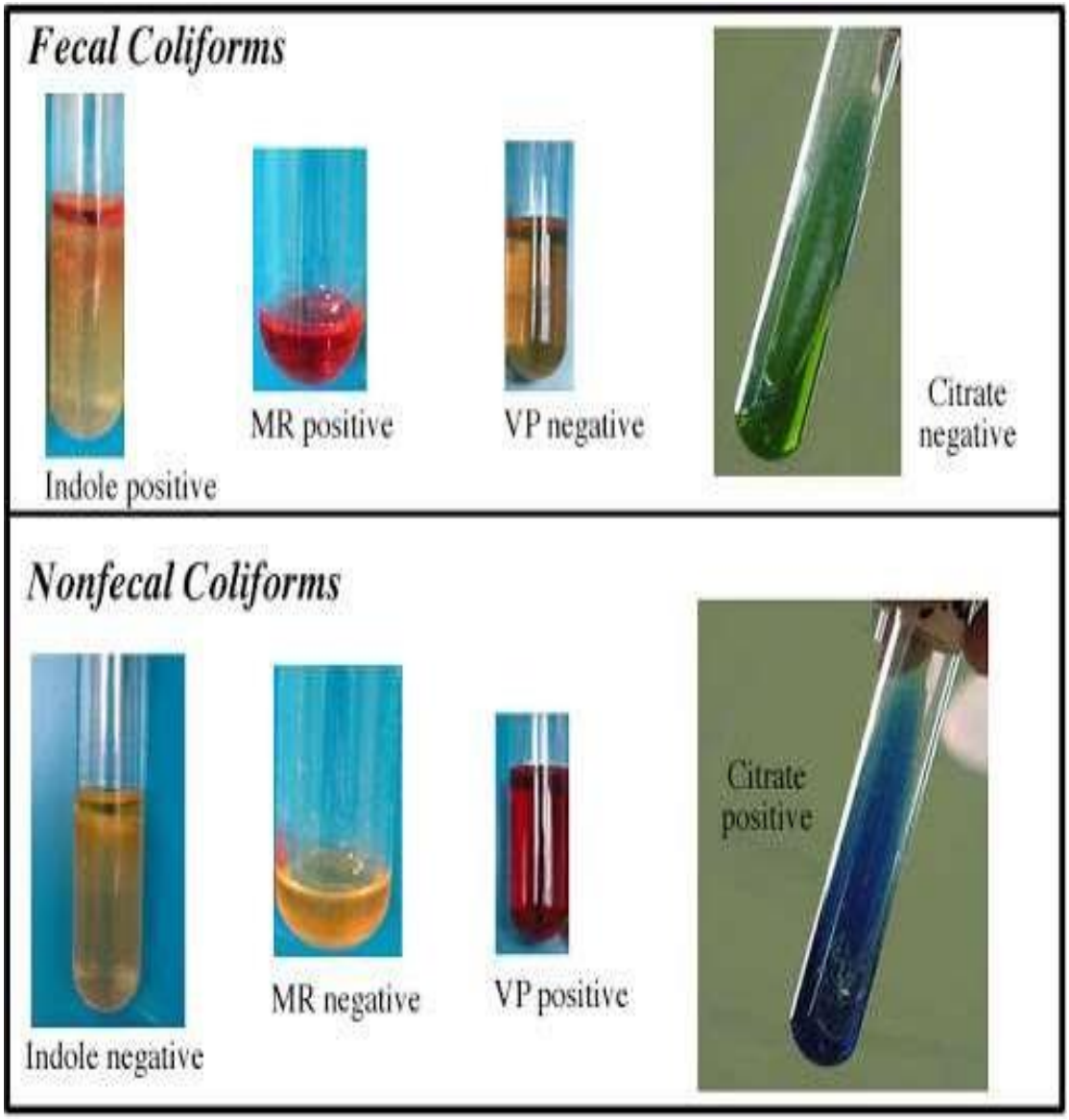
PH indicator: Bromothymol blue

Enzymes: Citrase

Substrate: Na-citrate

Positive result: Blue color

Negative result: NO growth



The IMViC results of some important species are shown below.

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

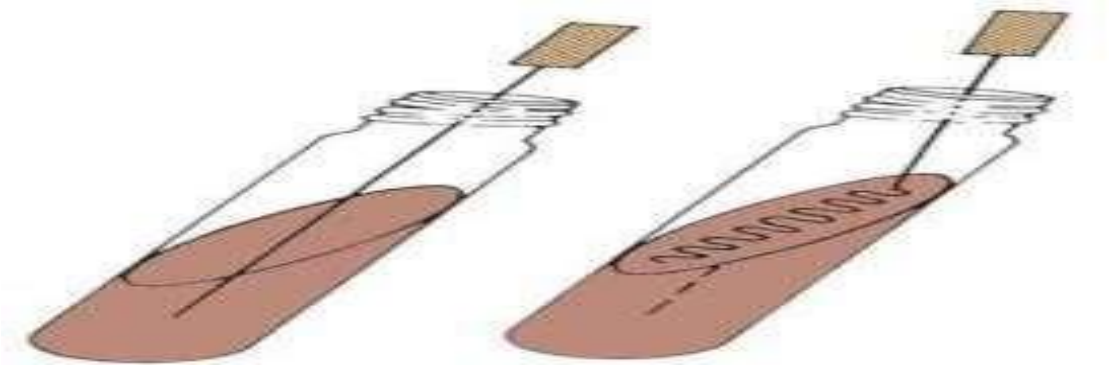
2- TSI agar (Triple Sugar Iron Agar) Test , Sugar fermentation , CO2 & H2S Production Test .

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

PH indicator = Phenol red

Iron= Ferric Ammonium Citrate

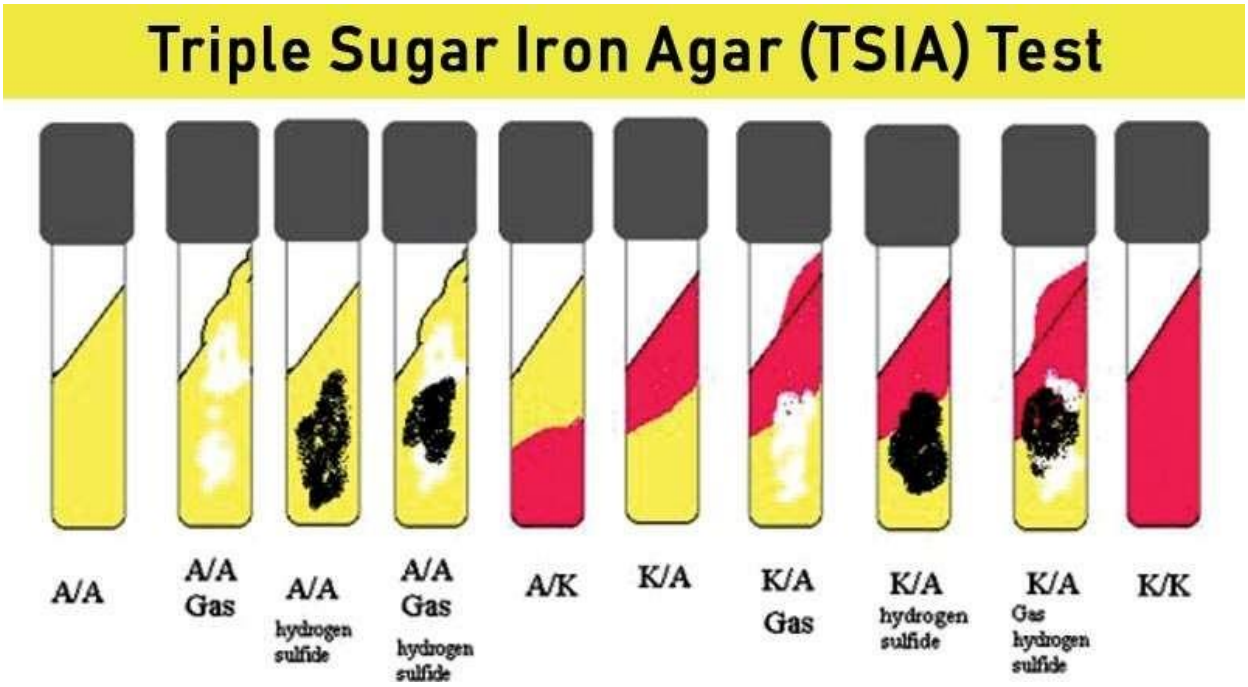
H2S indicator = Sodium Thiosulfate



Objectives of TSIA (Triple Sugar Iron Agar) Test

1- To determine a gram negative bacilli ferments glucose and lactose or sucrose and forms hydrogen sulfide (H_2S).

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



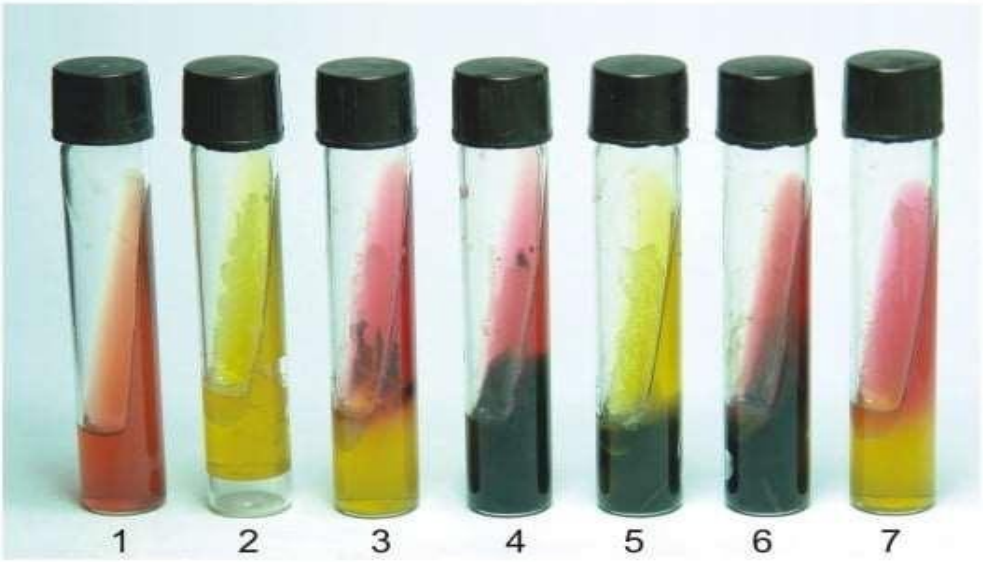
Principle of TSIA (Triple Sugar Iron Agar) Test

The Triple Sugar Iron agar (TSIA) test for differentiate among genera of the Enterobacteriaceae, which are all gram-negative bacilli capable of fermenting glucose and lactose or sucrose with the production of acid. . Phenol red and ferrous sulfate serve as indicators by the change in color of the medium from orange-red to yellow in the presence products of acids.

Glucose is utilized first by a fermentative organism and the entire medium becomes acidic (yellow) in 8 to 12 hours. The butt remains acidic because of the presence of organic acids resulting from.

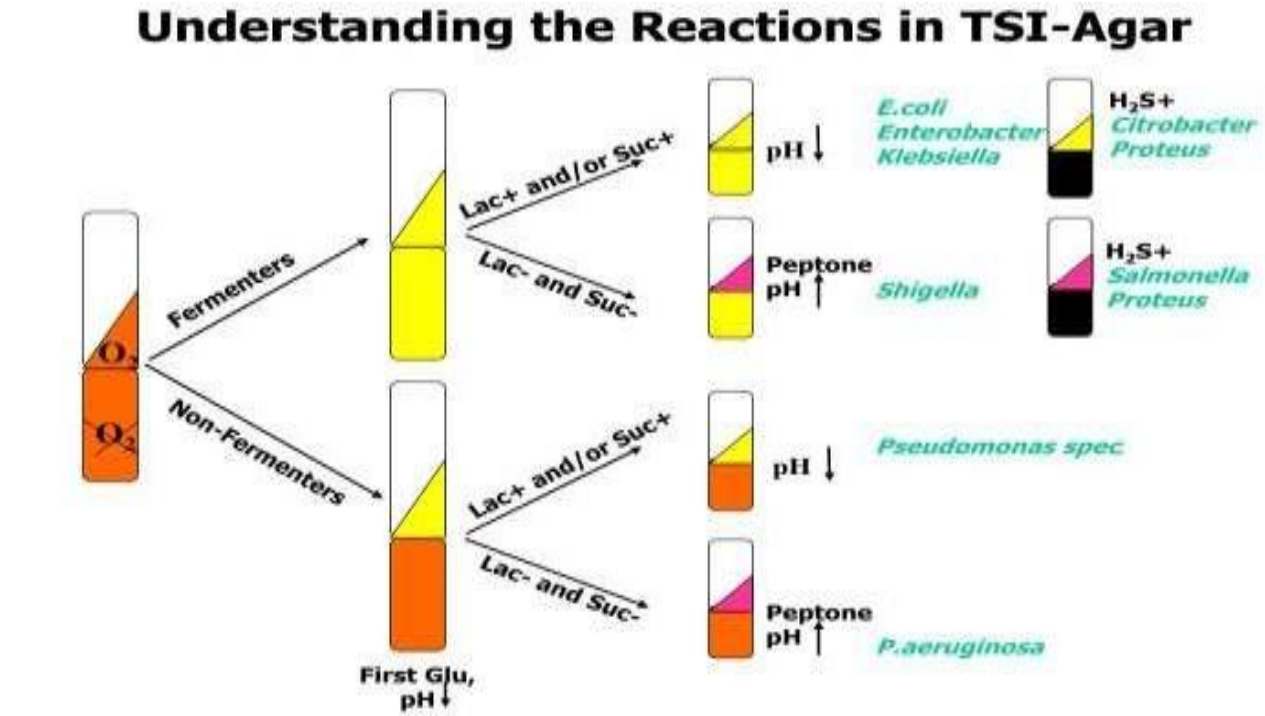
the fermentation of glucose under anaerobic conditions in the butt of the tube.

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. 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 CO_2 and hydrogen gas (H_2) 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 H_2S (sodium thiosulfate reduced to H_2S) requires an acidic environment, and reaction with the ferric ammonium citrate produces a blackening of theagar butt in the tube.



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



Result = Alkaline\ Acid, no CO2, no H2S \ Shigella. Spp

Procedure of TSIA (Triple Sugar Iron Agar) 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 in ambient air 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.

Quality Control of TSIA Test

Test organism	Slant	Butt	Gas production	H2S production
Escherichia coli ATCC25922	Yellow	Yellow	+	–
Pseudomonas aeruginosa ATCC27853	Red	Red	–	–
Salmonella enterica ATCC14028	Red	Yellow	+	+
Shigella sonnei ATCC9290	Red	Yellow	–	–

1- Urease Test

Urease is expressed enzyme that hydrolyzes urea to carbon dioxide and ammonia. Many organisms infect the urinary tract have a urease enzyme that is able to split urea in the presence of water to release ammonia and carbon dioxide.

The ammonia combines with carbon dioxide and water to form ammonium carbonate which turns the medium alkaline, turning the indicator phenol red from its original orange yellow color to bright pink.

Medium used for urease test:

(Christensen's urea agar), or (Stuart's urea broth). Urease test medium can be a sole medium or part of a panel like motility indole urease (MIU) test.

Microorganisms Tested

- **A. The urea test is part of the battery of tests to identify the following.**
- **Gram-negative enteric pathogens, including *Yersinia* spp.**
- **Fastidious Gram-negative rods—*Brucella,***
- ***H. pylori,* and *Pasteurella***
- **Gram-positive rod:**
  ***Corynebacterium* and *Rhodococcus* spp.**
- **Yeasts—*Cryptococcus* spp.**
- **B. Directly, this test is performed on gastric biopsysamples to detect the presence of *H. pylori***

Procedure for Urease test For Christensen's urea agar

1. **Streak the entire slant surface with a heavy inoculum froman 18-24 hour pure culture (do not stab the butt as it will serve as a color control).**
2. **Incubate tubes with loosened caps at 35°C.**
3. **Observe the slant for a color change at 6 hours and 24hours unless specified for longer incubation**

For Stuart's Urea Broth

1. **Inoculate the broth with a heavy inoculum from an 18-24hour 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 hours.**

Result and Interpretation

Organisms that hydrolyze urea rapidly (*Proteus* spp., *Morganella morganii,* and some *Providencia stuartii* strains) will produce strong positive reactions within 1or 6 hours of incubation; delayed positive organisms (e.g. *Klebsiella* spp and *Enterobacter* species) will produce weak positive reactions in the slant in 6 hours of incubation. The culture medium will remain a yellowish color if the organism is urease negative e.g. *Escherichia coli.*

- **If organism produces urease enzyme, the color of the slant changes from light orange to magenta.**
- **If organism does not produce urease the agar slant and buttremain light orange (medium retains original color).**

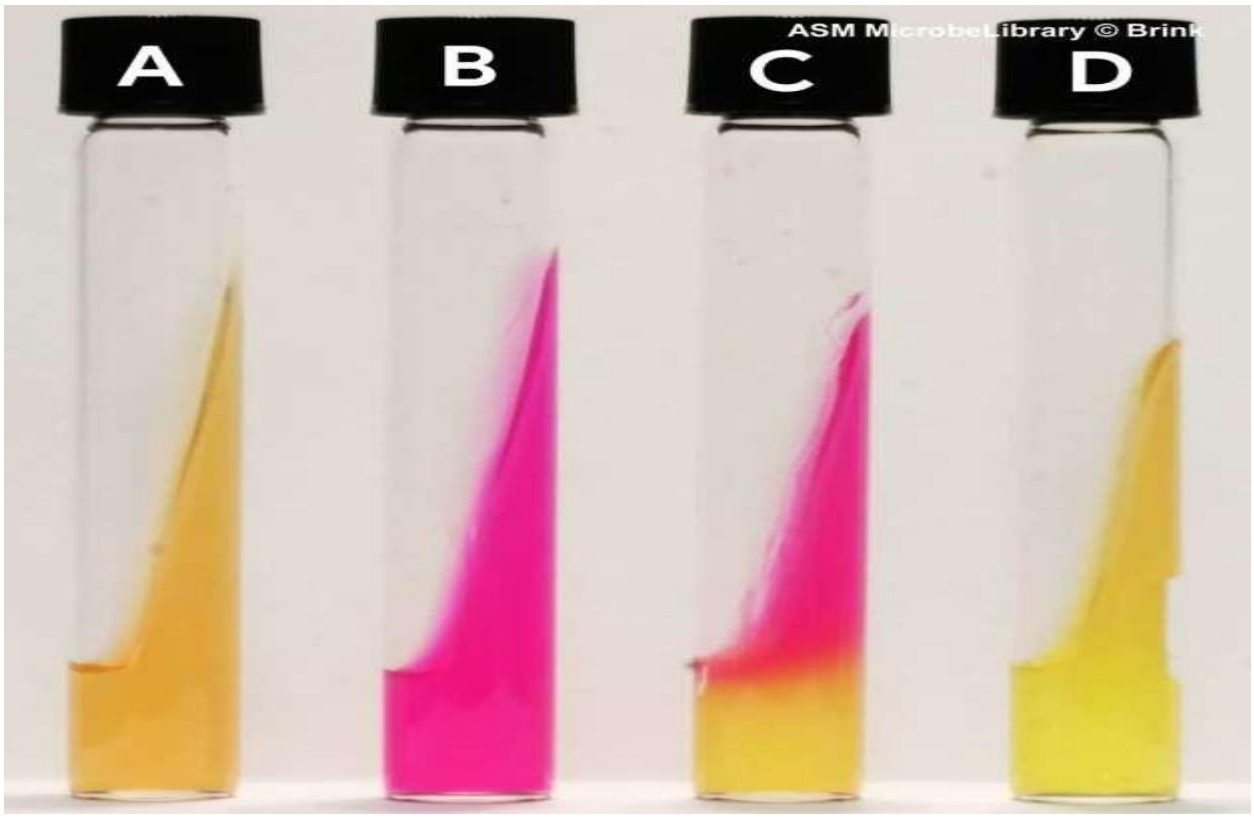


Figure: Urea agar test (a) uninoculated, (b) *Proteus mirabilis* (rapidly urease positive), (c) *Klebsiella pneumoniae* (delayed urease positive), (d) *Escherichia coli* urease negative).

Figure: positive result pink color (Urease positive)

Name of urease positive organisms

1- ***Proteus. spp***
2- ***Klebsiella .spp***

Urea agar

1- **Urea**
2- **PH- indicator = phenol red**
3- **Enzyme = Urease**