

Practical 8

Gram negative rods (Enterobacteriaceae)

Objective:

- 1- To identify *Enterobacteriaceae* from other Gram negative rods and to differentiate between lactose fermenter and non lactose fermenter by:
 - A. Detection of the presence of cytochrome oxidase in Gram-negative rods.
 - B. To determine the ability of bacteria to metabolize glucose oxidatively or fermentatively.
 - C. To know Gram reaction of *Enterobacteriaceae*.
 - D. Use of MacConkey agar to differentiate between Lactose fermenter and non lactose fermenter *Enterobacteriaceae*.

Required materials:

- 1- Overnight cultures of *E.coli*, *K. pneumoniae*, *Shigella*, *Salmonella*, *Pseudomonas*.
- 2- Gram stains dyes and reagents, filter paper, slides.
- 3- plates of MacConkey agar
- 4- tubes containing 5ml OF media
- 5- 10 ml of oxidase reagent

Experimental:

- 1- Gram stain of *Enterobacteriaceae*.
 - 2- Oxidase test.
 - 3- OF test.
 - 4- Inoculation of MacConkey agar with lactose fermenter and non lactose fermenter *Enterobacteriaceae*.
- **Oxidase** and **Oxidative-Fermentative (OF) tests** can be used in differentiation *Enterobacteriaceae* from other Gram negative rods as showed in the table:

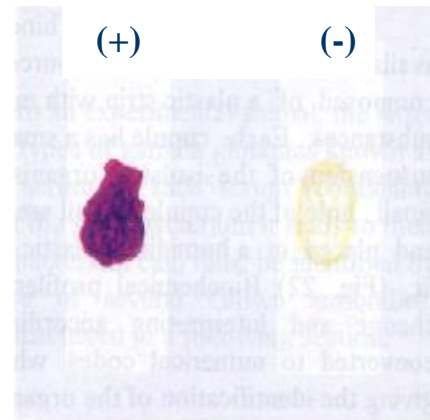
Microorganism	Oxidase test	OF test
<i>Enterobacteriaceae</i>	Negative	Fermentative
<i>Pseudomonads</i>	Positive	Oxidative
<i>Vibrio</i>	Positive	Fermentative



Gram stain of Enterobacteriaceae

Oxidase Test:

1. **Principle** - To determine the presence of cytochrome oxidase in bacteria. Recall that in aerobic bacteria the cytochrome serve as electron carriers during aerobic respiration. The detection of cytochrome is extremely beneficial in differentiating many groups of bacteria. All members of *Enterobacteriaceae* are oxidase negative, while other Gram-negative rods, such as *Pseudomonas*, are oxidase positive. The oxidase test is based on the ability of bacteria to turn (oxidize) a reagent (tetramethyl-p-phenylenediamine), which serves as an alternate substrate for the cytochrome oxidase, to a purple color.
2. **Materials:**
 - Overnight cultures of *E. coli* and *Pseudomonas aeruginosa*.
 - Oxidase reagent "tetramethyl-p-phenylenediamine".
 - Pieces of filter paper.
3. **Method** - hold a piece of the oxidase test paper with forceps and touch onto an area of heavy growth
4. **Results** - Color change to purple within:
 - 10 seconds = positive
 - 10 - 60 seconds = delayed positive
 - >60 seconds = negative
5. **Special Features**
 - Useful in differentiation of *Enterobacteriaceae* (-) and *Pseudomonas* (+).
 - An oxidase positive organism **will be** catalase positive.
 - Strict anaerobe organisms are oxidase negative.

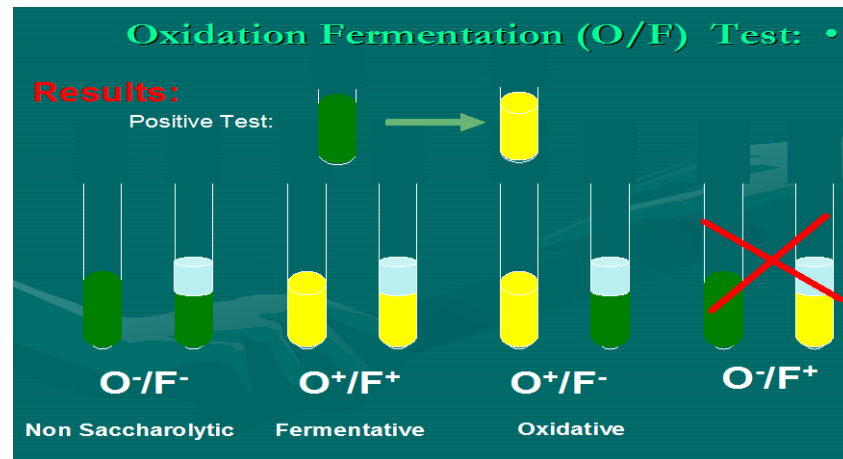


Oxidation-Fermentation test (O/F):

1. **Principle** - To determine the ability of bacteria to breakdown glucose oxidatively or fermentatively. The OF test is used to determine whether a bacterium has the enzymes necessary for the aerobic breakdown of glucose (i.e. oxidation) and/or for the fermentation of glucose.
2. **Materials:**
 - a- Overnight cultures of *E. coli* and *Pseudomonas aeruginosa*.
 - b- Tubes of OF medium/each student.
 - c- Sterile liquid paraffin.
3. **Method**
 - a- Inoculate two tubes of OF medium for each organism being tested. Inoculation is carried out as a stab to within 1 cm of the bottom of the tube.
 - b- Overlay one tube (covered) only with sterile paraffin oil to exclude all oxygen. Not overlay the second tube (open).
 - c- Incubate at 37°C for 24 hours.

4. Results

Open tube	Covered tube	Result
Yellow	Green	oxidation (O)
Yellow	Yellow	fermentation (F)
Green	Green	No action on glucose



5. **Special Features** - The test differentiates *Enterobacteriaceae* (F) from the *Pseudomonas* sp. (O), and *Micrococcus* sp. (O) from *Staphylococcus* sp. (F).
6. **Precautions in Interpretation** - Some organisms require prolonged incubation before acid production is visible.

Members of *Enterobacteriaceae* are characterized by:

- Small Gram-negative non-spore-forming enteric bacilli
- All *Enterobacteriaceae*:
 1. ferment glucose with acid production
 2. reduce nitrates into nitrites (NO_3 to NO_2 or all the way to N_2)
 3. are oxidase negative
- All are aerobic but can be facultative anaerobic
- Motile via peritrichous flagella **except** *Shigella* and *Klebsiella* which are non-motile
- Non-capsulated except *Klebsiella*
- Grow on ordinary medium i.e. non-fastidious as well as grow on bile containing media as MacConkey's medium, which used in primary classification depends on lactose fermentation on MacConkey's agar medium

Some members of the *Enterobacteriaceae* are true pathogens such as:

Salmonella spp.

Shigella spp.

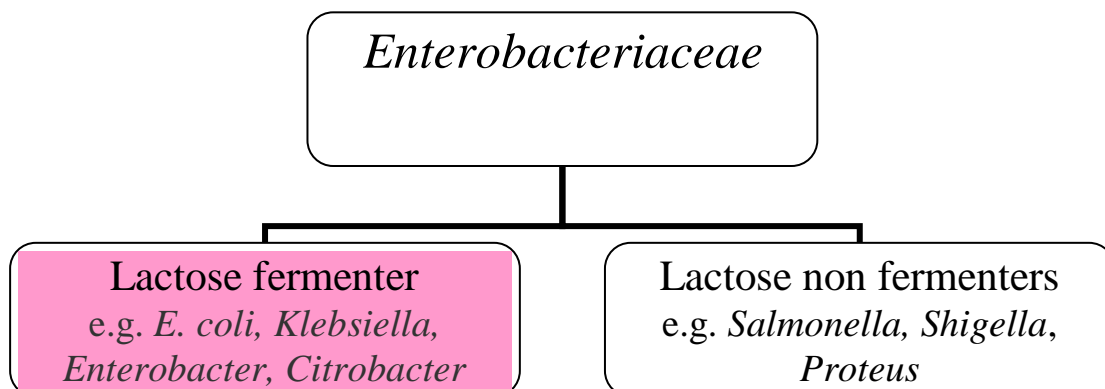
Yersinia spp.

Certain strains of *Escherichia coli* :

- ETEC = enterotoxigenic *E. coli*
- EIEC = enteroinvasive *E. coli*
- EPEC = enteropathogenic *E. coli*
- EHEC = enterohemorrhagic *E. coli*
- EaggEC = enteroaggregative *E. coli*
- UPEC = uropathogenic *E. coli*

Most members of the *Enterobacteriaceae* are opportunistic or cause secondary infections of wounds, the urinary and respiratory tracts, and the circulatory system e.g. *E. coli*.

***Primary classification of *Enterobacteriaceae* depends on lactose fermentation:**



Laboratory Identification:

Specimens whether pus, sputum, urine, feces, CSF should be cultured immediately or placed on special media to prevent overgrowth

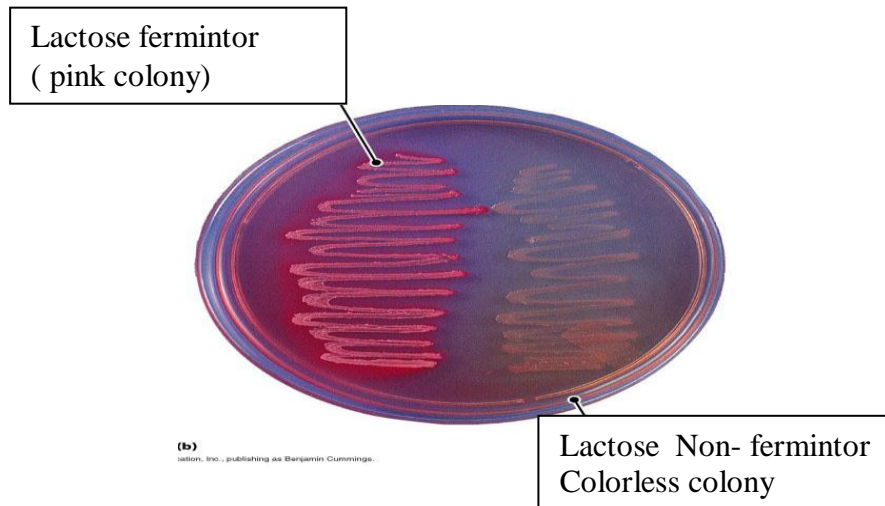
Culture:

Colony morphology: moist, gray (except *Serratia marcescans* which appears red) smooth colonies on non-selective media

Special differential and selective media used for separation of genera and species

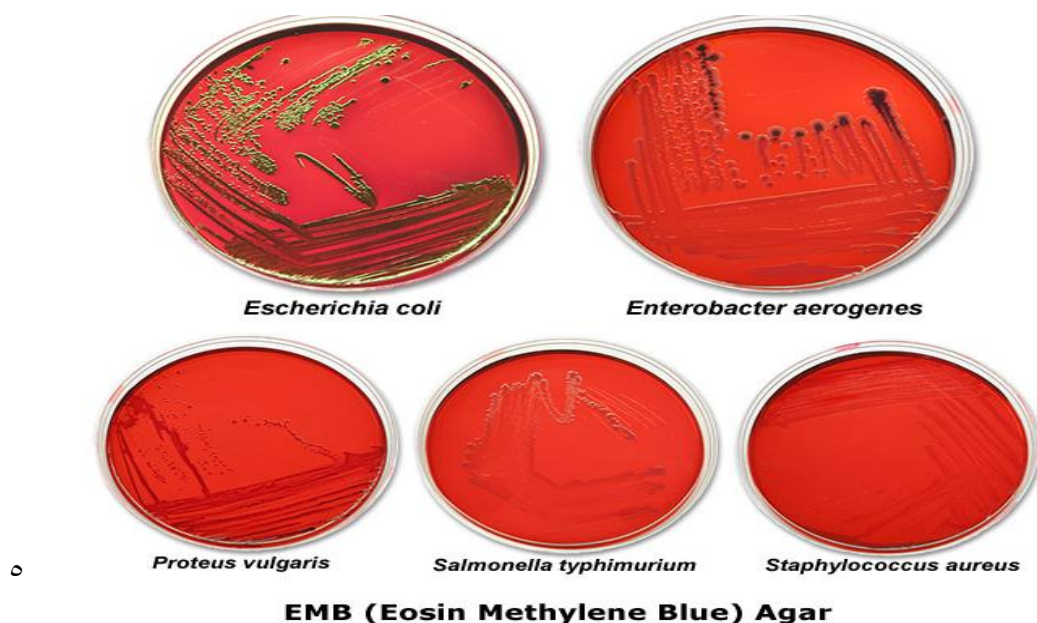
I- MacConkey agar medium:

- MacConkey agar is selective and differential medium for isolation of *Enterobacteriaceae*. The medium is made selective by the incorporation of **bile** and **crystal violet**, which inhibit gram-positive bacteria, especially staphylococci and enterococci. The medium is differential by use of combination of **neutral red** (in acidic pH the color turns red) and **lactose**. When an organism ferment lactose, the drop in pH (due to acid formation as end product of fermentation of lactose) causes the colony to take on a pink-red appearance whereas organism which unable to ferment lactose appears as pale yellow colonies.



II Eosin Methylene Blue (EMB) agar medium:

- EMB agar is a selective and differential medium used for the isolation and differentiation of enteric pathogens from contaminated clinical specimens. Eosin and methylene blue are the selective agents and inhibit gram-positive organisms. EMB contains lactose. Organisms that ferment lactose binds to dyes under acidic conditions and appear as blue-black colonies with a metallic sheen. Under less acidic conditions, other coliforms, such as *Klebsiella*, *Enterobacter*, and *Citrobacter*, appear as brown-pink "dark" colonies. Nonfermenters, such as *Salmonella*, *Shigella*, and *Proteus*, appear as the color of medium or transparent and colorless.



Salmonella-Shigella

(SS) agar: SS agar is a selective and differential medium used for isolation and differentiation of *Salmonella* and *Shigella* from clinical specimens. The selective agents are **bile salts**, and **brilliant green dye**, which inhibit gram-positive organisms. The medium contains only **lactose** and thus differentiates organisms on the basis of lactose fermentation. The formation of acid on fermentation of lactose causes the neutral red indicator to make red colonies. Non lactose fermenting organisms are clear on the medium. SS agar contains **sodium thiosulfate** and **ferric ammonium citrate** allows the differentiation of organisms that produce H_2S . Lactose fermenters, such as *E. coli*, have colonies which are pink with a precipitate, *Shigella* appears transparent or amber, and *Salmonella* appears transparent or amber with black centers.

A. *Klebsiella pneumoniae*

B. *Escherichia coli*

C : *Salmonella sp.*

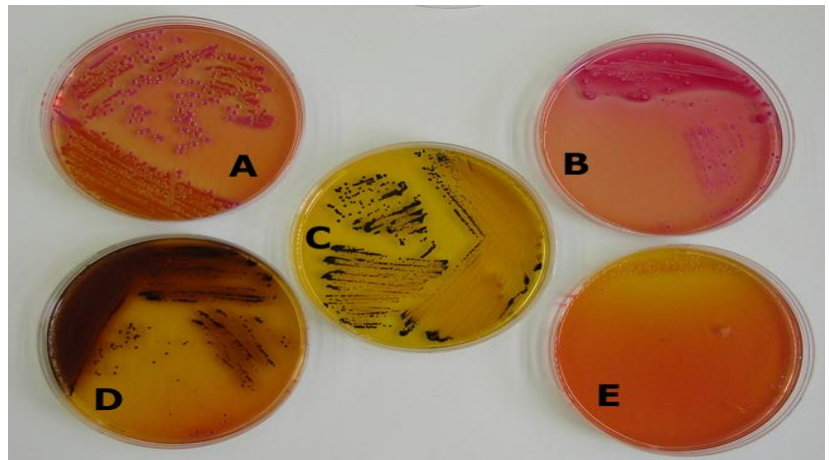
D : *Proteus mirabilis*

E : *Ps. aeruginosa*

A & B= Both are lactose fermenters

C & D = Both *Salmonella sp.* & *Proteus* product H_2S

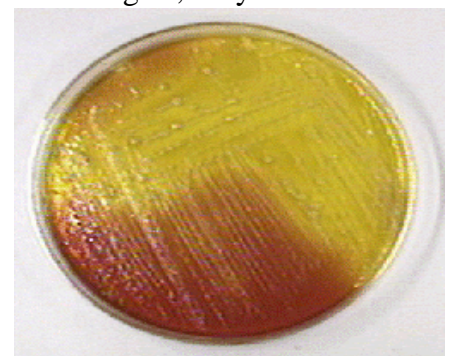
E= *Pseudomonas* colonies are nearly colorless



IV. Xylose-Lysine-Desoxycholate (XLD) agar:

XLD is a selective and differential medium used for the isolation and differentiation of enteric pathogens from clinical specimens. This medium is supportive of fastidious enteric organisms such as *Shigella*. The selective agent is **desoxycholate**, which inhibit gram-positive organisms. **Phenol red** is the color indicator. As with SS agar, **ferric ammonium citrate** (indicator) and **sodium thiosulfate** (sulfur source) allow identification of organisms that produce H_2S with appearance of colonies with black center. The medium contains **xylose**, which most of enteric organisms ferment. The most important exception is *Shigella*, the colonies appear to be transparent or the color of red medium. The **lysine** in the medium is utilized by the enteric organisms that contains lysine decarboxylase enzyme. For *Salmonella*, which contains lysine decarboxylase enzyme, this reaction converts the pH to an alkaline state and the colonies appear transparent or red with black center. The lactose and sucrose in the medium help to differentiate other enteric organisms. When other enteric organisms ferment these sugars, they maintain the pH at an acidic condition and the colonies appear yellow or yellow red.

***Escherichia coli* Growing on XLD Agar**



Acid from fermentation lowers the pH and turns the phenol red from red (alkaline) to yellow (acid). No hydrogen sulfide is produced.

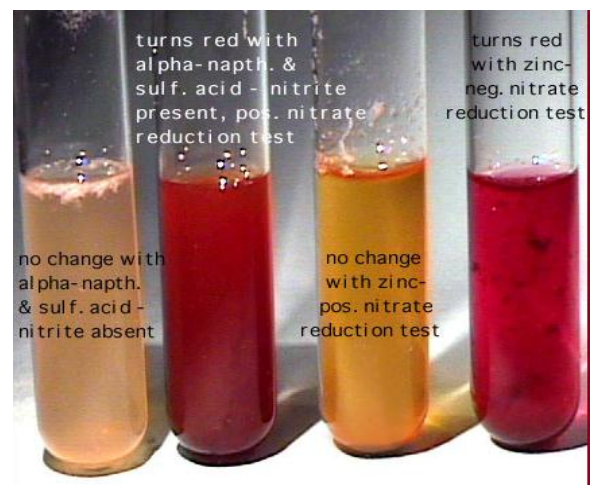
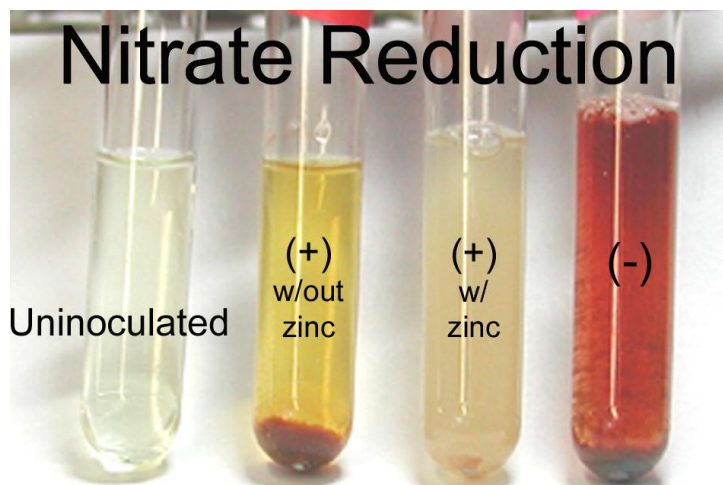
Enterobacteriaceae: I- Lactose Fermenters

● **Biochemical tests for identification of *Enterobacteriaceae*:**

All members of *Enterobacteriaceae* are ferment glucose, oxidase negative, and reduce nitrate into nitrites.

● **Nitrate Reduction:**

1. **Principle** - To determine the ability of an organism to reduce nitrate to nitrites or free nitrogen gas
2. **Method** - Inoculate a nitrate broth and incubate for 5 days at 37°C.
3. **Result**
 1. To each nitrate broth culture add 1 ml of sulphanilic acid and 1 ml of α -naphthylamine. The production of a red color occurs in the presence of nitrite indicates the ability of the organism to reduce nitrate to nitrite.
 2. To broths showing a negative reaction add a few particles of zinc. The appearance of a red color indicates that nitrate is still present and hence has not been reduced by the organism. If the solution does not change color the organism has reduced the nitrate through nitrite to nitrogen gas.
4. **Special Features** - Used in identification of *Enterobacteriaceae* (usually +).
5. **Precautions in Interpretation** - Interpret results immediately as the color produced in a positive reaction may fade quickly.

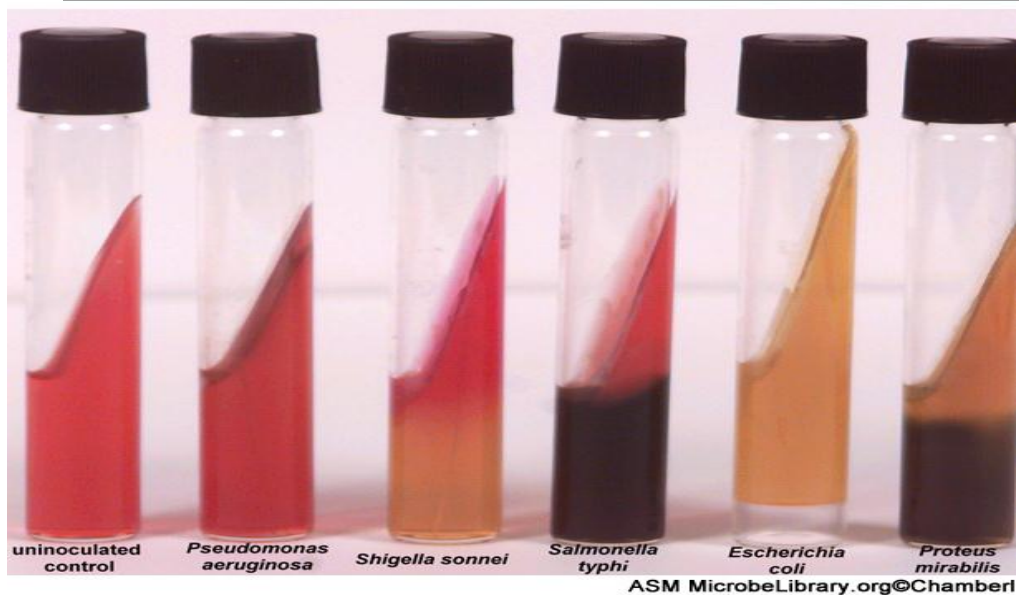


Triple Sugar Iron Agar (TSI) and H₂S production:

1. **Principle** - To determine the ability of an organism to attack a specific carbohydrate incorporated into a basal growth medium, with or without the production of gas, along with the determination of possible hydrogen sulphide (H₂S) production.
2. **Method** - Inoculate TSI medium with an inoculating needle by stabbing the butt and streaking the slant. Incubate at 37°C for 24 hours.

3.Results

Butt color	Slant color	H ₂ S	Result	Example
Yellow	Red	Negative	Glucose only fermented	Lactose non fermenter e.g. <i>Shigella</i>
Yellow	Red	Positive/blackening in butt	Glucose only fermented with H ₂ S production	Lactose non fermenter e.g. <i>Salmonella</i> & <i>Proteus</i>
Yellow	Yellow	Negative	Glucose fermented, also lactose and/or sucrose	Lactose fermenter e.g. <i>E. coli</i>
Red	Red	Negative	No action on glucose, lactose or sucrose	Non fermenter organisms e.g. <i>Pseudomonas</i>



H₂S production is indicated by the presence of a black precipitate

4. **Special Features** - H₂S production and carbohydrate fermentation patterns are generally characteristic for specific bacterial groups, especially the *Enterobacteriaceae*.
5. **Precautions in Interpretation** - An H₂S organism may produce so much of the black precipitate (ferrous sulphide) that the acidity produced in the butt is completely masked. However, if H₂S is produced, an acid condition does exist in the butt even if it is not observable.

🌈 **Indole, Methyl Red, Voges-Proskauer, Citrate (IMViC) Tests:**

- The following four tests comprise a series of important determinations that are collectively called the IMViC series of reactions (I= indole; M=methyl red; V=Voges Proskauer; and C=citrate). The IMViC series of reactions allows for the differentiation of the various members of *Enterobacteriaceae*.

Indole "Tryptophan Hydrolysis" Test:

1. **Principle** – Certain microorganisms can metabolize the amino acid tryptophan through the action of the enzyme tryptophanase. Once again, the activity of the enzyme is not measured directly, but rather, one of the end products (Indole) is detected. The enzymatic

degradation leads to the formation of pyruvic acid, indole and ammonia. The presence of indole is detected by addition of Kovac's reagent.

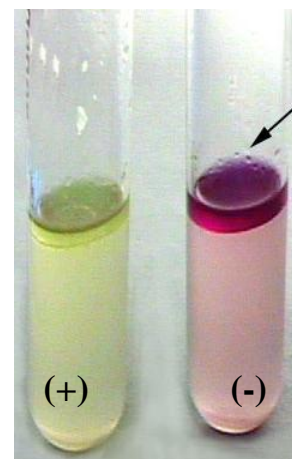
2. Materials

1. Tube of tryptone water
2. Tested microorganism { (such as *E. coli* (+) and *Klebsiella* (-)) }
3. Kovac's reagent

3. Method

1. Inoculate tryptone water with the tested microorganism
2. Incubate at 37°C for 24- 48 hours
3. After incubation interval, add 1 ml Kovacs reagent, shake the tube gently and read immediately.

Kovacs reagent	
p-dimethylaminobenzaldehyde	50 g
amyl or butyl alcohol	750 ml
HCl (conc.)	250 ml



4. **Result** - a bright pink color in the top layer (ring) indicates the presence of indole. The absence of color means that indole was not produced, and that the organism does not possess the tryptophanase enzyme i.e. indole is negative
5. **Special Features** - Used in the differentiation of genera and species. e.g. *E. coli* (+) from *Enterobacter* (-).
6. **Precautions in Interpretation**
 - Cultures to be tested for indole production must be incubated aerobically
 - The optimum pH for tryptophanase activity is one that is slightly alkaline (ph 7.4 - 7.8); a decrease in pH results in decreased indole production and a possible false negative.

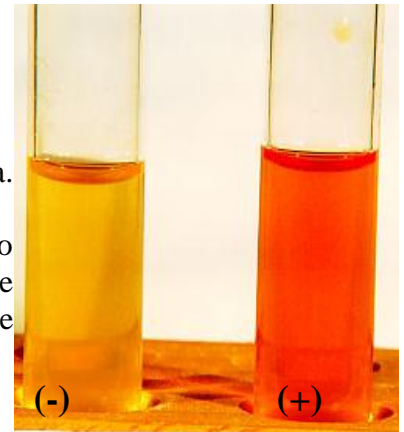
Methyl Red "Mixed acid Fermentation" Test:

1. **Principle** – All enteric bacteria can utilize sugar for their energy demands. Mixed acid fermenters such as *E. coli* ferment glucose to produce large amounts of acetic, formic and succinic acids as end products. The large amount of acid produced lowers the pH of the medium to below 5.0. By using the indicator methyl red (MR), the production of these acids as the end product of fermentation can be monitored. If pH drops below 4.5, the color of MR indicator will be red. If the pH is above 6.0, the color will be yellow/orange.
2. **Material**
 1. tubes of MRVP medium (Phosphate buffered glucose peptone water)
 2. Tested microorganism
 3. Methyl red indicator
3. **Method**
 1. Inoculate the tested organism into a tube of MRVP broth.
 2. Incubate the tube at 37°C for 24 hours.
 3. After incubation, add a few drops of MR solution to the culture. Read immediately.

4- Results

Red	MR positive
Yellow/orange	MR negative

4. **Special features** - The test is used to differentiate between genera. e.g. *E. coli* and *Citrobacter* (+) from *Klebsiella* and *Enterobacter* (-)
5. **Precautions in Interpretation** - If the MR test is performed too early, the results may be a false positive since MR negative organisms may not have had time to completely metabolize the initial acid products that accumulated from the glucose fermentation



• **Voges Proskauer "Butanediol Fermentation" Test:**

1. **Principle** – Some bacteria, rather than producing abundant acid in the fermentation of glucose, produce other products such as alcohols. All species of *Enterobacter* and *Klebsiella* will form products such as alcohol and 2,3-butanediol rather than the large amount of acid, as does *E. coli* and *Citrobacter*. A test for acetylmethylcarbinol (a precursor of 2-3, butanediol that appear in the growth medium) is performed. If Barrit's reagent is added, a pink color developing after few minutes indicates the presence of acetylmethylcarbinol and the test is positive.

2. Materials

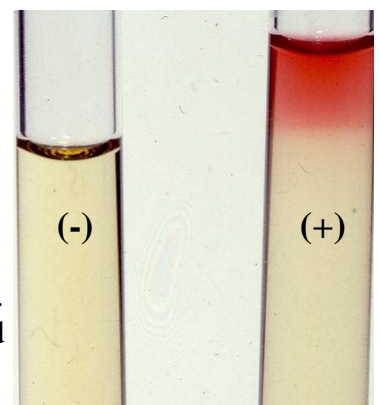
1. Tubes of MRVP
2. Tested microorganism
3. Barrit's solution A "40% KOH"
4. Barrit's solution B " α -Naphthol"

3. Method

1. Inoculate the tube by organism
2. Incubate the inoculated tube at 37°C for 24 h.
3. After it has become apparent that growth has occurred, add approximately 0.5 ml of α -naphthol, followed by 0.5 ml of 40% KOH.
4. Shake vigorously and let the tube stand for 1 to 2 h.

5. Results

Pink	VP(+)
No change	VP(-)

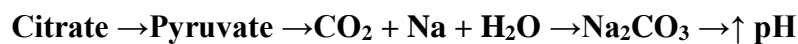


Special Features - The test is used to differentiate between genera. e.g. *K. pneumoniae* (+) and *Enterobacter* (+) from *E. coli* (-) and *Citrobacter*.

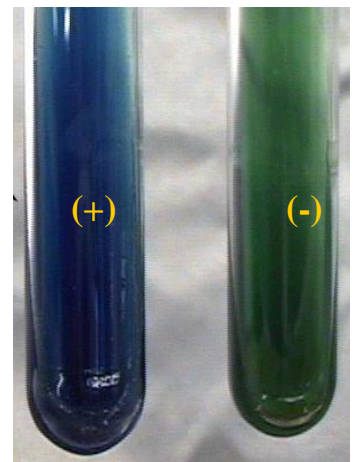
6. **Precautions in Interpretation** - After exposure to the reagents for over 2 hour, a negative VP culture may show a copper-like color due to the action of the KOH on the alpha naphthol. This is **not** a positive reaction.

Citrate Utilization Test

1. **Objective** – determine whether microorganism can utilize citrate as a sole source of carbon.
2. **Principle** – Some microorganisms can metabolize citrate as a sole of carbon source. Like many of the nutrients already used, citrate needs to be transported into the bacterial cells before it can be metabolized. This transport depends upon the enzyme **citrate permease**. Once inside the cell, the citrate is broken down to pyruvate and carbon dioxide. The carbon dioxide produced combines with sodium in the medium and water to form sodium carbonate, an alkaline product. The rise in pH is detected by the color change in bromothymol blue indicator present in the medium from green to deep blue.

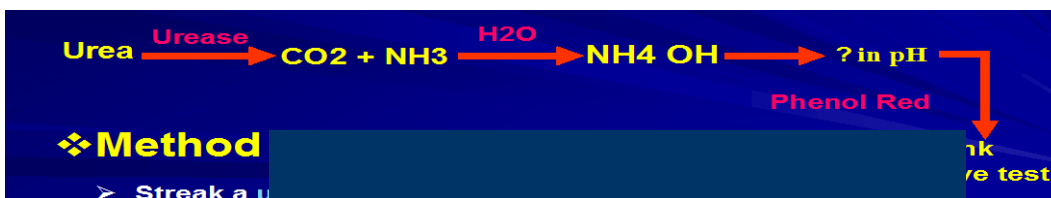


3. **Method** - Streak a Simmon's Citrate agar slant with the organism and incubate at 37°C for 24 hours.
4. **Result** - Examine for growth (+). Growth on the medium is accompanied by a rise in pH to change the medium from its initial green color to deep blue.
5. **Special Features** - Aids in the differentiation of genera and species.
6. **Precautions in Interpretation** - The medium must be lightly inoculated (from plate cultures, not from a broth) to avoid a carry over of nutrients, which may lead to a false positive result.

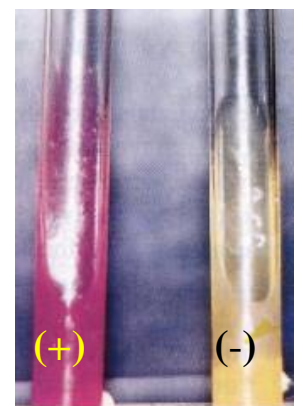


Urea Hydrolysis (Urease):

1. **Principle** – Urease is an enzyme that catalyzes the conversion of urea to CO₂ and NH₃. The urease test is particularly useful in identifying *Proteus*, *Klebsiella* and *Enterobacter*. The urea media contain the indicator phenol red. In the presence of urease, urea split and ammonia and carbon dioxide are produced. Ammonia combines with water to produce ammonium hydroxide, a strong base which raises the pH of the medium. This rise in the pH causes the phenol red indicator to turn a deep pink. This is indicative of a positive reaction for urease.



2. **Method** - Streak a urea agar tube with the organism and incubate at 37°C for 24 h
3. **Result** –If color of medium turns from yellow to pink indicates positive test. *Proteus* give positive reaction after 4 h while *Kelebsiella* and *Enterobacter* gave positive results after 24 h...
4. **Special Features** - Aids in the differentiation of members of *Proteus* also differentiates between *E. coli* (-) from *Klebsiella* (+) and *Enterobacter* (+).



5. Precautions in Interpretation - The test must be read after 4 h and 24 h

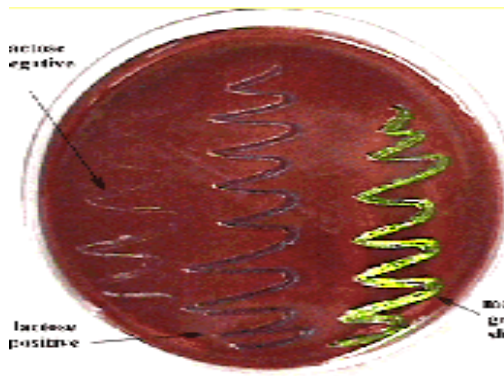
Item	Result	Example
Non-urease producer	Negative	<i>E. coli</i>
Weak urease producer	Positive after 18 hrs	<i>Klebsiella</i>
Strong urease producer	Positive after 2-6 hrs	<i>Proteus</i>

E.coli:

- Most significant species in the genus
- Important potential pathogen in humans
- Common isolate from colon flora
- Ferments glucose, lactose, trehalose, & xylose
- Positive indole and methyl red tests
- Does NOT produce H₂S
- Simmons citrate negative
- Usually motile
- Voges-Proskauer test negative
- Cause Gastrointestinal Infections, urinary tract infection, Septicemia & Meningitis



E.coli on nutrient agar



E.coli on EMB agar (metallic greenish colonies)

Klebsiella

- * Large gram negative bacilli
- * Usually found in GI tract
- * *K. pneumoniae* is mostly commonly isolated species
- * the colonies are large, moist and mucoid, due to possesses a polysaccharide capsule, which protects against phagocytosis
- * Has a distinctive “yeasty” odor

Frequent cause of nosocomial pneumonia

- Lactose positive(grow on MacConkey's agar & produce rose pink colonies)



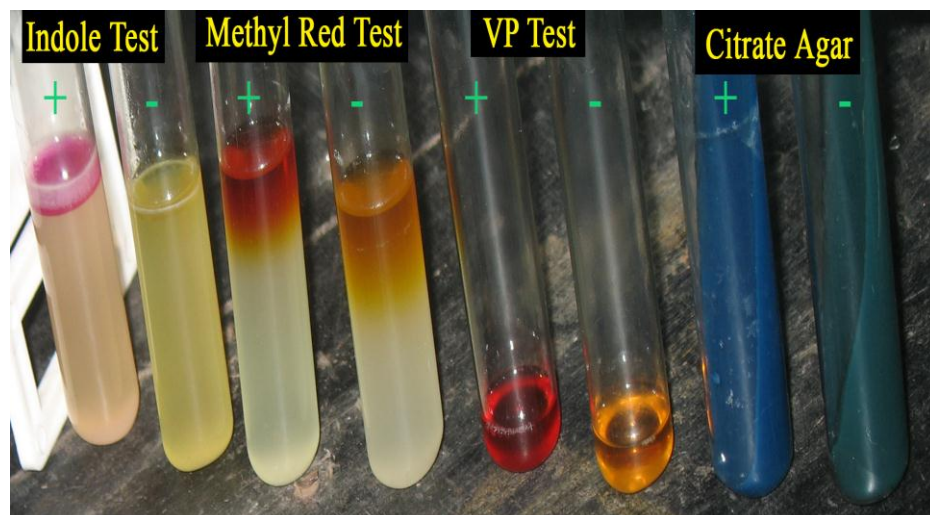
Klebsiella pneumoniae
on Macconkey Agar Plate

- Most are urease positive
- Non-motile
- Prominent polysaccharide capsule
- Facultative anaerobes,.

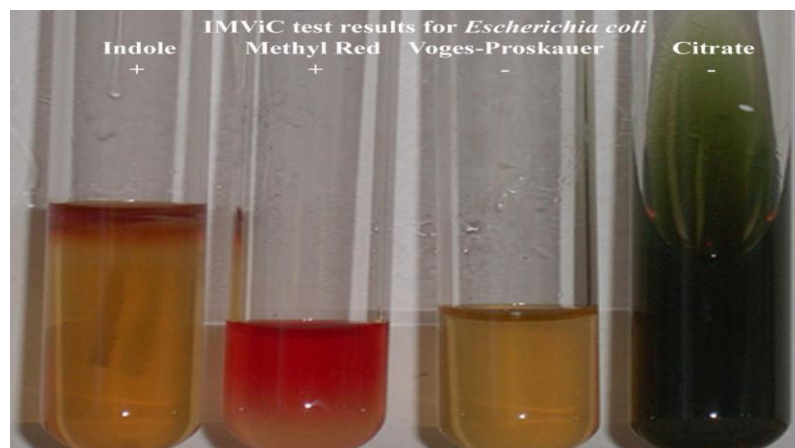
The differences between Lactose fermenter are summarized in the following table:

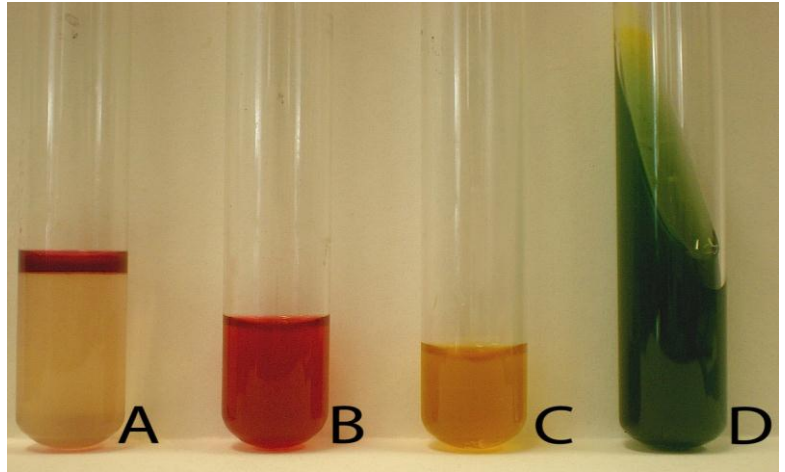
Items	<i>E. coli</i>	<i>Citrobacter</i>	<i>Klebsiella</i>	<i>Enterobacter</i>
MacConkey's	Rose pink colonies	Rose pink colonies	Rose pink colonies	Rose pink colonies
EMB	Metallic sheen	Dark colonies	Dark colonies	Dark colonies
Indole	+	+	-	-
MR	+	+	-	-
VP	-	-	+	+
CIT	-	+	+	+
Urease	-	+	+	+
Motility	Motile	Motile	Non motile	Motile

IMVIC test results



IMVIC test Result for *E.coli*



IMVIC test Result for *Proteus sp.***Summary of Enterobacteriaceae**

	<u>TSI</u>	<u>Indole</u>	<u>MR</u>	<u>VP</u>	<u>Citrate</u>	<u>Urease</u>	<u>Motility</u>
<u><i>E. coli</i></u>	<u>A/A/-</u>	<u>+ve</u>	<u>+ve</u>	<u>-ve</u>	<u>-ve</u>	<u>-ve</u>	<u>Motile</u>
<u><i>Citrobacter freundii</i></u> +	<u>A/A/-</u>	<u>+ve</u>	<u>+ve</u>	<u>-ve</u>	<u>+ve</u>	<u>-ve</u>	<u>Motile</u>
<u><i>Klebsiella pneumoniae</i></u>	<u>A/A/-</u>	<u>-ve</u>	<u>-ve</u>	<u>+ve</u>	<u>+ve</u>	<u>+ve</u>	<u>Non motile</u>
<u><i>Enterobacter cloacae</i></u>	<u>A/A/-</u>	<u>-ve</u>	<u>-ve</u>	<u>+ve</u>	<u>+ve</u>	<u>+ve</u>	<u>Motile</u>
<u><i>Salmonella typhi</i></u>	<u>A/Alk/+</u>	<u>-ve</u>	<u>+ve</u>	<u>-ve</u>	<u>+ve</u>	<u>-ve</u>	<u>Motile</u>
<u><i>Shigella boydii</i></u>	<u>A/Alk/-</u>	<u>-ve</u>	<u>+ve</u>	<u>-ve</u>	<u>-ve</u>	<u>-ve</u>	<u>Non motile</u>
<u><i>Proteus mirabilis</i></u>	<u>A/Alk/+</u>	<u>-ve</u>	<u>+ve</u>	<u>-ve</u>	<u>+ve</u>	<u>+ve</u>	<u>Motile</u>
							<u>Swarwing</u>
	<u>Gram stain</u>	<u>Oxidase</u>	<u>Nitrate reductase</u>	<u>O/F</u>	<u>MacConkey</u>	<u>SS</u>	<u>EMB</u>
<u><i>E. coli</i></u>	<u>-ve rod</u>	<u>-ve</u>	<u>+ve</u>	<u>O+/F+</u>	<u>LF</u>	<u>LF</u>	<u>Metallic sheen</u>
<u><i>Citrobacter</i></u>	<u>-ve rods</u>	<u>-ve</u>	<u>+ve</u>	<u>O+/F+</u>	<u>LF</u>	<u>LF</u>	<u>Dark</u>
<u><i>Klebsiella</i></u>	<u>-ve rods</u>	<u>-ve</u>	<u>+ve</u>	<u>O+/F+</u>	<u>LF</u>	<u>LF</u>	<u>Dark</u>
<u><i>Enterobacter</i></u>	<u>-ve rods</u>	<u>-ve</u>	<u>+ve</u>	<u>O+/F+</u>	<u>LF</u>	<u>LF</u>	<u>Dark</u>
<u><i>Salmonella</i></u>	<u>-ve rods</u>	<u>-ve</u>	<u>+ve</u>	<u>O+/F+</u>	<u>NLF</u>	<u>NLF/H2S</u>	<u>Colorless</u>
<u><i>Shigella</i></u>	<u>-ve rods</u>	<u>-ve</u>	<u>+ve</u>	<u>O+/F+</u>	<u>NLF</u>	<u>NLF</u>	<u>Colorless</u>
<u><i>Proteus</i></u>	<u>-ve rods</u>	<u>-ve</u>	<u>+ve</u>	<u>O+/F+</u>	<u>NLF</u>	<u>NLF/H2S</u>	<u>Colorless</u>

