

# Practical Bacteriology Laboratory Manual



CLS 413

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# Practical Bacteriology

CLS 413

# Laboratory Safety

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## General Safety Rules and Procedures

1. No food or drinks are permitted in the laboratory at any time.
2. Only closed-toe shoes are to be worn in the laboratory. Sandals are not permitted.
3. Keep hands and other objects away from your face, nose, eyes, ears, and mouth. The application of cosmetics in the laboratory is prohibited in the laboratory
4. Work areas/surfaces must be disinfected before and after use.
5. Laboratory coats must be worn and buttoned while in the laboratory.
6. Long hair should be secured behind your head.
7. Hands must be washed before leaving the laboratory.
8. All unnecessary books, purses, briefcases, etc., must be kept off the countertops.
9. Label all materials with your name, date, and any other applicable information (e.g., media, organism, etc.).
10. Dispose of wastes in their proper containers.
11. When handling chemicals, note the hazard code on the bottle and take the appropriate precautions indicated.
12. Do not pour chemicals down the sink.
13. Return all chemicals, reagents, cultures, and glassware to their appropriate places.
14. Flame (sterilize) transfer loops, wires, or needles before and immediately after use to transfer biological material.
15. Do not walk about the laboratory with transfer loops, wires, needles, or pipettes containing infectious material.
16. Turn off incinerators before leaving the laboratory.
17. Report any broken equipment.
18. If you are injured in the laboratory, immediately contact your course instructor or TA.

19. Follow all instructions given by your course instructor or TA in the lab.
20. Always wipe and clean the lenses of your microscope before putting it away. Use the appropriate tissue paper and cleaning solution for this purpose. And make sure to carry the microscope carefully in the correct manner.

# Culture Media

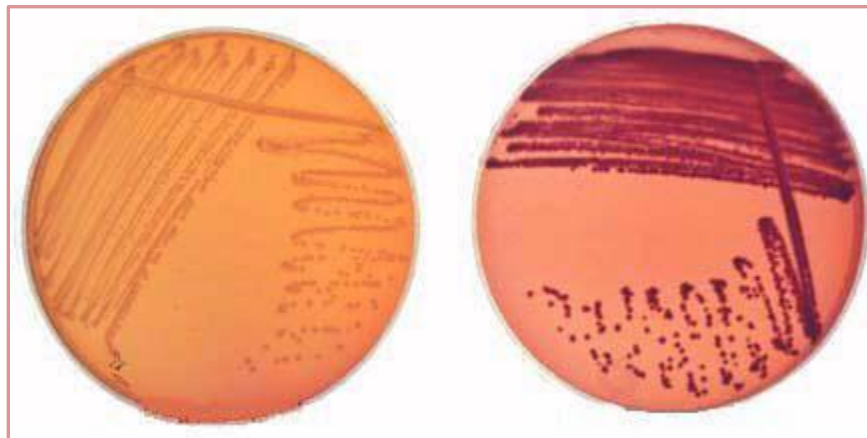
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The media discussed in this lab are the most commonly used media for culturing enterobacteriaceae species. The enterobacteriaceae bacteria include: *E.coli*, *Salmonella*, *Shigella*, *Proteus*, *Klebsiella*, *Yersinia pestis*, *Serratia*, *Citrobacter* and *Enterobacter*. In this practical we will only be dealing with the first five of the organisms mentioned.

## Mackonkey's agar (MAC):

This medium is a differential, low selective medium. It is selective for gram negative bacteria and it is used to differentiate between lactose fermenters and non-lactose fermenters. The inhibitory substance used for gram –ve selectivity is bile salts or crystal violet. For differentiation, the medium contains a PH indicator called neutral red. This indicator gives a pink/red color if acids have been produced due to lactose fermentation.

Lactose fermenters (*E.coli*, *Klebsiella*) give pink/red colonies in this medium. Why? Because these colonies produce acids when they ferment lactose, and when the PH is lowered it causes the color of the medium to change to pink/red giving the pink colonies appearance. Non lactose fermenters (*Salmonella*, *Shigella*) give colorless colonies on the medium. (There is no acid production here).



**Figure1:** Picture showing non- lactose fermenting colonies (left) and lactose fermenting colonies (right) on MAC agar.

## Cystein Lactose Electrolyte Deficient Agar (CLED):

This medium is a non-selective differential medium for lactose fermenters and non-lactose fermenters. Lactose fermenting bacteria produce yellow colonies on this medium, whereas non-lactose fermenters produce blue colonies.

Since CLED medium is electrolyte deficient (contains no sodium chloride), it inhibits the swarming of the bacterium *Proteus* causing it to produce single colonies on the plate. This medium contains beef extract and casein peptone to provide nitrogen, vitamins, minerals and amino acids for growth. Lactose is the fermentable carbohydrate providing carbon and energy. L-cystein is added as a growth supplement for cysteine dependent coliforms. Differentiation between lactose fermenters and non-lactose fermenters is achieved using bromothymol blue as PH indicator. Organisms that ferment lactose (*E.coli* and *Klebsiella*) lower the PH and change the medium color from green to yellow. *E.coli* colonies are smooth circular whereas *Klebsiella* colonies are circular and mucoid.

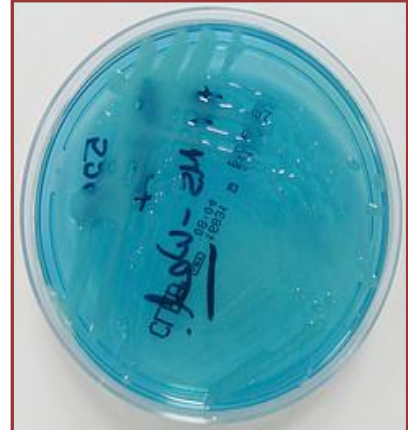
This agar is usually used for urine culture and all fastidious organisms grow on it. Fastidious organisms are those that grow only in a specially fortified enriched culture medium under certain conditions.



**Figure 2:** Lactose fermenting mucoid colonies of *Klebsiella pneumoniae* on CLED agar.



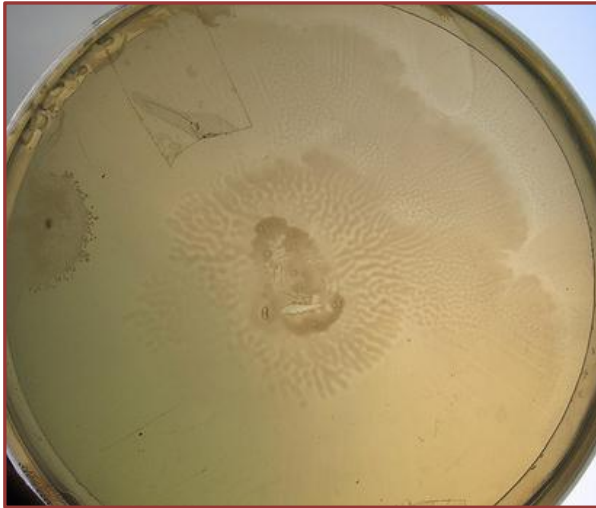
**Figure 3:** *E.coli* LF colonies on CLED agar.



**Figure 5:** NLF colonies on CLED medium

**Note:**

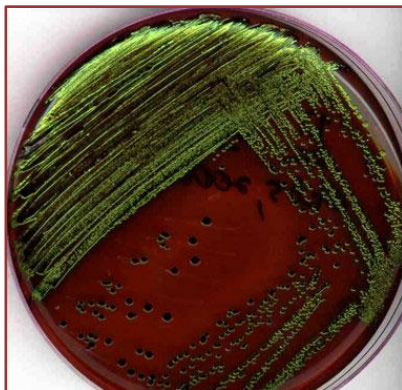
Swarming of proteus can be demonstrated on either nutrient or blood agar. It is done by performing a spot inoculation of the organism on a N.A petri-dish and incubating the plate at 37 C for 24 hours.



**Figure 6:** Swarming of Proteus on nutrient

**Eosin Methylene Blue Agar (EMB)**

It is a selective medium for gram negative organisms, and differential for *E.coli*. It contains neutral red as a PH indicator and eosin as an inhibitory substance. *E.coli* gives characteristic colonies on this medium. The colonies have a green metallic sheen.

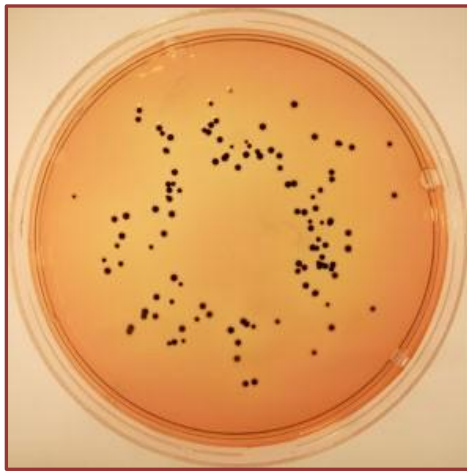


**Figure 7:** E.coli on EMB medium showing a green metallic

## Desoxycholate Citrate Agar (DCA)

This medium is selective for gram negative bacteria and differential for salmonella and shigella. The gram positive organisms are inhibited by an increased concentration of bile salts (sodium citrate and sodium desoxycholate). The medium also contains lactose as a fermentable sugar, and peptone as well as other substances to support the growth of bacteria. The PH indicator is phenol red or neutral red. Ferric citrate acids are added for the detection of H<sub>2</sub>S production.

On this medium, lactose fermenters (e.g. *E.coli*) produce pink/red colonies, whereas non-fermenters (e.g. *Salmonella*, *Shigella*) produce colorless colonies. H<sub>2</sub>S producers like *Salmonella typhi* give colonies with black centers, and non H<sub>2</sub>S producers like *Shigella* do not give black-centered colonies.



**Figure 8:** Salmonella in DCA medium gives colorless colonies with black centers.



**Figure 9:** Shigella in DCA medium gives colorless colonies.

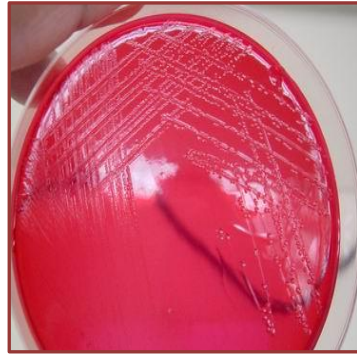
## Xylose Lysine Desoxycholate Agar (XLD)

It is a differential medium for *Salmonella* and *Shigella*. The medium contains three sugars: xylose, lactose and sucrose and an indicator phenol red. *Salmonella* and most gut bacteria are able to utilize the sugar xylose, lowering the pH and causing the medium to change its color from red to yellow. But because *Salmonella* can decarboxylate lysine by lysine decarboxylase, it causes the pH to revert back to alkaline (back to red color). *Shigella* does not utilize xylose so it does not lower the pH temporarily like *Salmonella* does. *Salmonella* produces red colonies with black spots due to H<sub>2</sub>S production and *Shigella* produces red colonies without black spots. Lactose fermenters produce yellow colonies on this medium.





**Figure 10:** Salmonella on XLD gives red colonies with black coloration due to H<sub>2</sub>S production.



**Figure 11:** Shigella on XLD gives red colonies.



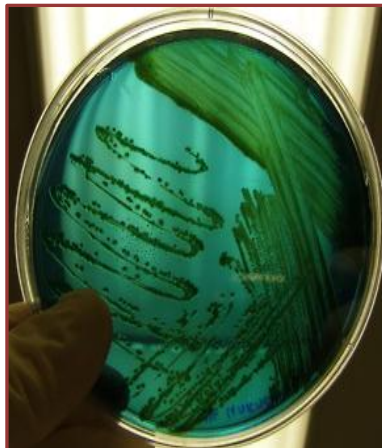
**Figure 12:** Yellow lactose fermenting colonies on XLD.

## Hektoen Enteric Medium (HE)

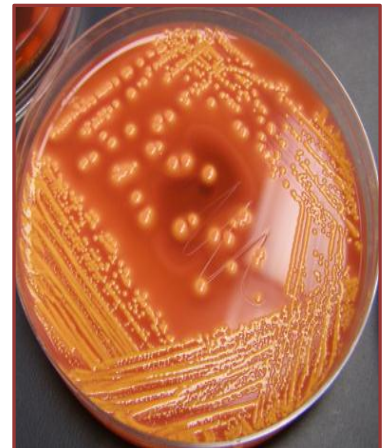
It is a selective medium for gram negative bacteria, and differential for salmonella and *Shigella*. It contains a pH indicator (bromothymol blue) and inhibitors (bile salts). This medium also contains two sugars; lactose and sucrose. Lactose fermenters like *E.coli* and *Klebsiella* give salmon orange colonies. Salmonella gives blue/green colonies with black spots due to hydrogen sulfide production, and *Shigella* produces blue-green colonies without black spots.



**Figure 13:** Salmonella producing blue colonies with black centers on HE medium.



**Figure 14:** Shigella producing blue-green colonies on HE medium.



**Figure 15:** Salmon orange LF colonies on HE agar.

**Table Showing the Colonial Morphology of Different Enteric Bacteria on Culture Media**

	<b>MAC</b>	<b>XLD</b>	<b>HE</b>	<b>DCA</b>	<b>CLED</b>	<b>EMB</b>
<b>e.coli</b>	Growth (+) LF, smooth Pink colonies	Growth (+) LF, smooth Yellow colonies	Growth (+) LF smooth ,Salmon orange colonies	Growth (+) LF,smooth, pink colonies	Growth(+) LF, smooth, yellow colonies	Growth (+) LF, green metallic sheen
<b>Klebsiella</b>	Growth(+) LF,pink mucoid colonies	Growth(+) LF, yellow mucoid colonies	Growth(+) LF,Salmon orange mucoid colonies	Growth(+) LF, pink mucoid colonies	Growth(+) LF,yellow mucoid colonies	Growth(+) LF,pink mucoid colonies
<b>Shigella</b>	Growth(+) NLF, colorless colonies	Growth (+) NLF, red colonies	Growth (+) NLF blue colonies	Growth(+) NLF,colorless colonies	Growth(+) NLF,colorles s colonies	Growth(+) NLF, colorless colonies
<b>Proteous</b>	Growth(+) NLF,Colorless colonies	Growth(+) NLF,colorless colonies +H2S production (black spots)	Growth(+) NLF,colorless colonies+ H2S production (black spots)	Growth(+) NLF, colorless colonies +H2S production (black spots)	Growth(+) NLF,colorles s colonies	Growth(+) NLF,colorless colonies
<b>Salmonella</b>	Growth(+) NLF,Colorless colonies	Growth (+) NLF, red colonies+H2S production (black spots)	Growth (+) NLF, blue colonies+H2S production (black spots)	Growth (+) NLF,colorlessc olonies+H2S production (black spots)	Growth (+) NLF,colorles s colonies	Growth (+) NLF,colorless colonies

# Biochemical Reactions of Enterobacteriaceae

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It is important to know that all members of the enterobacteriaceae are:

- Catalase positive
- Oxidase negative
- Nitrite positive
- Facultative anaerobes
- Glucose fermenters

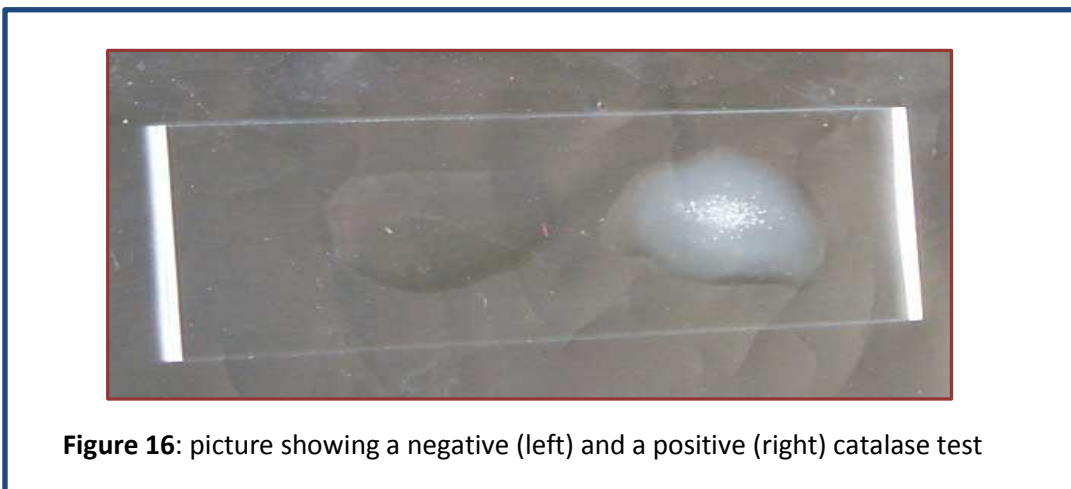
## Catalase Test

### Principle:

The test determines the ability of the organism to produce an enzyme called catalase which breaks down hydrogen peroxide into oxygen and water. It is performed by the emulsification of a bacterial colony in hydrogen peroxide solution. If catalase is present, air bubbles will form due to the production of oxygen.

### Procedure:

On a clean slide, put two drops of hydrogen peroxide. Using a loop, take a small amount of the bacterium and emulsify it on the slide. If bubbles form, the organism is catalase positive.



## Nitrite Test

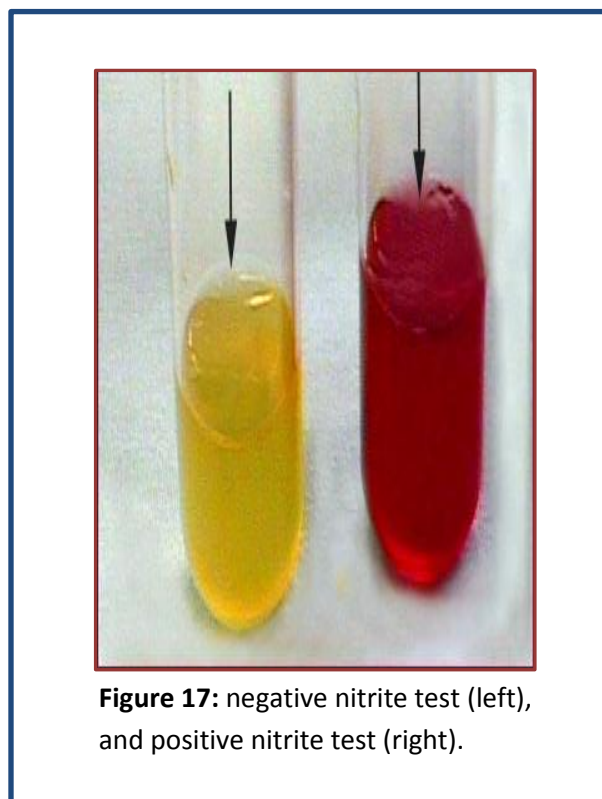
### Principle:

The test is performed in a broth tube. Enterobacteriaceae produce an enzyme called nitrate reductase that reduces nitrate into nitrite. Nitrite then reacts with salfanilic acid yielding a colorless complex (nitrite-salfanilic acid). This complex is detected with the help of two reagents by giving a red precipitate in the tube.

### Procedure:

Inoculate the organism of interest into the broth medium using a loop. Incubate at 37° C for 24 hrs then add two drops of each of the nitrite reagents. A positive result is indicated by a red color change. A negative result is indicated by no color change.

\*the original color of the medium is colorless-yellow.



## Oxidase Test

### Principle:

This test determines whether the organism produces an enzyme called cytochrome oxidase and therefore can use oxygen for energy production. The test uses swabs or filter paper impregnated with a reagent such as N,N dimethyl-p-phenylalanine, which is also a redox indicator. If the organism produces oxidase it will oxidize the reagent giving a purple color.

### Procedure:

In a clean petri-dish, place a piece of filter paper and add a few drops of the oxidase reagent on it. Using a clean slide or a cover slip, take some bacterial colonies and rub them on the wet filter paper. If a purple color appears, the organism is oxidase positive. If there is no change in color, the organism is oxidase negative.



**Figure 18:** picture showing oxidase test performed using filter paper. The result on the left is -ve and the one on the right is +ve



**Figure 19:** picture showing oxidase test performed using cotton swabs. The left swab is +ve and the right swab is -ve.

## Indole Test

### Principle:

This test detects the ability of the organism to split indole from the amino acid, tryptophan, by an enzyme called tryptophanase. Indole is then detected by the kovac reagent giving a red color or a red colored ring if performed in a test tube.

### Procedure:

To perform the test, we inoculate the organism into a liquid medium or “broth” containing either tryptone or peptone or both. Inoculation is done using a loop by taking a colony of the bacterium we want to test. The loop is then rubbed on the wall of the broth tube. The tube is capped and incubated at 37 C for 24 hours. After incubation, the kovac reagent is added. If a red ring appears this means the organism is indole positive. If there isn't a red ring, the organism is indole negative.

\*Indole positive organisms are E.coli and some Shigella sp.



**Figure 20:** picture showing negative indole test result (left) and positive indole test result (right)

## MR-VP Tests

Methyl red and Voges Proskaur broth is used for both tests. It is a broth that contains peptone, buffers and glucose or dextrose.

Principle:

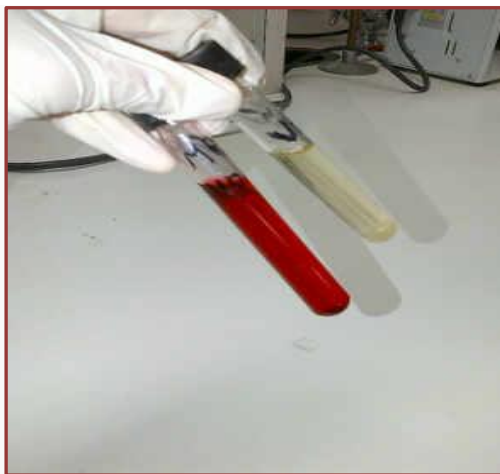
Different bacteria convert dextrose and glucose to pyruvate using different metabolic pathways. Some of these pathways produce unstable acidic products which quickly convert to neutral compounds. Some organisms use the butylene glycol pathway, which produces neutral end products, including acetoin and butanediol. Other organisms use the mixed acid pathway, which produces acidic end products such as lactic, acetic and formic acid. These acidic end products are stable and remain acidic.

The Methyl Red test involves adding the pH indicator methyl red to an inoculated tube of MR-VP broth. If the organism uses the mixed acidic fermentation pathway and produces stable acidic end products, the acids will overcome the buffers in the medium and produce an acidic environment in the medium. When methyl red is added, if acidic end products are present, the methyl red will stay red. A red color indicates a positive result.

The VP test detects organisms that utilize the butylene glycol pathway and produce unstable acids which convert to neutral end products like acetoin. When the VP reagents are added to MR-VP broth that has been inoculated with an organism that uses the butylene glycol pathway, a red color is produced. Therefore, red is a positive result. If, after the reagents have been added, a copper color is present, the result is negative.

Procedure:

The MR-VP broth is inoculated using a loop then incubating at 37° C for 24 hrs. Two drops of methyl red are added to the MR tube. And two drops of the VP reagent is added to the VP tube.



**Figure 21:** MR-VP tests. Left tube is a positive MR test and right tube is a negative VP test.

## Amino Acid Decarboxylase Test

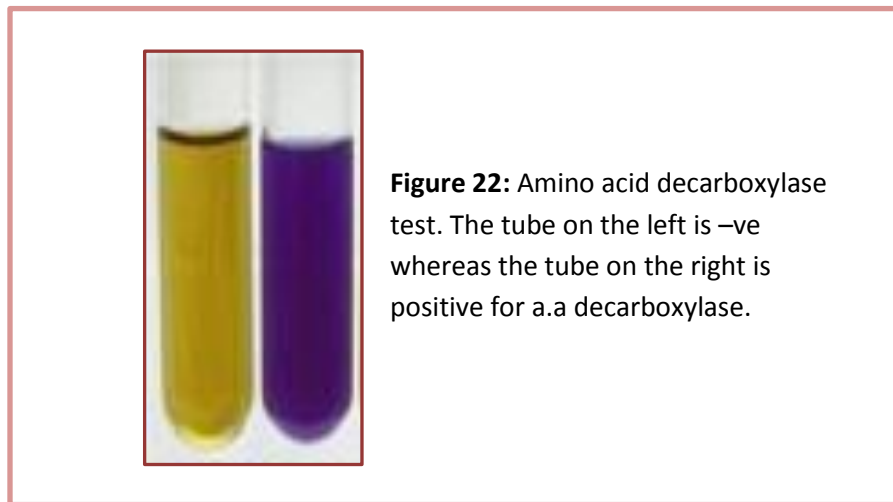
The media used is a broth medium which contains nutrients, dextrose and pH indicators (bromocresol purple and cresol red). A single amino acid (a.a) is added to tube of decarboxylase broth. The amino acids used are arginine, lysine and ornithine. The a.a content of them medium gives it an alkaline pH.

### Principle:

The test depends on the ability of bacteria to produce decarboxylase, an enzyme which removes a carboxyl group from amino acids. First, the organism utilizes dextrose to produce acids which change the pH of the medium from alkaline (purple) to acidic (colorless-light yellow). If the organism has the ability to decarboxylate the a.a in the tube, it will yield alkaline products like ammonia or an amine group changing the pH of the medium back to alkaline (purple). If the organism cannot decarboxylate the a.a, the medium will remain acidic and its color will stay colorless-light yellow.

### Procedure:

The organism is inoculated into the a.a decarboxylation broth using a loop. Paraffin oil is added to provide and anaerobic condition. Then the tubes are incubated at 37° C for 24 hrs. A purple color indicates a positive result. A colorless broth indicates a negative result.



## Citrate test

The medium used is a solid green slant medium containing citrate.

### Principle:

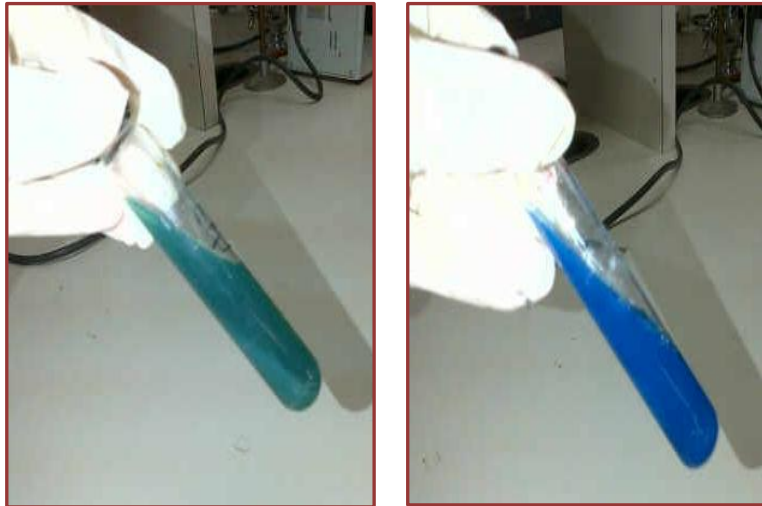
Organisms which can utilize citrate as their main source of carbon use the enzyme citratase to transport citrate into the bacterial cell. This utilization creates an alkaline environment in the medium causing its color to change from green to deep blue.



### Procedure:

The organism is inoculated using a straight inoculation needle by touching a colony of the bacterium and creating a zigzag line on the slope. The medium tube is capped and incubated at 37 C for 24 hrs. A green color is a negative result whereas a blue color is a positive result.

\*Citrate positive organisms include some *Proteus sp.*, *Klebsiella sp.* and most *Salmonella sp.*



**Figure 23:** Citrate test. The tube on the left shows a negative citrate test. The tube on the right shows a positive citrate test

### **Urease test**

The medium is an orange solid slant containing urea and an indicator, phenol red.

#### Principle:

Some organisms produce urease, an enzyme that converts urea to ammonium carbonate. This product gives a neon pink color in the medium. If the organism does not produce urease, it will not cause a change in the medium color. A neon pink color is a positive result. An orange color is a negative result.

#### Procedure:

The organism is inoculated in the medium and incubated at 37 C for 24 hrs.



**Figure 24:** Urease test. The left tube is +ve and the right tube is -ve.

## Triple Sugar Iron (TSI)

The medium is a solid slant containing glucose, lactose, sucrose, ferric acid and phenol red as an indicator. The slant contains lactose and the butt contains glucose.

### Principle:

The test determines the ability of bacteria to ferment the carbohydrates that are incorporated into the medium, with or without the production of gas, along with possible hydrogen sulfide production.

Fermentation is indicated by a change in the color of the medium from red to yellow. Organisms that ferment glucose only will give a K/A reaction (alkaline/acid = red/yellow). Those which ferment both glucose and lactose give an A/A reaction (acid/acid = yellow/yellow). Organisms that neither ferment lactose nor glucose give a K/K reaction (alkaline/alkaline = red/red). Gas production is indicated by cracks, bubbles or indentation. H<sub>2</sub>S production is indicated by a black color forming in the medium

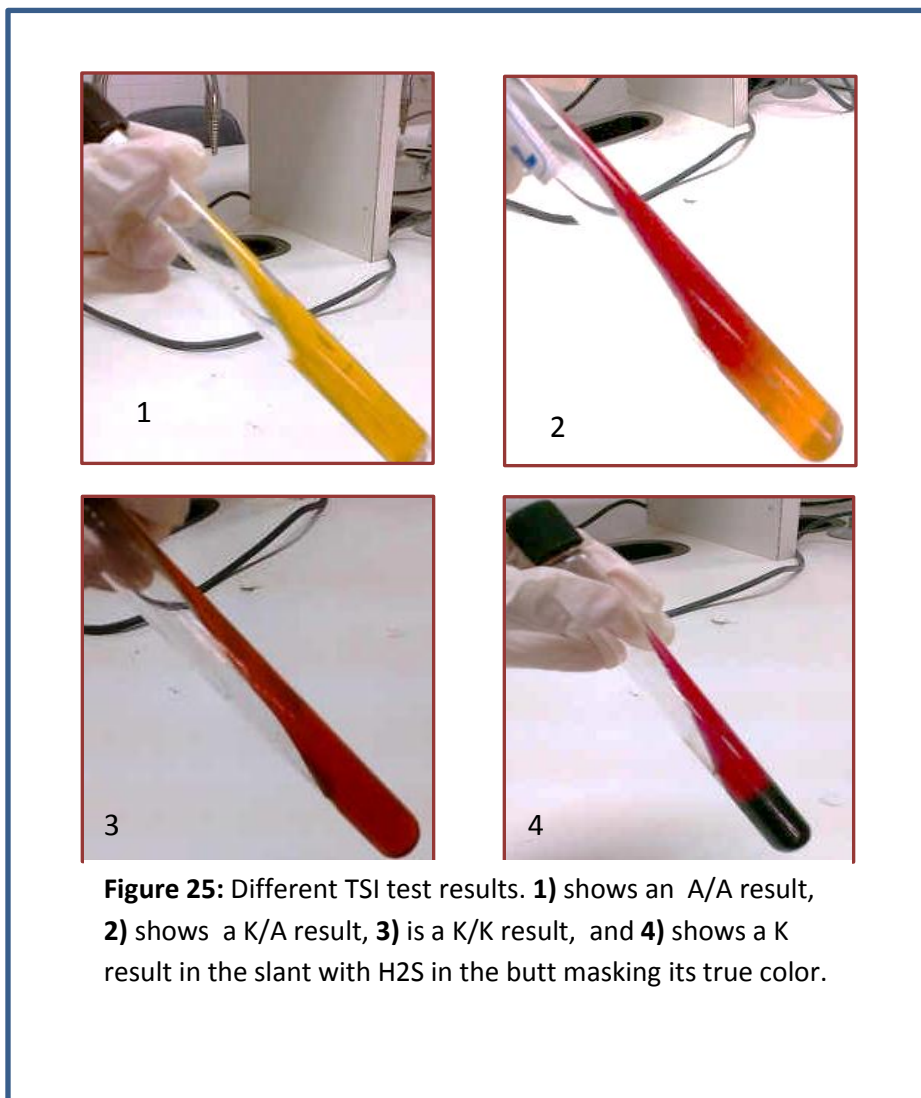
\*organisms giving a K/K reaction cannot be enterobacteriaceae because they lack the ability to ferment glucose.

### Procedure:

The organism is inoculated in the medium using a straight needle and incubated at 37 C for 24 hrs.

**Table summarizing the results of the TSI test:**

	Fermentation of glucose only (K/A)	Fermentation of glucose and lactose (A/A)	Neither lactose nor glucose are fermented
Slant	Red	Yellow	Red
Butt	Yellow	Yellow	Red



**Table Summarizing the Biochemical Reactions of Enterobacteriaceae**

	<b>Klebsiella</b>	<b>E.coli</b>	<b>Salmonella</b>	<b>Shigella</b>	<b>Proteus</b>
<b>Indole</b>	--	++	--	variable	<b>P. vulgaris ++</b> <b>P. mirabilis --</b>
<b>VP</b>	++	--	--	--	--
<b>MR</b>	--	++	variable	variable	++
<b>Citrate</b>	++	--	Most are ++ Except: S.typhi, S.paratyphi	--	++
<b>Urease</b>	++	--	--	--	++
<b>TSI (Butt)</b>	Yellow	Yellow	Yellow	Yellow	Yellow
<b>TSI (Slant)</b>	Yellow	Yellow	Red	Red	Red
<b>TSI (H<sub>2</sub>S)</b>	--	--	S.typhi ++ S.paratyphi --	--	++
<b>TSI (Gas)</b>	++	Mostly ++	S.typhi -- S.paratyphi ++	--	++
<b>Motility</b>	--	++	++	--	++
<b>Lysine Decarboxylase</b>	++	++	Typhi ++ Paratyphi --	--	--
<b>ONPG</b>	++	++	--	--	--

# Analytical Profile Index (API E 20) and ONPG

## Analytical Profile Index API E 20

The analytical profile index E 20 or (API E 20) provides an easy way to inoculate and read tests relevant to members of the family Enterobacteriaceae and associated organisms. It is a classification of bacteria based on experiments, allowing fast identification. The test systems are stored in 20 small reaction tubes, which contain the substrates. These small tubes are referred to as wells or cupules.

### Procedure:

#### 1) Prepare a suspension of the bacteria in normal saline

- Inoculate a large colony of the bacterium into the NaCl solution, making sure that the suspension is homogenous and without clumps of floating bacteria.

#### 2) Inoculate the API strip

- Holding the strip at a slight angle up from the table top, inoculate the bacterial suspension into each well with a sterile pipette. See figure A.
- Touch the end of the pipette to the side of the cupule, allowing capillary action to draw the fluid into the well as you slowly squeeze the bulb. This should eliminate any bubbles forming in the wells. Each well should be filled up to the neck. See figure B.
- CIT, VP and GEL have boxes around their names. These tests will be filled all the way up to the top of the well.
- LDC, ODC, ADH, H<sub>2</sub>S, and URE are filled up to the neck of the well. Then they are filled up to the top with oil.

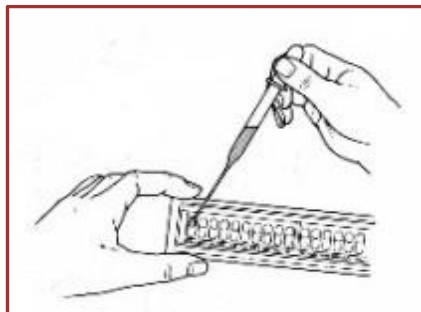


Figure A

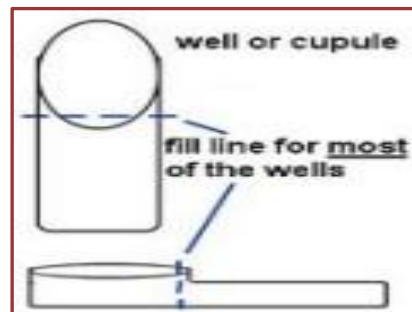


Figure B

### 3) Incubate the strip in its chamber

- The bottom of the incubation chamber has small indented wells in the bottom: fill it with water just enough to fill these indentations.
- Place the strip into this bottom. There should not be so much water that it slops onto the API strip.
- Place the top of the incubation chamber over the bottom, and label it.
- Place the strip at 37° C for 18-24 hours.



**Figure 26:** picture showing a strip of inoculated API E 20. This pattern belongs to a Salmonella

### Ortho-Nitrophenyle- $\beta$ -galactoside (ONPG)

This test detects an enzyme called ortho-nitrophenyl galactosidase. The enzyme ferments lactose and therefore, this test detects lactose fermentation. The substrate for the enzyme is ONPG. Lactose fermenters produce two enzymes: 1)  $\beta$ -galactosidase which is an intracellular enzyme and 2) permease, an extra cellular enzyme that regulates entry of lactose into the cell.

In the presence of ONPG, the enzyme  $\beta$ -galactosidase hydrolyzes ONPG to orthonitrophenol. The enzyme will also ferment lactose giving a yellow color.

In organisms that lack the permease enzyme and have only  $\beta$ -galactosidase, lactose will enter the cell through passive transport which leads to a slower fermentation of lactose. This is called delayed lactose fermentation.

\*Non lactose fermenters lack both enzymes and they do not ferment lactose.

\*This test is incorporated into the API E 20 testing system. There is a specific cupule for this test on the strip.

## Sulfide-Indole-Motility Medium

This is a semi solid medium containing ferrous sulfide. It detects H<sub>2</sub>S production, Indole and motility. The test is done by inoculation the organism into the media using a straight needle. To inoculate, take a bacterial colony using a straight needle and insert the needle vertically into the medium letting it go all the way through to the bottom. Then pull the needle out slowly and gently. Incubate the tube at 37° C for 24 hours

Motility is detected by diffused growth in the medium. If growth occurs only at the site of inoculation, the organism is non-motile. H<sub>2</sub>S production is indicated by a black color. Indole is detected by the formation of a red ring.

### Note:

In clinical labs, the abbreviation **IMViC** is used for the following group of tests: “I” for indole, “M” for methyl red, “V” for Voges Proskauer and “iC” for citrate.

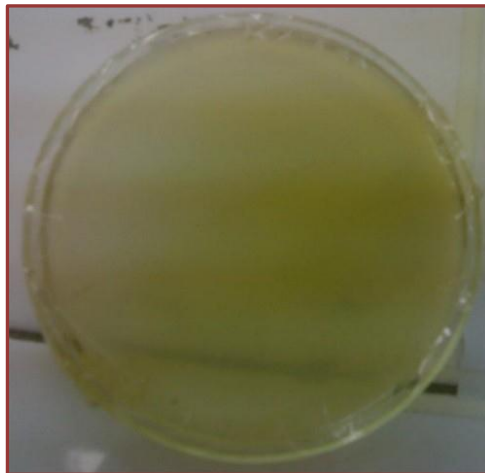
# Pseudomonas

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Pseudomonas is a gram negative pathogenic bacterium that doesn't belong to the Enterobacteriaceae family. It is highly resistant to antibiotics and causes various nosocomial infections. It is oxidase positive and doesn't ferment lactose or glucose.

## Gram Smear and Culture

- Gram stain: gram negative bacilli
- Culture: Strict aerobe, Produces green pigments on nutrient agar and dark colonies on blood agar due to pigmentation.



**Figure 27:** picture showing Pseudomonas pigment production on nutrient agar



# Hemophilus Influenzae

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Hemophilus species include; Hemophilus influenza, Hemophilus para influenza and Hemophilus duchrii.

## Gram Smear and Culture

- Gram reaction: gram negative coccobacilli, pleomorphic in shape
- Culture: fastidious organisms that need enrichment media (chocolate agar)

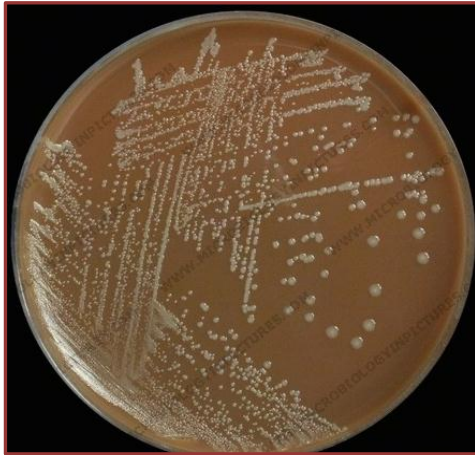
## Hemophilus on Chocolate Agar

### Preparation of the agar:

Prepare blood base broth. Then add 10% of sheep blood. Let it cool down at 45 degrees Celsius and put it in a water bath for 30 min at 75 degrees Celsius. This will cause the RBCs to lyse and release the X factor (heamin) and the V factor (porphorin) which are essential for the growth of Hemophilus Sp.

### Colonial Morphology:

Hemophilus Sp. give grey moist colonies on chocolate agar.



**Figure 28:** picture showing H.influenzae on chocolate agar. The colonies are grey and moist.

## Hemophilus on Nutrient Agar

On nutrient agar, V and X factor discs are added to support the growth of Hemophilus. Hemophilus influenzae requires both factors to grow whereas Hemophilus parainfluenzae requires only the X factor.



**Figure 29:** two pictures showing the growth of Hemophilus influenzae around the X&V factor disc on nutrient

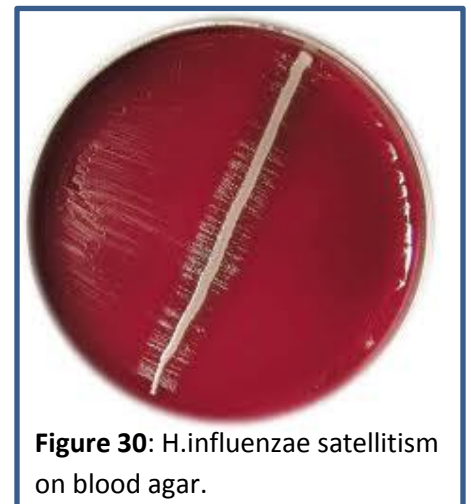
## Satellitism

### Principle:

Satellitism is the growth of one sort of bacteria in culture about the colonies of another sort that supplies needed micronutrients or growth factors. The Hemophilus influenzae satellitism test is performed on blood agar and it uses Staphylococcus aureus as a source of NAD (V factor). The X factor (heamin) is obtained from the agar itself since it's naturally one of the constituents of the agar. This test is used to differentiate between H.influenzae and H.parainfluenzae.

### Procedure:

- Using a cotton swab, pick up 2-3 colonies of H.influenzae and wipe the swab on the surface of the blood agar plate.
- Make a straight line streak of S.aureus across the middle of the plate.
- Hemophilus influenza colonies will grow all over the plate, but they will increase in size and number as they get closer to the colonies of S.aureus.



**Figure 30:** H.influenzae satellitism on blood agar.

# Campylobacter

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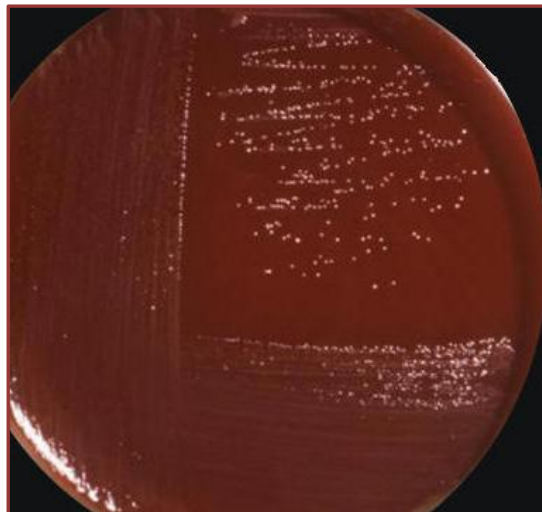
Campylobacter bacteria are a common cause of diarrhea and enteritis which makes them clinically significant. They are characterized as being microaerophilic (need CO<sub>2</sub> for growth), oxidase positive, catalase positive and thermophilic (require high temp. for growth 42°-43° C).

## Gram Smear and Culture

- Gram stain: gram negative curved seagull-shaped bacilli.
- Culture medium: Campylobacter is grown on a selective medium that prevents the growth of fecal normal flora. The medium is called Campy agar which is composed of blood agar incorporated with an antibiotic.

## Campy Agar

When culturing Campylobacter on campy agar, the plate should be incubated in a microaerophilic condition using an anaerobic jar and a gas generating kit. Also, incubation should be at a temperature of 42° – 43° C because the organism is thermophilic.



**Figure 31:** Campylobacter colonies on campy agar. Colonies are flat, droplet like and glistening.

## Hippurate Test

The Hippurate test is used to differentiate between two different species of *Campylobacter*: *Campylobacter jejuni* (hippurate +ve) and *Campylobacter coli* (hippurate –ve).

### Principle:

The test depends on the ability of the organism to produce an enzyme, hippuricase that hydrolyzes hippurate to produce glycine. A reagent called ninhydrin is added to detect the presence of glycine by reacting with it to give a purple color. If the broth turns purple, the organism is hippurate positive.

### Procedure:

Hippurate broth is inoculated with the organism and then incubated. After incubation ninhydrin is added to check if glycine is present. A purple color means that glycine is present and this indicates that hippurate has been hydrolyzed and that the organism is a hippuricase producer.



**Figure 32:** Hippurate test. A purple color indicates a positive hippurate result.

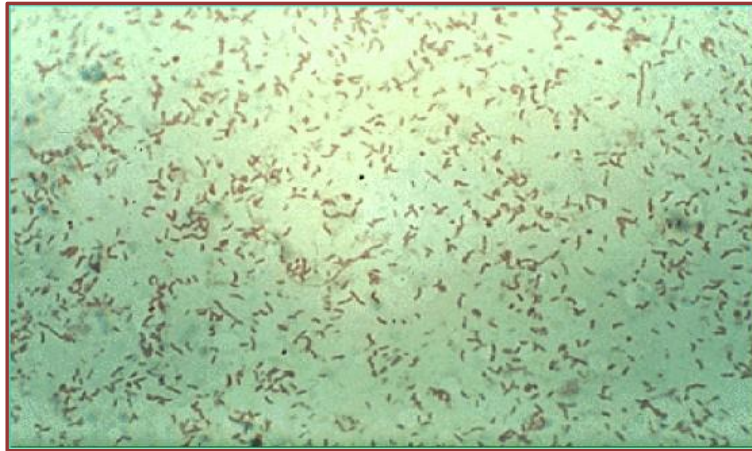
# Vibrio Species

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There are many different species of Vibrio bacteria. In this lab we will discuss: Vibrio cholera and Vibrio parahaemolyticus. V.cholerae causes the well-known cholerae disease and V.parahaemolyticus causes food poisoning. Vibrio species are oxidase positive, facultative anaerobes, motile and can grow at a wide range of temperatures (16° C- 40° C).

## Gram Smear and Culture

- Gram stain: gram negative comma shaped bacilli
- Culture: the medium Thiosulphate citrate bile sucrose (T.C.B.S) is used for culturing Vibrio. It is a selective medium which inhibits the growth of normal fecal flora.



**Figure 33:** gram stain of Vibrio sp. showing gram negative comma-shaped bacilli.

### Thiosulphate Citrate Bile Sucrose Agar (T.C.B.S)

*Vibrio cholera* grows at 8.2 pH and ferments sucrose which causes the colonies to turn yellow in color. *Vibrio parahemolyticus* does not ferment lactose and produces blue-green colonies on T.C.B.S. Both species are non-lactose fermenting and they both give moist colonies.



**Figure 34:** *Vibrio cholera* on T.C.B.S medium. The colonies are yellow due to sucrose fermentation.

# Anaerobes

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## Clostridia

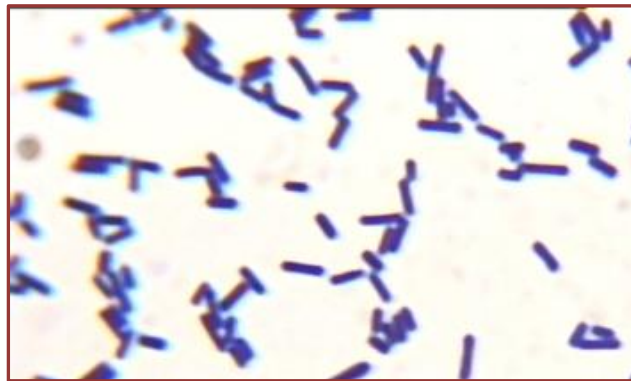
These organisms are spore forming anaerobic bacteria. Some are commensals of the human gut and others cause diseases such as gas gangrene, tetanus, botulism and food poisoning. The most common pathogens are: Clostridium perfringens, Clostridium tetani, Clostridium botulinum and Clostridium difficile.

## Clostridium Perfringens

This bacterium is the causative agent of gangrene. It is spore forming and non-motile.

### Gram Stain:

Gram positive brick-shaped bacilli.

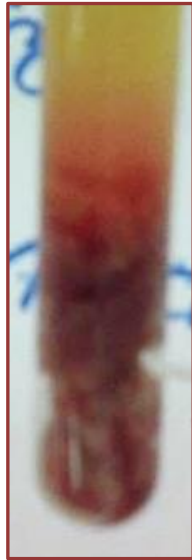


**Figure 35:** gram stain showing brick shaped gram positive bacilli of C.perfringens.

### Culture:

**Blood agar:** colonies produce double zones of hemolysis and a very foul smell. The organism is inhibited by metronidazole discs.

**Cooked Meat Agar:** this medium is composed of chopped pieces of heart muscle. Clostridium perfringens ferments the sugars of the medium causing the color of the medium to turn red. This reddening is called a saccharolytic reaction.



**Figure 36:** saccharolytic (reddening) reaction of *Clostridium perfringens* in cooked meat medium.

## **Clostridium Tetani**

This motile, endospore forming organism is the causative agent of the well-known disease tetanus.

### Gram stain:

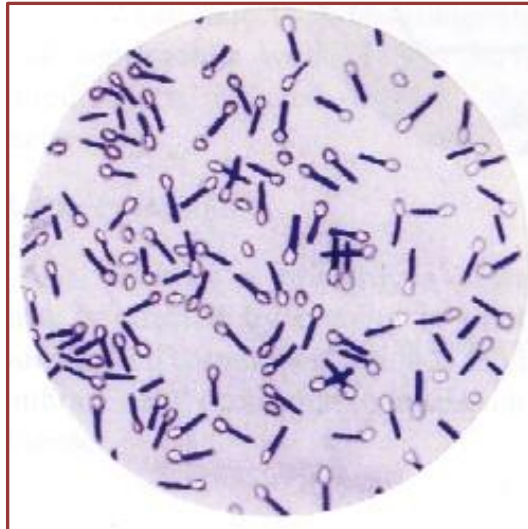
Gram positive slender bacilli with rounded ends (terminal spores) giving a drumstick appearance.

### Culture:

**Blood Agar:** on blood agar *C.tetani* gives a fine layer of featherlike swarming colonies due to motility. The colonies are none hemolytic and they produce a foul smell.

**Cooked Meat Medium:** the bacterium produces a proteolytic reaction, blackening of the medium due to the digestion of the proteins in the medium.





**Figure 37:** gram stain of *C.tetani* showing slender gram +ve rods with terminal spores.



**Figure 38:** Proteolytic (blackening) reaction of *Clostridium tetani* in cooked meat medium.

## **Bacteroides**

Bacteroides are gram negative non-spore forming bacilli that make up a substantial portion of our gastrointestinal flora. They are sensitive to the antibiotic metronidazole.

Gram stain: Gram negative bacilli

Culture: They grow on blood agar giving non hemolytic moist colonies

# Spirochetes

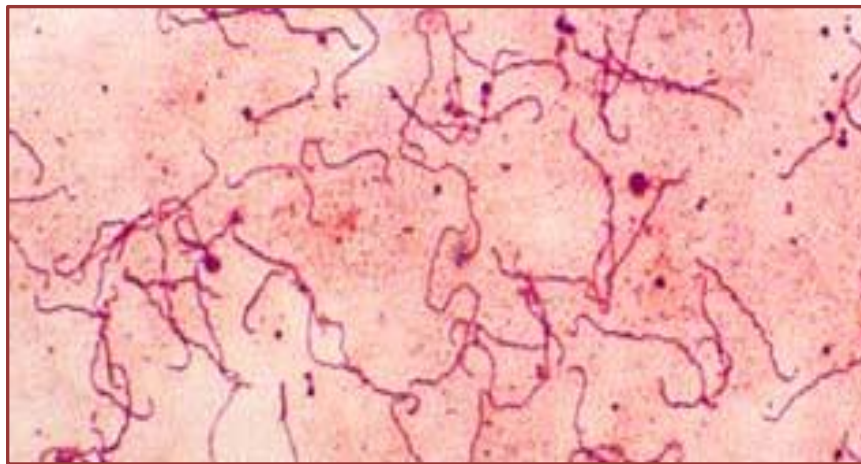
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## Borrelia

- Normal oral flora
- Gram stain: gram negative spirochetes

## Treponema Pallidum

- Treponema pallidum is the causative agent of the sexually transmitted disease syphilis.
- Gram Stain: gram negative spirochetes.



**Figure 39:** Picture showing the gram negative spirals of Treponema on gram stain.

## Screening Tests for Syphilis

### Venereal Disease Research Laboratory (VDRL)

Venereal Disease Research Laboratory (VDRL) Test is a serological slide flocculation test used in the diagnosis of syphilis. Since the antigen used in this test is cardiolipin, which is a lipoidal extracted from ox heart, it is not a specific test. For this reason the test is classified as non-specific or non-treponemal.

#### Principle:

Patients suffering from syphilis produce antibodies that react with cardiolipin antigen in a slide flocculation test, which is read using a microscope. It is not known if the antibodies that react with cardiolipin are produced against some lipid component of Treponema pallidum or as a result of tissue injury following infection. The test sample is serum.

Procedure:

- Serum should be heated in a water bath to inactivate nonspecific inhibitors such as complement.
- A drop of inactivated serum is added into the well
- Then a drop of cardiolipin Ag is added
- The well is then rotated gently for 4 minutes manually or on a rotator.
- After 4 minutes, the test is examined microscopically for flocculation.
- If flocculation is present, the test is reactive (positive).

### Rapid Plasma Reagin (RPR)

This test shares the same principle of the VDRL test with some modifications. In the RPR test, the serum does not require heating and the result can be viewed without a microscope. The cardiolipin Ag is mixed with visible carbon particles which enable the test to be performed macroscopically.

Procedure:

- Serum is added to the card well
- Cardiolipin Ag mixed with carbon particles is added to the well
- The card is rotated for 4 minutes
- If agglutination occurs, the test is said to be reactive (positive)
- No agglutination indicates a non-reactive (negative) result.



**Figure 40:** Picture showing a non-reactive RPR result (left) and a reactive RPR

## Picture References:

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- <http://www.sigmaaldrich.com/technical-documents/articles/analytix/differentiation-of.html>