Pathogenic Bacteria (practical)

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References:

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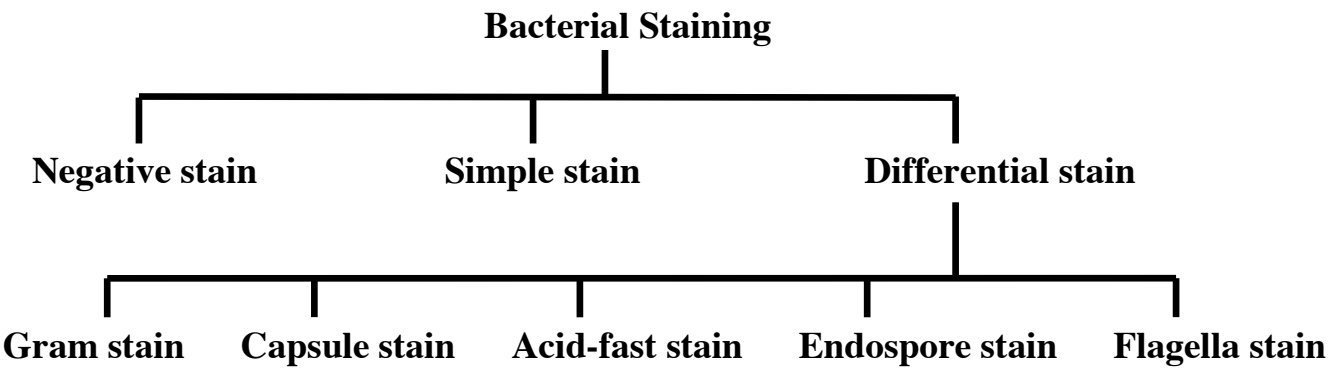
Bacterial Staining



Since living bacteria are generally colorless and almost invisible because of their lack of contrast with the water in which they may reside, staining is necessary in order to make them readily visible for observation of intracellular structures as well as overall morphology.

Negative Staining

Sometimes it is convenient to determine overall bacterial morphology without the use of harsh staining or heat-fixing techniques that change the shape of cells. Negative staining is also good for viewing capsules

Principles : Negative, indirect, or background staining is achieved by mixing bacteria with an acidic stain such as nigrosin, India ink, or eosin, and then spreading out the mixture on a slide to form a film. The above stains will not penetrate and stain the bacterial cells due to repulsion between the negative charge of the stains and the negatively charged bacterial wall. Instead, these stains either produce a deposit around the bacteria or produce a dark background so that the bacteria appear as unstained cells with a clear area around them.

Procedure: as in the figure 1:

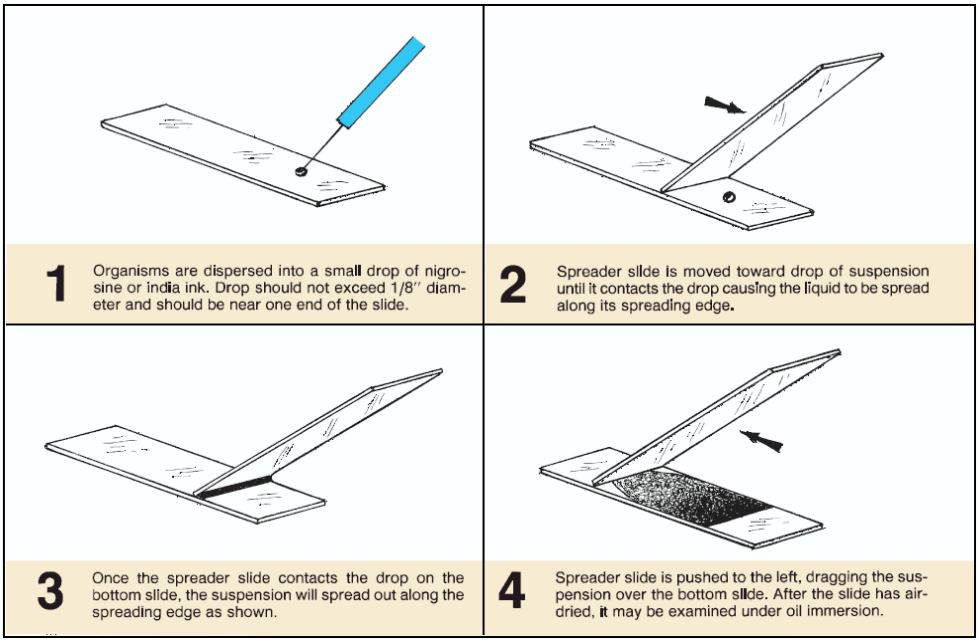


Figure 1: Negative staining technique

Smear Preparation and Simple Staining

Principles: While negative staining is satisfactory when making simple observations on bacterial morphology and size, more specific stains are necessary if bacterial detail is to be observed. One way of achieving this detail involves smear preparation and simple staining. A bacterial smear is a dried preparation of bacterial cells on a glass slide. The use of a single stain or dye to create contrast between the bacteria and the background is referred to as simple staining. Simple staining is often employed when information about cell shape, size, and arrangement is desired. Basic dyes such as crystal violet, carbolfuchsin, or methylene blue are often used, **Procedure of Smear Preparation as in the figure 2, Procedure of simple stain as in the figure 3 :**

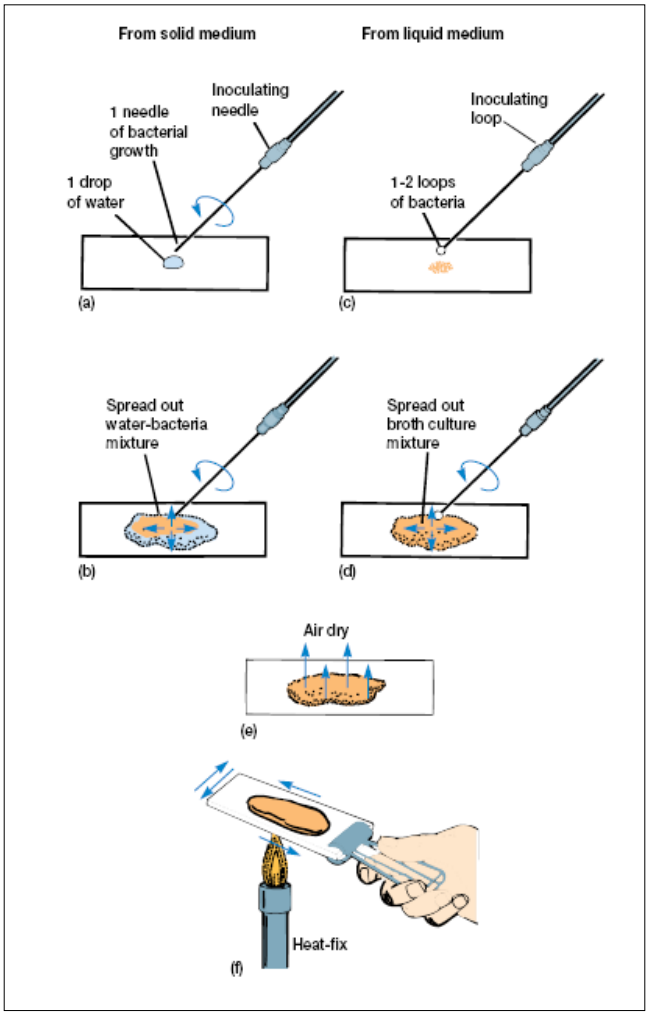


Figure 2: bacterial smear preparation

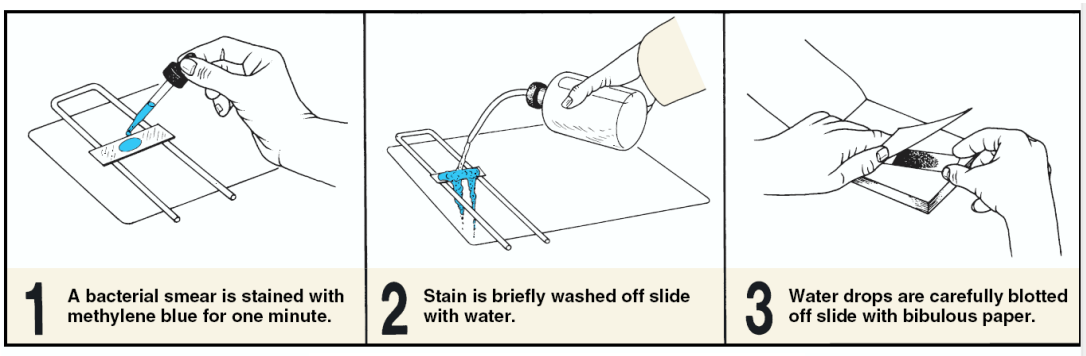


Figure 3: simple stain procedure

Gram Stain

Gram staining is the single most useful test in the clinical microbiology laboratory. It is the differential staining procedure most commonly used for the direct examination of specimens and bacterial colonies because it has a broad staining spectrum. The Gram stain is the first differential test run on a bacterial specimen brought into the laboratory for specific identification. The staining spectrum includes almost all bacteria, many fungi, and parasites.

Principles: Simple staining depends on the fact that bacteria differ chemically from their surroundings and thus can be stained to contrast with their environment. Bacteria also differ from one another chemically and physically and may react differently to a given staining procedure. This is the principle of differential staining. Differential staining can distinguish between types of bacteria. The Gram stain is the most useful and widely employed differential stain in bacteriology. It divides bacteria into two groups **gram negative and gram positive.** The first step in the procedure involves staining with the basic dye crystal violet. This is the primary stain. It is followed by treatment with an iodine solution, which functions as a mordant; that is, it increases the interaction between the bacterial cell and the dye so that the dye is more tightly bound or the cell is more strongly stained. The smear is then decolorized by washing with an agent such as 95% ethanol or isopropanol-acetone. Gram-positive bacteria retain the crystal violet-iodine complex when washed with the decolorizer, whereas gram-negative bacteria lose their crystal violet-iodine complex and become colorless.

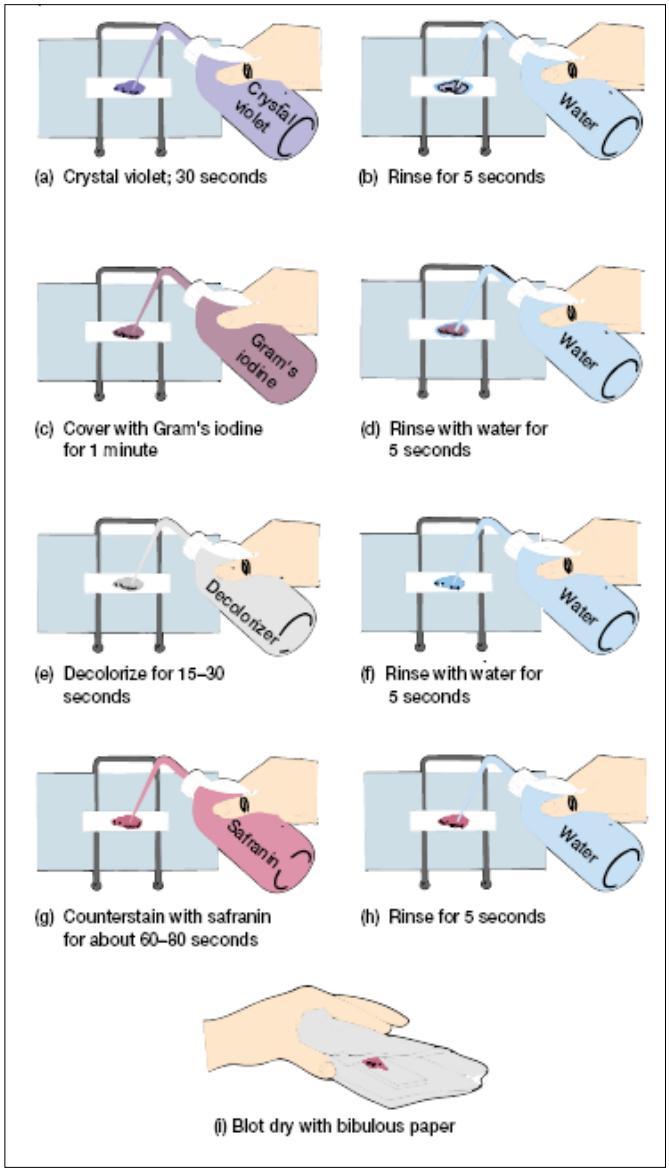


Figure 4 : Capsule Staining procedure

Finally, the smear is counterstained with a basic dye, different in color than crystal violet. This counterstain is usually safranin. The safranin will stain the colorless, gram-negative bacteria pink but does not alter the dark purple color of the gram-positive bacteria. **The end result is that gram-positive bacteria are deep purple in color and gram-negative bacteria are pinkish to red in color.**

Capsule Staining

Principles: Many bacteria have a slimy layer surrounding them, which is usually referred to as a capsule. The capsule's composition, as well as its thickness, varies with individual bacterial species. Polysaccharides, polypeptides, and glycoproteins have all been found in capsules. Often, a pathogenic bacterium with a thick capsule will be more virulent than a strain with little or no capsule since the capsule protects the bacterium against the phagocytic activity of the host's phagocytic cells. Two convenient procedures for determining the presence of a capsule are Anthony's capsule staining method (figure 5) and the Graham and Evans procedure. **Anthony's procedure** employs two reagents. The primary stain is crystal violet, which gives the bacterial cell and its capsular material a dark purple color. Unlike the cell, the capsule is nonionic and the primary stain cannot adhere. Copper sulfate is the decolorizing agent. It removes excess primary stain as well as color from the capsule. At the same time, the copper sulphate acts as a counterstain by being absorbed into the capsule and turning it a light blue or pink. In this procedure, smears should not be heat-fixed since shrinkage is likely to occur, because the capsule is a highly hydrated polymer, it will shrink dramatically when heat is applied or during other procedures in the staining process, and create a clear zone around the bacterium, which can be mistaken for a capsule.

Procedure Capsule Staining (Anthony's) (Figure 5)

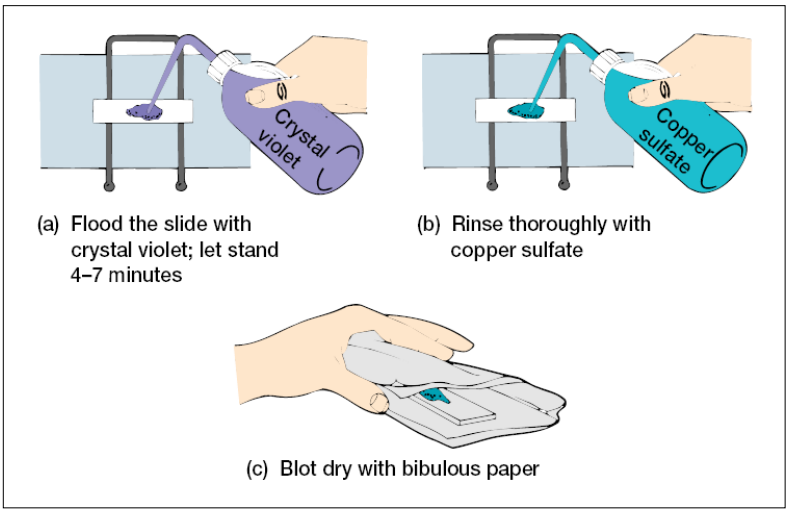


Figure 5: Capsule Staining procedure

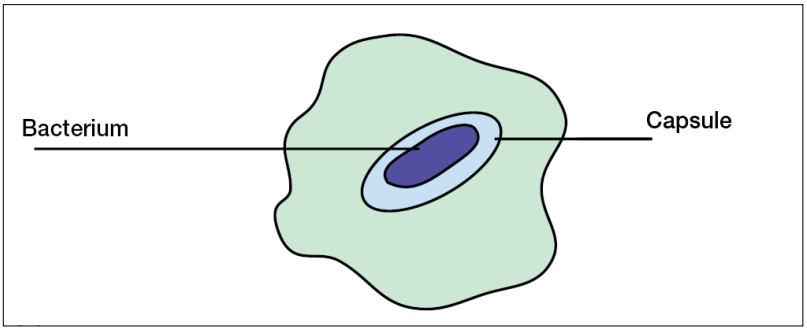
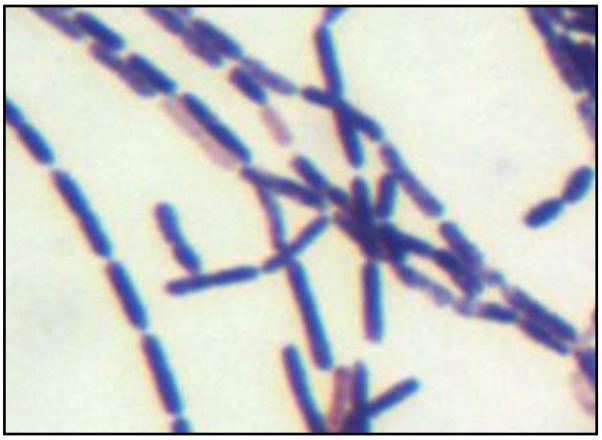
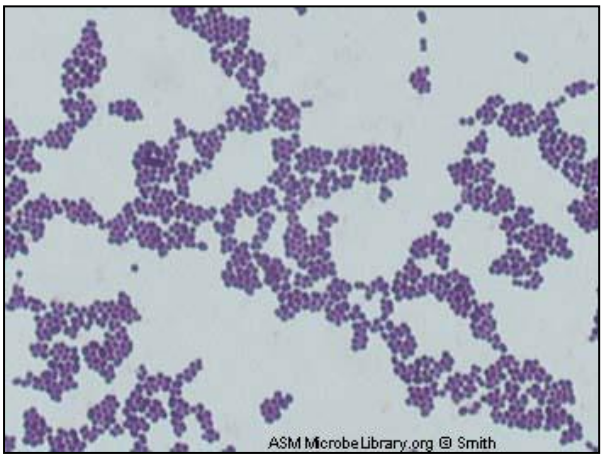


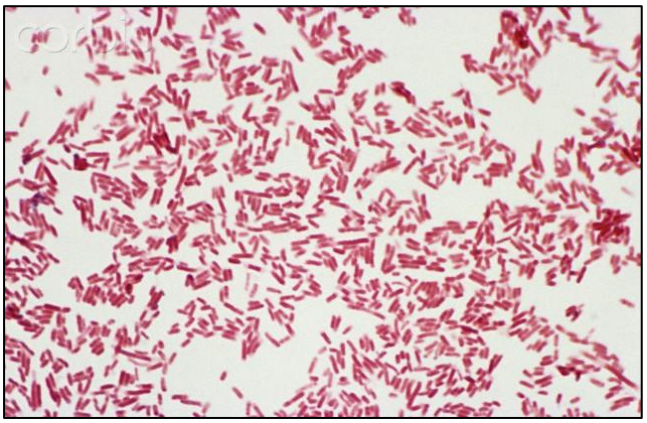
Figure 6: Anthony's Capsule Staining Method. Drawing of a single bacterium, capsule,and background material.



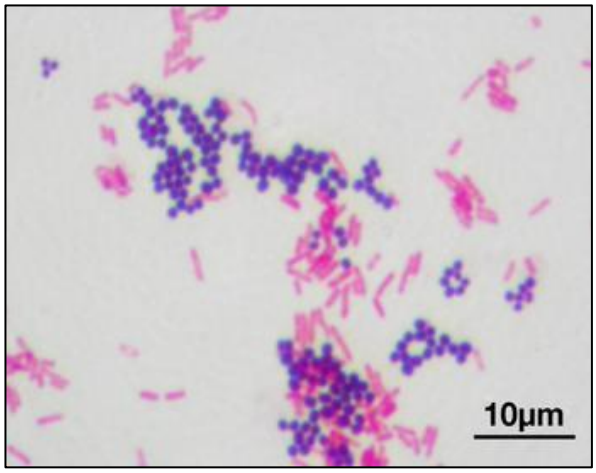
Gram stain - gram positive bacilli



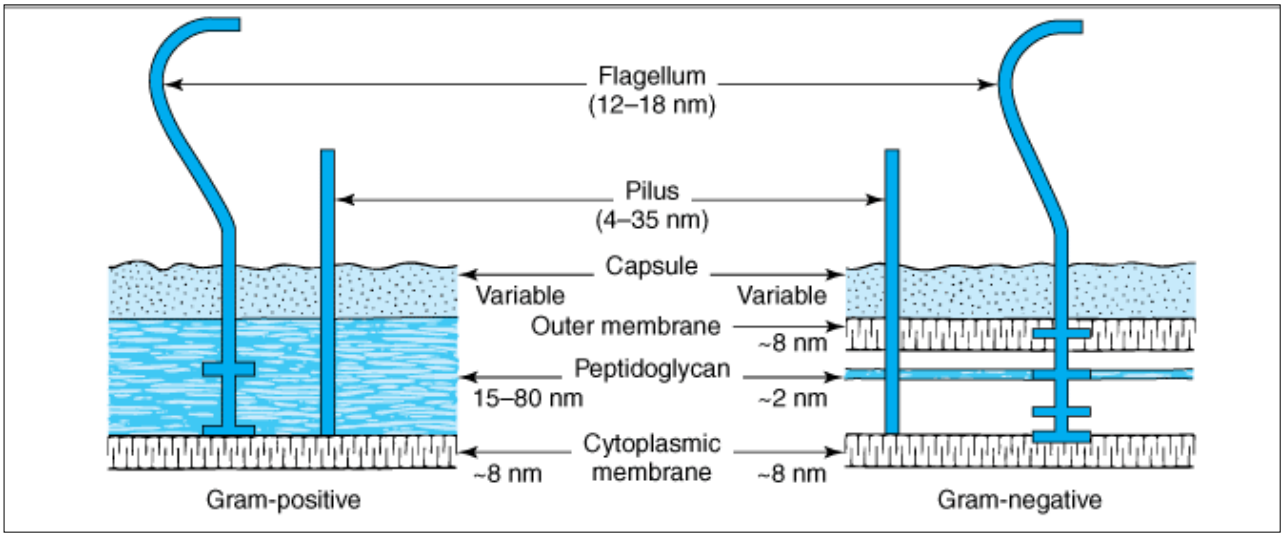
Gram stain - gram positive cocci



Gram stain - gram negative bacilli



a Gram-stained mixture of gram-positive (purple cocci) and gram-negative (pink rods).



Comparison of the structures of gram-positive and gram-negative cell envelopes. The region between the cytoplasmic membrane and the outer membrane of the gram-negative envelope is called the periplasmic space.

Family: Staphylococcaeae

Genus I: ***Staphylococcus***

- ***S. aureus***
- ***S. epidermidis***
- ***S. saprophyticus***
- ***S. lugdunensis***
- ***S. warneri***
- ***S. hominis***

Genus II: ***Micrococcus***

General Characteristics of Staphylococcus: The staphylococci are gram-positive cocci, spherical cells, usually arranged in grape-like irregular clusters. Single cocci, pairs, tetrads, and chains are also seen in liquid cultures. Staphylococci are non-motile and do not form spores. Oxidase negative and Catalase positive which differentiates them from the streptococci. Staphylococci are relatively resistant to drying, heat (they withstand 50 °C for 30 minutes), and 9% sodium chloride but are readily inhibited by certain chemicals, eg, 3% hexachlorophene. Some are members of the normal flora of the skin and mucous membranes of humans; others cause suppuration, abscess formation, a variety of pyogenic infections, and even fatal septicemia. Nasal carriage of *S aureus* occurs in 20–50% of humans.The pathogenic staphylococci often hemolyze blood, coagulate plasma, and produce a variety of extracellular enzymes and toxins. Micrococcus species often resemble staphylococci. They are found free-living in the environment and form regular packets of four or eight cocci. Their colonies can be yellow, red, or orange.

Culture and Growth Characteristics: *Staphylococci* grow readily on many types of media under aerobic or microaerophilic conditions. They grow most rapidly at 37 °C but form pigment best at room temperature (20–25 °C). Colonies on solid media are round, smooth, raised, and glistening. And are active metabolically, fermenting carbohydrates and producing pigments that vary from white to deep yellow. *S. aureus* usually forms gray to deep golden yellow colonies. *S. epidermidis* colonies usually are gray to white on primary isolation. Various degrees of hemolysis are produced by *S. aureus* and occasionally by other species.

Pathogenesis: The three major species included. ***S. aureus, S. epidermidis and S. saprophyticus*** the last two species are a virulent however under special circumstances where a suitable portal of enter is provided, they become virulent.

- ***S. epidermidis:*** may be etiological agent for skin lesion and endocarditis.
- ***S. saprophyticus:*** has been implicated in some urinary tract infection (UTI).
- ***S. aureus:*** are often cause:

1. Skin infection.
2. Abscess formation (pus- producing lesion).
3. Boils, acne and impetigo.
4. Infections of deeper tissue and organs include pneumonia, bacteremia, sepsis, endocarditis, meningitis osteomylitis and cystitis.
5. Enteritis due to enterotoxins contamination of food.
6. Toxic shock syndrome.

***Enzymes and Toxins*:**

1. **Catalase.**
2. **Coagulase and Clamping factor:** *S aureus* produces coagulase, an enzyme-like protein that clots oxalated or citrated plasma. Coagulase binds to prothrombin; together they become enzymatically active and initiate fibrin polymerization. Coagulase may deposit fibrin on the surface of staphylococci, perhaps altering their ingestion by phagocytic cells or their destruction within such cells.

Coagulase + prothrombin

Fibrinogen ⟶ **Fibrin clot**

activation

Clumping factor: is a surface *S aureus* compound that is responsible for adherence of the organisms to fibrinogen and fibrin. When mixed with plasma, *S aureus* forms clumps.

Clumping factor

Fibrinogen ⟶ **Fibrin clot**

3. **Nuclease (DNase):** extracellular enzyme which hydrolyse DNA or RNA to nucleotides which dissolve in acid. The enzyme act on phosphodiester bonds. DNase enzyme is medically important specially for ***S. epidermidis*** because it determines the pathogenicity of the strain. Normally ***S. epidermidis*** is non-pathogenic, it is normal floral of skin, and also can be isolated from upper respiratory tract, they are opportunistic and they can cause fatal infection if they reach the blood stream and colonized in heart causing endocarditis. Strain which produce DNase are pathogenic, non pathogenic strain do not produce DNase. All strain of ***S. aureus*** produce DNase**.**
4. **Hyaluronidase, or spreading factor.**
5. **Staphylokinase**
6. **Proteinases:** (gelatinase)
7. **Lipases.**
8. **β-lactamase:** which cause cleavage of β-lactam ring in the β-lactam antibiotics.
9. **Haemolysin:** lysis of RBCs.
10. **Lecocidin:** lysis of WBCs.
11. **Enterotoxin:** causes enteritis and food poisoning.
12. **Toxic Shock syndrome toxin and Exfoliative toxin**

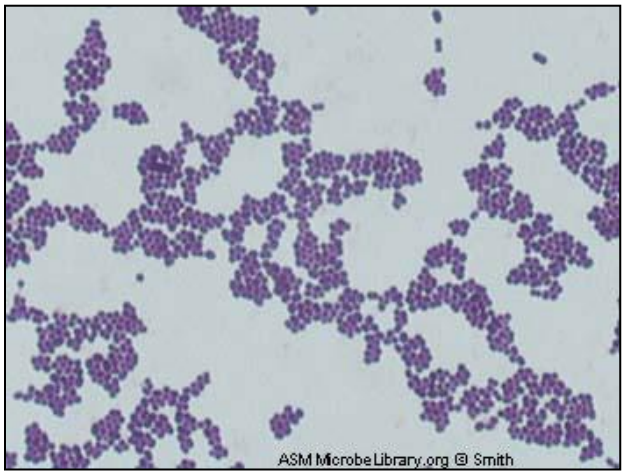
Specimens: Surface swab pus, blood, stool, sputum or tracheal aspirate, or spinal fluid for culture, depending upon the localization of the process.

Laboratory diagnostic tests:

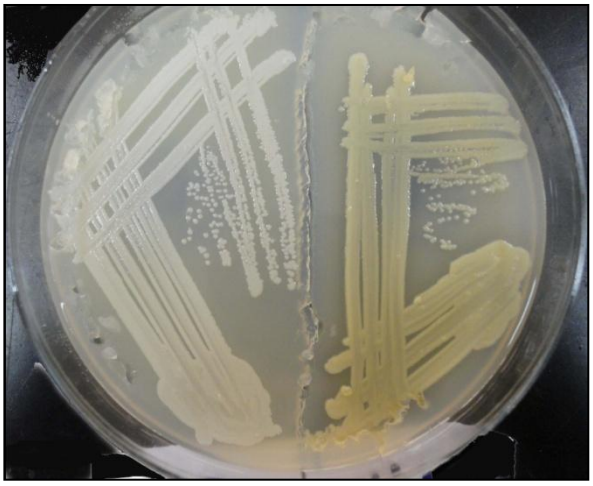
1. **Gram stain** (gram-positive cocci grape-like irregular clusters)

2. **Blood agar** (for haemolysis)
3. **Milk agar** (for pigments production)
4. **Staph 110** (selective media because it contains 7.5% NaCl).
5. **Mannitol salt agar (selective and differential media)** ***selective*** because it contains **7.5% NaCl.** ***Differential*** because it contains **mannitol sugar and phenol red as pH indicator** which differentiated between mannitol fermented Staphylococci e.g. *S. aureus* and mannitol non-fermented e.g. *S. epidermidis*.
6. **Gelatin liquefaction test**
7. **Catalase & Oxidase test**
8. **Coagulase test:** Citrated rabbit (or human) plasma diluted 1:5 is mixed with an equal volume of broth culture or growth from colonies on agar and incubated at 37 °C. A tube of plasma mixed with sterile broth is included as a control. If clots form in 1–4 hours, the test is positive.
9. **DNase test:** Heavily spot-inoculate DNase agar plate with Staphylococci bacteria over a 0.5-cm area, Incubate for 18 to 24 hours at 35°C. Then flood the DNase test agar plate with 1 N HCl. A zone of clearing around the colony indicates a positive DNase test. This clearing occurs because the large DNA molecule has been degraded the enzyme, and the end products dissolve in the added acid.

Test		*S. aureus*	*S. epidermidis*	*S. saprophyticus*
Pigment		**Yellow, Golden**	**White**	**White, Light yellow**
Mannitol salt agar	**Growth**	**+**	**+**	**+**
	Fermentation	**+**	**-**	**+/-**
Staph 110		**+**	**+**	**+**
Coagulase		**+**	**-**	**-**
DNase		**+**	**-/weak**	**-**
Haemolysis		**β**	**-/weak**	**-**
Catalase		**+**	**+**	**+**
Gelatin liquefaction		**+**	**-**	**-**



***Staphylococcus* Gr+ve cocci**
grape-like irregular clusters

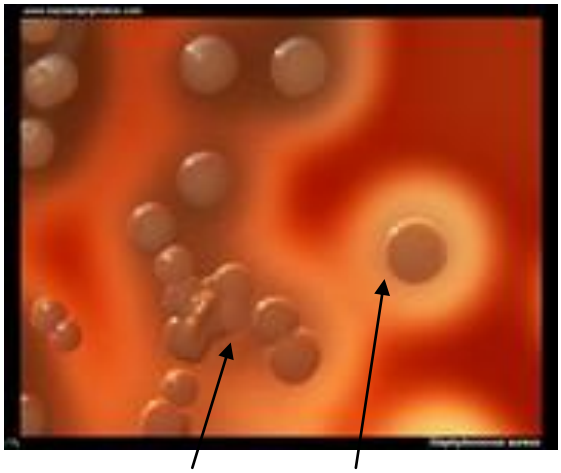


Pigments production on Milk agar

***S. epidermidis* & *S. aureus* on**
Mannitol salt agar

***S. aureus* on Mannitol salt agar**

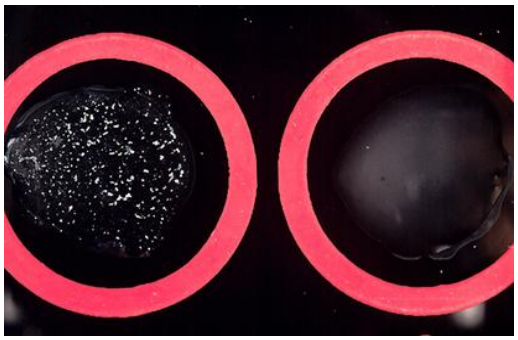
(α, β and γ) Haemolysis on Blood agar



α- Haemolysis **β-Haemolysis**

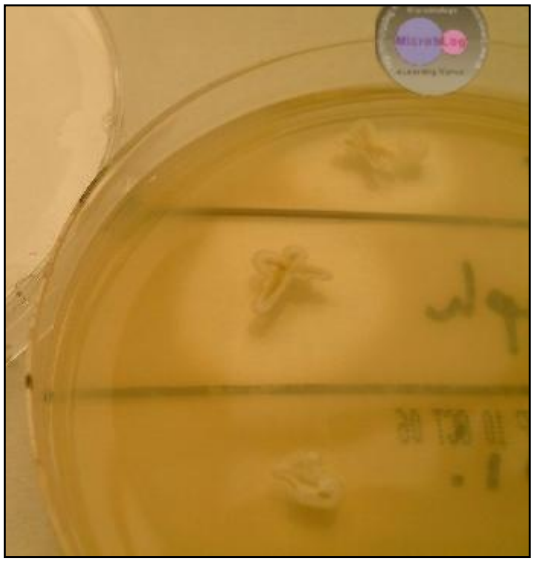
Coagulase Test (tube test)

Positive Coagulase Test

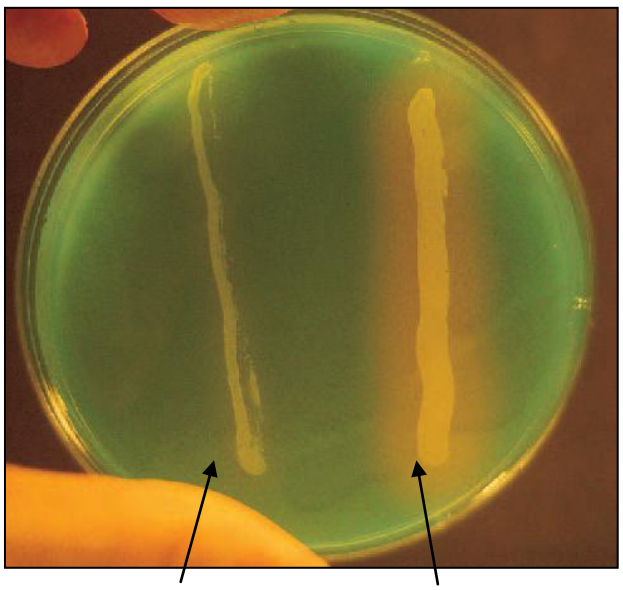


+ **-**

Clumping factor



DNase Test



S. epidermidis ***S. aureus***

DNase Test

Family: Streptococcaceae

Genus: Streptococcus

- ***S. pyogenes***
- ***S. agalactiae***
- ***S. equi***
- ***S. bovis***
- ***S. salvarius***
- ***S. mitis***
- ***S. mutans***

S.pneumonia

Family: Enterococcaceae

Genus: *Enterococcus faecalis*

General Characteristics: The streptococci are gram-positive cocci arranged in chains; however, as a culture ages and the bacteria die, they lose their gram-positivity and can appear to be gram-negative, this can occur after overnight incubation. Catalase and oxidase negative. They are widely distributed in nature. Some are members of the normal human flora. Several species of streptococci are normal inhabitants of the pharynx, they can also be isolated from surfaces of the teeth, the saliva, skin, colon, rectum, and vagina. Others are associated with important human diseases attributable in part to infection by streptococci. The pneumococci (*S. pneumoniae*) are diplococci, often lancet-shaped or arranged in chains, possessing a capsule of polysaccharide that permits typing with specific antisera. Pneumococci are readily lysed by surface-active agents, which probably remove or inactivate the inhibitors of cell wall autolysins.

Culture and Growth Characteristics: The colonies on the blood agar are circular, small, semi-translucent gray-white colonies. Growth of streptococci tends to be poor on solid media or in broth unless enriched with blood or tissue fluids. Nutritive requirements vary widely among different species. Growth and hemolysis are aided by incubation in 10% CO_2. Most pathogenic hemolytic streptococci grow best at 37°C. Most streptococci are facultative anaerobes and grow under aerobic and anaerobic conditions.

Classification and Pathogenesis: Streptococci are classified according to:

1. Their haemolytic activity (type of haemolysis α, β, γ)
2. Immunologic properties (the serological classification of Lancefield) serologic specificity of the cell wall group-specific substance and other cell wall or capsular antigens. According to this classification there are many serogroup associated with human disease that are groups A, B, C & D.
3. Resistance to chemical and physical factors.

4. Ecologic features: Molecular genetics have also been used to study the Streptococci.

Secimens: Specimens to be obtained depend upon the nature of the streptococcal infection. A throat swab, pus, or blood is obtained for culture. Serum is obtained for antibody determinations. CSF and sputum are collected for demonstration of pneumococci.

Laboratory diagnostic tests:

1. **Gram stain (**gram-positive cocci)
2. **Blood agar** (for type haemolysis)
3. **Carbohydrates fermentation** (glucose, inulin, mannitol & lactose)
4. **Bile solubility: in the** presence of bile salts (Na-deoxycholate or Na-taurocholate), the surface tension of this salts cause release of autolytic enzyme that cause lysis of the cells.

- ✓ Positive result: cell lysis (no growth.
- ✓ Negative result: growth of cell.

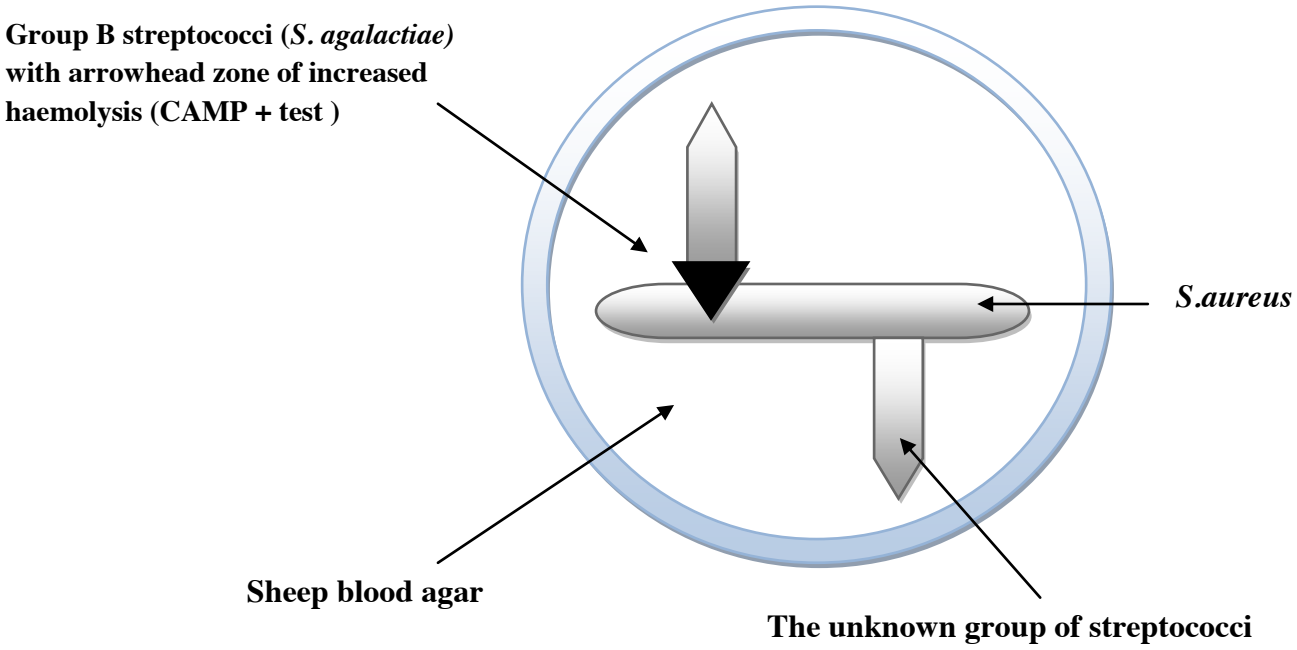
5. **Streptokinase test:** Streptokinase transforms the plasminogen of human plasma into plasmin, an active proteolytic enzyme that digests fibrin and other proteins. Indicator for this test is the lysis of the plasma clot (inverse coagulase), to make a clot in plasma $CaCl_2$ was added to the plasma then the growth of bacteria were added to see the Streptokinase production.

- ✓ Positive result: no clot
- ✓ Negative result: clot

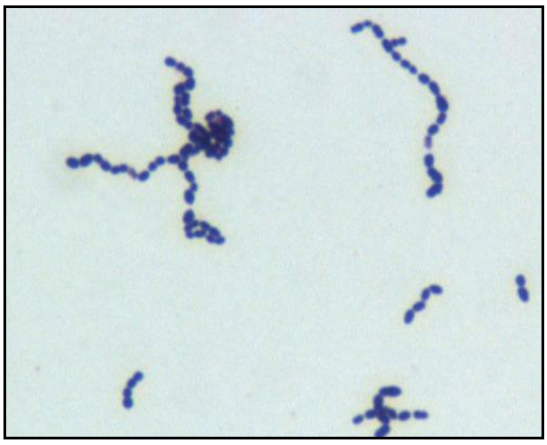
All species of streptococcus produce Streptokinase except ***S. pneumoniae***

6. **Bacitracin and Optochin Susceptibility:** ***S. pyogenes*** can be distinguished from viridans streptococci by means of the **bacitracin antibiotic disk (*S. pyogenes*** inhibited by Bacitracin)**. Optochin disks** are used for differentiation of the alpha hemolytic viridans streptococci from the pneumococci. The pneumococci are sensitive to these disks; the viridans organisms are resistant.
7. **Quelling reaction "capsule swelling test":** this test is performed for capsulated ***S. pneumoniae.*** When pneumococci are mixed with specific antiserum (anti-capsular Ag) on a microscope slide, the capsule swells, and the organisms agglutinate by cross-linking of the antibodies, indicating the positive result
8. **Salt Tolerance (6.5% NaCl):** All enterococci of group D produce heavy growth in 6.5% NaCl broth, none of the nonenterococci, group D, grow in this medium. This test, then, provides us with a good method for differentiating the two types of group D streptococci.
9. **CAMP Reaction:** Group B streptococci (***S.agalactiae***) can be distinguished from other β-hemolytic streptococci by their production of a substance called the CAMP factor. This factor is a peptide that acts together with the β-hemolysin produced by some strains of ***S. aureus***, enhancing the effect of the β-hemolysin on a sheep blood agar plate. With an inoculating loop, streak a strain of *S. aureus* down the centre of a blood agar plate. On one side of the plate, inoculate a standard strain of Group B streptococci (*S. agalactiae*) by making a streak at a 90° angle, starting 5 mm away from the *S.aureus* and extending outward to the edge of the agar. On the other side of the plate, inoculate the unknown strain of streptococci. This streak should not be directly opposite the Group B streak. Incubate the plate at 35°C for

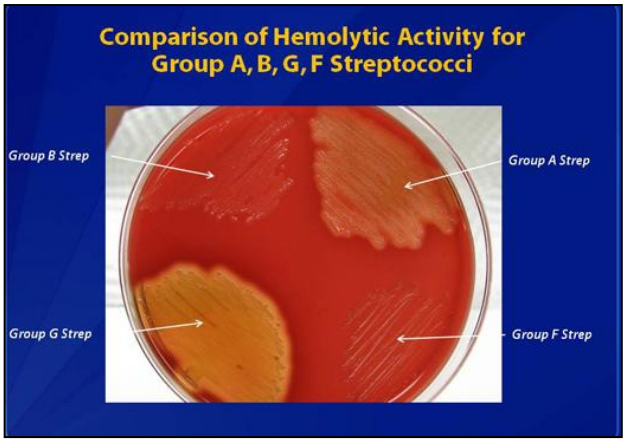
24 hours. Haemolysis of ***S.aureus*** interact with haemolysis of Group B streptococci (***S.agalactiae*****)** to form a flame shape or arrow head zone of increased haemolysis between the two genera, other groups of *Streptococcus* spp. does not show this shape.



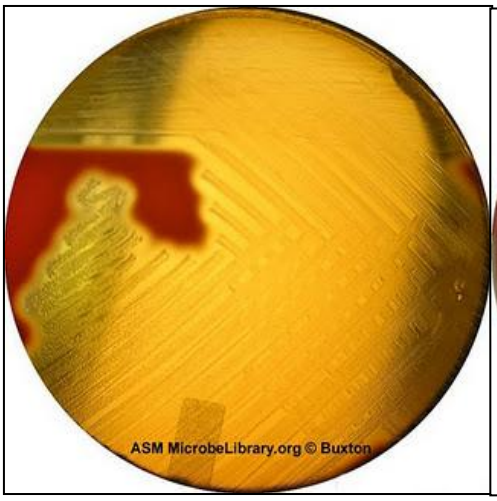
Test	*S. pneumoniae*	*S. salvarius*	*Enterococcus faecalis*	*S. pyogenes*
Inulin	**+**	**+/-**	**-**	**-**
Lactose	**-**	**+**	**+**	**+ no gas**
Mannitol	**-**	**-**	**+**	**+ no gas**
Glucose	**-**	**+**	**+**	**+ no gas**
Bile salt solubility	**+ no growth**	**- growth**	**- growth**	**- growth**
Optochin	**+ no growth**	**- growth**	**- growth**	**- growth**
Bacitracin	**- growth**	**- growth**	**- growth**	**+ no growth**
CAMP reaction	**-**	**-**	**-**	**-**
Haemolysis	**α**	**α**	**α**	**β**
Growth at 6.5% NaCl	**- no growth**	**- no growth**	**+ growth**	**+/-**
Streptokinase	**- clot**	**+ no clot**	**+ no clot**	**+ no clot**



Streptococci gram-positive cocci arranged in chains

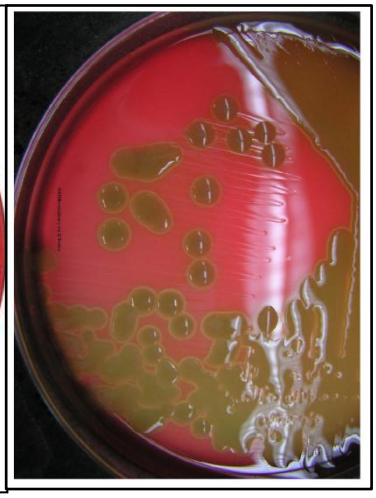


Types of blood haemolysis

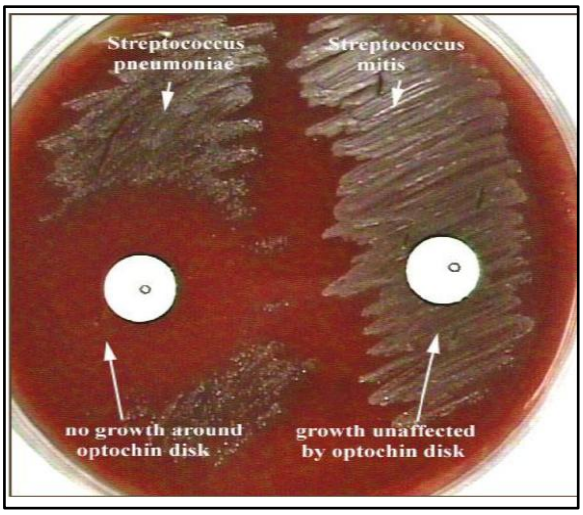


S. pyogenes

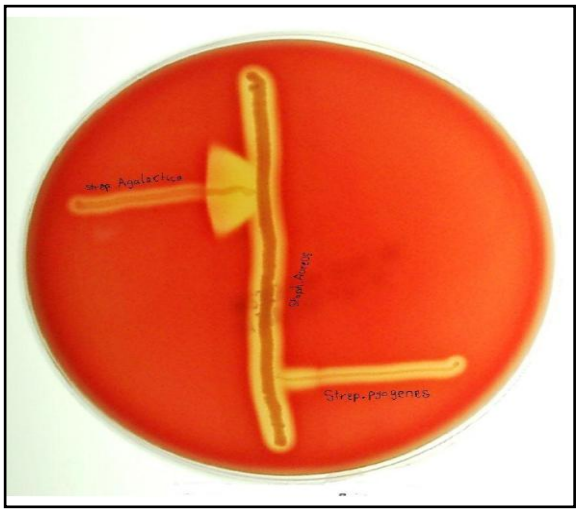
S.pneumoniae



Viridans streptococci



Optochin Susceptibility



CAMP Reaction

Family: Bacillaceae

Phylum: Firmicutes

Class: Bacilli

Order: Bacillales

Family: Bacillaceae

Genus: *Bacillus*

- ***B. anthracis***
- ***B. cereus***
- ***B. subtilis***

General Characteristics: The genus *bacillus* includes large aerobic, gram-positive rods have square ends and are arranged in long chains; spores are located in the center of the nonmotile bacilli. Most members of this genus are saprophytic organisms prevalent in soil, water, and air and on vegetation, such as *Bacillus cereus* and *Bacillus subtilis*. Some are insect pathogens. *B. cereus* can grow in foods and produce an enterotoxin or an emetic toxin and cause **food poisoning**. *B. anthracis,* which causes **anthrax,** is the principal pathogen of the genus. The spores are resistant to environmental changes, withstand dry heat and certain chemical disinfectants for moderate periods, and persist for years in dry earth. Animal products contaminated with anthrax spores (eg, hides, bristles, hair, wool, bone) can be sterilized by autoclaving.

Culture and Growth Characteristics: When grown on blood agar plates, the organisms produce non hemolytic gray to white round colonies with a rough texture and have a "cut glass" appearance in transmitted light. Comma-shaped outgrowths (Medusa head) may project from the colony. Hemolysis is uncommon with *B. anthracis* but common with the saprophytic bacilli. Carbohydrate fermentation is not useful. In semisolid medium, anthrax bacilli are always nonmotile, whereas related nonpathogenic organisms (eg, *B cereus*) exhibit motility by "swarming." Gelatin is liquefied, and growth in gelatin stabs resembles an inverted fir tree. The saprophytic bacilli utilize simple sources of nitrogen and carbon for energy and growth. Demonstration of capsule requires growth on bicarbonate-containing medium in 5–7% carbon dioxide.

Pathogenesis:

- ***B. anthracis*** causes **anthrax:** In humans, the infection is usually acquired by the entry of spores through injured skin (cutaneous anthrax) or rarely the mucous membranes (gastrointestinal anthrax), or by inhalation of spores into the lung (inhalation anthrax).
- ***B. cereus*** cause **Food poisoning,** eye infections, severe keratitis, endophthalmitis, panophthalmitis, localized infections and systemic infections, including endocarditis, meningitis, osteomyelitis, and pneumonia.

Specimens: Specimens to be examined are fluid or pus from a local lesion, blood, and sputum.

Laboratory diagnostic tests:

1. **Gram stains** (chains of large gram-positive rods).
2. **Blood agar**
3. **Pathogenicity in mouse:** Virulent anthrax cultures kill mice or guinea pigs upon intraperitoneal injection.
4. **Starch Hydrolysis: (*B. subtilis* is α-amylase positive)**

The starch molecule consists of two constituents: amylose, an unbranched glucose polymer and amylopectin, a large branched polymer. Both amylopectin and amylose are rapidly hydrolyzed by certain bacteria, using their α-amylases, to yield dextrins, maltose, and glucose, as follows:

Starch **α-amylase**

[Amylose + Amylopectin] $\longrightarrow$ **Dextrins + Maltose + Glucose**

(Large polysaccharide) **H_2O** **(Intermediate Polysaccharides)** **(Disaccharide)** **(Monosaccharide)**

Gram's iodine can be used to indicate the presence of starch. When it contacts starch, it forms a blue to brown complex. Hydrolyzed starch does not produce a color change. If a clear area appears after adding Gram's iodine to a medium containing starch and bacterial growth, α-amylase has been produced by the bacteria. If there is no clearing, starch has not been hydrolyzed.

Propriety	*B. anthracis*	*B. cereus*	*B. subtilis*
Motility	-	+	+
Capsule	+	+	-
Optimal growth temperature	37 ºC	30 ºC	37 ºC
Pathogenicity in mouse	+++	+	-

Treatment: Ciprofloxacin, penicillin G, along with gentamicin or streptomycin. Some other gram-positive bacilli, such as *B. cereus,* are resistant to penicillin by virtue of β-lactamase production. Doxycycline, erythromycin, or ciprofloxacin may be effective alternatives to penicillin.

Family: Clostridiaceae

Phylum: Firmicutes

Class: Clostridia

Order: Clostridiales

Family: Clostridiaceae

Genus: *Clostridium*

- ***C. botulinum***
- ***C. tetani***
- ***C. perfringens***
- ***C difficile***

General Characteristics: The clostridia are large anaerobic, gram-positive, motile rods. Many decompose proteins or form toxins, and some do both. Their natural habitat is the soil or the intestinal tract of animals and humans, where they live as saprophytes**.** Spores of clostridia are usually wider than the diameter of the rods in which they are formed. In the various species, the spore is placed centrally, subterminally, or terminally. Most species of clostridia are motile and possess peritrichous flagella.

Culture and Growth Characteristics: the clostridia grow well on the blood-enriched media used to grow anaerobes and on other media used to culture anaerobes as well. Some clostridia produce large raised colonies (eg, *C. perfringens*); others produce smaller colonies (e.g, *C. tetani*). Some clostridia form colonies that spread on the agar surface. Many clostridia produce a zone of haemolysis on blood agar. *C. perfringens* typically produces multiple zones of haemolysis around colonies. Clostridia can ferment a variety of sugars; many can digest proteins. Milk is turned acid by some and digested by others and undergoes "stormy fermentation" (ie, clot torn by gas) (e.g, *C. perfringens*). Various enzymes are produced by different species.

Pathogenesis:

- ***C. botulinum*** causes **botulism.**
- ***C. tetani*** causes **tetanus.**
- ***C. perfringens*** can produce invasive infection (including **myonecrosis** and **gas gangrene**) if introduced into damaged tissue. An enterotoxin of *C. perfringens* is a common cause of food poisoning.
- ***C. difficile*** causes **Pseudomembranous Colitis**

Laboratory diagnostic tests:

1. ***C. botulinum:*** Toxin can often be demonstrated in serum from the patient, and toxin may be found in leftover food. Mice injected intraperitoneally die rapidly. The antigenic type of toxin is identified by neutralization with specific antitoxin in mice. *C botulinum* may be grown from food remains and tested for toxin

production, but this is rarely done and is of questionable significance. Toxin may be demonstrated by passive hemagglutination or radioimmunoassay.

2. ***C. tetani*** **:**

- ***Specimens:*** wounds swb, exudates in tissue from wound, gram staining shows gram positive bacilli with drum-stick appearance.
- ***Culture:*** Specimens are inoculated on the blood agar or on cooked meat medium under anaerobic conduction, *C. tetani* produce swarming growth after 1-2 days of incubation.

3. ***C. perfringens:***

- ***Specimens:*** Specimens consist of material from wounds, pus, and tissue. The presence of large gram-positive rods in Gram-stained smears suggests gas gangrene clostridia; spores are not regularly present.

- ***Culture:*** Material is inoculated into **chopped meat-glucose** medium and **thioglycolate** medium and onto **blood agar** plates incubated anaerobically. *C perfringens* rarely produces spores when cultured on agar in the laboratory.
- ***Haemolysis on blood agar***
- ***Litmus milk reaction:*** The growth from one of the media is transferred into milk. A clot torn by gas in 24 hours is suggestive of *C. perfringens*.
- ***biochemical reactions*** (various sugars in thioglycolate)
- ***Lecithinase activity*** is evaluated by the precipitate formed around colonies on egg yolk media.

- ***Final identification*** **rests on toxin production and neutralization by specific antitoxin.**

Litmus milk reaction:

Medium: **Litmus milk broth contains:**

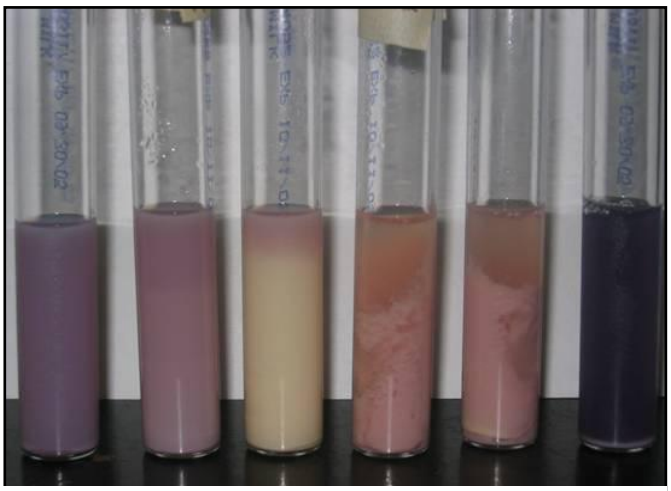
- **Milk that contains sugar lactose and protein casein**
- **Litmus: as pH indicator (purple color turn to pink color in acidic pH and to blue in alkaline pH) and oxidation-reduction indicator.**

Result:

1. **No color change**: remain purple, no change in the texture of the fluid.
2. **Acid production** (lactose fermentation as a result of production β-galactosidase enzyme): pink color
3. **Acid followed by reduction**: continued incubation may produce a white color at the bottom of the tube (reduced litmus) with a pink band at the top of the tube.
4. **Acid followed by reduction, followed by crud formation:** continued incubation may produce a crud at the bottom of the tube, as a result of produce a rennin enzyme from bacteria that causes casein to coagulate and form a rennet crud (clot) along with acid production and gas formation (CO_2 and/or H_2) and these gases may seen as separation of the crud, the presence of bubbles in the crud, or developed of tracts or

fissures in the crud. Some bacteria, such as clostridia, produce so much gas that the crud is torn to shreds. This is known as **stormy fermentation.**

5. **Proteolysis:** decrease in turbidity of milk due to loss of the colloid casein. Purple band at the top of the tube and a brown color throughout the rest of the tube. The milk also becomes watery.
6. **Alkaline reaction:** change in the color from purple to blue or dark blue as a result of decarboxylation or deaminationof the casein amino acids.

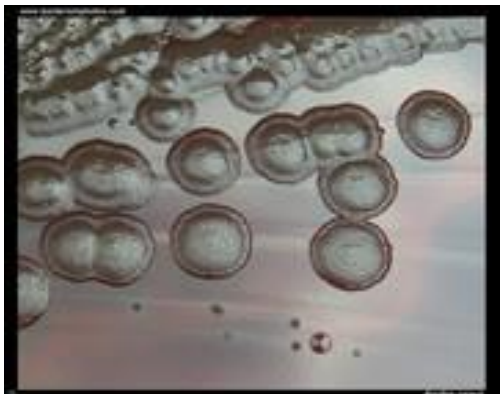


Control , pink=acid , white=reduction , stormy fermentation , blue=alkaline

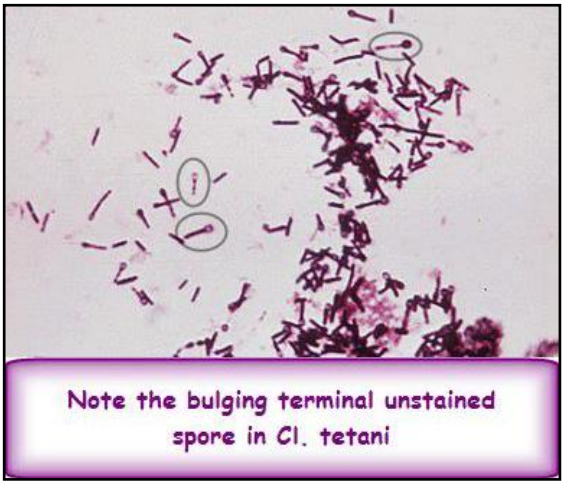
Litmus milk reaction

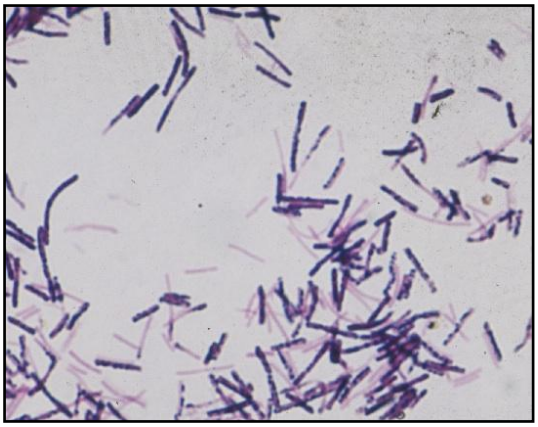
Colonies of on blood agar *B. cereus*

***Bacillus* chains of large gram-positive**



Colonies *B. cereus* of on blood agar





***Clostridium ssp.* gram-positive rods**

Family: Enterobacteriaceae

Domain: **Bacteria**
Phylum: **Proteobacteria**
Class: **Gammaproteobacteria**
Order: **Enterobacteriales**
Family: **Enterobacteriaceae**

Genus: ***Escherichia, Shigella, Salmonella, Enterobacter, Klebsiella, Serratia, Proteus***

General Characteristics **:** The Enterobacteriaceae are a large, heterogeneous group of gram-negative rods whose natural habitat is the intestinal tract of humans and animals. They are gram-negative rods, either motile or nonmotile; grow well on MacConkey's agar; grow aerobically and anaerobically (are facultative anaerobes); are catalase-positive, oxidase-negative, and reduce nitrate to nitrite Capsules are large and regular in *klebsiella*, less so in *enterobacter*, and uncommon in the other species.

Enterobacteriaceae can be subdivided into:

Enterobacteriaceae

Lactose fermented rapidly	**Lactose fermented slowly**	**Lactose non-fermenter**
Escherichia coli	***Edwardsiella***	***Salmonella* species**
Enterobacter aerogenes	***Serratia***	***Shigella* species**
***Klebsiella* species**	***Citrobacter***	***Proteus* species**
	Arizona	
	Providencia	
	Erwinia	

Lactose fermenter Enterobacteriaceae

Escherichia coli*:* gram-negative rods, motile, flat, nonviscous colonies. ***Can cause:***

1. Infants epidemic diarrhoea, indicate faecal contamination.
2. Urinary tract infection (UTI).
3. Wound infection.
4. Bloody diarrhoea.
5. Sepsis.
6. Meningitis.

Klebsiella:

- ***K. pneumoniae****:* very viscous, mucoid growth, nonmotile.

It causes**:** 1. a small proportion (about 1%) of bacterial pneumonias.
2. produce urinary tract infection
3. bacteremia.
4. hospital-acquired infections.

- ***K. oxytoca***: ***causes:*** hospital-acquired infections.

- ***Klebsiella ozaenae*** **and** ***Klebsiella rhinoscleromatis:*** ***<u>are associated</u>:*** with inflammatory conditions of the upper respiratory tract.

Enterobacter aerogenes: This organism has small capsules, may be found free-living as well as in the intestinal tract. ***<u>Causes</u>:***

1. Urinary tract infections**.**
2. Sepsis.

Serratia: S. marcescens is a common opportunistic pathogen in hospitalized patients. ***Serratia*** (usually nonpigmented) ***<u>causes</u>:***

1. Pneumonia.
2. Bacteremia.
3. Endocarditis.

Only about 10% of isolates form the red pigment (prodigiosin) that has long characterized *S. marcescens*.

Providencia: Providencia **species (*Providencia rettgeri, Providencia alcalifaciens,* and *Providencia stuartii*)** are members of the normal intestinal flora. ***<u>All cause</u>*** urinary tract infections and occasionally other infections and are often resistant to antimicrobial therapy.

Citrobacter: Citrobacter <u>can cause:</u> urinary tract infections and sepsis.

<u>Specimens</u> : Specimens included urine, blood, pus, spinal fluid, sputum, or other material, as indicated by the localization of the disease process. Specimens are plated on both blood agar and differential media.

<u>Culture and Growth Characteristics</u> : *E. coli* and most of the other enteric bacteria form circular, convex, smooth colonies with distinct edges. *Enterobacter* colonies are similar but somewhat more mucoid. *Klebsiella* colonies are large and very mucoid and tend to coalesce with prolonged incubation. Some strains of *E coli* produce haemolysis on blood agar.

<u>Classification</u> :

1. Serological classification upon antigens (O-Ag, H-Ag, K-Ag).
2. Biochemical classification and sugar fermentation.
3. DNA-DNA hybridization / G:C ratio.

<u>Laboratory diagnostic tests:</u>

1. **Gram stain** (Gram-negative rods).

2. **MacConkey agar (Selective and Differential media)**

<u>Selective because contains</u>: bile salts and crystal violet that inhibited the growth of Gram-positive bacteria and some fastidious Gram-negative bacteria.
<u>Differential because contains</u>: lactose as source of carbon and pH indicator neutral red (yellow at alkaline pH, pink at acid pH) that differential between lactose fermenter and non lactose fermenter.
Lactose fermenting bacteria produce a mixed of acids that conversion of the neutral red to red color so that produce pink to red colonies.

Lactose non fermenting bacteria produce colonies appear colorless or transparent.

3. Eosin methylene blue agar (EMB) (Selective and Differential media)

Selective because contains: the aniline dyes (eosin and methylene blue) that inhibited the growth of Gram-positive bacteria and some fastidious Gram-negative bacteria.

Differential because contains: the aniline dye also combines to form a precipitate at acid pH (appearing as a metallic green sheen) thus serving as indicator of acid production from lactose.

4. Triple Sugar Iron Agar (TSI):

The triple sugar iron (TSI) agar test is generally used for the identification of enteric bacteria (Enterobacteriaceae). It is also used to distinguish the Enterobacteriaceae from other gram-negative intestinal bacteria.

TSI agar slants contain:

- 0.1% glucose, 1% lactose, and 1% sucrose,
- pH indicator: phenol red (pink at alkaline pH, yellow at acid pH).
- Substrate for H_2S production: sodium thiosulfate (Na-thiosulfate)
- Indicator for H_2S production: ferrous sulphate ($FeSO_4$)

Na-thiosulfate + H_2 $\longrightarrow$ H_2S

$H_2S + FeSO_4 \longrightarrow FeS\downarrow$ (black precipitate)

Once H_2S is produced, it combines with the ferrous sulfate, forming an insoluble, black ferrous sulfide precipitate.

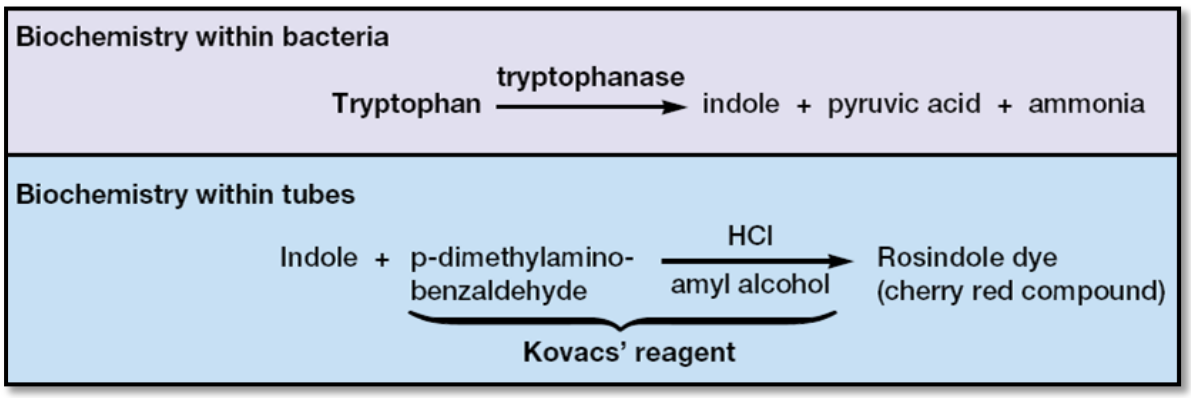
The results:

1. Yellow butt (A) and red slant (K) due to the fermentation of glucose. The slant remains red (alkaline) (K) because of the limited glucose in the medium and, therefore, limited acid formation, which does not persist.
2. Yellow butt (A) and slant (A) due to the fermentation of lactose and/or sucrose due to the high concentration of these sugars leading to excessive acid formation in the entire medium.
3. Red butt (K) and slant (K) indicates that none of the sugars were fermented and neither gas nor H_2S were produced.
4. Gas formation (CO_2) noted by bubbles or splitting of the agar.
5. Gas formation (H_2S) seen by blackening of the agar (black precipitate).

5. IMViC test (indole, methyl red, Voges-Proskauer, and citrate) (the "i" is for ease of pronunciation)

This test is using to the differentiation and identification of Family Enterobacteriaceae.

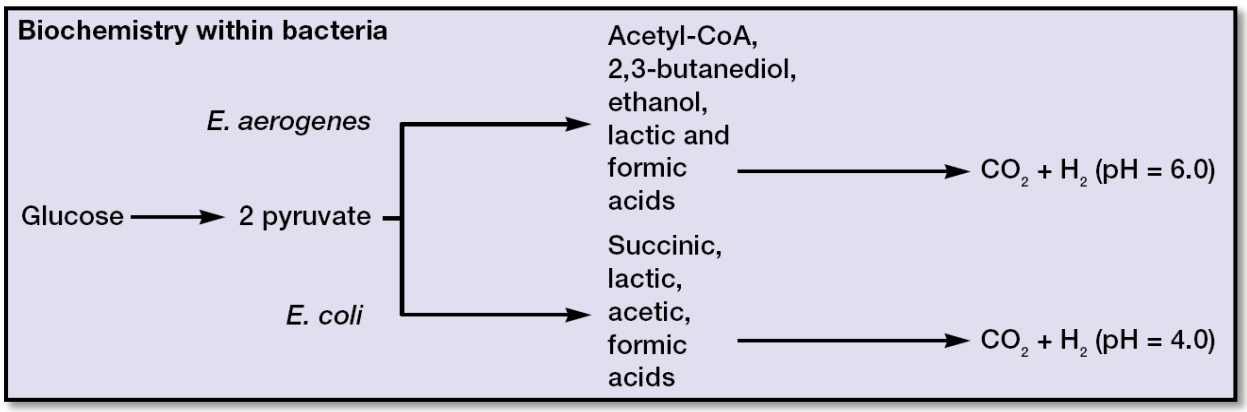
- **Indole Production:** This test is using to detect the production tryptophanase enzyme that can hydrolyze tryptophan (amino acid) to its metabolic products, namely, indole, pyruvic acid, and ammonia. The pyruvic acid and ammonia are using nutrition of bacteria while indole is not used and accumulates in the medium. Kovacs' reagent reacts with the indole, producing a bright red compound on the surface of the medium.
  - ***Medium: peptone water or tryptic soy broth.***
  - ***Substrate: tryptophan***
  - ***Reagent: Kovacs' reagent***



The results:

- ✓ Positive (+): red layer on the surface of the medium (red ring).
- ✓ Negative (-): the absence of a red color (no change).

- **Methyl Red test (MR):** glucose full fermentation (Mixed acids fermentation and thus acidify the medium). This test distinguishs between *E. coli* (a mixed acid fermenter) and *E. aerogenes* (a butanediol fermenter).
  - ***Medium: MR-VP broth medium***
  - ***Substrate: glucose***
  - ***Indicator: methyl red (yellow at alkaline pH, red at acid pH)***

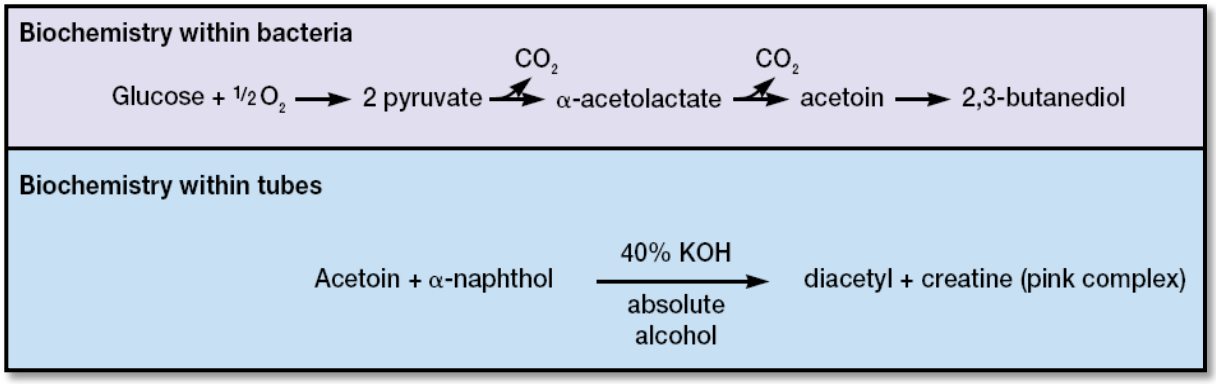


The results:

- ✓ Positive (+): red color
- ✓ Negative (-): yellow color

- **Voges-Proskauer test (VP):** glucose partial fermentation (ferment glucose, leading to 2,3-butanediol accumulation, the pH of the medium does not fall as low as during mixed acid fermentation). This test detect the presence of acetoin - a precursor in the synthesis of 2,3-butanediol.
  - ***Medium: MR-VP broth medium***
  - ***Substrate: glucose***

- ***Indicator: 40% KOH and 5% solution of alpha-naphthol in absolute ethanol (Barritt's reagent)***



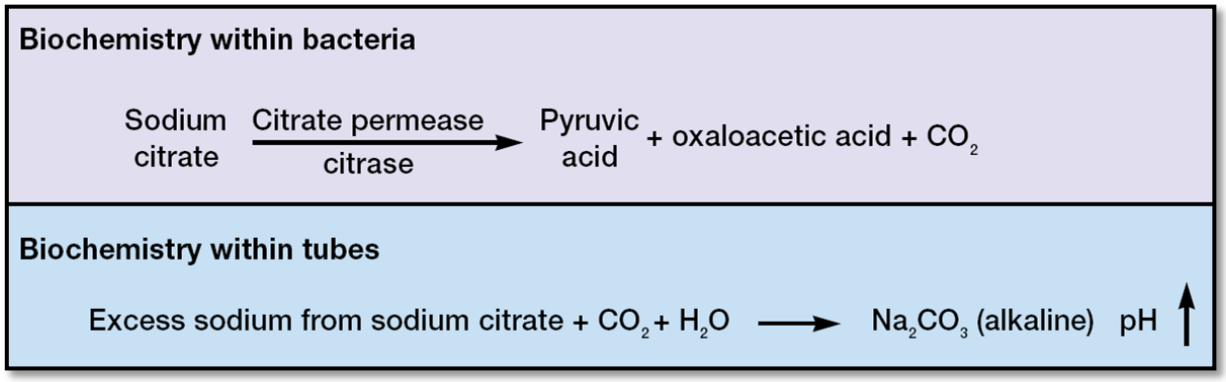
The results:

- ✓ Positive (+): Development of a red color in 15 minutes
- ✓ Negative (-): absence of a red color

➢ **Citrate Utilization Test:** This test determines the ability of bacteria to use citrate as a sole carbon source for their energy needs. This ability depends on the presence of a citrate permease that facilitates transport of citrate into the bacterium.

- ***Medium: Simmons citrate agar slants***
- ***Substrate: citrate***
- ***pH Indicator: bromothymol blue (blue at alkaline pH, yellow at acid pH)***

When bacteria oxidize citrate, they remove it from the medium and liberate CO_2. CO_2 combines with sodium (supplied by sodium citrate) and water to form sodium carbonate an alkaline



product. This raises the pH, turns the pH indicator to a blue color.

The results:

- ✓ Positive (+): blue color
- ✓ Negative (-): no color change and no growth in the medium

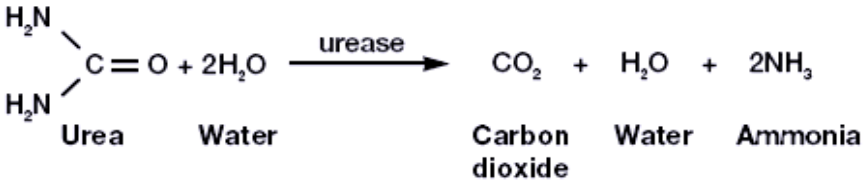
6. Urease test :

Some bacteria are able to produce an enzyme called urease that attacks the nitrogen and carbon bond in amide compounds such as urea, forming the end products ammonia, CO_2, and water. When urea is hydrolyzed, ammonia accumulates in the medium and makes it alkaline (increase in pH).

- ***Medium: urea broth***

- ***Substrate: urea***
- ***pH Indicator: phenol red***

Biochemistry within bacteria:



Biochemistry within tubes:

Ammonia + phenol red ⟶ **deep pink or purplish red**

The results:

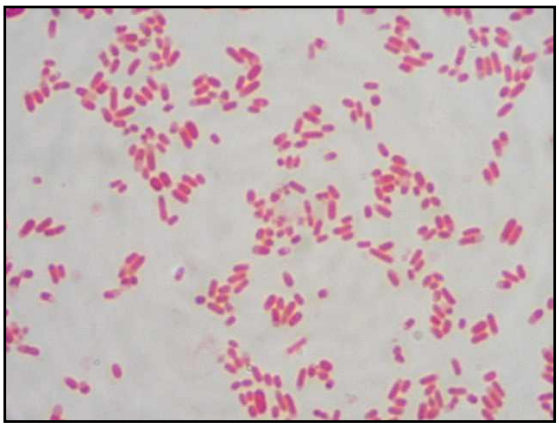
- ✓ Positive (+):deep pink or purplish red
- ✓ Negative (-): no deep pink color or (yellow color)

7. **Motility test:** stab method in semisolid media

8. **Sensitivity test:** on Mueller-Hinton Agar

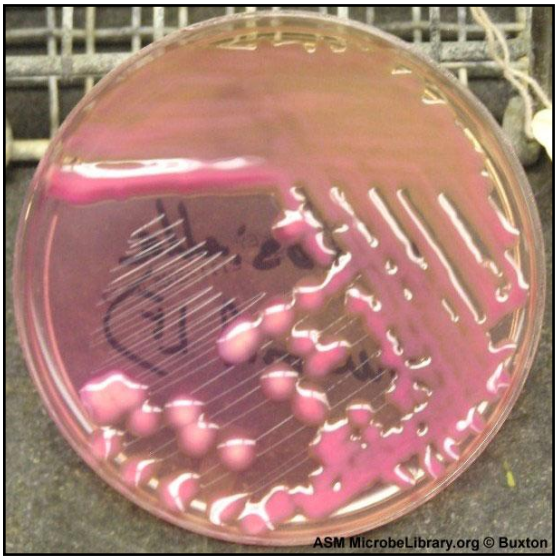
The sulfonamides, ampicillin, cephalosporins, fluoroquinolones, and aminoglycosides have marked antibacterial effects against the enterics, but variation in susceptibility is great.

Test	*E.coli*	*K. pneumoniae*	*Enterobacter*
MacConkey agar	**Pink colonies, smooth**	**Pink colonies, mucoid, larger than *E.coli***	**Pink colonies**
EMB	metallic green sheen	No metallic green sheen	No metallic green sheen
TSI	**A/A + -**	**A/A + -**	**A/A + -**
Indole	**+**	**-**	**-**
MR	**+**	**-**	**-**
VP	**-**	**+**	**+**
Citrate	**-**	**+**	**+**
Uraese	**-**	**+**	**-**
Motility	**+**	**-**	**+**

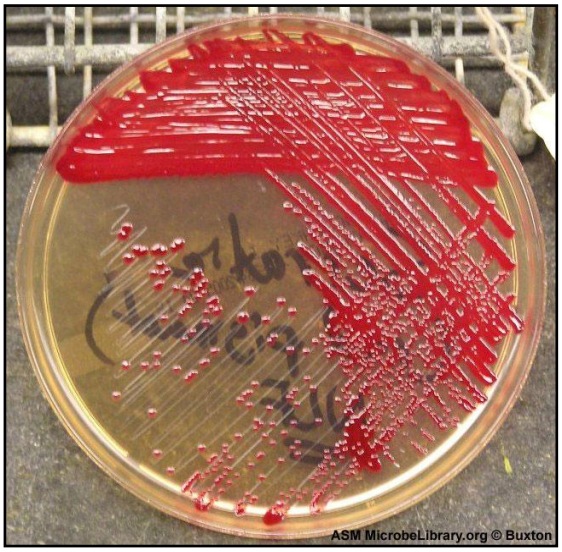


Gram stain (Gram-negative rods)

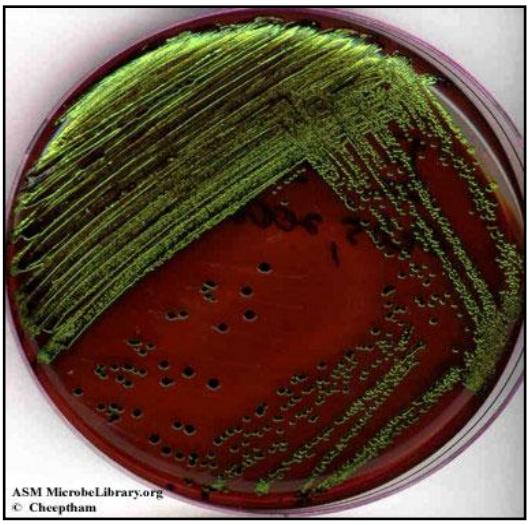
E. coli **on MacConkey agar (Lactose fermenter)**



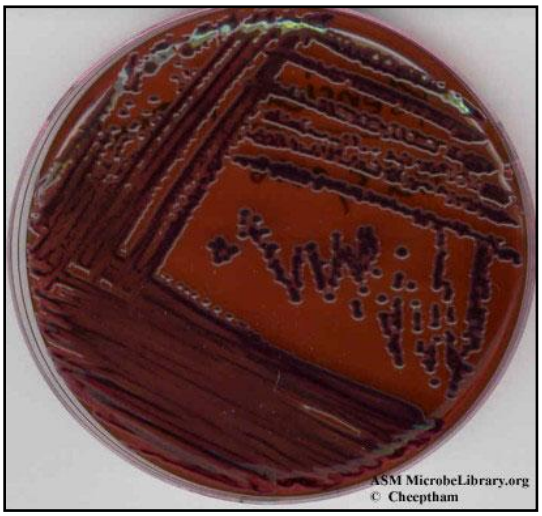
K. pneumoniae **on MacConkey agar (Lactose fermenter)**



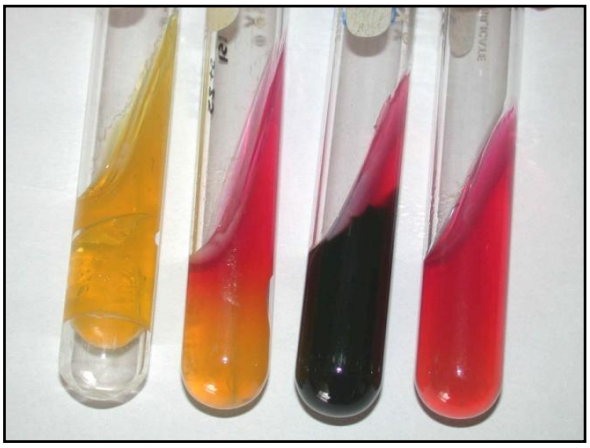
Serratia marcescens **on MacConkey agar (Lactose fermenter) with red pigment (prodigiosin)**



E. coli **on Eosin methylene blue agar (EMB) (Metallic green sheen)**

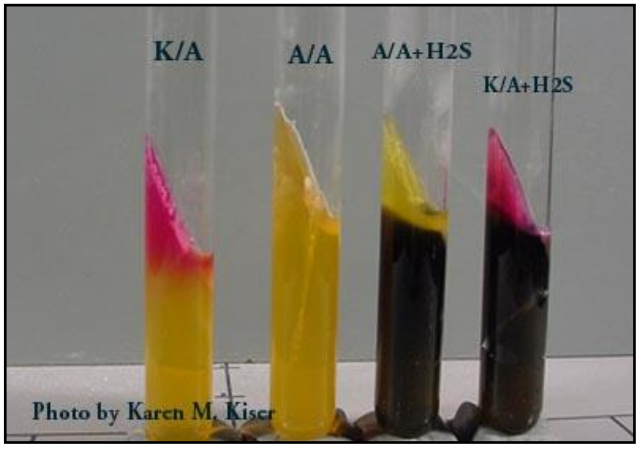


K. pneumoniae **on EMB agar (No metallic green sheen)**

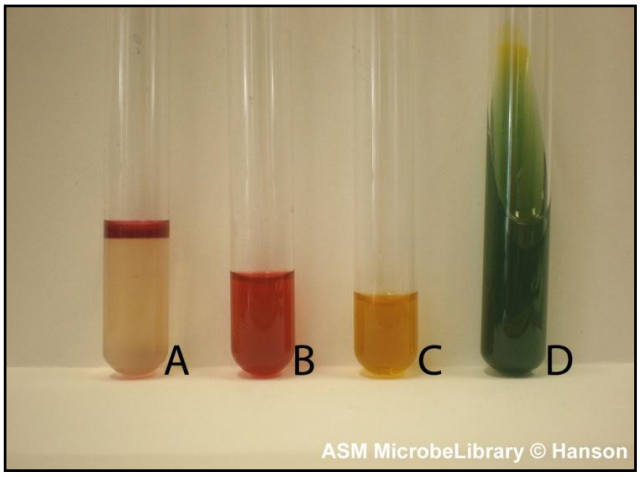


A/A + - K/A- - K/A- + K/K- -

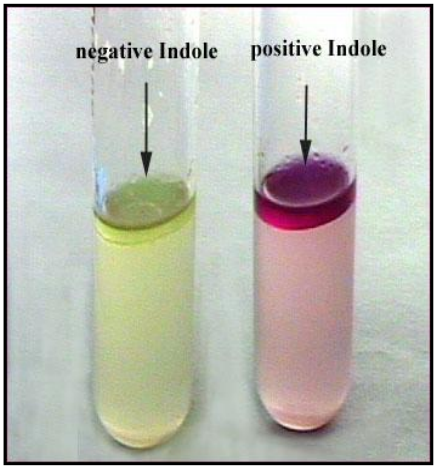
Triple Sugar Iron Agar (TSI)



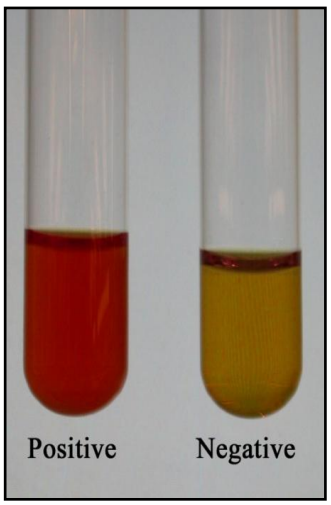
Triple Sugar Iron Agar (TSI)



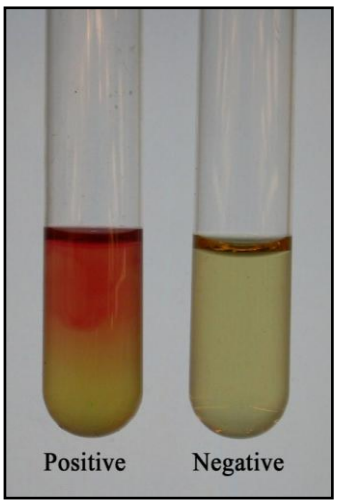
IMViC test



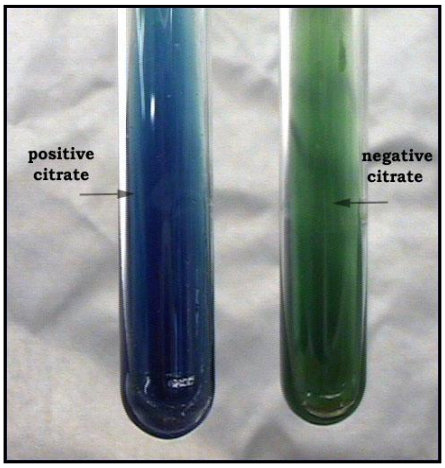
Indole test



Methyl red test



Voges-Proskauer test



Citrate Utilization Test

Family: Enterobacteriaceae

Lactose non-fermenter Enterobacteriaceae

Genus: *Proteus*

- ***Proteus vulgaris***
- ***P. mirabilis***
- ***P. penneri***

General Characteristics ***:*** gram-negative rods, pleomorphic, lactose non-fermenter, non capsulated, *Proteus* species move very actively by means of peritrichous flagella, resulting in "swarming" on solid media unless the swarming is inhibited by chemicals, eg, phenylethyl alcohol or CLED (cystine-lactose-electrolyte-deficient) medium, non capsulated, *Proteus* species are urease-positive . *Proteus* species produce urease, resulting in rapid hydrolysis of urea with liberation of ammonia. Thus, in urinary tract infections with *proteus*, the urine becomes alkaline, promoting stone formation and making acidification virtually impossible. The rapid motility of *proteus* may contribute to its invasion of the urinary tract. Strains of proteus vary greatly in antibiotic sensitivity. *P mirabilis* is often inhibited by penicillins; the most active antibiotics for other members of the group are aminoglycosides and cephalosporins.

Proteus species produce infections in humans only when the bacteria leave the intestinal tract. ***They are cause:***

1. urinary tract infections
2. bacteremia
3. pneumonia
4. and focal lesions in debilitated patients or those receiving intravenous infusions.
5. ***P. mirabilis*** is the major cause of human urinary tract infections and occasionally other infections.
6. ***P. vulgaris*** is important nosocomial pathogens.

Specimens: Specimens included urine, blood, pus, spinal fluid, sputum, or other material, as indicated by the localization of the disease process. Specimens are plated on both blood agar and differential media.

Laboratory diagnostic tests:

1. **Gram stain:** gram-negative rods, pleomorphic.
2. **Inoculation on selective and differential media e.g. MacConkey agar (Lactose non-fermenter)**
3. **Blood agar: to test swarming and haemolysis.**
4. **TSI.**
5. **IMViC test.**
6. **Urease test (positive): members of the genus *Proteus* can be distinguished from other enteric non lactose-fermenting bacteria (*Salmonella, Shigella*) by their fast urease activity.**
7. **Gelatin liquefaction tset.**
8. **Phenylalanine Deamination test.**
9. **Maltose and glucose fermentation.**

- ***Gelatin liquefaction test:*** When boiled in water, the connective tissue collagen (which is stringy, insoluble, and indigestible) changes into elatine, a soluble mixture of polypeptides. Certain bacteria are able to hydrolyze elatine by secreting a proteolytic enzyme called gelatinase. The resulting amino acids can then be used as nutrients by the bacteria. The ability of some bacteria to digest elatine is an important characteristic in their differentiation Gelatin hydrolysis can also be used to assess the pathogenicity of certain bacteria. The production of gelatinase can often be correlated with the ability of a bacterium to break down tissue collagen and spread throughout the body of a host.

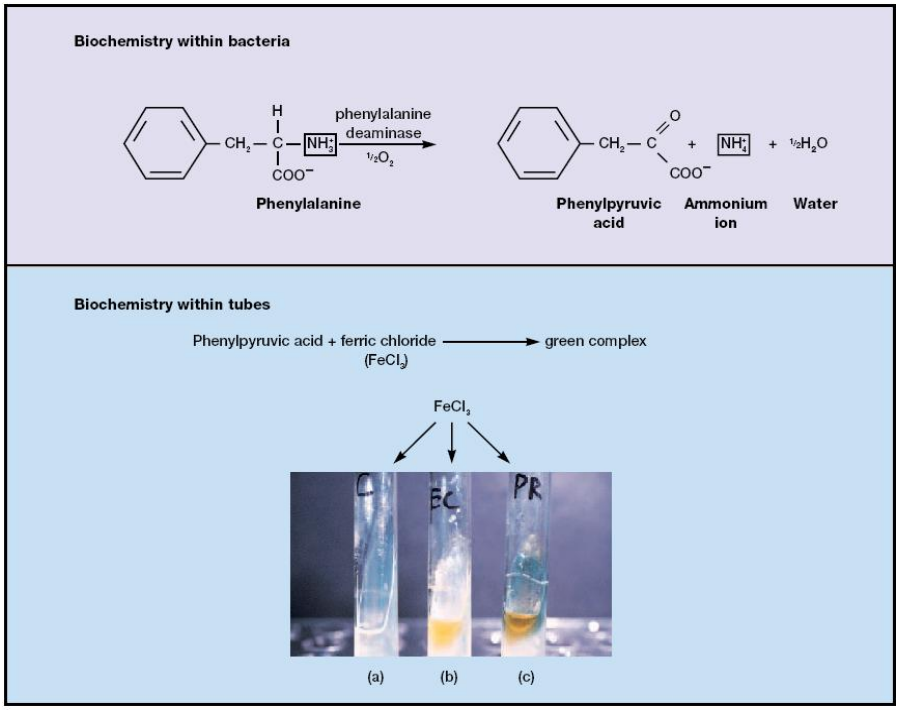
Gelatin liquefaction (the formation of a liquid) can be tested by:

1. Stabbing nutrient elatine deep tubes.
2. Following incubation, the cultures are placed in a refrigerator or ice bath at 4°C until the bottom resolidifies.
3. If elatine has been hydrolyzed, the medium will remain liquid after refrigeration. If elatine has not been hydrolyzed, the medium will resolidify during the time it is in the refrigerator.

Nutrient elatine may require up to a 14-day incubation period for positive results.

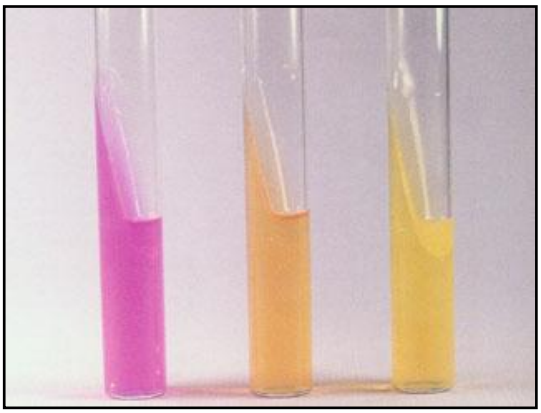
- ***Phenylalanine Deamination test***: Phenylalanine deaminase catalyzes the removal of the amino group (NH3+) from phenylalanine. This test can be used to differentiate among enteric bacteria such as *E. coli* (-) and *P. vulgaris*.(+) *P. vulgaris* produces the enzyme phenylalanine deaminase, which deaminates phenylalanine, producing phenylpyruvic acid. When ferric chloride is added to the medium, it reacts with phenylpyruvic acid, forming a green compound (figure 1).
- ***Medium: phenylalanine deaminase agar slants***
- ***Substrate: phenylalanine***
- ***pH Indicator: bromothymol blue***
- ***Reagent: ferric chloride (10% $FeCl_3$)***

Test	*P. vulgaris*	*P. mirabilis*
Urease	+	+
TSI	A/A + + K/A + +	A/A + + K/A + +
MacConkey agar	L.N.F.	L.N.F.
Indol	+	-
MR	+	+
VP	-	-
Citrate	+	+
Gelatin liquefaction	+	+
Phenylalanine deaminase	+	+
Maltose	+	-
Glucose	+	+
Motility	+	+
Blood agar	Swarming, haemolysis	Swarming, haemolysis



Phenylalanine Deamination. (a) Uninoculated control. (b) Phenylalanine negative. (c) Phenylalanine positive.

Gelatin liquefaction tset



+ control -

Urease test

***Proteus* spp. on MacConkey agar (Lactose non-fermenter)**

***Proteus* spp. on Blood agar (swarming)**

Family: Enterobacteriaceae

Lactose non-fermenter Enterobacteriaceae

Genus: *Salmonellae*

- ***Salmonella enterica* subspecies *enterica* serotype Typhimurium (*Salmonella* Typhimurium)**
- ***Salmonella* Typhi**
- ***Salmonella* Paratyphi A**
- ***Salmonella* Paratyphi B**
- ***Salmonella* Choleraesuis**
- ***Salmonella* Enteritidis**

General Characteristics ***:*** gram-negative rods, lactose non-fermenter, non capsulated, *Salmonellae* vary in length. Most isolates are motile with peritrichous flagella. *Salmonellae* grow readily on simple media, but they almost never ferment lactose or sucrose. They form acid and sometimes gas from glucose and mannose. They usually produce H_2S. urease negative. They survive freezing in water for long periods. *Salmonellae* are resistant to certain chemicals (eg, brilliant green, sodium tetrathionate, sodium deoxycholate) that inhibit other enteric bacteria; such compounds are therefore useful for inclusion in media to isolate *salmonellae* from feces.

Salmonellae are often pathogenic for humans or animals when acquired by the oral route. They are transmitted from animals and animal products to humans, where ***they cause***:

1. **The "Enteric Fevers" (Typhoid Fever):** Four serotypes of *salmonellae* that cause enteric fever can be identified in the clinical laboratory by biochemical and serologic tests. They are as follows: *Salmonella* Paratyphi A, *Salmonella* Paratyphi B, *Salmonella* Choleraesuis, and *Salmonella* Typhi.
2. **Bacteremia with Focal Lesions:** This is associated commonly with *S choleraesuis* but may be caused by any salmonella serotype.
3. **Enterocolitis.**

Specimens: included faeces (stool), urine, blood.

Laboratory diagnostic tests:

1. **Bacteriologic Methods for Isolation of *Salmonellae***

    - **Selective and Differential Medium Cultures: EMB**, **MacConkey's** and **deoxycholate medium** permits rapid detection of lactose non-fermenters (not only *salmonellae* and *shigellae* but also *proteus, serratia, pseudomonas*, etc). Also the specimen is plated on **salmonella-shigella agar (S.S agar) : *Selective*** because it contains bile salts for G+ve bacteria inhibition and G-ve other than Enterobacteriaceae and brilliant green for the inhibition of coliform and other Enterobacteriaceae. ***Differential*** because it contains Na-thiosulfate, ferric citrate for H_2S production and neutral red as indicator (dose not need autoclaving because it includes inhibitors)**, Hektoen enteric agar, XLD (xylose lysine deoxycholate)** contain (xylose, lactose, sucrose and phenol red as indicator), **DCA (deoxycholate-citrate agar)** which favor growth of *salmonellae* and *shigellae* over other

Enterobacteriaceae. **Bismuth sulfite medium** permits rapid detection of *salmonellae* which form black colonies because of H_2S production.

- **Enrichment Cultures:** The specimen (usually stool) also is put into **selenite F** or **tetrathionate broth**, both of which inhibit replication of normal intestinal bacteria and permit multiplication of *salmonellae*. After incubation for 1–2 days, this is plated on differential and selective media.

2. **Gram stain:** gram-negative rods
3. **TSI.**
4. **IMViC test.**
5. **Urease test**
6. **Mannitol and glucose fermentation**
7. **Serologic Methods**

- **Agglutination Test:** In this test, known sera (commercial kits) and unknown culture are mixed on a slide. Clumping, when it occurs, can be observed within a few minutes. This test is particularly useful for rapid identification of cultures.
- **Tube Dilution Agglutination Test (Widal Test):** Serum agglutinins rise sharply during the second and third weeks of *Salmonella* Typhi infection. The Widal test to detect these antibodies against the O and H antigens has been in use for decades. Serial dilutions of unknown sera are tested against known antigens from representative *salmonellae*. A titer against the O antigen of > 1:320 and against the H antigen of > 1:640 is considered positive. High titer of antibody to the Vi antigen occurs in some carriers.

Test	*S.* Typhi	*S.* Paratyphi A	*S.* Paratyphi B	*S.* Typhimurium
TSI	**K/A + +**	**K/A + +**	**K/A + +**	**K/A + +**
MacConkey agar	**L.N.F.**	**L.N.F.**	**L.N.F.**	**L.N.F.**
Indol	**-**	**-**	**-**	**-**
MR	**+**	**+**	**+**	**+**
VP	**-**	**-**	**-**	**-**
Citrate	**-**	**+**	**+**	**+**
Glucose	**+ no gas**	**+ no gas**	**+ no gas**	**+ no gas**
Mannitol	**+ no gas**	**+ with gas**	**+ with gas**	**+ with gas**
Motility	**+**	**+**	**+**	**+**
Urease	**-**	**-**	**-**	**-**
S.S. agar	**L.N.F. (colorness) with H_2S**	**L.N.F. (colorness) with H_2S**	**L.N.F. (colorness) with H_2S**	**L.N.F. (colorness) with H_2S**

Genus: ***Shigellae***

- ***S. dysenteriae***
- ***S. flexneri***
- ***S. boydii***
- ***S. sonnei***

General Characteristics **:** *Shigellae* are slender gram-negative rods; coccobacillary forms occur in young cultures, non-motile, do not produce H_2S, lactose non-fermenter, non capsulated. Facultative anaerobes but grow best aerobically. Convex, circular, transparent colonies with intact edges reach a diameter of about 2 mm in 24 hours. All shigellae ferment glucose, with the exception of *Shigella sonnei*. *Shigellae* form acid from carbohydrates but rarely produce gas. They may also be divided into those that ferment mannitol and those that do not. Urease negative. The natural habitat of *shigellae* is limited to the intestinal tracts of humans and other primates. ***They cause:***

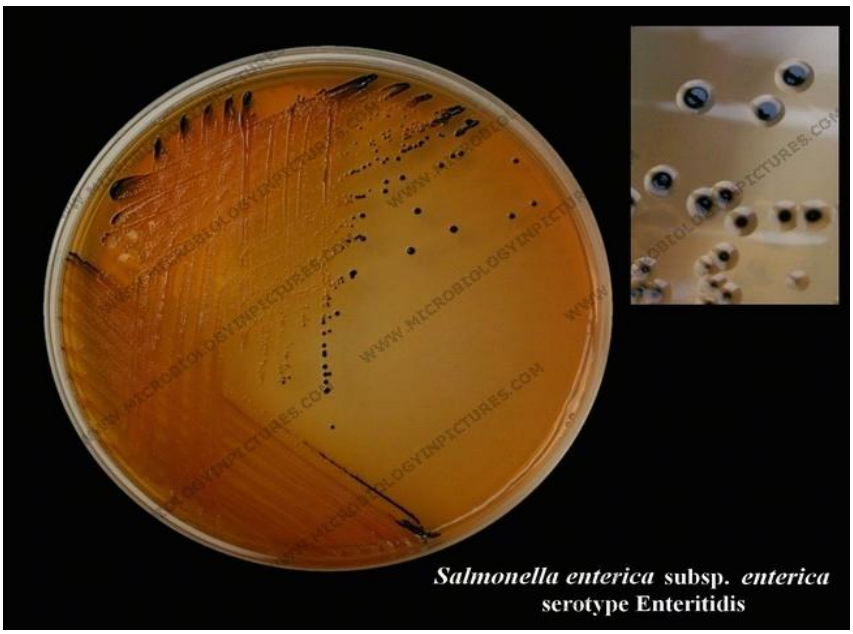
- ✓ Bacillary dysentery.
- ✓ Shigella infections are almost always limited to the gastrointestinal tract; bloodstream invasion is quite rare.

Specimens: included fresh stool, mucus flecks, and rectal swabs for culture. Large numbers of fecal leukocytes and some red blood cells often are seen microscopically.

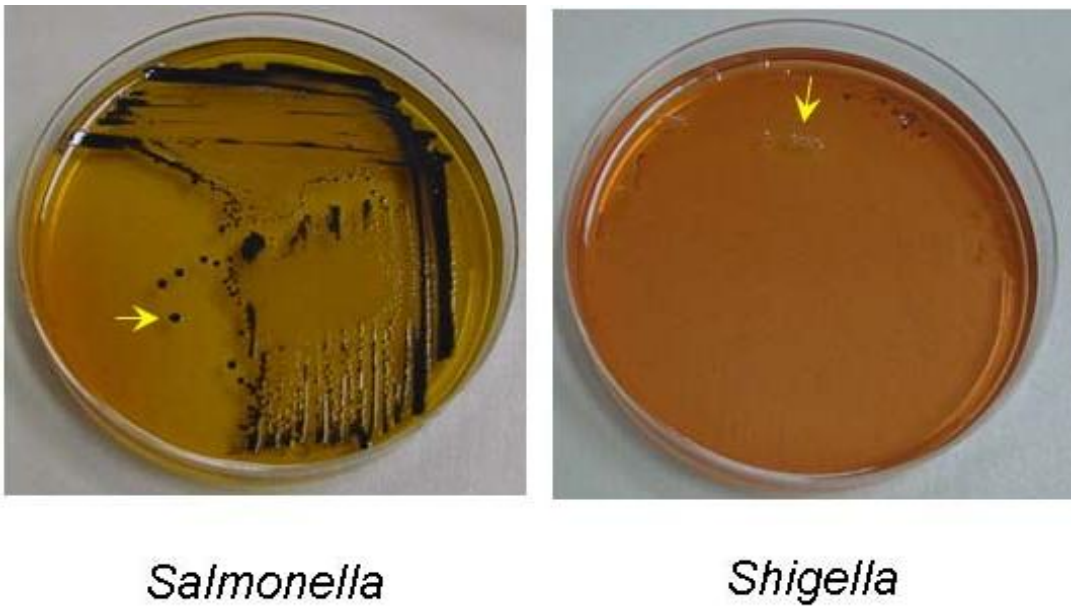
Laboratory diagnostic tests:

1. **Selective and Differential Medium Cultures:** (eg, MacConkey's or EMB agar, Hektoen enteric agar or salmonella-shigella agar, XLD, DCA)
2. **Gram stain:** gram-negative rods
3. **TSI.**
4. **IMViC test.**
5. **Urease test**
6. **Motility test (non-motile).**

Test	*S.dysenteriae*	*S. flexneri*	*S. sonnei*	*S. boydii*
TSI	K/A - -	K/A - -	K/A - -	K/A - -
MacConkey agar	L.N.F.	L.N.F.	L.N.F.	L.N.F.
Indol	+/-	+/-	-	+/-
MR	+	+	+	+
VP	-	-	-	-
Citrate	-	-	-	-
Glucose	+ no gas	+ no gas	-	+ no gas
Mannitol	-	+ no gas	+ no gas	+ no gas
Motility	-	-	-	-
Urease	-	-	-	-
S.S. agar	L.N.F. transparent	L.N.F. transparent	L.N.F. transparent	L.N.F. transparent



Salmonella enterica **on Salmonella-Shigella agar (S.S. agar)**



Salmonella-Shigella agar (S.S. agar)

Family: Pseudomonadaceae

Phylum: Proteobacteria

Class: Betaproteobacteria

Order: Pseudomonadales

Family: Pseudomonadaceae

Genus: *Pseudomonas*

Pseudomonas **Fluorescent group**	***Pseudomonas aeruginosa***
	Pseudomonas fluorescens
	Pseudomonas putida
Nonfluorescent group	***Pseudomonas stutzeri***
	Pseudomonas mendocina

Pseudomonas aeruginosa

General Characteristics : *P. aeruginosa* is widely distributed in nature and is commonly present in moist environments in hospitals. *P aeruginosa* is **gram-negative, Motile by one or several polar flagella and rod-shaped**. It is occurs as single bacteria, in pairs, and occasionally in short chains. It is **catalase-positive**, **oxidase-positive**, **strict aerobic.**

Culture and Growth Characteristics: *P. aeruginosa* is an obligate aerobe that grows readily on many types of culture media, sometimes producing a sweet or grape-like or corn taco-like odor. Some strains hemolyze blood. *P. aeruginosa* forms smooth round colonies with a fluorescent greenish color. It often produces the nonfluorescent bluish pigment **pyocyanin,** which diffuses into the agar. Other *Pseudomonas* species do not produce pyocyanin. Many strains of *P. aeruginosa* also produce the fluorescent pigment **pyoverdin,** which gives a greenish color to the agar. Some strains produce the dark red pigment **pyorubin** or the black pigment **pyomelanin.** *P. aeruginosa* grows well at 37–42 °C; its growth at 42 °C helps differentiate it from other *Pseudomonas* species in the fluorescent group. It does not ferment carbohydrates, but many strains oxidize glucose. Specimens are plated on blood agar and the differential media commonly used to grow the enteric gram-negative rods. Pseudomonads grow readily on most of these media, but they may grow more slowly than the enterics. *P aeruginosa* does not ferment lactose and is easily differentiated from the lactose-fermenting bacteria.

Pathogenesis:

1. *P. aeruginosa* produces infection of wounds and burns, giving rise to blue-green pus.
2. Meningitis.
3. Urinary tract infection,
4. Necrotizing pneumonia.
5. It may cause invasive (malignant) otitis externa in diabetic patients.
6. Infection of the eye, which may lead to rapid destruction of the eye, occurs most commonly after injury or surgical procedures.
7. *P aeruginosa* may invade the bloodstream and result in fatal sepsis.

Enzymes and Toxins: Most *P*. *aeruginosa* isolates from clinical infections produce extracellular enzymes, including elastases, proteases, and haemolysins: a heat-labile phospholipase C and a heat-stable glycolipid. Many strains of *P*. *aeruginosa* produce exotoxin A, which causes tissue necrosis and is lethal for animals when injected in purified form. The toxin blocks protein synthesis

Classification:

1. *P aeruginosa* can be typed by lipopolysaccharide immunotype.
2. By pyocin (bacteriocin) susceptibility.
3. rRNA/DNA homology.
4. Serological (H-Ag, O-Ag).
5. Phage typing.

Specimens: Skin lesions, pus, urine, blood, spinal fluid, sputum, and other material should be obtained as indicated by the type of infection.

Laboratory diagnostic tests:

1. **Gram stain (Gram-negative bacilli).**
2. **Culture on blood agar (for haemolysis)**
3. **Culture on milk agar (for pigmentation)**
4. **Culture on MacConkey agar (lactose-non fermenter)**
5. **Culture on Selective and Differential media (e.g.; King A, King B, Cetrimide agar)**
6. **TSI**
7. **IMViC test**
8. **Motility test**
9. **Nitrate reduction test**
10. **OF test (Oxidation-Fermentation) [Hugh & Leifson (HL)]**
11. **Oxidase test (+ ve)**
12. **Catalase test (+ ve)**
13. **Sensitivity test**: A penicillin, ticarcillin or piperacillin is used in combination with an aminoglycoside, tobramycin. Other drugs active against *P aeruginosa* include aztreonam, imipenem, and the newer quinolones, including ciprofloxacin. cephalosporins, ceftazidime and cefoperazone.

Catalase test: Some bacteria contain flavoproteins that reduce O_2, resulting in the production of hydrogen peroxide (H_2O_2) or superoxide (O_2^-). These are extremely toxic because they are powerful oxidizing agents and destroy cellular constituents very rapidly. A bacterium must be able to protect itself against such O_2 products or it will be killed. Many bacteria possess enzymes that afford protection against toxic O_2 products. Obligate aerobes and facultative anaerobes usually contain the enzymes superoxide dismutase, which catalyzes the destruction of superoxide, and either catalase or peroxidase, which catalyze the destruction of hydrogen peroxide as follows:

$$2O^-_2 + 2H^+ \xrightarrow{\text{Superoxide dismutase}} \underset{\text{Oxygen}}{O_2} + \underset{\text{Hydrogen peroxide}}{H_2O_2}$$

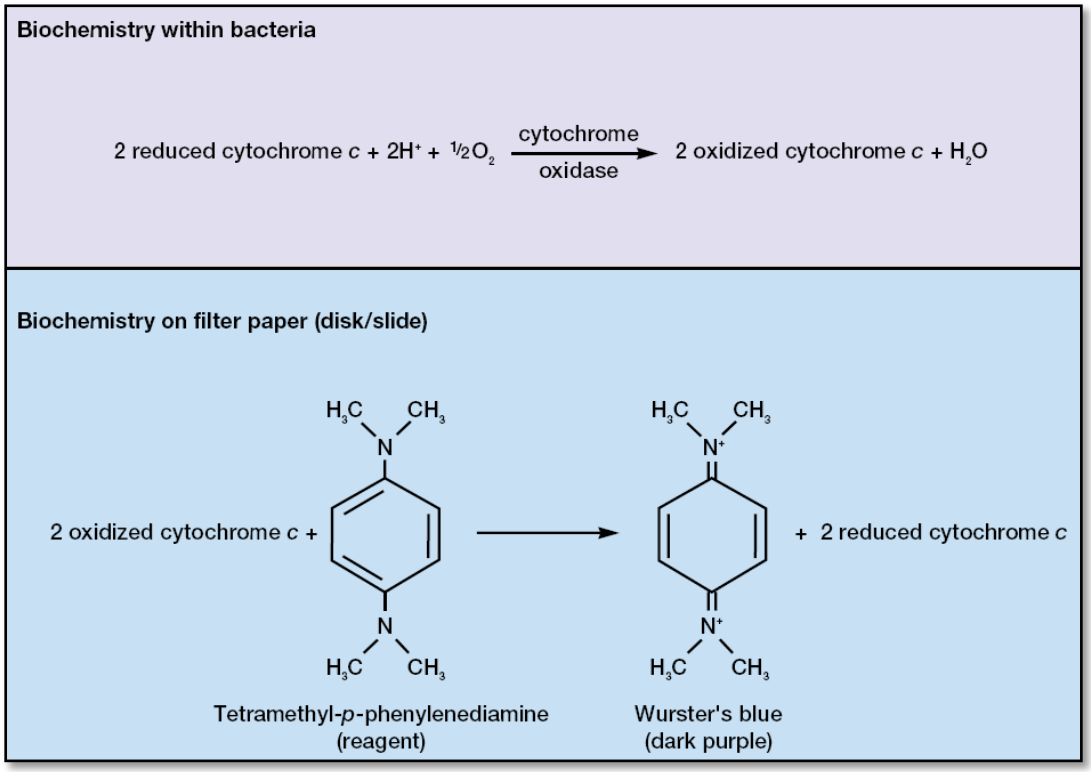
$$2H_2O_2 \xrightarrow{\text{Catalase or peroxidase}} \underset{\text{Water}}{2H_2O} + \underset{\text{Free oxygen}}{O_2}\uparrow \text{(Bubbles)}$$

Most strict anaerobes lack both enzymes and therefore cannot tolerate O2.

Substrate: **H_2O_2 3%**
Enzyme: ***Catalase***

- ✓ **Positive catalase test: Bubbles of free O_2 gas.**
- ✓ **Negative catalase test: The absence of bubble formation.**

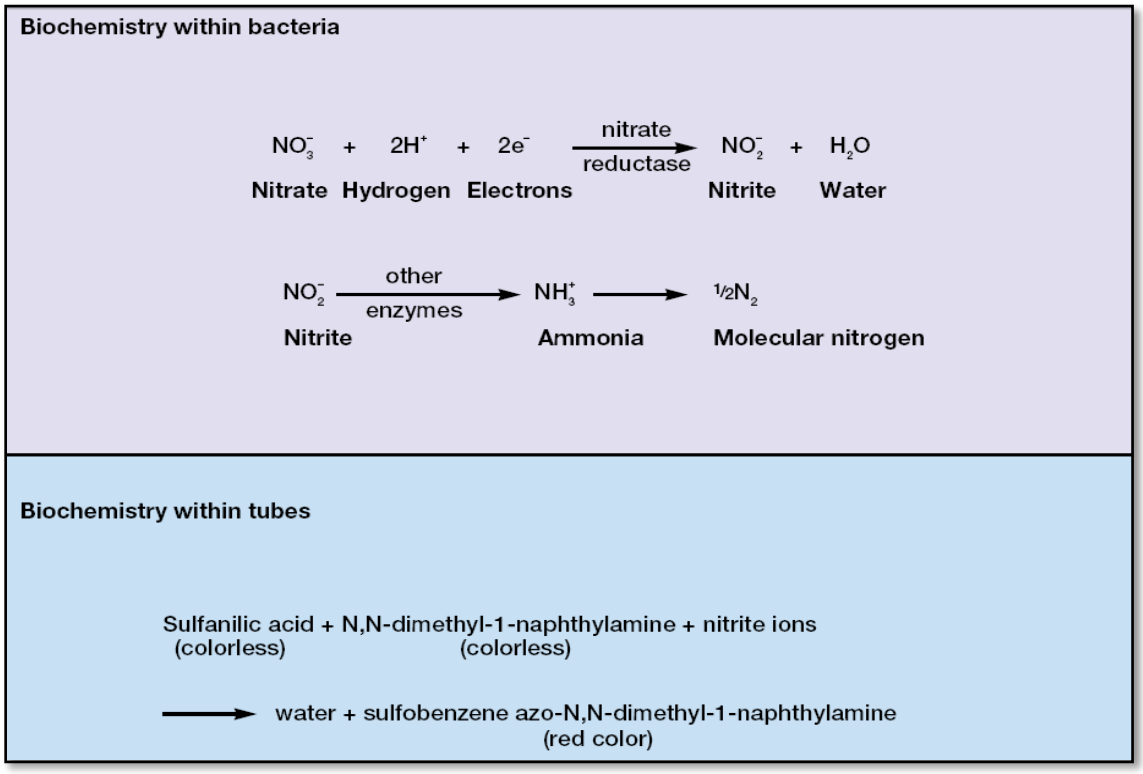
Oxidase test: Oxidase enzymes play an important role in the operation of the electron transport system during aerobic respiration. Cytochrome oxidase uses O_2 as an electron acceptor during the oxidation of reduced cytochrome c to form water and oxidized cytochrome c.



Substrate: Tetramethyl-p-phenylenediamine dihydrochloride **(*oxidase test reagent serves as an artificial substrate electrons to cytochrome oxidase)***
Enzyme: Cytochrome oxidase
Reagent: (Tetramethyl-p-phenylenediamine dihydrochloride)

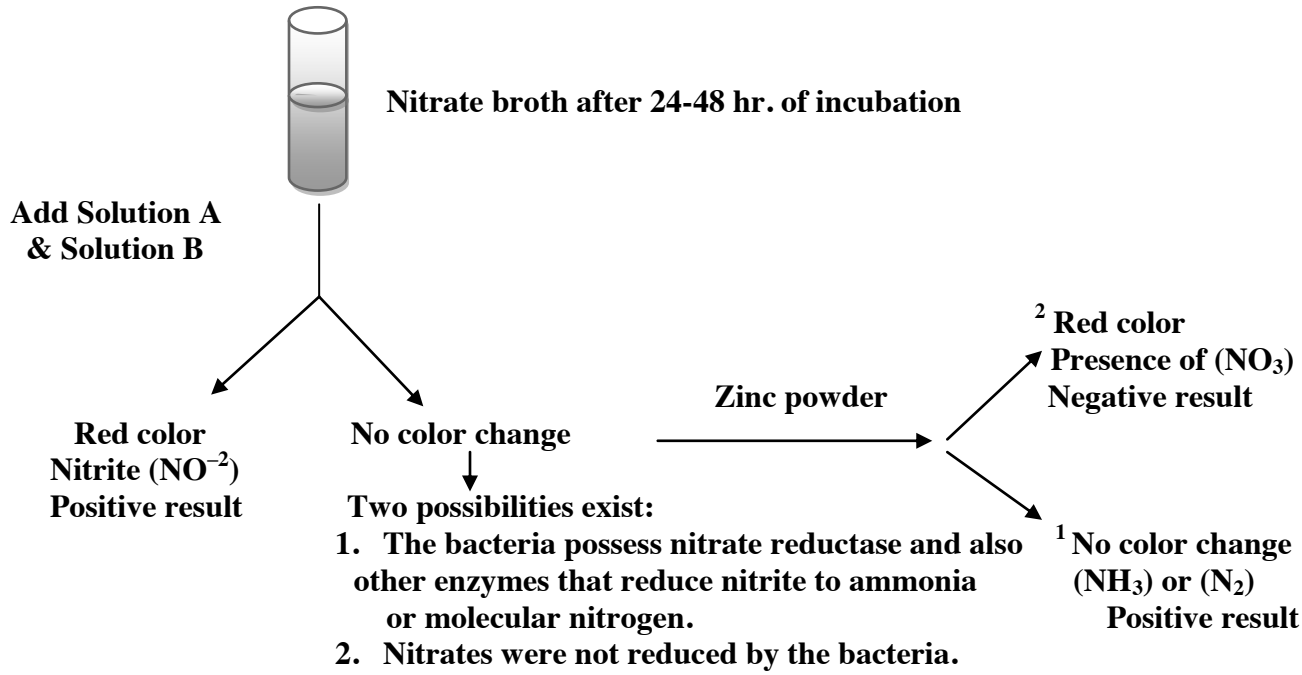
- ✓ **Positive oxidase test: Dark purple**
- ✓ **Negative oxidase test: No color change or a light pink**

Nitrate reduction test: Many chemoorganoheterotrophs (bacteria that require organic compounds for growth; the organic compounds serve as sources of carbon and energy) can use nitrate (NO^{-3}) as a terminal electron acceptor during anaerobic respiration. In this process, nitrate is reduced to nitrite (NO^{-2}) by nitrate reductase. Some of these bacteria possess the enzymes to further reduce the nitrite to either the ammonium ion or molecular nitrogen as illustrated:



Medium: Nitrate Broth
Substrate: Nitrate (NO_3)
Enzyme: Nitrate reductase
Reagents:

- ***Solution A: sulfanilic acid***
- ***Solution B: N,N-dimethyl-1-naphthylamine***
- ***Zinc powder***



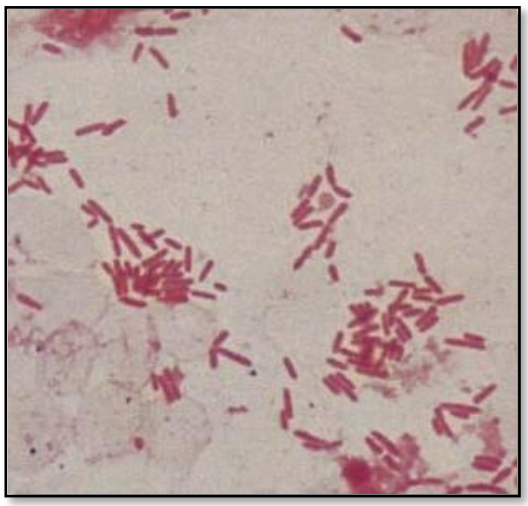
OF test (Oxidation-Fermentation): Two tubes were used, added to one of them paraffin on the surface to produce anaerobic conduction, inoculated by stabbing then incubation at 37ºC.

Medium: Hugh & Leifson (HL) Contains; peptone, glucose, K_2HPO_4, NaCl, agar-agar and Bromothymol blue as pH indicator.

<u>The results:</u>

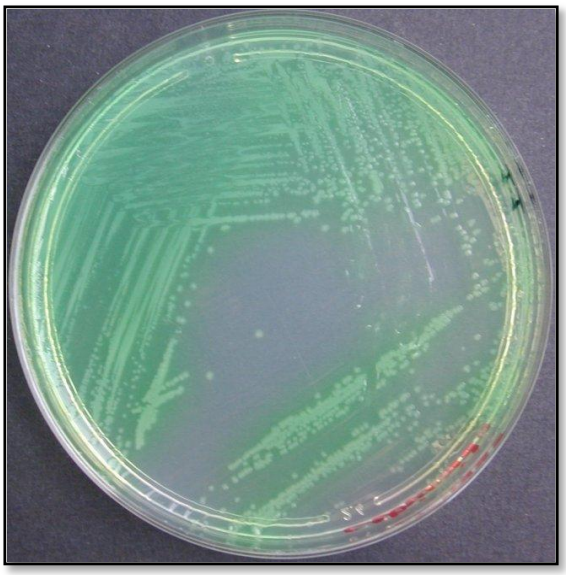
- ✓ Yellow color in the tube without paraffin and green color in the tube with paraffin mean: oxidation positive and fermentation negative.
- ✓ Green color in the tube without paraffin and yellow color in the tube with paraffin mean: oxidation negative and fermentation positive.
- ✓ Yellow color in the both tubes mean: oxidation and fermentation positive.
- ✓ Green color in the both tubes mean: oxidation and fermentation negative (Inert bacteria).

Test	*P. aeruginosa*	*P. fluorescence*
Indole	**-**	**-**
MR	**-**	**-**
VP	**-**	**-**
Citrate	**+**	**+**
TSI	**K/K - -**	**K/K - -**
Nitrate reduction test	**+**	**+**
Motility	**+**	**+**
Growth at 42ºC	**+**	**-**
Growth at 4ºC	**-**	**+**
King A	**+ Pyocyanin**	**± Pyocyanin**
King B	**+ Flourescen**	**+ Flourescen**
MacConkey agar	**L.N.F transparence, irregular**	**L.N.F transparence, irregular**
Oxidase	**+**	**+**
Catalase	**+**	**+**
OF medium	**Oxidation + Fermentation -**	**Oxidation + Fermentation -**

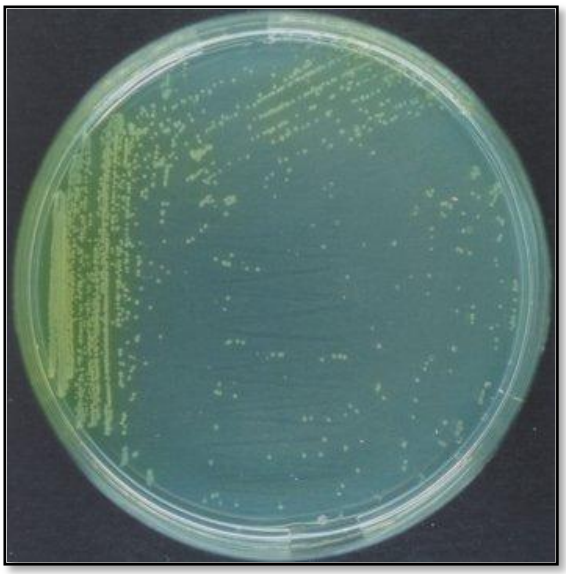


***Pseudomonas aeruginosa* (Gram-negative bacilli)**

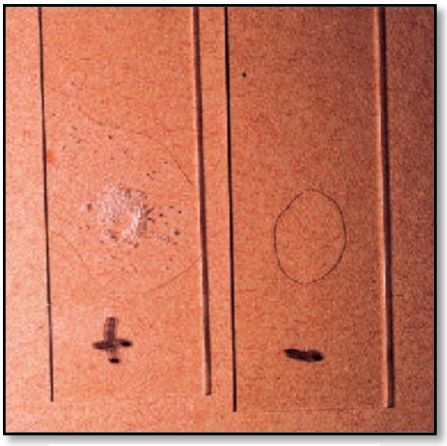
***P. aeruginosa* on MacConkey agar**



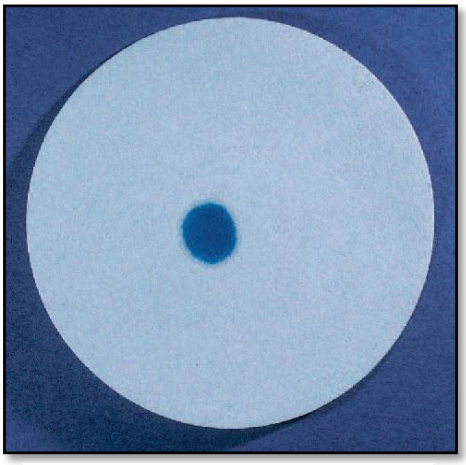
***Pseudomonas aeruginosa* on Cetrimide agar**



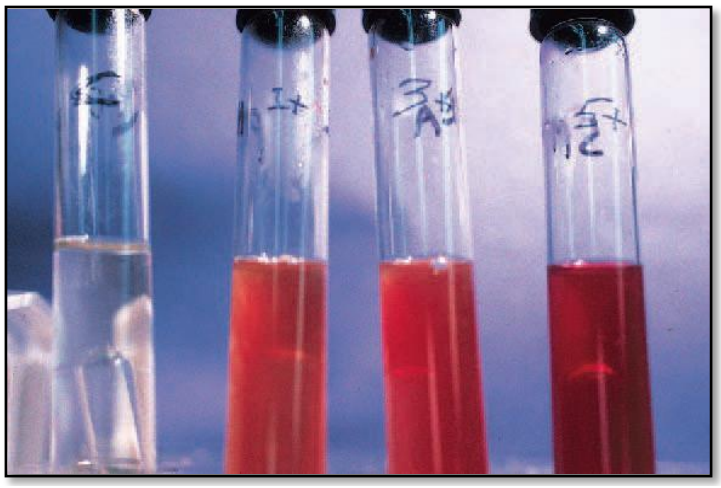
***Pseudomonas fluorescens* on Kings B agar**



Catalase test



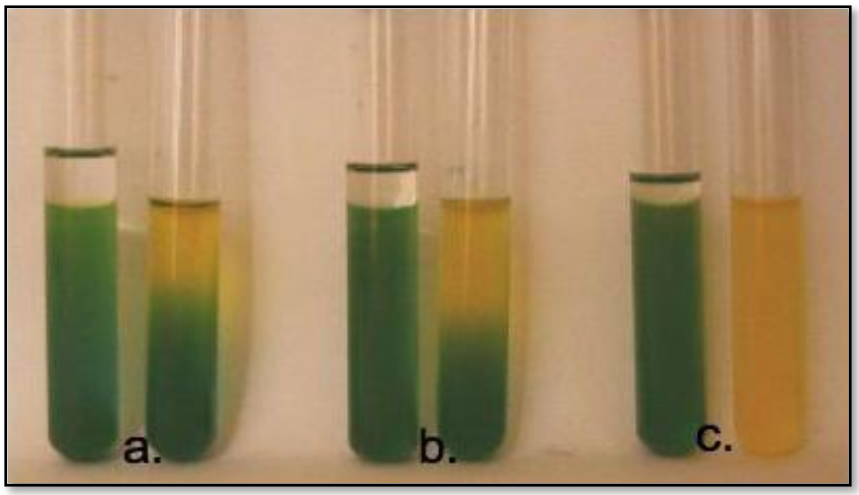
Positive oxidase test



Nitrate reduction test : Control +1 +3 +5

Oxidative-fermentative (OF) test (Control)

Oxidative-fermentative test inoculated with *E. coli*. Acid production in both the open and oil-covered tubes



Oxidative - fermentative test inoculated with *P. aeruginosa*. Acid production in the open tube and not the oil-covered tube indicates an oxidative result. (a) *P. aeruginosa* incubated for 24 hours. Note pH change in the top of the open tube only. (b) *P. aeruginosa* incubated for 48 hours. Note the diffusion of the acid down the tube. (c) *P. aeruginosa* incubated for 5 days. Note the diffusion of the acid throughout the tube. The result is best if read within 24 to 48 hours

Family: Vibrioaceae

Phylum: Proteobacteria

Class: Gammaproteobacteria

Order: Vibrionales

Family: Vibrioaceae

Genus: *Vibrio*

- ***V. cholerae***
- ***V. parahaemolyticus***

General Characteristics : *V. cholerae* is a comma-shaped, curved rod. It is actively motile by means of a polar flagellum. On prolonged cultivation, vibrios may become straight rods that resemble the gram-negative enteric bacteria. Vibrios are among the most common bacteria in surface waters worldwide. They are curved aerobic rods and are motile, possessing a polar flagellum. *V. cholerae* serogroups O1 and O139 cause cholera in humans, while other vibrios may cause sepsis or enteritis. *V. cholerae* regularly ferments sucrose and mannose but not arabinose. Vibrios are oxidase-positive, which differentiates them from enteric gram-negative bacteria. Characteristically, vibrios grow at a very high pH (8.5–9.5) and are rapidly killed by acid. Most *Vibrio* species are halotolerant, and NaCl often stimulates their growth. Some vibrios are halophilic, requiring the presence of NaCl to grow.

Culture and Growth Characteristics: Growth is rapid in peptone agar, on blood agar with a pH near 9.0, or on TCBS agar, and typical colonies can be picked in 18 hours. For enrichment, a few drops of stool can be incubated for 6–8 hours in taurocholate-peptone broth (pH 8.0–9.0); organisms from this culture can be stained or subcultured. *V. cholerae* produces convex, smooth, round colonies that are opaque and granular in transmitted light. *V. cholerae* and most other vibrios grow well at 37 °C on many kinds of media, including defined media containing mineral salts and asparagine as sources of carbon and nitrogen. *V. cholerae* grows well on **thiosulfate-citrate-bile-sucrose (TCBS)** agar, on which it produces yellow colonies that are readily visible against the dark-green background of the agar.

Antigenic Structure & Biologic Classification:

- *V. cholerae* has O lipopolysaccharides that confer serologic specificity. There are at least 139 O antigen groups. *V. cholerae* strains of O group 1 and O group 139 cause classic cholera; occasionally, non-O1/non-O139 *V. cholerae* causes cholera-like disease.
- The *V. cholerae* serogroup O1 antigen has determinants that make possible further typing Two biotypes of epidemic *V. cholerae* have been defined, **classical** and **El Tor.**

El Tor	Classical
Haemolytic	**Non-Haemolytic**
Resistant to polymyxin B.	**Sensitive**
Cause haemoagglutination of SRBCs	**Dose not**

- Molecular techniques can also be used to type *V. cholerae*.

Pathogenesis:

The Medically Important Vibrios.	
Organism	**Human Disease**
***V. cholerae* serogroups O1 and O139**	**Epidemic and pandemic cholera**
***V. cholerae* serogroups non-O1/non-O139**	**Cholera-like diarrhea; mild diarrhea; rarely, extraintestinal infection**
V. parahaemolyticus	**Gastroenteritis, perhaps extraintestinal infection**
Others ***V. mimicus, V. vulnificus, V. hollisae, V. fluvialis, V. damsela, V. anginolyticus, V. metschnikovii***	**Ear, wound, soft tissue, and other extraintestinal infections, all uncommon**

Vibrio parahaemolyticus: is a halophilic bacterium that causes acute gastroenteritis following ingestion of contaminated seafood such as raw fish or shellfish. After an incubation period of 12–24 hours, nausea and vomiting, abdominal cramps, fever, and watery to bloody diarrhea occur. Fecal leukocytes are often observed. The enteritis tends to subside spontaneously in 1–4 days with no treatment other than restoration of water and electrolyte balance. The disease occurs worldwide, with highest incidence in areas where people eat raw seafood. *V parahaemolyticus* does grow well on blood agar. It also grows well on TCBS, where it yields green colonies.

Specimens: Specimens for culture consist of mucus flecks from stools.

Treatment: The most important part of therapy consists of water and electrolyte replacement to correct the severe dehydration and salt depletion. Many antimicrobial agents are effective against *V cholerae*. Oral tetracycline tends to reduce stool output in cholera and shortens the period of excretion of vibrios.

Laboratory diagnostic tests:

1. **Gram stain** (Gram-negative comma-shaped, curved rod).
2. **Culture on blood agar**
3. **Culture on Selective and Differential media like TCBS agar.**
4. ***V. cholerae* organisms are identified by slide agglutination tests using anti-O group 1 or group 139 antisera**
5. **TSI**
6. **IMViC test**
7. **Nitrate reduction test**
8. **Gelatin liquefaction test.**
9. **Peptone water pH= 9, NaCl= 7%**
10. **Cholera Red Reaction (Nitroso endol test): to test the bacteria ability to produce deaminase enzyme**

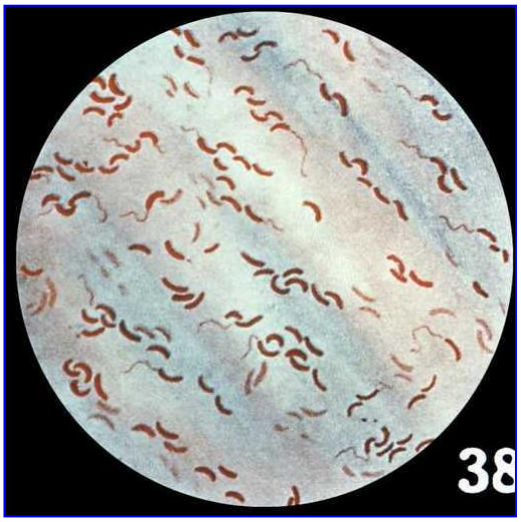
Tryptophane + H_2SO_4 (4 drops) ⟶ Nitrosoindol (red color)

Tryptophane $\xrightarrow{\text{Deaminase}}$ NH_3

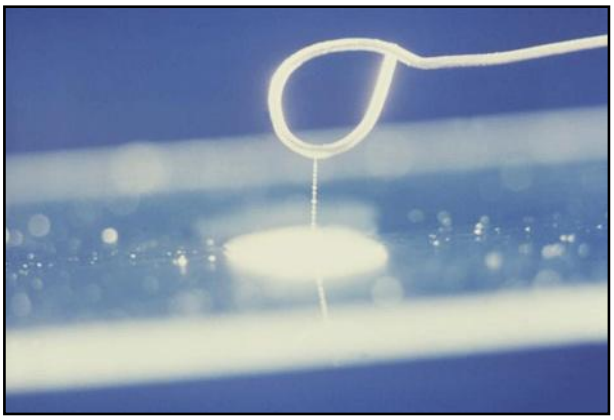
NH_3 + H_2SO_4 ⟶ Nitrosoindol (red color)

14. String test: add 0.5% Na-deoxycholate solution to 1 drop of culture, the culture convert to thread like when draws by loop disappear after 45-60 sec.

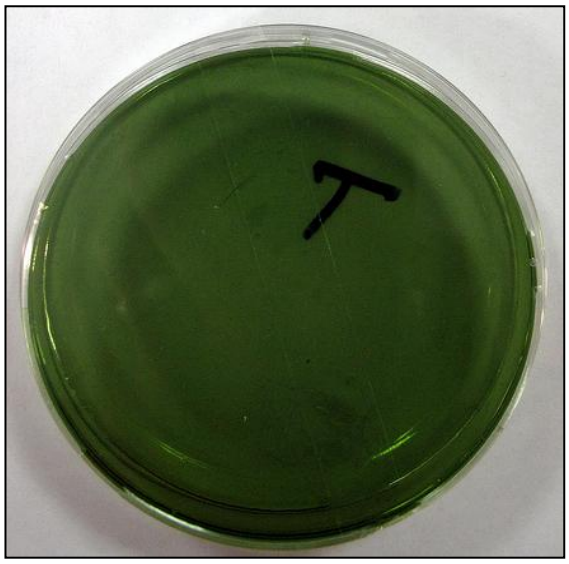
Test	*V. cholerae*	*V. parahaemolyticus*
Catalase	+	+
Oxidase	+	+
NO_3 reduction	+	+
Indole	+	+
MR	+ weak	-
VP	-	-
Citrate	+/-	+/-
Peptone water + 7% NaCl	-	+
Peptone water + 0% NaCl	+	-
TSI	A/A - -	K/A - -
Motility	+	+
Cholera red	+	-
Mannitol	+ weak	+ weak
String test	+	+
OF medium	Oxidation-fermentation	Oxidation-fermentation
TCBS medium	Yellow colonies	Green colonies



Vibrio cholerae

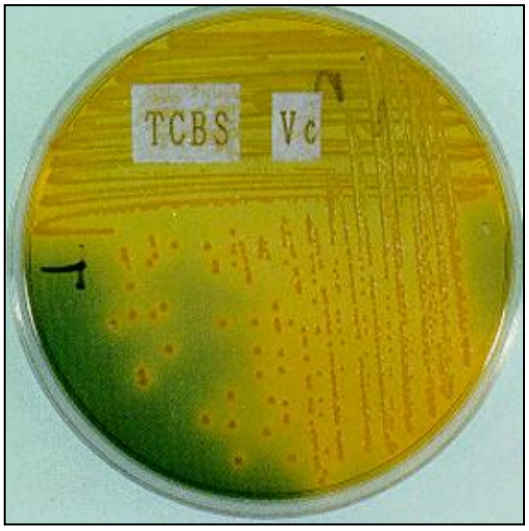


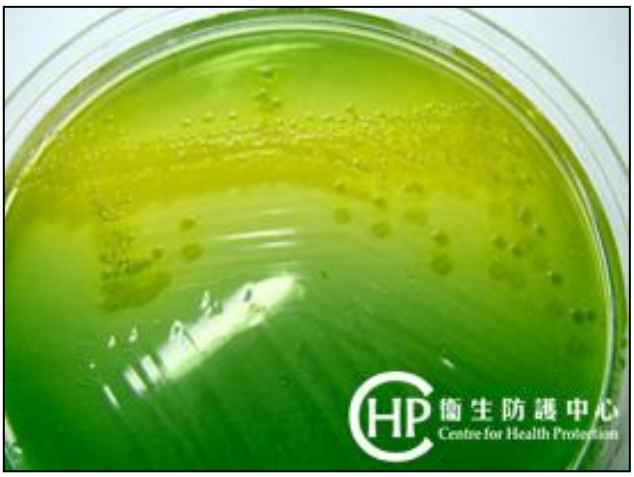
The String Test



TCBS medium

***Vibrio parahaemolyticus* on TCBS medium**





***Vibrio cholerae* on TCBS medium**

Family: Neisseriaceae

Phylum: Proteobacteria

Class: Betaproteobacteria

Order: Neisseriales

Family: Neisseriaceae

- **Genus I:** ***Neisseria***
- **Genus II:** ***Kingella***
- **Genus III:** ***Eikenella***
- **GenusIV:** ***Simonsiella***
- **GenusV:** ***Alysiella***

Neisseria

General Characteristics: is a gram-negative, nonmotile diplococcus, Individual cocci are kidney-shaped; when the organisms occur in pairs, the flat or concave sides are adjacent. *Neisseria gonorrhoeae* (gonococci) and *Neisseria meningitidis* (meningococci) are pathogenic for humans and typically are found associated with or inside polymorphonuclear cells. Some neisseriae are normal inhabitants of the human respiratory tract, rarely if ever cause disease, Meningococci have polysaccharide capsules, whereas gonococci do not, and meningococci rarely have plasmids whereas most gonococci do. The neisseriae produce oxidase and give positive oxidase reactions; the oxidase test is a key test for identifying them.

Culture and Growth Characteristics: In 48 hours on enriched media (eg, Mueller-Hinton, modified Thayer-Martin, "chocolate" agar), gonococci and meningococci form convex, glistening, elevated, mucoid colonies 1–5 mm in diameter. Colonies are transparent or opaque, nonpigmented, and nonhemolytic. The neisseriae grow best under aerobic conditions, but some will grow in an anaerobic environment. They have complex growth requirements. Most neisseriae ferment carbohydrates, producing acid but not gas, and their carbohydrate fermentation patterns are a means of distinguishing them. Meningococci and gonococci grow best on media containing complex organic substances such as heated blood, hemin, and animal proteins and in an atmosphere containing 5% CO_2 (e.g, candle jar). To avoid overgrowth by contaminants, the selective medium contains antimicrobial drugs (eg, vancomycin, colistin, amphotericin B, and trimethoprim). A modified Thayer-Martin medium with antibiotics (vancomycin, colistin, amphotericin) favors the growth of neisseriae, inhibits many other bacteria, and is used for nasopharyngeal cultures. The organisms are rapidly killed by drying, sunlight, moist heat, and many disinfectants. They produce autolytic enzymes that result in rapid swelling and lysis in vitro at 25 °C and at an alkaline pH. Gonococci ferment only glucose and differ antigenically from the other neisseriae. Gonococci usually produce smaller colonies than those of the other neisseriae

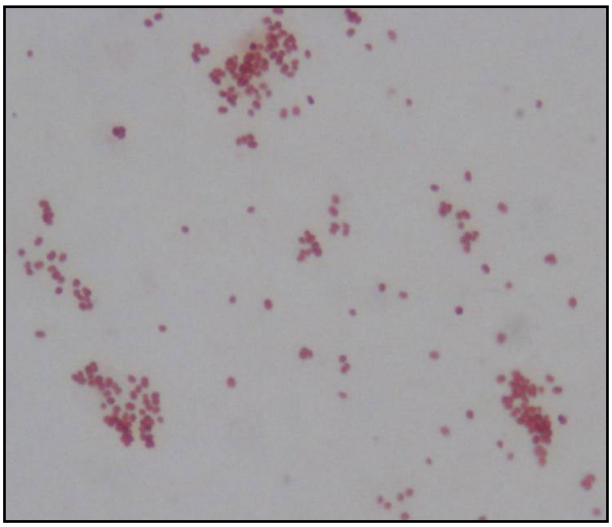
Pathogenesis:

- *Neisseria meningitidis* (Meningococci) typically are found in the upper respiratory tract and cause meningitis,
- while *Neisseria gonorrhoeae* (gonococci) cause genital infections (gonorrhoeal disease, sexual transmitted disease)

Specimens: Pus and secretions are taken from the urethra, cervix, rectum, conjunctiva, throat, and spinal fluid for culture and smear. Blood culture is necessary in systemic illness.

Laboratory diagnostic tests:

1. **Gram stain (G-ve diplococci).**
2. **Enriched media** (eg, Mueller-Hinton, modified Thayer-Martin, "chocolate" agar).
3. **Oxidase test (+ve)**
4. **Carbohydrate fermentation.**
5. **Nitrate reduction test.**



***Neisseria* gram-negative cocci**

Test	*Neisseria gonorrhoeae*	*Neisseria meningitidis*
Glucose	+	+
Maltose	-	+
Fructose	-	-
Lactose	-	-
Pigments	Greyish white	Greyish white
CO_2 requirement	Necessary	Necessary
Growth at 22ºC	-	-
Growth at 35ºC	+	+
NO_3 reduction	-	+