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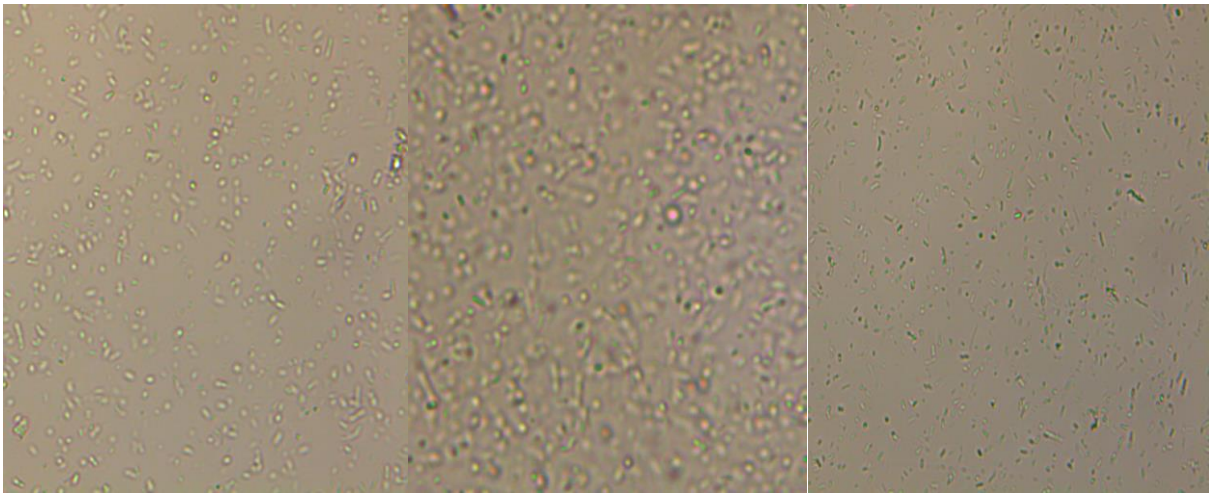

# SPECIAL BACTERIOLOGY
# BASIC LABORATORY TESTS

# Preface

Special Bacteriology – Basic Laboratory Tests is intended above all for medical students. The book includes standard procedures commonly used in microbiological laboratory. We have tried to present principles of laboratory tests to make them easier to understand.

Authors

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# 1 STAPHYLOCOCCI

Staphylococci are typical Gram-positive bacteria forming irregular clusters of cocci. Staphylococci are widespread in nature, although they are mainly found on the skin, skin glands and mucous membranes of mammals and birds, but can cause infection under certain circumstances. [1]*S. aureus* is more pathogenic than the other common members of the genus, *S. epidermidis* and *S. saprophyticus*. *S. epidermidis* has been known to cause various hospital-acquired infections (such as prosthetic or indwelling devices), whereas *S. saprophyticus* is mainly associated with urinary tract infections in young females who are sexually active. Disease processes with *S. aureus* are numerous. The portal of entry is variable, since they gain access to the body via the skin, the respiratory tract or the genito-urinary tract. *Staphylococcus aureus* expresses many potential **virulence factors**:

1. **surface proteins** - promote colonization of host tissues
2. **leukocidin**, **kinases**, **hyaluronidase** - invasins that promote bacterial spread in tissues
3. **capsule**, **Protein A** - surface factors that inhibit phagocytic engulfment
4. **carotenoids**, **catalase** - enhance staphylococcal survival in phagocytes
5. **protein A**, **coagulase** - immunological disguises
6. **hemolysins**, **leukotoxin**, **leukocidin** - membrane-damaging toxins that lyse eucaryotic cell membranes
7. [2]**TSST**, [3]**ET** - exotoxins that damage host tissues or otherwise provoke symptoms of disease
8. inherent and acquired **resistance to antimicrobial agents**.

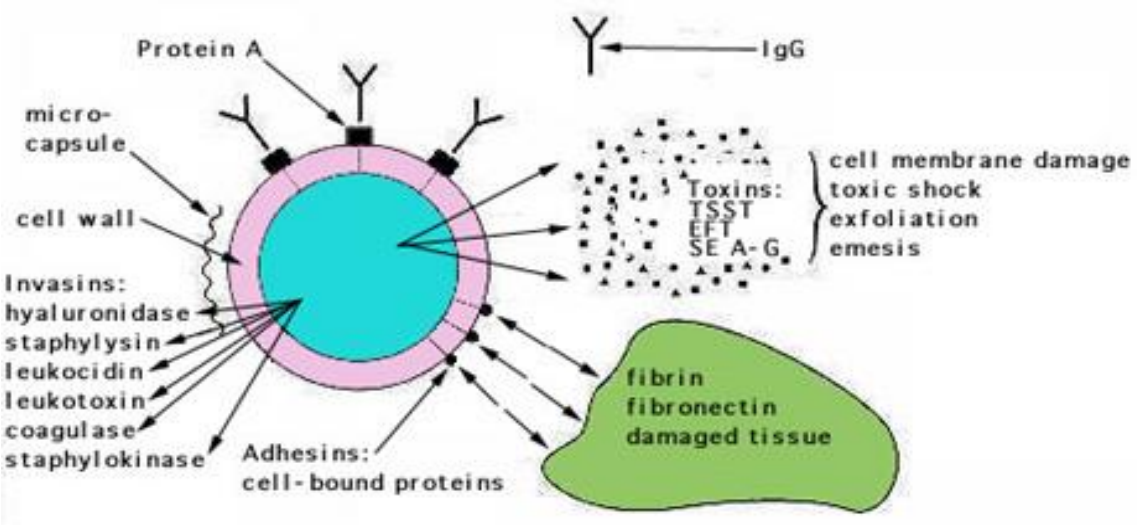



**Fig. 1 Virulence determinants of *Staphylococcus aureus*.**

[1] S. - Staphylococcus
[2] TSST - Toxic Shock Syndrome Toxin
[3] ET - Exfoliatin Toxin

**Staphylococci can cause many forms of infection:**

1. *S. aureus* causes superficial skin lesions (boils) and localized abscesses in other sites.
2. *S. aureus* causes deep-seated infections, such as osteomyelitis and endocarditis and more serious skin infections (furunculosis).
3. *S. aureus* is a major cause of hospital acquired (nosocomial) infection of surgical wounds and, with *S. epidermidis*, causes infections associated with indwelling medical devices.
4. *S. aureus* causes food poisoning by releasing enterotoxins into food.
5. *S. aureus* causes toxic shock syndrome by release of superantigens into the blood stream.
6. *S. saprophyticus* causes urinary tract infections, especially in girls.
7. Other species of staphylococci (*S. lugdunensis, S. haemolyticus, S. warneri, S. schleiferi, S. intermedius*) are infrequent pathogens.

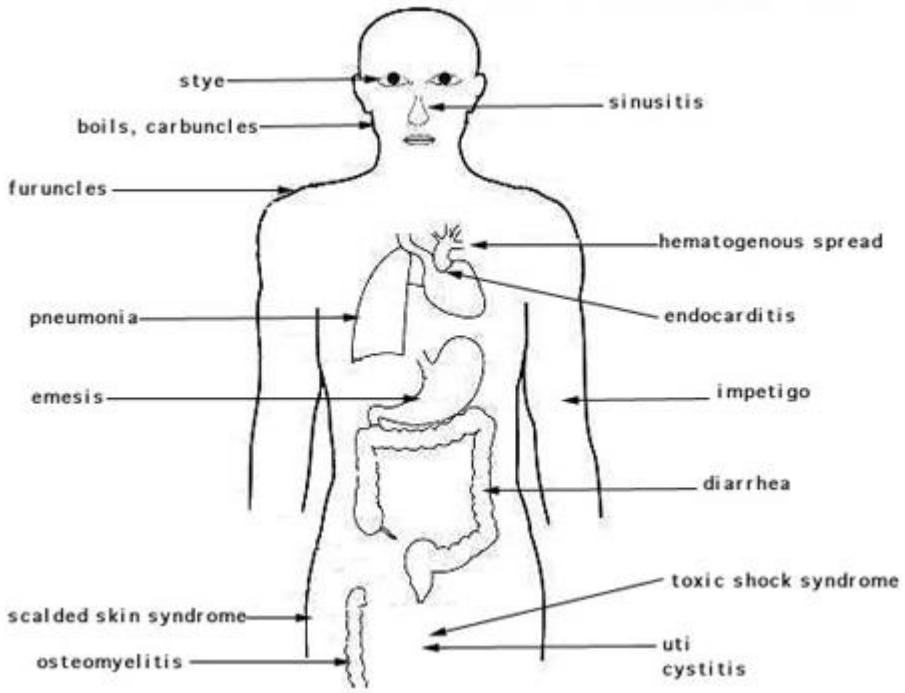



**Fig. 2 Sites of infection and diseases caused by *Staphylococcus aureus.***

Although strains of *Staphylococcus aureus* resistant to penicillin have caused infections for many years, isolates resistant to methicillin, oxacillin, and other ß-lactams have become predominant-primarily in the last 20 years.

Diagnosis of staphylococcal infections begins with attempting to culture the bacteria from an infected site. Any area with pus, crusty drainage, or blisters should be cultured. Blood from patients with sepsis, toxic shock syndrome, or pneumonia should be cultured. Standard microbiological techniques include a positive coagulase test. *S. aureus* lyses red blood cells in blood agar plates (hemolytic staphylococci) while *S. epidermidis* does not (nonhemolytic staphylococci). All staphylococci should be further tested to see if the bacteria are resistant to the antibiotic methicillin (and other antibiotics) and thus determine if the organisms are [4]MRSA.

[4] MRSA – Methicillin Resistant *Staphylococcus aureus*

## 1.1 GRAM STAIN

*Staphylococcus* is a genus of bacteria that is characterized by a round shape (coccus or spheroid shaped), Gram-positive (purple), and found as either single cells, in pairs, or more frequently, in clusters that resemble a bunch of grapes. The genus name *Staphylococcus* is derived from Greek terms (*staphyle* and *kokkos*) that mean "a bunch of grapes,"

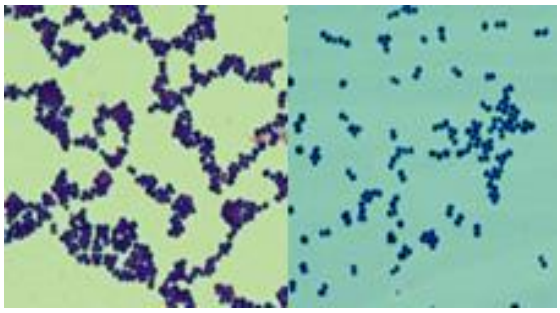


**Fig. 3 *S. aureus* (left), *S. epidermidis* (right) - Gram stain.**

## 1.2 STAPHYLOCOCCI - BLOOD AGAR CULTURE

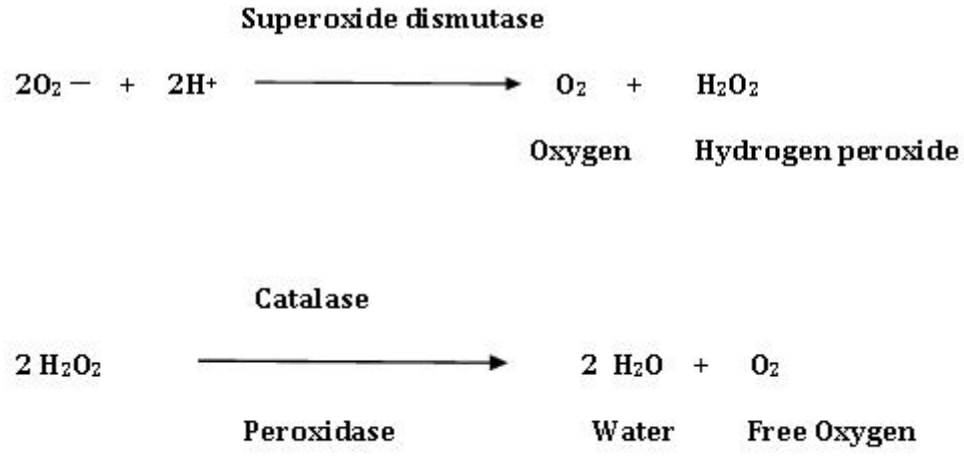
Blood agar is both differential and enriched medium. The blood that is incorporated into this medium is an enrichment ingredient for the cultivation of fastidious organisms. On blood agar, *S. aureus* usually displays a light to golden yellow pigment, whereas *S. epidermidis* has a white pigment and *S. saprophyticus* either a bright yellow or white pigment. However, pigmentation is not always a reliable characteristic. On blood agar, *S. aureus* is usually beta-hemolytic, *S. epidermidis* and *S. saprophyticus* are almost always nonhemolytic.

**Fig. 4 *S. aureus* (left) and *S. epidermidis* (right) - colonies on blood agar.**

***S. aureus*** - individual colonies on agar are round, convex, and 1-4 mm in diameter with a sharp border. On blood agar plates, colonies of *Staphylococcus aureus* are frequently surrounded by zones of clear beta-hemolysis. The golden appearance of colonies of some strains is the etymological root of the bacteria's name; aureus meaning "golden" in Latin. ***Staphylococcus epidermidis*** - showing γ-haemolytic, porcelin-white colonies as compared to *S. aureus* on the same medium. This clear distinction in colony color is not seen at all times.

## 1.3 CATALASE TEST

Some bacteria contain flavoproteins that reduce oxygen ($O_2$), resulting in the production of hydrogen peroxide ($H_2O_2$) and, in some cases, an extremely toxic superoxide ($O_2$–). Accumulation of these substances will result in death of the organism as they are powerful oxidizing agents and destroy cellular constituents very rapidly unless they can be enzymatically degraded. These substances are produced when aerobes, facultative anaerobes, and microaerophiles use the aerobic respiratory pathway, in which oxygen is the final electron acceptor, during degradation of carbohydrates for energy production. 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. Facultative anaerobes and obligate aerobes usually contain the enzymes superoxide dismutase, which has the ability to catalyze the destruction of superoxide, and either catalase or peroxidase, which catalyze the destruction of hydrogen peroxide as follows:




The inability of strict anaerobes to synthesize catalase, peroxidase, or superoxide dismutase may explain why oxygen is poisonous to these microorganisms. In the absence of these enzymes, the toxic concentration of $H_2O_2$ cannot be degraded when these organisms are cultivated in the presence of oxygen. Organisms capable of producing catalase rapidly degrade hydrogen peroxide which is a tetramer containing four polypeptide chains, which are usually 500 amino acids long. It also contains four porphyrin heme groups (ie., iron groups) that will allow the enzyme to react with the hydrogen peroxide.

The enzyme catalase is present in most cytochrome-containing aerobic and facultative anaerobic bacteria. Catalase is the enzyme which has one of the highest turnover numbers compared to all other enzymes; one molecule of catalase has the ability to convert millions of molecules of hydrogen peroxide to water and oxygen in each second. Catalase production and activity can be detected either by adding the substrate $H_2O_2$ to an appropriately incubated (18 to 24 hours) tryptic soy agar slant culture or by slide test. Organisms which produce the enzyme break down the hydrogen peroxide, and the resulting $O_2$ production produces bubbles in the reagent drop, indicating a positive test. Organisms lacking the cytochrome system also lack the catalase enzyme and are unable to break down hydrogen peroxide, into $O_2$ and water

and are catalase negative.
The catalase test is primarily used to distinguish among Gram-positive cocci. Members of the genus *Staphylococcus* are catalase-positive, and members of the genera *Streptococcus* and *Enterococcus* are catalase-negative.

**Procedure of Catalase Test (Slide Test)**

1. Transfer a small amount of bacterial colony to a surface of clean, dry glass slide using a loop or sterile wooden stick
2. Place a drop of 3% $H_2O_2$ on to the slide and mix.
3. A positive result is the rapid evolution of oxygen (within 5-10 sec.) as evidenced by bubbling (Fig. 5).
4. A negative result is no bubbles or only a few scattered bubbles.

**Fig. 5 Catalase - slide test.**

## 1.4 MANNITOL SALT AGAR CULTURE

Mannitol salt agar (MSA) is both a selective and differential media used for the isolation of *Staphylococci* from mixed cultures.

**MSA Components**

**7,5% NaCl** – selects for species of S*taphylococcus*. This concentration of salt is too high for most other bacteria to withstand and, therefore, inhibits their growth.
**Mannitol** – alcohol of the carbohydrate mannose. Mannitol fermentation produces acid end products which turn the medium yellow. Yellow indicates mannitol positive and no color change indicates mannitol negative.
**Phenol red pH indicator** – yellow in acid pH (the same indicator that is used in phenol red carbohydrate fermentation broths).
On MSA, only pathogenic *Staphylococcus aureus* produces small colonies surrounded by yellow zones. The reason for this color change is that *S. aureus* have the ability to ferment the mannitol, producing an acid, which changes the indicator color from red to yellow. The growth of other types of bacteria is usually inhibited. This growth differentiates *S. aureus* from *S. epidermidis*, which forms colonies with red zones.

**Fig. 6 Mannitol Salt Agar.**

## Expected Results

1. On MSA, pathogenic *Staphylococcus aureus* ferments mannitol, thereby changing the colour of the medium from red to yellow.

**Fig. 7 *Staphylococcus aureus* on Mannitol Salt Agar.**

2. *Staphylococcus epidermidis* grows on MSA, but does not ferment mannitol (media remains light pink in color, colonies are colorless).

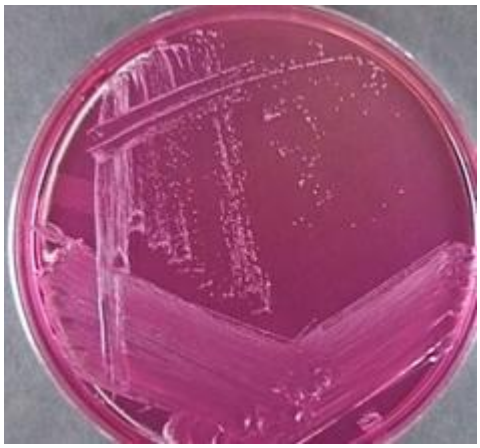


**Fig. 8 *Staphylococcus epidermidis* on Mannitol Salt Agar.**

## 1.5 COAGULASE TEST

Coagulases are enzymes that clot blood plasma by a mechanism that is similar to normal clotting. The coagulase test identifies whether an organism produces this exoenzyme. This enzyme clots the plasma component of blood. The only significant disease causing bacteria of humans that produce coagulase enzyme are *Staphylococcus aureus*. Thus this enzyme is a good indicator of the pathogenic potential of *S. aureus.*

In human host, the action of coagulase enzyme produces clotting of the plasma by converting fibrinogen to fibrin in the immediate vicinity of the bacterium as a means of protection by itself. The fibrin meshwork that is formed by this conversion surrounds the bacterial cells or infected tissues, protecting the organism from non-specific host resistance mechanisms such as phagocytosis and the anti staphylococcal activity of normal serum. This enables the bacterium to persist in the presence of a host immune response, which can lead to the establishment of infection. Thus, coagulase is described as a virulence factor (disease-causing factor) of *Staphylococcus aureus*. Citrate and EDTA (Ethylenediaminetetraacetic acid**)** are usually added to act as anticoagulants and prevent false-positive results. Most strains of *S.aureus* produce one or two types of coagulase; free coagulase and bound coagulase. Bound coagulase is localized on the surface of the cell wall and reacts with α- and β-chains of the plasma fibrinogens to form a coagulate. Free coagulase is an enzyme that is secreted extracellularly and bound coagulase is a cell wall associated protein. Free coagulase can be detected in tube coagulase test and bound coagulase can be detected in slide coagulase test.

Slide coagulase test may be used to screen isolates of *S.aureus* and tube coagulase may be used for further confirmation. There are seven antigenic types of free coagulase, but only one antigenic type of bound coagulase exists. Free coagulase is always heat labile while bound coagulase is heat stable.

In the test, the sample is added to rabbit plasma and held at 37° C for a specified period of time. Clot formation occurs within 4 hours is interpreted as a positive result and indicative of a virulent *Staphylococcus aureus* strain. The absence of coagulation after 24 hours of incubation is a negative result, indicative of an avirulent strain.

## Detection of Bound Coagulase - Slide Test

This method measures bound coagulase. The bound coagulase is also known as clumping factor. It cross-links the α and β chain of fibrinogen in plasma to form fibrin clot that deposits on the cell wall. As a result, individual coccus stick to each other and clumping is observed.

1. Divide the slide into two sections with grease pencil. One should be labeled as „test" and the other as „control".
2. Place a small drop of distilled water on each area.
3. Emulsify one or two colonies of Staphylococcus on blood agar plate on each drop to make a smooth suspension.
4. The test suspension is treated with a drop of citrated plasma and mixed well with a needle.
5. Do not put anything in the other drop that serves as control. The control suspension serves to rule out false positivity due to auto agglutination.
6. Clumping of cocci within 5-10 seconds is taken as positive (Fig. 9).

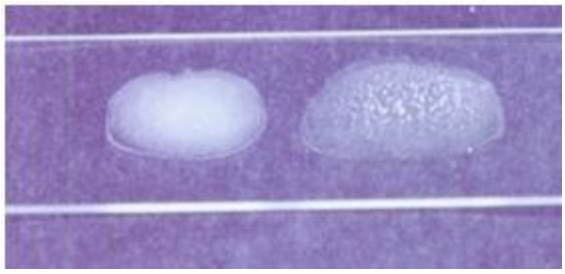


**Fig. 9 Slide Coagulase Test.**

Some strains of *S.aureus* may not produce bound coagulase, and such strains must be identified by tube coagulase test

## Detection of Free Coagulase - *Tube Coagulase Test*

Most strains of *S.aureus* produce one or two types of coagulase; free coagulase and bound coagulase. Free coagulase is an extracellular enzyme which reacts with prothrombin and its derivatives. This method helps to measure free coagulase. The free coagulase secreted by *S.aureus* reacts with coagulase reacting factor (CRF) in plasma to form a complex, which is thrombin. This converts fibrinogen to fibrin resulting in clotting of plasma (Fig. 10).

1. Three test tubes are taken and labeled "test", "negative control" and "positive control".
2. Each tube is filled with 1 ml of 1 in 10 diluted rabbit plasma.
3. To the tube labeled test, 0.2 ml of overnight broth culture of test bacteria is added.
4. To the tube labeled positive control, 0.2 ml of overnight broth culture of known *S. aureus* is added.
5. To the tube labeled negative control, 0.2 ml of sterile broth is added.
6. All the tubes are incubated at 37°C.
7. Positive result is indicated by gelling of the plasma, which remains in place even after inverting the tube.

8. If the test remains negative until four hours at 37°C, the tube is kept at room temperature for overnight incubation.

**Fig. 10 Tube Coagulase Test.**

The coagulase test is used to distinguish between pathogenic and nonpathogenic members of the genus Staphylococcus. All pathogenic strains of *S. aureus* are coagulase positive whereas the nonpathogenic species (*S. epidermidis*) are coagulase negative.
While slide coagulase test is useful in screening, tube coagulase test is useful in confirmation of coagulase test. Samples must be observed for clotting within 24 hours. This is because some strains that produce coagulase also produce an enzyme called fibrinolysin, which can dissolve the clot. Therefore, the absence of a clot after 24 hours is no guarantee that a clot never formed. The formation of a clot by 12 hours and the subsequent disappearance of the clot by 24 hours could produce a so-called false negative if the test were only observed at the 24-hour time.

**Sources:**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# 2 STREPTOCOCCI

Streptococci are Gram-positive, nonmotile, nonsporeforming, catalase-negative cocci that occur in pairs or chains. Older cultures may lose their Gram-positive character. Most streptococci are facultative anaerobes, and some are obligate (strict) anaerobes. Most require enriched media (blood agar).

Streptococci are subdivided into groups by antibodies that recognize surface antigens (Fig. 11). These groups may include one or more species. Serologic grouping is based on antigenic differences in cell wall carbohydrates (groups A to V), in cell wall pili-associated protein, and in the polysaccharide capsule in group B streptococci. Rebecca Lancefield developed the serologic classification scheme in 1933. **β-hemolytic** strains possess group-specific cell wall antigens, most of which are carbohydrates. These antigens can be detected by immunologic assays and have been useful for the rapid identification of some important streptococcal pathogens. The most important groupable streptococci are A, B and D. Among the groupable streptococci, infectious disease (particularly pharyngitis) is caused by group A. Group A streptococci have a hyaluronic acid capsule. *Streptococcus pneumoniae* (a major cause of human pneumonia) and *Streptococcus mutans* and other so-called viridans streptococci (among the causes of dental caries) do not possess group antigen. *Streptococcus pneumoniae* has a polysaccharide capsule that acts as a virulence factor for the organism, more than 90 different serotypes are known, and these types differ in virulence.

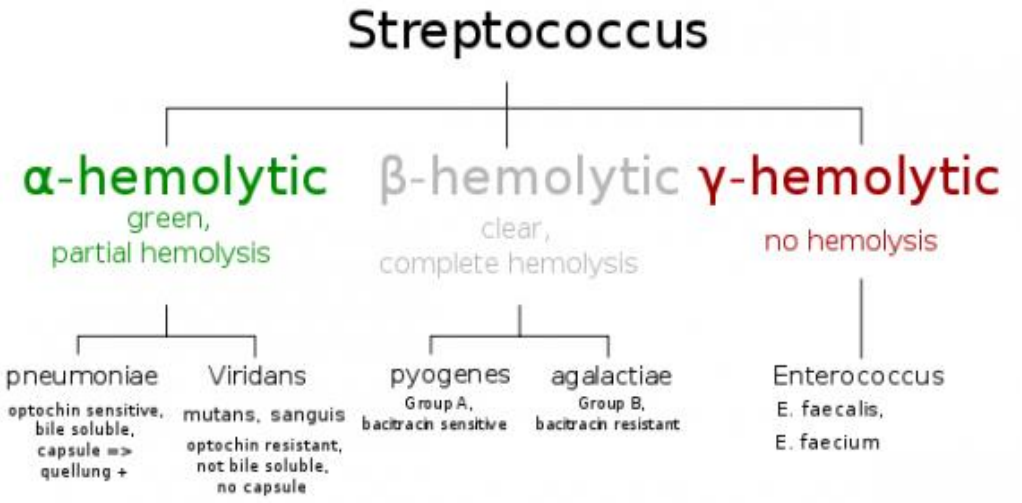


**Fig. 11 Streptococci - classification.**

**Group A streptococci** causes:

- Strep throat - a sore, red throat, sometimes with white spots on the tonsils
- Scarlet fever - an illness that follows strep throat. It causes a red rash on the body.
- Impetigo - a skin infection
- Toxic shock syndrome
- Cellulitis and necrotizing fasciitis (flesh-eating disease)

**Rheumatic fever** is a nonsuppurative complication of *S. pyogenes* pharyngitis. Rheumatic fever is an inflammatory disease affecting primarily the heart and joints. Although severe, it can take an extended period of time to develop. The mechanism of chronic immunopathology of rheumatic fever is not resolved. M protein cross-reacts with heart myosin leading to autoimmunity. Also the group A streptococcal cell wall is highly resistant to degradation in the host. These antigens persist for months *in vivo* and experimentally elicit diseases that resemble rheumatic arthritis and carditis. Rheumatic arthritis should not be confused with the most common rheumatic disease - rheumatoid arthritis. Early termination of throat infections with penicillin therapy decreases the incidence of the subsequent development of rheumatic carditis. **Acute glomerulonephritis** is an immune complex disease of the kidney.
**Group B streptococci** can cause blood infections, pneumonia and meningitis in newborns. Adults can also get group B strep infections, especially if they are elderly or already have health problems. Strep B can cause urinary tract infections, blood infections, skin infections and pneumonia in adults.
**Group D streptococci** is now classified as an Enterococcus. Enterococci are distantly related to other streptococci and have been moved into the genus *Enterococcus*; the most commonly isolated are *E. (S[5].) faecalis* and *E. (S.) faecium. E. (S.) faecalis* can cause nosocomial infections, urinary tract infections, bacteremia, endocarditis, meningitis, and can be found in wound infections along with many other bacteria.

***Streptococcus pneumoniae*** causes pneumonia, acute sinusitis, otitis media, meningitis, bacteremia, sepsis, osteomyelitis, septic arthritis, endocarditis, peritonitis, pericarditis, cellulitis, and brain abscess. *S. pneumoniae* is the most common cause of bacterial meningitis in adults and children, and is one of the top two isolates found in ear infection, otitis media.

## 2.1 STREPTOCOCCI - GRAM STAIN

The genus *Streptococcus* is a diverse collection of **Gram-positive cocci** typically arranged in **pairs** or **chains** (in contrast to the clusters formed by *Staphylococcus*) (Fig. 12). *Streptococcus pneumoniae* are lancet shaped (ovoid) cocci in short chains, diplococci and single cocci (Fig 13).

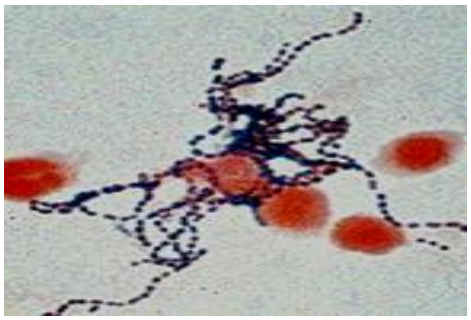

**Fig. 12 *S. pyogenes* - Gram stain.**

[5] S. - streptococcus

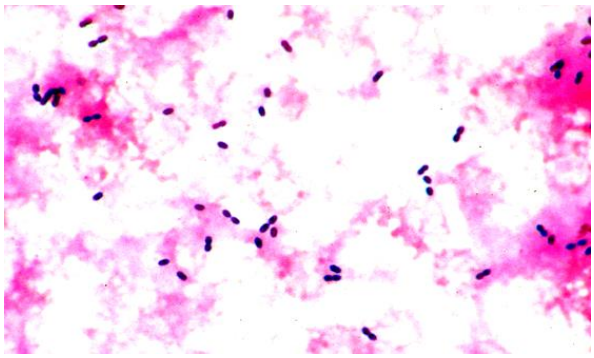

**Fig. 13 *S. pneumoniae* - Gram stain.**

## 2.2 STREPTOCOCCI - BLOOD AGAR CULTURE

Most species of streptococci are facultative anaerobes, and some grow only in an atmosphere enhanced with carbon dioxide (capnophilic growth). The classification of species within the genus is complicated because three different schemes are used:
- **hemolytic patterns:** complete (β) hemolysis, incomplete (α) hemolysis, and no hemolysis (γ);
- **serologic properties**: Lancefield groupings (originally A to W);
- **biochemical** (physiologic) properties.

### HEMOLYTIC PATTERNS

Streptococci are divided into three groups (Fig. 11):
**β-hemolytic streptococci**, which are classified by Lancefield grouping,
**α-hemolytic** and **γ-hemolytic streptococci**, which are classified by biochemical testing.

#### Alpha-hemolysis

***Streptococcus pneumoniae, Streptococcus salivarius, viridans*** are referred to collectively as **viridans streptococci**, a name derived from *viridis* (Latin for "green"), referring to the green pigment formed by the partial, α-hemolysis of blood agar. Encapsulated, virulent strains of *S. pneumoniae* often forming highly mucoid, glistening colonies (production of capsular polysaccharide) surrounded by a zone of α -hemolysis.
When **α-hemolysis** is present, the agar under the colony is dark and greenish. *Streptococcus pneumoniae* and a group of oral streptococci (*Streptococcus* viridans or viridans streptococci) display alpha hemolysis. This is sometimes called *green hemolysis* because of the color change in the agar. Other synonymous terms are *incomplete hemolysis* and *partial hemolysis*. Alpha hemolysis is caused by hydrogen peroxide produced by the bacterium, oxidizing hemoglobin to green methemoglobin. Alpha-hemolytic colonies with depressions in their centers are characteristic of pneumococci (Fig. 14). Some strains produce high ammounts of capsular polysaccharide which gives glistening appearance.

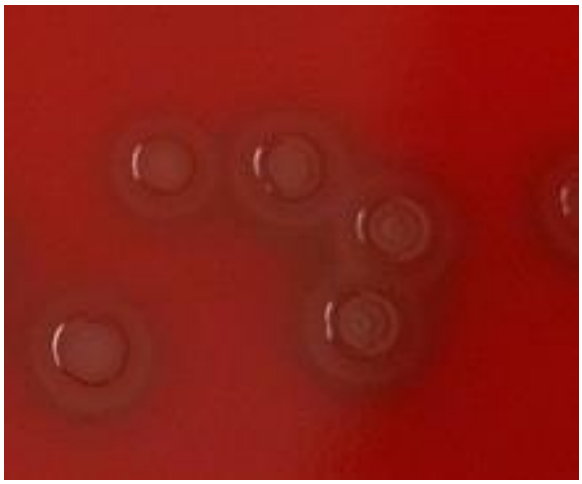

**Fig. 14 *Streptococcus pneumoniae* – alfa hemolysis.**

### Beta Hemolysis

*Streptococcus pyogenes*, or **G**roup **A** beta-hemolytic **S**treptococci (**GAS**), and *Streptococcus agalactiae*, or **G**roup **B** beta-hemolytic **S**treptococci (**GBS**) blood agar cultures display beta hemolysis (Fig. 15, Fig. 16). **Beta hemolysis** (β-hemolysis), sometimes called *complete hemolysis*, is a complete lysis of red blood cells in the media around and under the colonies: the area appears lightened (yellow) and transparent.

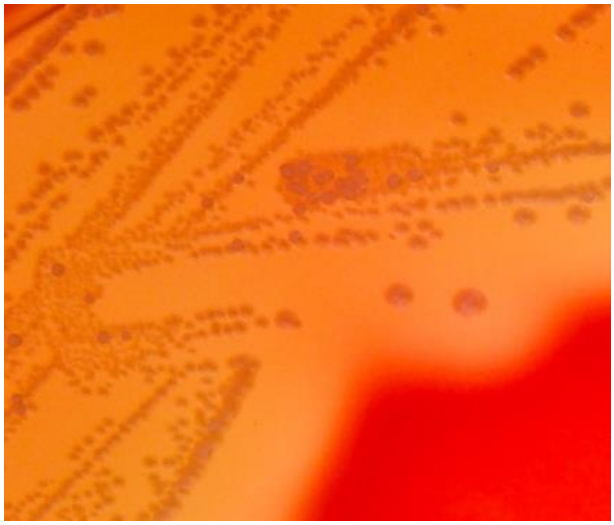

**Fig. 15 *Streptococcus pyogenes* - beta hemolysis.**

Streptolysin, an exotoxin, is produced by the bacteria which causes the complete lysis of red blood cells. Streptolysin O is oxygen-sensitive cytotoxin, secreted by most GAS, and interacts with cholesterol in the membrane of eukaryotic cells (mainly red and white blood cells, macrophages, and platelets), and usually results in β-hemolysis under the surface of blood

agar. Colonies of group B streptococci often have less pronounced zones of beta-hemolysis than do other beta-hemolytic streptococci (Fig. 16).

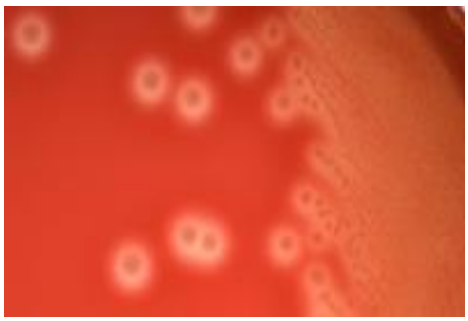

**Fig. 16 *Streptococcus agalactiae* - beta hemolysis.**

### Gamma Hemolysis

If an organism does not induce hemolysis, the agar under and around the colony is unchanged, and the organism is called *non-hemolytic* or said to display **gamma hemolysis** (γ-hemolysis). *Enterococcus faecalis* (formerly called Group D Streptococci) displays gamma hemolysis.

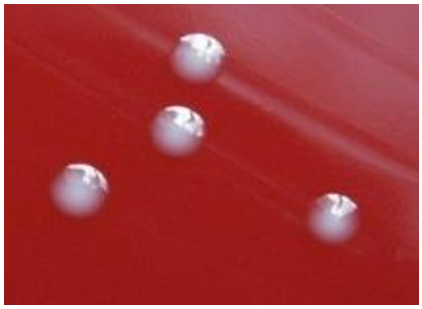

**Fig. 17 *Enterococcus faecalis* - gamma hemolysis.**

*E. faecalis* typically exhibits gamma-hemolysis on blood agar, but some strains are alfa-hemolytic or even beta-hemolytic (a plasmid-encoded hemolysin, called the cytolysin).

## 2.3 STREPTOCOCCI - SEROLOGIC PROPERTIES TESTING

### Bacitracin Sensitivity Test (β-hemolytic streptococci Group A, GAS)

**Bacitracin** is a polypeptide antibiotic interfering with the synthesis of peptidoglycan, a unique chemical fabric bacteria include in their cell walls. Bacitracin is common in topical ointments or inhaled into the lungs, but is very rarely given by injection. In a clinical laboratory, bacitracin is useful in helping identify *Streptococci* and other Gram-positive bacteria. Two concentrations of bacitracin-impregnated disks are made for this purpose, with

each being useful in distinguishing among select groups of Gram-positive cocci. Different types of bacteria have different degrees of susceptibility to bacitracin. This test determines whether the bacterium is either sensitive (susceptible) to bacitracin or resistant to the drug. Knowledge about bacitracin susceptibility is valuable in identification of Gram positive cocci, some of which are susceptible and others of which are resistant.

**Principle**: Bacitracin test is used to determine the effect of a small amount of bacitracin (0.04 U[6]) on an organism. *Streptococcus pyogenes* (GAS) is inhibited by the small amount of bacitracin in the disk (visible zone of inhibition of growth, Fig. 18); other beta-hemolytic streptococci usually are not.

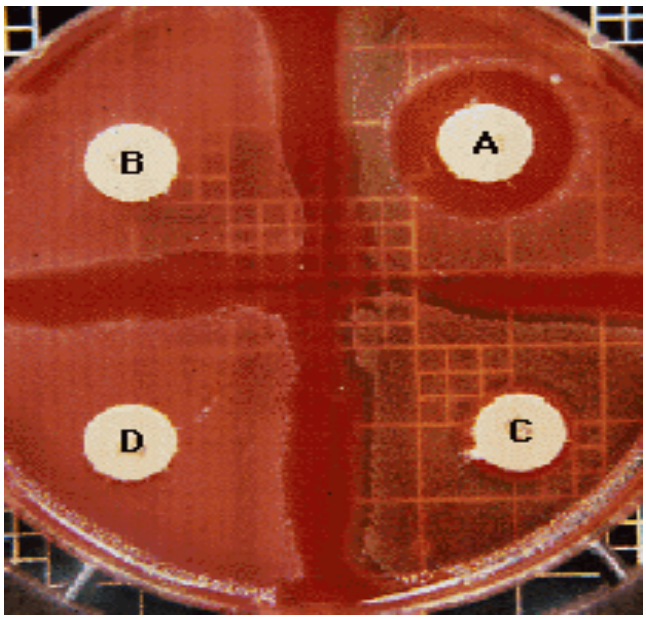


**Fig. 18 Bacitracin ensitivity test.**

### CAMP Test (β-hemolytic streptococci Group B, GBS)

***S. agalactiae*** is the only species that has the group B antigen. This organism was first recognized as a cause of puerperal sepsis. Although this disease is now relatively uncommon, ***S. agalactiae*** has become better known as an important cause of septicemia, pneumonia, and meningitis in newborn children, as well as a cause of serious disease in adults. **CAMP** - it is an acronym for "Christie, Atkins, Munch-Petersen", for the three researchers who discovered the phenomenon. The CAMP test is a test to identify Group B β-hemolytic streptococci based on their formation of a substance (CAMP factor) that enlarges the area of hemolysis formed by β-hemolysin from *Staphylococcus aureus*. It can be used to identify *Streptococcus agalactiae* (GBS) Though not strongly beta-hemolytic on its own, it presents with a wedge-shape in the presence of *Staphylococcus aureus.*

**Principle**: The majority of group B streptococci produce a diffusable extracellular protein (CAMP factor) that acts synergistically with staphylococcal beta lysin to lyse erythrocytes.

[6] U - unit

**Fig. 19 CAMP test - positive result.**

CAMP test showing the arrow-shaped zone of enhanced hemolysis (positive result) of *Streptococcus agalactiae* (group B) and the negative result of *Streptococcus pyogenes* (group A) when tested against *Staphylococcus aureus* (Fig 19, Fig 20).

**Fig. 20 CAMP test - positive (up) and negative (down) result.**

## 2.4 STREPTOCOCCI - PHYSIOLOGIC (BIOCHEMICAL) PROPERTIES TESTING

***Streptococcus pneumoniae, Streptococcus salivarius, Streptococcus viridans*** are referred to collectively as **viridans streptococci**, a name derived from *viridis* (Latin for "green"), referring to the green pigment formed by the partial hemolysis of blood agar. The α-hemolytic (viridans) streptococci are classified by biochemical testing.

### Optochin Test

**Optochin** (or ethylhydrocupreine) is a chemical used in cell culture techniques for the of *Streptococcus pneumoniae*, which is optochin-sensitive (positive result), from other alpha-hemolytic streptococci such as *Streptococcus viridans* which are resistant. Optochin Differentiation Disks are recommended for use in the presumptive identification of *Streptococcus pneumoniae* from other alpha-hemolytic streptococci. The growth of bacteria

that are optochin sensitive will be inhibited around optochin disc, while the growth of bacteria that are optochin resistant will not be affected. A zone of inhibition of ≥ 15 mm around the disk is considered a susceptible result (Fig. 21). In vitro a solution of as little as 1: 10,000,000 may be inhibitory to the pneumococcus, and 1: 500,000 is bactericidal.
Strains of other viridans streptococci have been found to be resistant to optochin in laboratory testing.

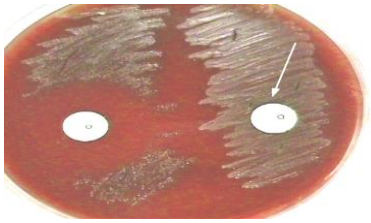

**Fig. 21 Optochin Test - positive result (left).**

### Bile Solubility Test

The bile (sodium deoxycholate) solubility test distinguishes *Streptococcus pneumoniae* from all other alpha-hemolytic (viridans) streptococci. *Streptococcus pneumoniae* is bile soluble whereas all other alpha-hemolytic streptococci are bile resistant. Sodium deoxycholate (2% in water) will lyse the pneumococcal cell wall. A clearing of the turbidity in the bile tube indicates a positive test (Fig. 22).

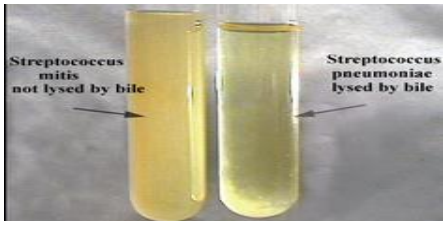


**Fig. 22 Bile solubility test - positive result (right).**

## 2.5 ANTISTREPTOLYSIN O (ASO)

**Anti-streptolysin O** (ASO) is the antibody made against streptolysin O, an immunogenic, oxygen-labile hemolytic toxin produced by most strains of group A and many strains of groups C and G streptococci. The O in the name stands for *oxygen-labile*, the other related toxin being oxygen-stable streptolysin-S. When the body is infected with streptococci, it produces antibodies against the various antigens that the streptococci produce. A raised or rising levels can indicate past or present infection. Historically it was one of the first bacterial markers used for diagnosis and follow up of rheumatic fever or scarlet fever. Its importance in this regard has not diminished. Since these antibodies are produced as a delayed antibody

reaction to the above mentioned bacteria, there is no normal value. The presence of these antibodies indicates an exposure to these bacteria. However, as many people are exposed to these bacteria and remain asymptomatic, the presence of ASO does not indicate disease.

Acceptable values, where there is no clinical suspicion of rheumatism are as follows:

- Adults: less than 200 units
- Children: less than 400 units

This titre has a significance only if it is greatly elevated (>200), or if a rise in titre can be demonstrated in paired blood samples taken days apart. The antibody levels begin to rise after 1 to 3 weeks of streptococcal infection, peaks in 3 to 5 weeks and falls back to insignificant levels in 6 months. Values need to be correlated with a clinical diagnosis.

## 2.6 C-REACTIVE PROTEIN (CRP)

C-reactive protein (CRP) is found in the blood and is a response to inflammation in the body. It can also be an indicator of the presence of infection, trauma or serious illness. Chronic inflammation can keep CRP levels elevated, which can increase the risk of cardiovascular conditions such as heart attacks or stroke. C-reactive protein is produced by the liver. The level of CRP rises when there is inflammation throughout the body. It is not a specific test.

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# 3 NEISSERIA

Of the eleven species of *Neisseria* that colonize humans, only two are pathogens. [7]***N. gonorrhoeae*** (the gonococcus) is the causative agent of gonorrhoea and is transmitted via sexual contact. Symptoms of infection with *N. gonorrhoeae* differ depending on the site of infection. Infection of the genitals can result in a purulent (or pus-like) discharge from the genitals which may be foul smelling, inflammation, redness, swelling, dysuria and a burning sensation during urination. *N. gonorrhoeae* can also cause conjunctivitis, pharyngitis, proctitis or urethritis, prostatitis and orchitis. Conjunctivitis is common in neonates and silver nitrate or antibiotics are often applied to their eyes as a preventive measure against gonorrhoea. Neonatal gonorrheal conjunctivitis is contracted when the infant is exposed to *N. gonorrhoeae* in the birth canal, and can result in corneal scarring or perforation. Disseminated *N. gonorrhoeae* infections can occur, resulting in endocarditis, meningitis or gonococcal dermatitis-arthritis syndrome.

***Neisseria meningitidis*** (the meningococcus) causes significant morbidity and mortality in children and young adults worldwide through epidemic or sporadic meningitis and/or septicemia. *N. meningitidis* is exclusive human pathogen. The epidemiological profile of *N. meningitidis* is variable in different populations and over time and virulence of the meningococcus is based on a transformable/plastic genome and expression of certain capsular polysaccharides (serogroups A, B, C, W-135, Y and X) and non-capsular antigens. *N. meningitidis* colonizes mucosal surfaces using a multifactorial process involving pili, twitching motility, and surface proteins. Certain clonal groups have an increased capacity to gain access to the blood, evade innate immune responses, multiply, and cause systemic disease. Although new vaccines hold great promise, meningococcal infection continues to be reported in both developed and developing countries, where universal vaccine coverage is absent and antibiotic resistance increasingly more common.

## 3.1 NEISSERIA - GRAM STAIN

***Neisseria meningitidis*** is a Gram-negative, either an encapsulated or unencapsulated, aerobic diplococcus with a "kidney" or "coffee-bean" shape. *N. meningitidis* may occur intracellularly or extracellularly in [8]PMN leukocytes *N. meningitidis* is a fastidious bacterium, dying within hours on inanimate surfaces.

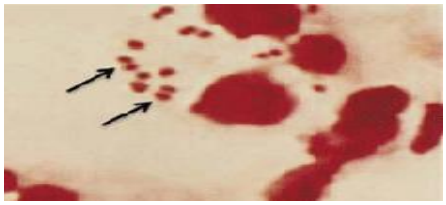

**Fig. 23 Gram stain of *N. meningitidis* in cerebro-spinal fluid with associated PMNs.**

[7] N. – Neisseria
[8] PMN - Polymorphonuclear

***Neisseria gonorrhoeae,*** also known as gonococcus, is a species of Gram-negative coffee bean-shaped diplococci that typically appear in pairs with the opposing sides flattened. Bacteria are responsible for the sexually transmitted disease gonorrhea.

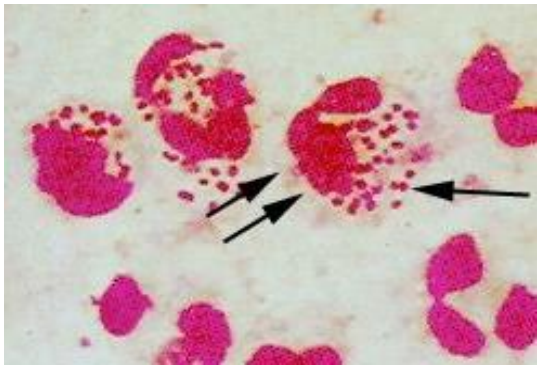

**Fig. 24 *Neisseria gonorrhoeae* - Gram-negative diplococci.**

## 3.2 NEISSERIA - BLOOD AGAR CULTURE

For cultivation of pathogenic *Neisseria* [9]spp. are used special media for cultivation and isolation of nutritionally fastidious microorganisms.
***Neisseria meningitidis*** grows on them without hemolysis. Colonies of *Neisseria meningitidis* are unpigmented and appear round, smooth, glistening, and convex, with a clearly defined edge. Some strains may produce larger, grey, opaque colonies. Cultivation 24 hours in an aerobic atmosphere enriched with 5% carbon dioxide, 37°C.
The organism grows on different media such as blood agar, trypticase soy agar, supplemented chocolate agar, and Mueller-Hinton agar.

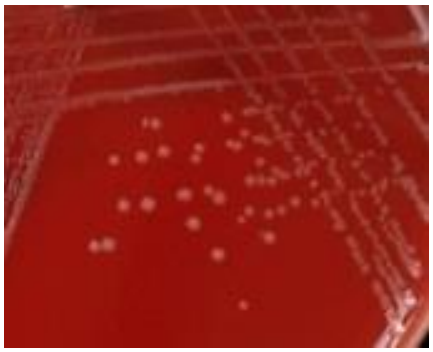

**Fig. 25 *Neisseria meningitidis* - Blood agar culture.**

***Neisseria gonorrhoeae*** are the most fastidious of the *Neisseria* species, require complex growth media and are highly susceptible to toxic substnces (e.g., fatty acids). Gonococci are not able to grow on common blood agar. Colonies are positive by the oxidase test and the result is confirmed with carbohydrate reactions (meningococci oxidize glucose and usually maltose, but not sucrose and lactose).

[9] spp. - species

## 3.3 NEISSERIA - CHOCOLATE AGAR CULTURE

**Chocolate agar** (CHOC) or C**hocolate blood agar** (CBA) - is a non-selective, enriched growth medium used for isolation of pathogenic bacteria. It is a variant of the blood agar plate, containing red blood cells that have been lysed by slowly heating to 80°C. Chocolate agar is used for growing fastidious respiratory bacteria, such as *Haemophilus influenzae* and *Neisseria meningitidis*. Chocolate agar with the addition of bacitracin becomes selective, most critically, for the genus *Haemophilus*.

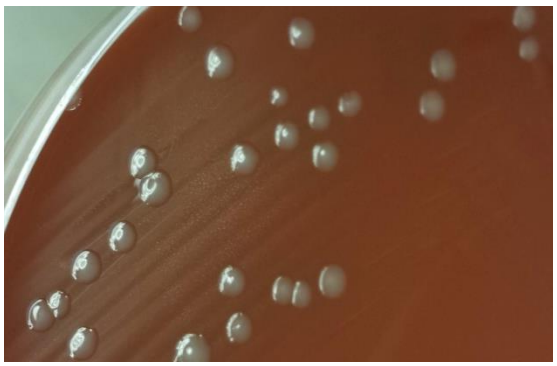

**Fig. 26 *Neisseria meningitidis* on Chocolate Agar.**

Media for *N. gonorrhoeae* contain antimicrobials that inhibit the growth of organisms other than *N. gonorrhoeae*; typically vancomycin (inhibits Gram-positive bacteria), colistin (inhibits gram-negative bacteria including the commensal *Neisseria* spp.), trimethoprim (inhibits swarming of *Proteus* spp.) and nystatin or amphotericin B (antifungal agents). Often are used media resembling chocolate agar in appearance (e.g., modified Thayer-Martin agar (MTM) or Martin-Lewis agar (ML).

**Fig. 27 *Neisseria gonorrhoeae* on Chocolate Agar.**

Plates are incubated in a $CO_2$-enriched, humid atmosphere (some gonococci require $CO_2$ for growth, the growth of all species is enhanced by carbon dioxide). Colonies of *N. gonorrhoeae* vary in diameter from 1.0 to 4.0 mm after 48 hours. The colonies are smooth and nonpigmented (Fig. 27). Some strains may produce atypical small colonies.

## 3.4 NEISSERIA - BIOCHEMICAL PROPERTIES TESTING

Further testing to differentiate the species includes testing for oxidase (all *Neisseria* show a positive reaction) and the carbohydrates maltose, sucrose, and glucose test in which *N. gonorrhoeae* will only oxidize (that is, utilize) the glucose.

### Kovac's Oxidase Test

Kovac's oxidase test (cytochromoxidase test) determines the presence of cytochrome oxidase. Kovac's oxidase reagent, tetramethyl-p-phenylenediamine dihydrochloride, is turned into a purple compound by organisms containing cytochrome c as part of their respiratory chain. This test aids in the recognition of *N. meningitidis*, but other members of the genus *Neisseria,* as well as unrelated bacterial species, may also give a positive reaction. Positive and negative quality control strains should be tested along with the unknown isolates to ensure that the oxidase reagent is working properly. Positive reactions will develop within 10 seconds in the form of a purple color. Negative reactions will not produce a color change.

### Oxidase Test - Filter paper method

1. Grow the isolate to be tested for 18-24 hours on a blood agar plate (BAP) at 35-37°C with 5% $CO_2$ (or in a candle-jar).
2. On a nonporous surface (Petri dish or glass plate), wet a strip of filter paper with a few drops of Kovac's oxidase reagent.
3. Let the filter paper strip air dry before use.
4. Use a disposable plastic loop, a platinum inoculating loop, or a wooden applicator stick to pick a portion of a colony from overnight growth on the BAP and rub it onto the treated filter paper.
5. Observe the filter paper for color change to purple (Fig. 28).

**Fig. 28 Oxidase Test - Filter paper method.**

### Oxidase Test - Plate method

1. Grow the isolate to be tested for 18-24 hours on a blood agar plate (BAP) at 35-37°C with 5% $CO_2$ (or in a candle-jar).

2. Dispense a few drops of Kovac's oxidase reagent directly on top of a few suspicious colonies growing on the 18-24 hour BAP (Fig. 29).
3. Tilt the plate and observe colonies for a color change to purple (Fig. 29).

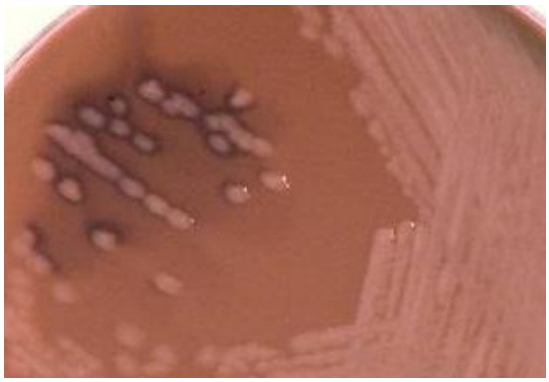

**Fig. 29 Oxidase Test - Plate method.**

### Identification of *Neisseria* with a Commercial Kit (NEISSERIAtest, Erba Lachema).

The NEISSERIAtest is a miniaturized version of conventional procedures for the identification of *Neisseria* species. It is a ready-to-use microwell plate system designed for performance of 7 biochemical tests:

- acid production from **glucose**, **maltose**, **fructose** and **sucrose**;
- detection of **γ-glutamyl transferase**, hydrolysis of **tributyrin** and **synthesis of polysacharide**

(To evaluate the colour reactions of the tests follow the table "Interpretation of reactions" and/or the colour reaction of the control strains).

Any change of the colour reactions of these sugars in comparison to negative control means positive reaction (Fig. 30, Table 1).

*Neisseria* spp. produce acid from carbohydrates by oxidation, not fermentation.

***N. meningitidis* oxidizes glucose and maltose, but not lactose or sucrose.**

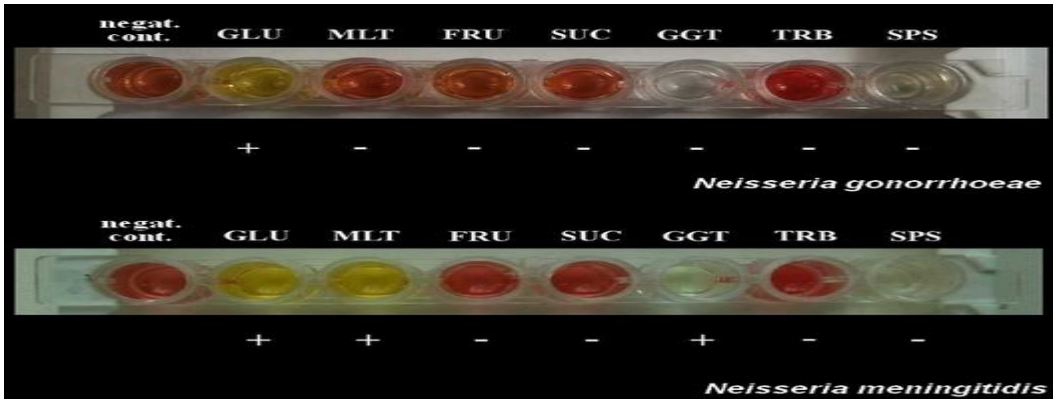


**Fig. 30 NEISSERIAtest.**

| ***N. meningitidis:*** | ***N. gonorrhoeae:*** |
|---|---|
| Negative control | Negative control |
| GLU - Glucose [10]POS | **GLU - Glucose POS** |
| **MLT - Maltose POS** | MLT – Maltose NEG |
| FRU - Fructose [11] NEG | FRU - Fructose NEG |
| SUC - Sucrose NEG | SUC - Sucrose NEG |
| **GGT - γ-glutamylaminopeptidase POS** | GGT - γ-glutamylaminopeptidase NEG |
| TRB - Tributyrin hydrolysis NEG | TRB - Tributyrin hydrolysis NEG |
| SPS - Polysaccharide synthesis NEG | SPS - Polysaccharide synthesis NEG |

**Table 1. NEISSERIAtest - results.**

[10] POS - positive
[11] NEG - negative

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# 4 HAEMOPHILUS

***Haemophilus*** is a genus of Gram-negative, pleomorphic, coccobacilli bacteria belonging to the Pasteurellaceae family. The genus includes commensal organisms along with some significant pathogenic species such as [12]*H. influenzae* - a cause of sepsis and bacterial meningitis in young children, and *H. ducreyi*, the causative agent of chancroid (Fig. 31). Other *Haemophilus* species cause disease less frequently. *Haemophilus parainfluenzae* sometimes causes pneumonia or bacterial endocarditis. *Haemophilus aphrophilus* is a member of the normal flora of the mouth and occasionally causes bacterial endocarditis. *Haemophilus aegyptius*, which causes conjunctivitis and Brazilian purpuric fever, and *Haemophilus haemolyticus* used to be separated on the basis of their ability to agglutinate or lyse red blood cells, but both are now included among the nontypable *H influenzae* strains.

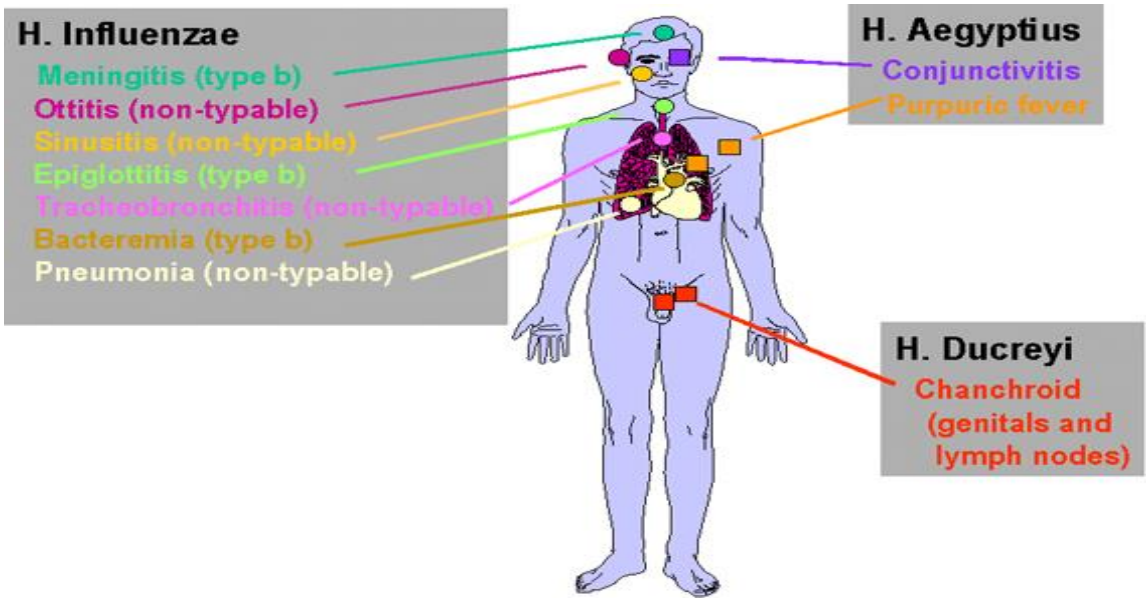


**Fig. 31 Diseases caused by *Haemophilus* species.**

There are two major groups ***H. influenzae*:**

1. The encapsulated group. This group consists of types a,b,c,d and f. The capsule has a role in virulence as it gives protection from phagocytosis, so the non-capsulated strains of *H. influenzae* are usually less invasive.
2. Non-encapsulated group. The non-encapsulated strain of *H. influenzae* is present in the nasopharynx of approximately 75% of healthy children and adults so *H. influenzae* cultured from the nasopharyngeal cavity or sputum would not indicate *H. influenzae* disease, because these sites are colonized in disease-free individuals. However, *H. influenzae* isolated from cerebrospinal fluid or blood would indicate *H. influenzae* infection.

[12] H - Haemophilus

*H. influenzae* type b (**Hib**) is the most common bacterium that cause disease such as bacteremia, pneumonia, epiglottitis and acute bacterial meningitis. The bacterial transmission spread person-to-person by direct contact or through respiratory droplets like coughing and sneezing.

## 4.1 HAEMOPHILUS - GRAM STAIN

Gram stain and microscopic observation of a specimen of *H. influenzae* will show Gram-negative, rod shaped bacteria with no specific arrangement. The rounded ends of short (0.5-1.5 μm) bacilli make many appear round, hence the term coccobacilli.

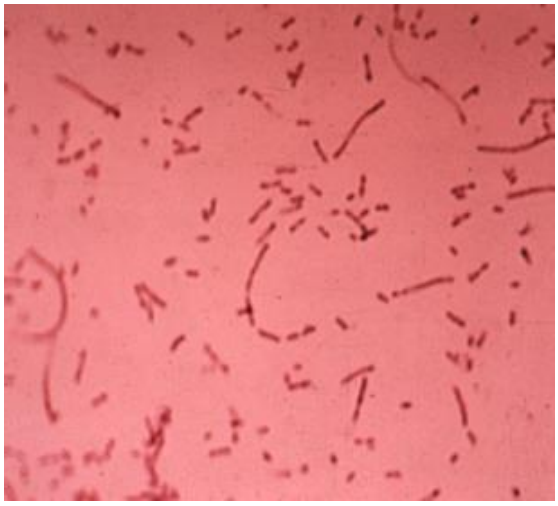

**Fig. 32 *H. influenzae* - Gram stain.**

Non-encapsulated organisms from sputum are pleomorphic and often exhibit long threads and filaments (Fig. 32). The organism may appear Gram-positive unless the Gram stain procedure is very carefully carried out.
*H. parainfluenzae* are Gram-negative pleomorphic rods (Fig. 33).

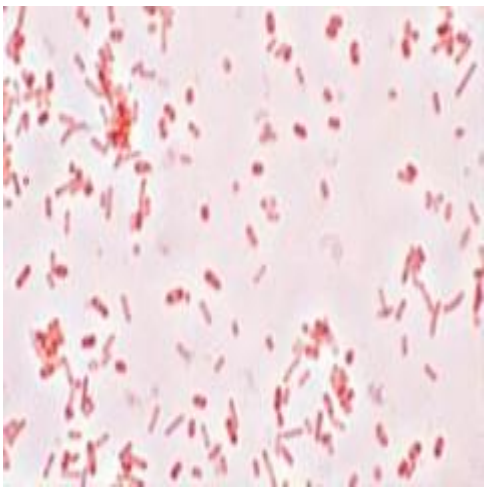

**Fig. 33 *H. parainfluenzae* - Gram stain.**

## 4.2 HAEMOPHILUS – CULTIVATION ON BLOOD AGAR

***H. influenzae*** can't grow on blood agar as it lacks the growth factors [13]X and [14]V but in special case the growth is only achieved as a satellite phenomenon around other bacteria.

**Satellite Phenomenon**

*H. influenzae* will grow in the hemolytic zone of *Staphylococcus aureus* on blood agar plates, the hemolysis of cells by *S.aureus* releases factor V which is needed for its growth. *H. influenzae* will not grow outside the hemolytic zone of *S. aureus* due to the lack of nutrients such as factor V in these areas.

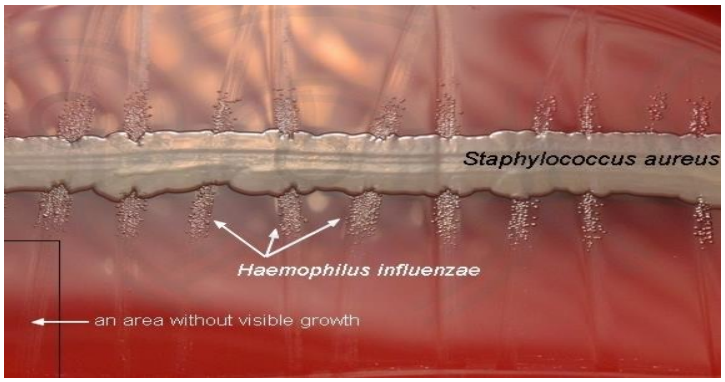


**Fig. 34 *Haemophilus influenzae* - Satellite phenomenon.**

*Haemophilus influenzae* will grow in the hemolytic zone of *Staphylococcus aureus* on blood agar plates (Fig. 34). The hemolysis of erythrocytes by *S. aureus* releases nutrients vital to the growth of *H. influenzae* (NAD, factor V). The NAD diffuses into the surrounding medium and stimulates the growth of *Haemophilus influenzae* in the vicinity of the staphylococcus. This is known as satelliting. For *Haemophilus* spp., the satellite test substitutes for the V factor test.

Colonies of *H. influenzae* appear as convex, smooth, pale, grey or transparent colonies. Encapsulated strains may produce larger colonies with a glistening mucoid quality, mouse nest odor is typical. *H. parainfluenzae* colonies morphology: medium to large, smooth, and translucent, nonhemolytic on rabbit or horse blood agar, appear as "schools of fish"

## 4.3 HAEMOPHILUS - CULTIVATION ON CHOCOLATE AGAR

***Haemophilus influenzae*** requires X (hemin) and V (NAD) factors for growth so *H. influenzae* culture is performed on chocolate agar, which contain X and V factors and the plate is placed at 37°C in a $CO_2$-enriched incubator.

[13] Factor X - hemin
[14] Factor V - nicotinamide adenine dinucleotide **(NAD)**

**Chocolate agar** (CHOC) or **chocolate blood agar** (CBA) - is a non-selective, enriched growth medium used for isolation of pathogenic bacteria. It is a variant of the blood agar plate, containing red blood cells that have been lysed by slowly heating to 80°C. Chocolate agar is used for growing fastidious respiratory bacteria, such as *Haemophilus influenzae* and *Neisseria meningitidis*. Chocolate agar with the addition of bacitracin becomes selective, most critically, for the genus *Haemophilus*.

**Fig. 35 *Haemophilus influenzae* - colonies on Chocolate agar.**

## 4.4 X AND V FACTOR DISCS FOR DIFFERENTIATION OF HAEMOPHILUS SPECIES

*Haemophilus influenzae* requires two accessory growth factors: **factor X** (haemin or other porphyrins) and **V factor** (NAD). The X and V factor requirement is usually demonstrated by the absence of growth on porphyrin and NAD deficient but otherwise nutritionally adequate media except near paper disc impregnated with X and V factors.

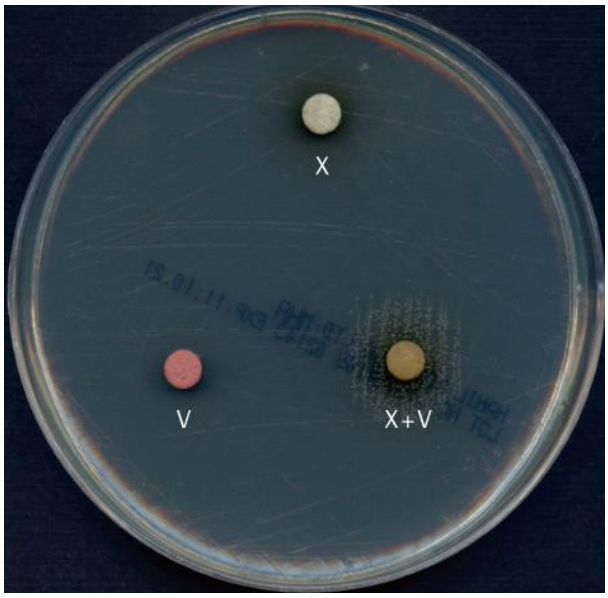


**Fig. 36 *H. influenzae* - X and V factor disc test.**

**X and V Factor Disks** are paper disks impregnated with X (hemin) and V (nicotinamide adenine dinucleotide) growth factors. They are used for the differentiation of ***Haemophilus*** **species** (Fig. 36, Table 2). We usually use Mueller Hinton Agar for the disk test. ***Haemophilus influenzae*** require both X and V factor to grow.

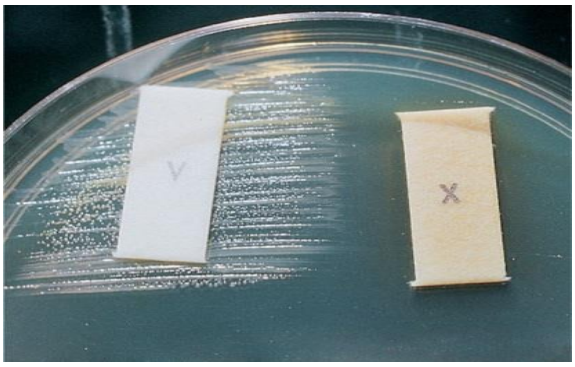


**Fig. 37 *H. parainfluenzae* - X and V factor disc test.**

***Haemophilus parainfluenzae*** requires V factor only for growth.

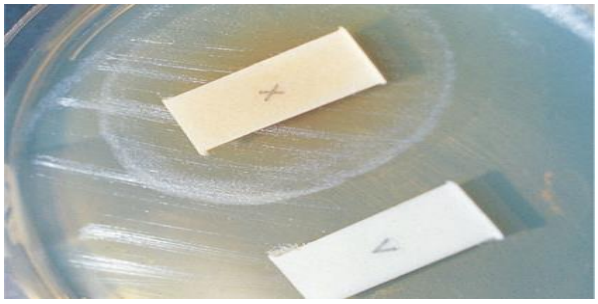


**Fig. 38 *H. ducreyi* - X and V factor disc test.**

***Haemophilus ducreyi*** requires only X factor without need of V factor.

| ***Haemophilus*** species | Growth Around Disk | | |
|---|---|---|---|
| | X | V | XV |
| *Haemophilus aegyptius* | - | - | + |
| *Haemophilus aphrophilus* | v/- | - | + |
| *Haemophilus ducreyi* | + | - | + |
| *Haemophilus haemolyticus* | - | - | + |
| *Haemophilus influenzae* | - | - | + |
| *Haemophilus parahaemolyticus* | - | + | + |
| *Haemophilus parainfluenzae* | - | + | + |

**Table 2 *Haemophilus* species - growth around X an V discs.**

## 4.5 QUELLUNG TEST

Quellung Test is an increase in the opacity and visibility of the capsule of encapsulated organisms resulting from exposure to specific, agglutinating, anticapsular antibodies. This test is also called Neufeld reaction or quellung reaction. The Quellung reaction is a biochemical reaction in which antibodies bind to the bacterial capsule of *Haemophilus influenzae* (or *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Neisseria meningitidis*) and thus allow them to be visualized under a microscope. If the reaction is positive, the capsule becomes opaque and appears to enlarge.

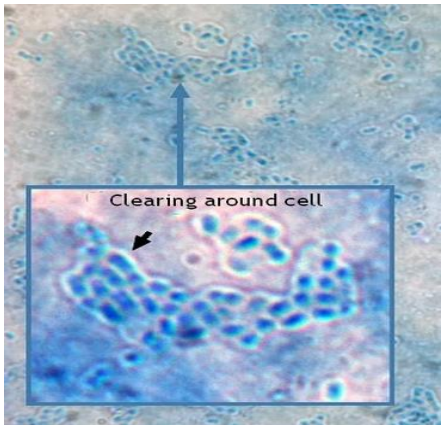


**Fig. 39 Quellung reaction - swelling of bacterial capsules.**

**SOURCES:**


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http://www.microbiologyinpictures.com/bacteria-photos/haemophilus-influenzae-images/satellite-test.html

*http://www.medical-labs.net/x-and-v-factor-disks-for-the-differentiation-of-haemophilus-species-2649/*

*http://www.medical-labs.net/x-and-v-factor-disks-for-the-differentiation-of-haemophilus-species-2649/ISBN 978-1-904455-34-9.*

http://www.snipview.com/q/Quellung_reaction

# 5 ENTEROBACTERIACEAE

Enterobacteriaceae family contains a large number of genera that are biochemically and genetically related to one another. This group of organisms includes several that cause primary infections of the human gastrointestinal tract. Members of this family are major causes of opportunistic infection (including septicemia, pneumonia, meningitis and urinary tract infections). Examples of genera that cause opportunistic infections are: *Citrobacter, Enterobacter, Escherichia, Hafnia, Morganella, Providencia* and *Serratia*.

***Escherichia coli*** live in the human gut and are usually harmless but some are pathogenic causing diarrhea and other symptoms as a result of ingestion of contaminated food or water.
Enteropathogenic *E. coli* (EPEC). Certain serotypes are commonly found associated with infant diarrhea.
Enterotoxigenic *E. coli* (ETEC) produce diarrhea resembling cholera but much milder in degree. They also cause "traveler's diarrhea".
Enteroinvasive *E. coli* (EIEC ) produce a dysentery (indistinguishable clinically from shigellosis, see bacillary dysentery).
Enterohemorrhagic *E. coli* (EHEC). These are usually serotype O157:H7. These organisms can produce a hemorrhagic colitis (characterized by bloody and copious diarrhea with few leukocytes in afebrile patients). The organisms can disseminate into the bloodstream producing systemic hemolytic-uremic syndrome (hemolytic anemia, thrombocytopenia and kidney failure) which is often fatal.
The commonest community acquired ("ascending") urinary tract infection is caused by *E. coli*.

***Shigella*** (4 species; *S. flexneri, S. boydii, S. sonnei, S. dysenteriae*), all cause bacillary dysentery or shigellosis, (bloody feces associated with intestinal pain). The organism invades the epithelial lining layer but does not penetrate. Usually within 2 to 3 days, dysentery results from bacteria damaging the epithelial layers lining the intestine, often with release of mucus and blood (found in the feces) and attraction of leukocytes (also found in the feces as "pus"). However, watery diarrhea is frequently observed with no evidence of dysentery. Shiga toxin (chromosomally-encoded), which is neurotoxic, enterotoxic and cytotoxic, plays a role.

***Salmonella*** infections most often cause vomiting or diarrhea, sometimes severe. In rare cases, Salmonella illness can lead to severe and life-threatening bloodstream infections. Salmonellosis, the common salmonella infection, is caused by a variety of serotypes (most commonly *S. enteritidis*) and is transmitted from contaminated food (such as poultry and eggs). It does not have a human reservoir and usually presents as a gastroenteritis (nausea, vomiting and non-bloody stools). The disease is usually self-limiting (2-5 days). Like *Shigella,* these organisms invade the epithelium and do not produce systemic infection.
***Salmonella enterica*** **serovar** ***typhi*** is transmitted from a human reservoir or in the water supply (if sanitary conditions are poor) or in contaminated food. It initially invades the intestinal epithelium. The organisms penetrates (usually within the first week) and passes into

the bloodstream where it is disseminated in macrophages. Typical features of a systemic bacterial infection are seen. The Vi (capsular) antigen plays a role in the pathogenesis of typhoid. Antibiotic therapy is essential.

***Yersinia enterocolitica*** - the organisms are invasive (usually without systemic spread). Typically the infection is characterized by diarrhea, fever and abdominal pain. *Y. enterocolitica* infections are seen most often in young children. *Y. enterocolitica* can be transmitted by fecal contamination of water or milk by domestic animals or from eating meat products.

***Klebsiella pneumoniae*** is often involved in respiratory infections. The organism has a prominent capsule aiding pathogenicity.

***Proteus*** is another common cause of urinary tract infection; the organism produces a urease that degrades urea producing an alkaline urine.

## 5.1 ENTEROBACTERIACEAE - GRAM STAIN

***Enterobacteriaceae*** are Gram-negative, short rods, non-sporulating, facultative anaerobes.

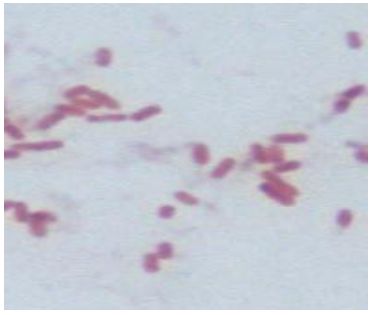


**Fig. 40 *Escherichia coli* – Gram stain.**

***Escherichia coli***, a Gram-negative bacillus, stained according to Gram stain protocol, the small rods characteristic of this organism.

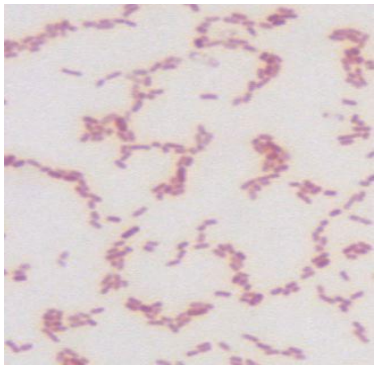


**Fig. 41 *Klebsiella pneumoniae* – Gram stain.**

*Klebsiella pneumoniae* is a Gram-negative, non-motile, encapsulated rod shaped bacterium found in the normal flora of the mouth, skin, and intestines.

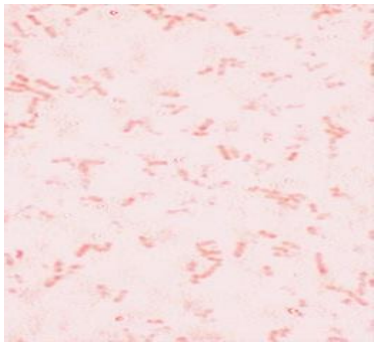


**Fig. 42 *Shigella flexneri* – Gram stain.**

*Shigella* is Gram-negative, nonmotile, nonspore forming, rod-shaped bacteria closely related to *Escherichia coli* and *Salmonella*. The causative agent of human shigellosis, *Shigella* causes disease in primates, but not in other mammals. It is only naturally found in humans and apes. During infection, it typically causes dysentery.

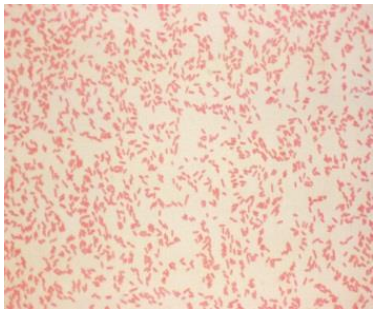


**Fig. 43 *Yersinia enterocolitica* – Gram stain.**

*Yersinia enterocolitica* is a species of gram-negative coccobacillus-shaped bacterium, belonging to the family *Enterobacteriaceae*. *Y. enterocolitica* infection causes the disease yersiniosis which is a zoonotic disease occurring in humans as well as a wide array of animals such as cattle, deer, pigs, and birds.

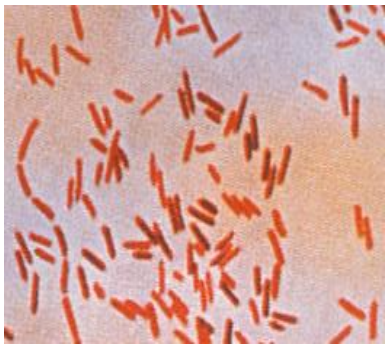


**Fig. 44 *Salmonella* Typhi – Gram stain.**

***Salmonella enterica* serovar *typhi*** are motile by means of peritrichous flagella. *Salmonella typhi* lives only in humans. Persons with typhoid fever carry the bacteria in their bloodstream and intestinal tract. In addition, a small number of persons, called carriers, recover from

typhoid fever but continue to carry the bacteria. Both ill persons and carriers shed *Salmonella typhi* in their feces (stool).

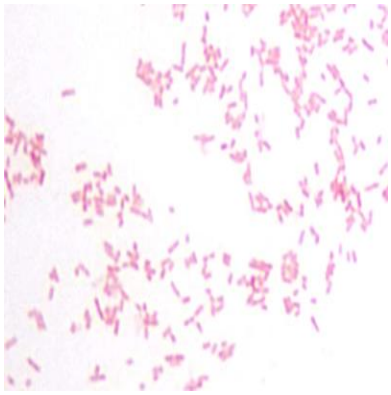


**Fig. 45 *Proteus vulgaris* – Gram stain.**

***Proteus vulgaris*** is Gram-negative bacterium that inhabits the intestinal tracts of humans and animals. It can be found in soil, water, and fecal matter. It is known to cause wound infections and other species of its genera are known to cause urinary tract infections.

## 5.2 BURRI´S INDIA INK METHOD (CAPSULE VISUALISATION)

Most bacterial capsules are composed of polysaccharide however some genera produce polypeptide capsules. The polymers which make up the capsule tend to be uncharged and as such they are not easily stained. For this reason we use a **negative stain** to visualize them. That is, we use a stain which stains the background against which the uncolored capsule can be seen. Burri´s India ink method, uses India ink to color the background and crystal violet (or safranin) to stain the bacterial cell.

### Capsule Stain Procedure

1. Place a single drop of India ink on a clean microscope slide, adjacent to the frosted adge.
2. Using a flamed loop and sterile technique, remove some *K. pneumoniae* (or the organism you want to stain) from your tube or plate and mix it into the drop of India ink.
3. Place the end of another clean microscope slide at an angle to the end of the slide containing the organism. Spread out the drop out into a film.
4. Allow the film to air dry.
5. Saturate the slide with crystal violet for 1 minute.
6. Rinse the slide gently with water.
7. Allow the slide to air dry.
8. Observe the slide under the microscope.

**Burri´s India ink method – results:**
The background will be **dark.**
The bacterial cells will be **stained purple.**
The capsule (if present) will appear **clear** against the dark background (Fig. 46).

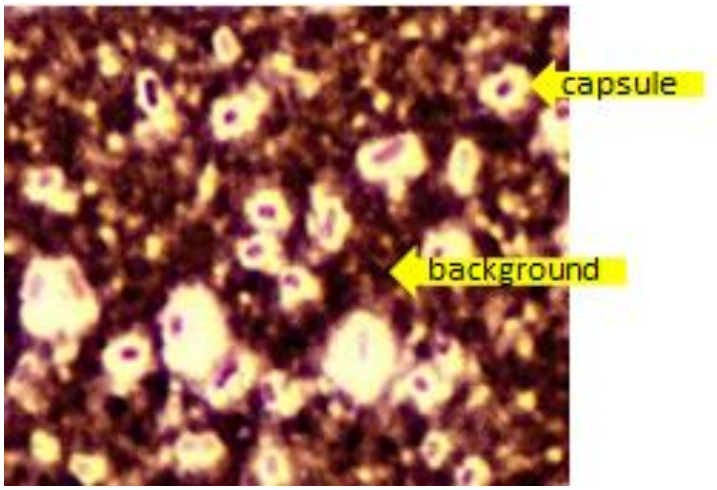


**Fig. 46 *K. pneumoniae* - Burri´s India ink method.**

## 5.3 ENTEROBACTERIACEAE - CULTIVATION ON BLOOD AGAR

Enteric organisms cultivated on blood agar usually reveal large, smooth, shiny, circular, raised colonies which mayor may not be hemolytic or pigmented. Proteus species often exhibit swarming.


**Fig. 47 *Escherichia coli*.**

*Escherichia coli* cultivated on blood agar. Colonies are without hemolysis but many strains isolated from infections are beta-hemolytic.

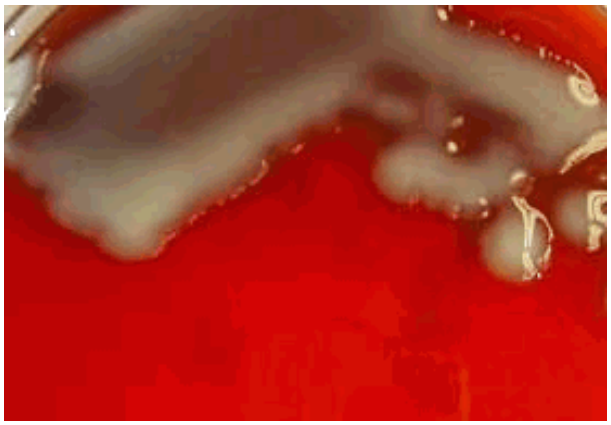


**Fig. 48 *Klebsiella pneumoniae*.**

**Non-hemolytic (gamma-hemolytic), mucous colonies** of *Klebsiella pneumoniae* on blood agar.

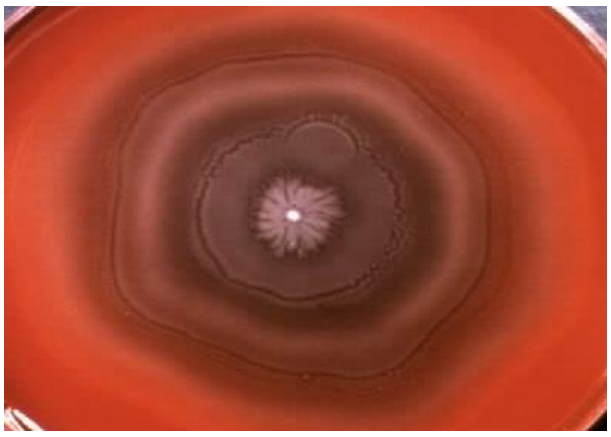


**Fig. 49 *Proteus mirabilis*.**

*Proteus mirabilis* on blood agar - for most strains of *P. mirabilis* and *P. vulgaris* is typical their **ability to swarm** (**RAUSS phenomenon**) over the surfaces of solid cultivation media (the spreading growth covers other organisms in the culture and thus delays their isolation).

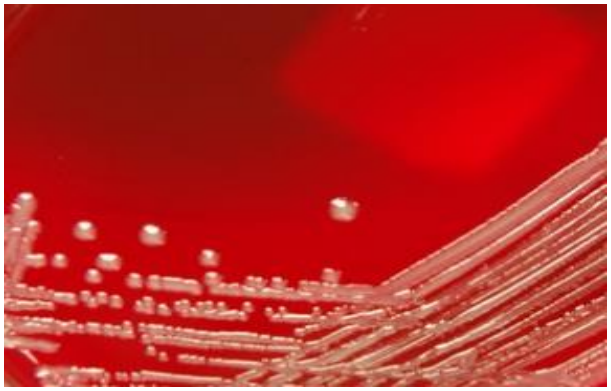


**Fig. 50 *Salmonella enterica* - non-hemolytic colonies on blood agar.**

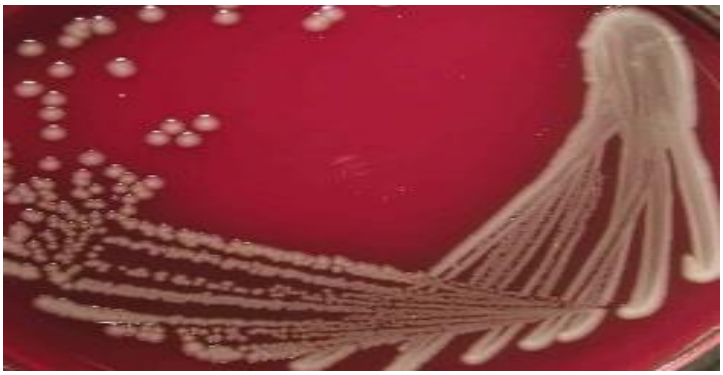
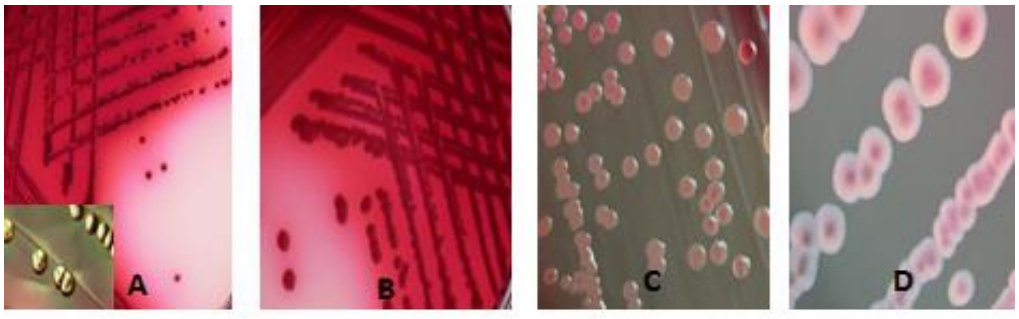


**Fig. 51 *Shigella* sp. - non-hemolytic colonies on blood agar.**

## 5.4 ENTEROBACTERIACEAE – CULTIVATION ON ENDO AGAR

Endo agar is a differential and slightly selective culture medium for the detection of coliform and other enteric microorganisms. The selectivity of Endo agar is due to the sodium sulfite/basic fuchsin combination which results in the suppression of gram-positive microorganisms. Endo agar is culture medium for the differentiation of lactose fermenters from the nonfermenters. Coliforms ferment the lactose producing pink to rose-red colonies and similar coloration of the medium. The colonies of organisms which do not ferment lactose are colorless to faint pink against the pink background of the medium.

**Typical colonial morphology on Endo Agar is as follows:**
*E. coli*.....pink to rose-red, green metallic sheen
*Enterobacter/Klebsiella*.....large, mucoid, pink
*Proteus*.....colorless to pale pink
*Salmonella*.....colorless to pale pink
*Shigella*.....colorless to pale pink
Gram-positive bacteria .....no growth to slight growth




**A - Lactose positive** colonies of *Escherichia coli* on Endo agar, metallic sheen.
**B - Lactose positive** colonies of *Klebsiella pneumoniae* on Endo agar.
**C - Lactose negative** colonies of *Shigella flexneri* on Endo agar.
**D - Lactose negative** colonies of *Salmonella enterica* on Endo agar.

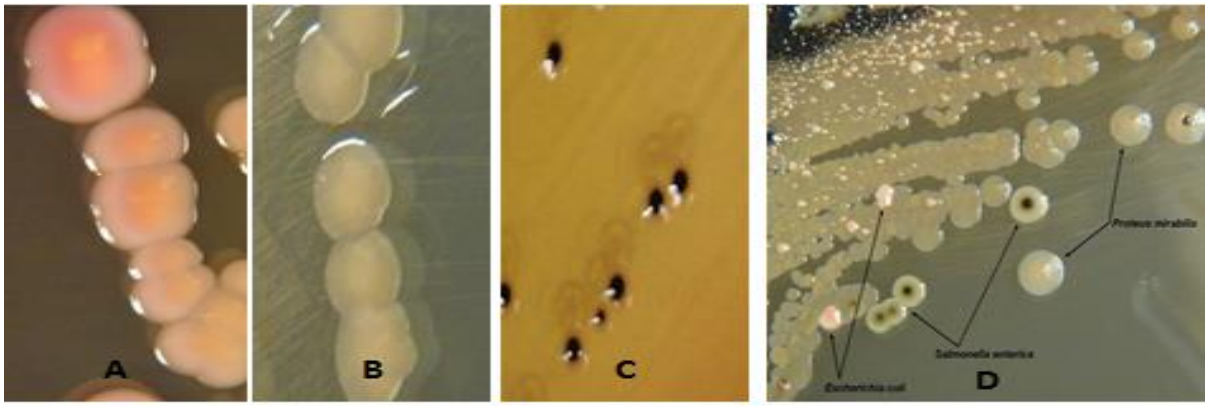
**Fig. 52 Enterobacteria - growth on Endo agar.**

| **GROWTH** | | **INHIBITION OF GROWTH** |
|---|---|---|
| **LACTOSE POSITIVE** examples | **LACTOSE NEGATIVE** examples | |
| • ***Escherichia coli*** (most strains)<br>• ***Klebsiella pneumoniae***<br>• *Entrobacter cloacae* | • ***Salmonella enterica* ssp. *Enterica***<br>• ***Shigella* spp.**<br>• ***Proteus*** spp.<br>• *Citrobacter freundii* (some strains)<br>• *Morganella morganii*<br>• *Providencia* spp. | • staphylococci<br>• streptococci<br>• enterococci<br>Inhibition of Gram-positive microorganisms is achieved by the **sodium sulfite** and **basic fuchsin** contained in the formulation |

**Table 3 Examples of Lactose positive and Lactose negative enterobacteria.**

## 5.5 ENTEROBACTERIACEAE – CULTIVATION ON DEOXYCHOLATE CITRATE AGAR

**Deoxycholate Citrate Agar** is a selective medium recommended for the isolation of enteric pathogens particularly *Salmonella* and *Shigella* species. This medium is selective for enteric pathogens owing to increased concentrations of both citrate and deoxycholate salts. Sodium deoxycholate at pH 7.3 to 7.5 is inhibitory for Gram-positive bacteria. Citrate salts, in the concentration included in the formulation, are inhibitory to gram-positive bacteria and most other normal intestinal organisms. Lactose helps in differentiating enteric bacilli, as lactose fermenters produce red colonies while lactose non-fermenters produce colourless colonies. Coliform bacteria, if present form pink colonies on this medium. The degradation of lactose causes acidification of the medium surrounding the relevant colonies and the pH indicator neutral red changes its colour to red. *Salmonella* and *Shigella* species do not ferment lactose but *Salmonella* may produce $H_2S$, forming colorless colonies with or without black centers (Fig. 53).




A - ***Klebsiella pneumoniae*** on Deoxycholate Citrate Agar - **lactose positive**.
B - ***Proteus mirabilis*** on Deoxycholate Citrate Agar - **lactose negative.**
C - ***Salmonella enterica*** subsp. *enterica* on Deoxycholate Citrate Agar – **lactose positive, $H_2S$ positive**.
D - **Lactose negative, $H_2S$ positive** colonies of *Salmonella enterica* (DCA).
**Lactose negative** colonies of *Proteus mirabilis* (DCA).
**Lactose positive** colonies of *Escherichia coli* (DCA).

**Fig. 53 Enterobacteria on Deoxycholate Citrate Agar.**

## 5.6 ENTEROBACTERIACEAE BIOCHEMICAL PROPERTIES TESTING – TRIPLE SUGAR IRON AGAR (TSI, HAJN)

The **Triple Sugar-Iron (TSI) agar** test is designed to differentiate among the different groups or genera of the Enterobacteriaceae. To facilitate the observation of carbohydrate utilization patterns, TSI Agar contains three fermentative sugars, **lactose** and **sucrose** in 1% concentrations and **glucose** in 0.1% concentration. Due to the building of acid during fermentation, the pH falls.

The acid base indicator (phenol red) is incorporated for detecting carbohydrate fermentation that is indicated by the change in color of the carbohydrate medium from orange red to yellow in the presence of acids. In case of oxidative decarboxylation of peptone, alkaline products are built and the pH rises. This is indicated by the change in colour of the medium from orange red to deep red.

**Sodium thiosulfate** and **ferrous ammonium sulfate** present in the medium detects the **production of hydrogen sulfide.** Sodium thiosulfate is reduced to hydrogen sulfide, and hydrogen sulfide reacts with an iron salt yielding the typical black iron sulfide. Ferric ammonium citrate is the hydrogen sulfide ($H_2S$) indicator.

**Principle**

Carbohydrate fermentation is indicated by the production of gas and a change in the colour of the pH indicator from red to yellow. To facilitate the detection of organisms that only ferment glucose, the glucose concentration is one-tenth the concentration of lactose or sucrose.

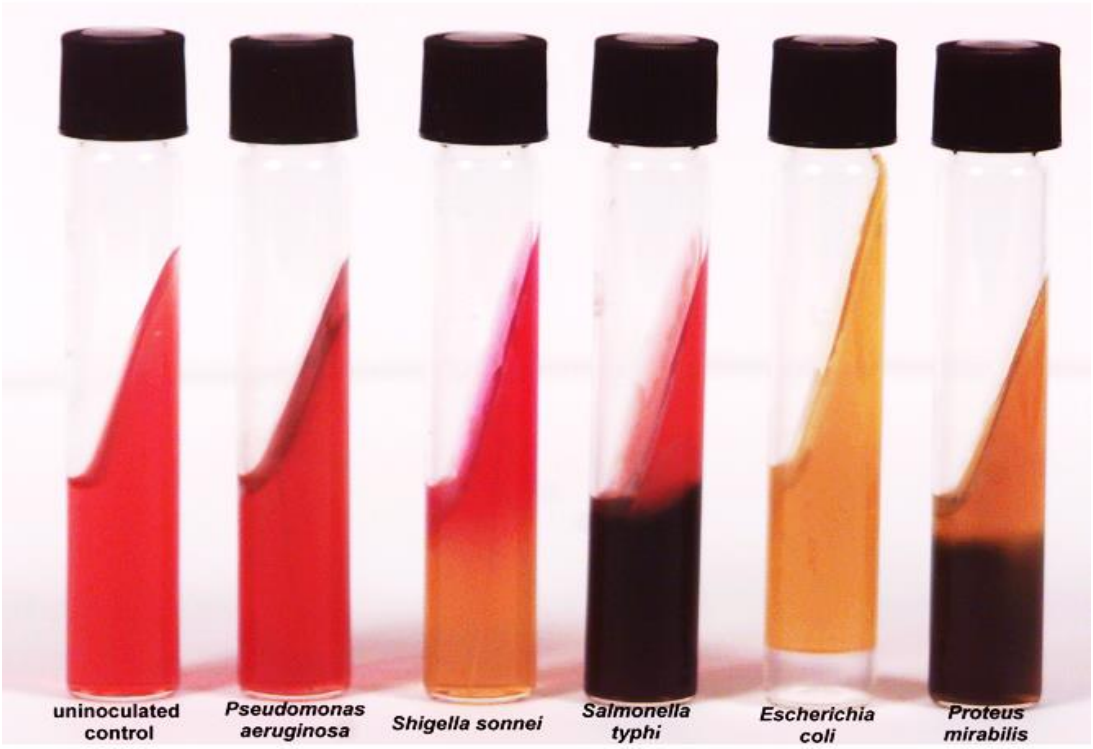



**Fig. 54 Triple Sugar Iron Agar.**

The meagre amount of acid production in the slant of the tube during glucose fermentation oxidizes rapidly, causing the medium to remain orange red or revert to an alkaline pH. In contrast, the acid reaction (yellow) is maintained in the butt of the tube since it is under lower oxygen tension.

| **Result** | **Interpretation** |
|---|---|
| Red/Yellow | Glucose fermentation only, peptone catabolized. |
| Yellow/Yellow | Glucose and lactose and/or sucrose fermentation. |
| Red/Red | No fermentation, Peptone catabolized. |
| Yellow/Yellow with bubbles | Glucose and lactose and/or sucrose fermentation, Gas produced. |
| Red/Yellow with bubbles | Glucose fermentation only, Gas produced. |
| Black precipitate | Glucose fermentation only, Gas produced, $H_2S$ produced. |
| Yellow/Yellow with bubbles and black precipitate | Glucose and lactose and/or sucrose fermentation, Gas produced, $H_2S$ produced. |
| Red/Yellow with black precipitate | Glucose fermentation only, $H_2S$ produced. |
| Yellow/Yellow with black precipitate | Glucose and lactose and/or sucrose fermentation, $H_2S$ produced. |

**Table 4 Triple Sugar Iron Agar - interpretation of results.**

## 5.7 UREASE TEST

This test is used to identify bacteria capable of hydrolyzing urea using the enzyme urease. The hydrolysis of urea forms the weak base, ammonia, as one of its products. This weak base raises the pH of the media above 8.4 and the pH indicator, phenol red, turns from yellow to pink.

**Fig. 55 Urease Test.**

***Proteus mirabilis*** is a rapid hydrolyzer of urea (center tube). The tube on the right was inoculated with a urease negative organism and the tube on the left was uninoculated (Fig. 55).
***Helicobacter pylori*** has urease activity that hydrolyse urea (making so a good environment– $NH_4$ – for surviving in acidic environment - stomach).

## 5.8 SIMMON´S CITRATE AGAR

This is a defined medium used to determine if an organism can use citrate as its sole carbon source. It is often used to differentiate between members of *Enterobacteriaceae*. In organisms capable of utilizing citrate, the **enzyme citrase** hydrolyzes citrate into oxaloacetic acid and acetic acid. If $CO_2$ is produced, it reacts with components of the medium to produce an alkaline compound. The alkaline pH turns the pH indicator (bromthymol blue) from green to blue. This is a positive result- the tube on the right is citrate positive (Fig.56).

**Fig. 56 Simmon´s citrate Agar.**

*Klebsiella pneumoniae* and *Proteus mirabilis* are examples of citrate positive organisms. *Escherichia coli* and *Shigella dysenteria*e are citrate negative.

## 5.9 WIDAL REACTION

Widal reaction is specific reaction consisting in agglutination of typhoid bacilli when mixed with serum from a patient having typhoid fever or other salmonella infection and constituting a test for the disease. Widal test is an agglutination test which detects the presence of serum agglutinins in patients serum with typhoid and paratyphoid fever. When facilities for culturing are not available, the Widal test is the reliable and can be of value in the diagnosis of typhoid fevers in endemic areas. It was developed by Georges Ferdinand Widal in 1896.
The patient's serum is tested for O and H antibodies (agglutinins) against *Salmonella* antigens.

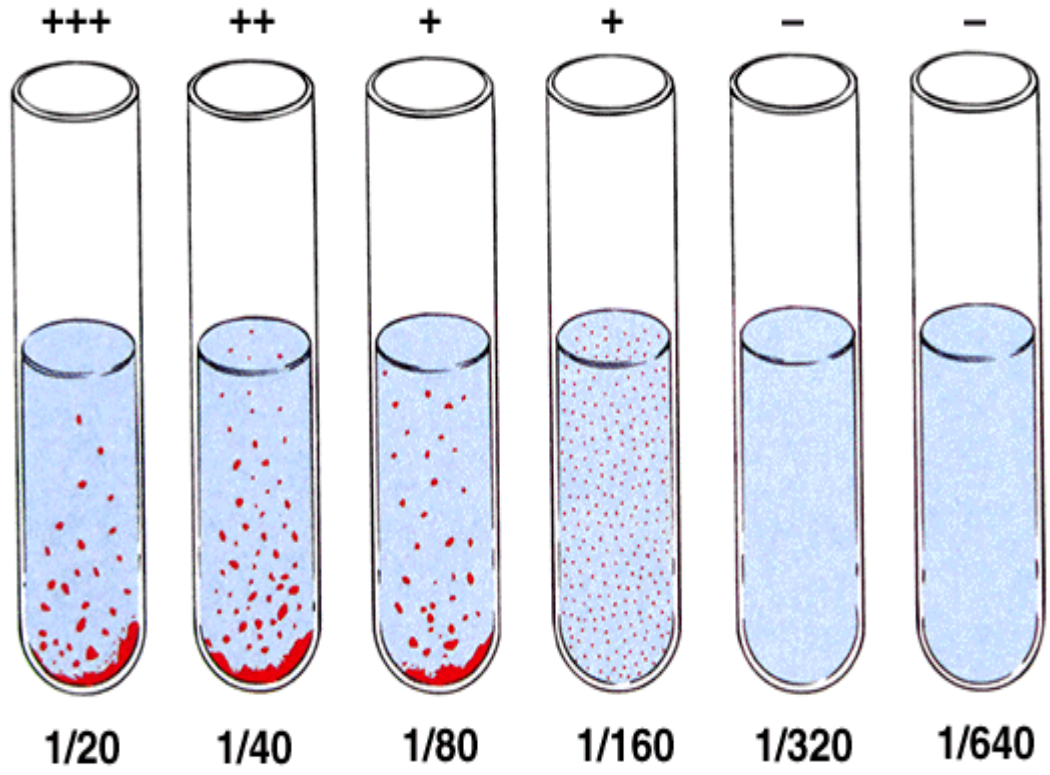



**Fig. 57 Widal Test.**

The main principle of widal test is that if homologous antibody is present in patients serum, it will react with antigen in the reagent and gives visible agglutination in the tube. The antigens used in the test are H and O antigens of *Salmonella* Typhi.
The titre of the patient serum using Widal test antigen suspensions is the highest dilution of the serum sample that gives a positive result (Titers are expressed by the denominator only, for example 1:256 is written 256). The sample which shows the titre of 100 or more for O agglutinations and 200 or more for H agglutination should be considered as clinically significant (active infection). Demonstration of 4-fold rise between the two is diagnostic. H

agglutination is more reliable than O agglutinin. Agglutinin starts appearing in serum by the end of 1st week with sharp rise in 2nd and 3rd week and the titre remains steady till 4th week after which it declines.

## 5.10 BACTERIAL SEROTYPING

**Serotyping (serological typing)** is based on the long-standing observation that microorganisms from the same species can differ in the antigenic determinants expressed on the cell surface. Serotyping is one of the classic tools for epidemiological study and is applied to numerous species that express different serotypes, such as: *Escherichia coli*, *Salmonella* species, *Shigella* species, *Yersinia.*

Antisera for serotyping (examples):

**Fig. 58 Polyvalent antisera.**

Polyvalent antisera to *Escherichia coli* serotypes 026, 055, 086, 0111, 0119, 0125, 0126, 0127, and 0128 for bacterial serotyping.

**Fig. 59 Monovalent antisera.**

**Monovalent antiserum** to *Escherichia coli* serotype 0111for bacterial serotyping.

Where polyvalent and monovalent antisera are available, start by testing agglutination with polyvalent sera, then with the specific monovalent sera corresponding to the mixture giving marked agglutination.

### Principle of Bacterial Serotyping Test

Antigen-antibody complexes are formed when a bacterial culture is mixed with a specific antiserum directed against bacterial surface components (agglutination). The complexes are usually visible to the naked eye which allows for easy determination of O and H antigens by slide agglutination. After full serotyping of the Salmonella culture the name of the serotype can be determined by using the Kauffmann-White Scheme

### Slide Agglutination – Procedure

The slide agglutination test is done on a glass slide and read with the naked eye in front of a light source against a black background.
1. Add a small drop of antiserum on a glass slide and mix it with the Salmonella culture.
2. Tilt the slide for 5 - 10 seconds.
3. A positive reaction is seen as visible agglutination (Fig. 60 – left), whereas a negative reaction is seen as homogeneous milky turbidity (Fig. 60 – right).

**Fig. 60 Serotyping - Slide Agglutination.**

## 5.11 ENTEROTEST - PRINCIPLE

The final identification of enterobacteriaceae studies the phenotypical demonstration of those biochemical properties, which are specific for the individual pathogens. Enterotest is a system that permits the differentiation of *Enterobacteriaceae* by several simultaneous biochemical reactions. Microorganisms are identified by colour change after 18 - 24 hours of incubation at 35 ± 2°C (according to Colour Scale for ENTEROtest 16) (Fig. 60).

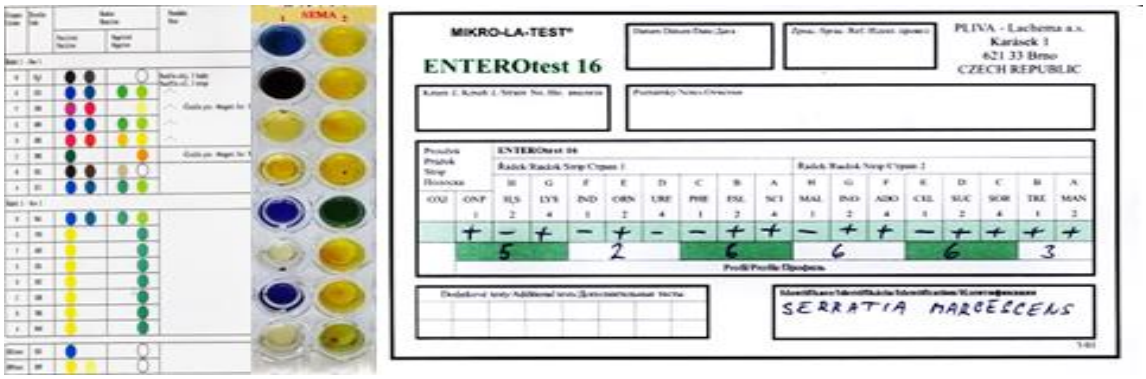



**Fig. 61 ENTEROtest 16.**

Group of biochemical tests aligned that they allow numeric identification based on statistical probability of the result of one test. In the positive result the well is attributed the cipher according to the position in the triplet ( 1, 2 or 4).
Addition of ciphers in triplet gives the number and each result of the triplet gives a subsequent one position of the code that is the combination of numbers of tested triplets. This code is corresponding to one bacteria (526663 – *Serratia marcescens*). For the identification use the differentiation table, or the Code book or the Identification programme.

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# 6 CAMPYLOBACTER, HELICOBACTER

These two groups of Gram-negative organisms are both curved or spiral shaped and are genetically related.

## 6.1 CAMPYLOBACTER JEJUNI

Campylobacteriosis is one of the commonest bacterial disease causing diarrhea. The organism infects the intestinal tract of several animal species (including cattle and sheep) and is a major cause of abortions. It is transmitted to man in milk and meat products. The organism is invasive but generally less so than *Shigella*. The sites of tissue injury include the jejunum, the ileum, and the colon. [15]*C. jejuni* appears to achieve this by invading and destroying epithelial cells. Bacteremia is observed in a small minority of cases. Treatment with antibiotics, therefore, depends on the severity of symptoms. Erythromycin can be used in children, and tetracycline in adults. Quinolones are effective if the organism is sensitive. Trimethoprim/Sulfamethoxazole and ampicillin are ineffective against *Campylobacter*.

## 6.2 CAMPYLOBACTER - GRAM STAIN

*Campylobacter jejuni* is a small spiral/curved ("seagull-shaped") non-spore forming, Gram-negative pathogen (0.2-0.5 μm), stain very lightly with carbolfuchsin.

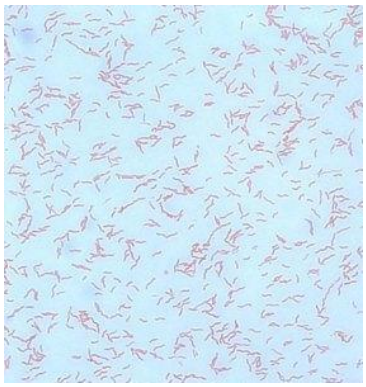

**Fig. 62 *Campylobacter jejuni*.**

## 6.3 CAMPYLOBACTER - CULTIVATION

*Campylobacter* infection is diagnosed when a culture of a stool specimen yields the bacterium. *Campylobacter jejuni* are oxygen sensitive and can grow only under conditions of reduced oxygen tension and grows best at 42°C. They have a single polar flagellum at one

[15] C - Campylobacter

or both ends of the cell. It is frequently isolated using selective media. It is also, catalase, oxidase and $H_2S$ positive.

**Fig. 63 *Campylobacter jejuni* - colonies.**

Colonies of *C. jejuni* are small, nonhemolytic, mucoid, usually grayish, and flat with irregular edges. Campylobacter CSM Agar (Charcoal-Based Selective Medium) is a blood free selective medium for the primary isolation of *Campylobacter* species from human fecal specimens (Fig. 61).

## 6.4 HELICOBACTER PYLORI

Approximately one-half of the world's population is estimated to be infected with *Helicobacter pylori* (*H. pylori*), a Gram-negative, curved bacterial rod, which has been associated with symptoms ranging from peptic ulcer disease and dyspepsia to gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma. Due to the potentially severe consequences of infection, accurate diagnosis and prompt initiation of therapy are important for successful disease resolution.

## 6.5 HELICOBACTER - GRAM STAIN

Microscopy of Gram-stained smears – *H. pylori* are curved Gram-negative rods. The spiral morphology and flagellar motility assist in penetration into the viscous mucus layer, where the more pH-neutral conditions allow growth of the gastric *Helicobacter* species.

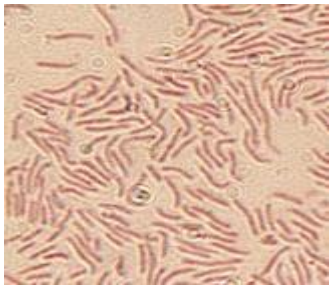

**Fig. 64 *Helicobacter pylori* - Gram stain.**

## 6.6 HELICOBACTER - CULTIVATION

Isolation of *H. pylori* by culture of a biopsy specimen is definitive evidence of active infection and isolates can subsequently be tested for susceptibility to various antimicrobial agents.

**Fig. 65 *Helicobacter pylori* - colonies.**

*H. pylori* can grow on different solid media containing blood or blood products (blood or lysed blood agar plates). *H. pylori* is a fastidious microorganism and requires complex growth media. Often these media are supplemented with blood or serum. These supplements may act as additional sources of nutrients and possibly also protect against the toxic effects of long-chain fatty acids. Commonly used solid media for routine isolation and culture of *H. pylori* consist of Columbia or Brucella agar supplemented with either (lysed) horse or sheep blood. A key feature of *H. pylori* is its microaerophilicity, with optimal growth at $O_2$ levels of 2 to 5% and the additional need of 5 to 10% $CO_2$ and high humidity. *H. pylori* forms small (1 mm), translucent smooth colonies (Fig. 65).

## 6.7 UREASE TEST

Urease test is used for the presumptive evidence of the presence of *Helicobacter pylori* in tissue biopsy material. This is done by placing a portion of crushed tissue biopsy material directly into urease broth.


**Fig. 66 Urease test results.**

A positive urease test is considered presence of *Helicobacter pylori*. Many organisms have a urease enzyme which is able to split urea in the presence of water to release ammonia and carbon dioxide. The ammonia combines with carbon dioxide and water to form ammonium carbonate which turns the medium alkaline, turning the indicator phenol red from its original orange yellow color to bright pink (Fig. 66).

## 6.8 UREA BREATH TEST

Urea breath test, a common noninvasive test to detect *Helicobacter pylori* also based on urease activity. This is highly sensitive and specific test.

**Principle of Urea Breath Test:** Patient ingests radioactively labeled (13C) Urea. If infection is present, the urease produced by *Helicobacter pylori* hydrolyzes the urea to form ammonia and labeled bicarbonate that is exhaled as $CO_2$. The labeled $CO_2$ is detected either by a scintillation counter or a special spectrometer (Fig. 67).

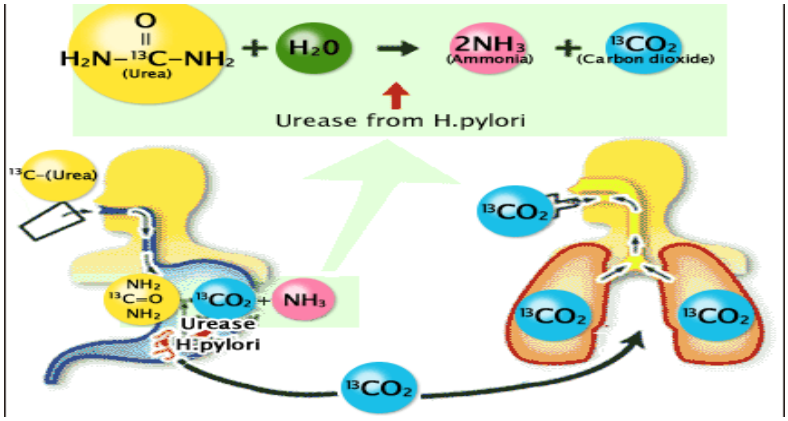


**Fig. 67 Urea Breath Test - principle.**

## 6.9 HELICOBACTER HEILMANNII

The diverse species *Helicobacter heilmannii* was originally designated *Gastrospirillum hominis* and is a *Helicobacter* species with a wide host range. It has been isolated from several domestic and wild animals, including dogs, cats, and nonhuman primates, and is also observed in a small percentage of humans with gastritis.

**Fig. 68 *Helicobacter heilmannii*.**

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# 7 CORYNEBACTERIA

The genus *Corynebacterium* consists of a diverse group of bacteria including animal and plant pathogens, as well as saprophytes. Some corynebacteria are part of the normal flora of humans, finding a suitable niche in virtually every anatomic site, especially the skin and nares. Corynebacteria are Gram-positive, aerobic, nonmotile, rod-shaped bacteria classified as **Actinobacteria**. Corynebacteria are related phylogenetically to mycobacteria and actinomycetes. "Diphtheroids" or "coryneform" bacteria are recognized as causing opportunistic disease under specific circumstances, such as in patients who are immunocompromised, have prosthetic devices, or have been in hospitals/nursing homes for long-term periods of time.

The best known and most widely studied species is ***Corynebacterium diphtheriae***, the causal agent of the disease **diphtheria**.

[16]*C. pseudodiphteriae* has been associated mainly with respiratory disease and less commonly with endocarditis, prostheses or wound infections or colonizations.

Most of the respiratory disease occurred in immunosuppressed hosts. Other organisms such as *C. ulcerans*, *C. pseudotuberculosis* and *C. xerosis* may also be able to cause infection of the nasopharnyx and skin.

## 7.1 CORYNEBACTERIUM DIPHTERIAE

The pathogenicity of *Corynebacterium diphtheriae* includes two distinct phenomena:

**1. Invasion** of the local tissues of the throat, which requires colonization and subsequent bacterial proliferation. Little is known about the adherence mechanisms of *C. diphtheriae*, but the bacteria produce several types of pili. The diphtheria toxin, as well, may be involved in colonization of the throat.

**2. Toxigenesis -** bacterial production of the toxin. The diphtheria toxin causes the death of eucaryotic cells and tissues by inhibition protein synthesis in the cells. Although the toxin is responsible for the lethal symptoms of the disease, the virulence of *C. diphtheriae* cannot be attributed to toxigenicity alone, since a distinct invasive phase apparently precedes toxigenesis. However, it has not been ruled out that the diphtheria toxin plays an essential role in the colonization process due to short-range effects at the colonization site.

**The role of B-phage**.

Only those strains of *Corynebacterium diphtheriae* that are lysogenized by a specific Beta phage produce diphtheria toxin. A phage lytic cycle is not necessary for toxin production or release. The **phage contains the structural gene for the toxin molecule**.

The zoonotic agents *Corynebacterium pseudotuberculosis* (the cause of caseous lymphadenitis primarily in sheep and goats), transmitted to humans by contact with diseased animals and *C. ulcerans*, historically thought to cause disease in humans after contact with

[16] C. - Corynebacterium

contaminated milk or farm animals, but more recently linked to transmission between humans and their companion pets such as cats and dogs, are also able to produce diphtheria toxin and cause diphtheria-like disease in humans. It is therefore recommended that those in frequent contact with animals (veterinarians, animal care technologists, or farm workers) ensure that they maintain adequate vaccine coverage against diphtheria.
Coryneform bacteria are increasingly being recognized as causing opportunistic disease under specific circumstances, such as in patients who are immunocompromised, have prosthetic devices, or have been in hospitals/nursing homes for long-term periods of time. The most significant pathogen of this group remains *Corynebacterium diphtheriae*, the primary cause of diphtheria, a disease which has essentially disappeared from developed countries after implementation of universal vaccination that targets the primary virulence factor, the diphtheria toxin (DT).

## 7.2 CORYNEBACTERIA - GRAM STAIN

Corynebacteria are Gram-positive, catalase positive, non-spore-forming, non-motile, rod-shaped bacteria that are straight or slightly curved, with clubbed ends (from the Greek *koryne*, club).

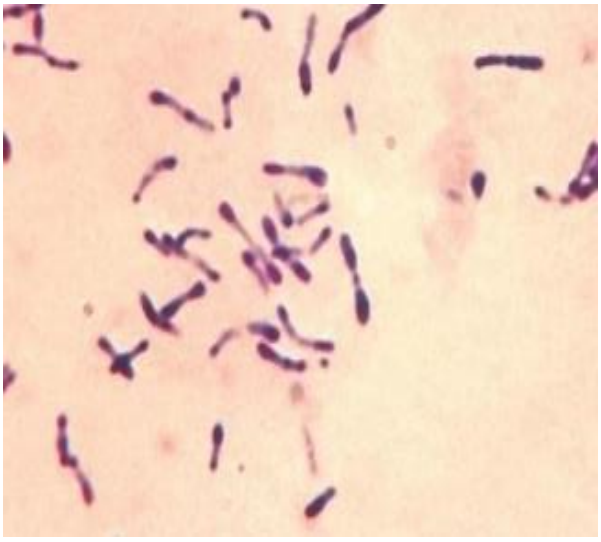

**Fig. 69 *Corynebacterium diphtheriae* - Gram stain.**

Corynebacteria are pleomorphic rods that occur in angular arrangements. They undergo snapping movements just after cell division, which brings them into characteristic forms resembling „Chinese-letters“ or „palisades“. This is due to the incomplete separation of the daughter cells after binary fission. Their size falls between 2-6 micrometers in length and 0.5 micrometers in diameter. Corynebacteria do not form spores or branch as do the actinomycetes.

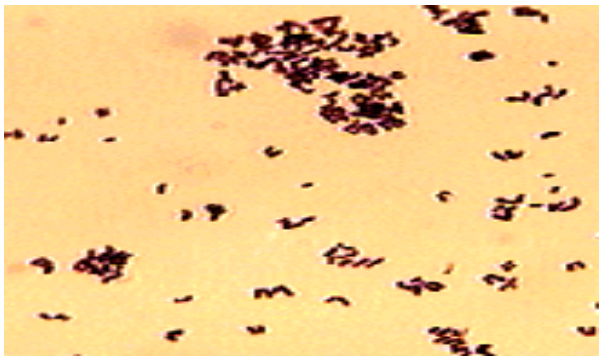

**Fig. 70 *Corynebacterium pseudodiphtheriae* - Gram stain.**

***Corynebacterium pseudodiphteriae*** are pleiomorphic, Gram-positive rods, irregularly shaped, they are arranged as single cells, in pairs, in V forms, in palisades, or in clusters with Chinese-letter appearance.

## 7.3 CORYNEBACTERIA – ALBERT STAIN (METACHROMATIC GRANULES)

**Metachromatic granules** are irregularly sized granules found in the protoplasm of numerous bacteria. It stains a different color from that of the dye used. Granules are usually present in Corynebacteria, representing stored phosphate regions. They are composed of complex polyphosphate, lipid, and nucleoprotein molecules (volutin) and serve as an intracellular phosphate reserve. Called also Babès-Ernst body or granule.

**Abert´s Stain Procedure**

1. Prepare a smear on clean grease free slide.
2. Air dry and heat fix the smear.
3. Treat the smear with Albert's stain and allow it to react for about 7 minutes.
4. Drain of the excess stain do not water wash the slide with water.
5. Flood the smear with Albert's iodine for 2 minutes.
6. Wash the slide with water, air dry and observe under oil immersion lens.

Corynebacterium diphtheriae appears green coloured rod shaped bacteria with bluish black metachromatic granules at the poles.

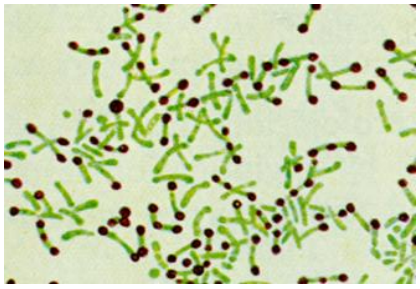

**Fig. 71 *Corynebacterium diphteriae* - Albert stain.**

## 7.4 CORYNEBACTERIA – CULTIVATION ON BLOOD AGAR

The causative agent of diphtheria is an aerobe or a facultative anaerobe. The optimal temperature for growth is 37° C and the organism does not grow at temperatures below 15°C and above 40°C. The pH of medium is 7.2-7.6. *Corynebacterium diphtheriae* usually grows on media with blood with a weak beta-hemolysis (*C. diphtheriae* biotype mitis and gravis) or is nonhemolytic (biotype intermedius).

**Fig. 72 *Corynebacterium diphteriae* - Blood agar culture.**

Three strains of *Corynebacterium diphtheriae* are recognized, **gravis**, **intermedius** and **mitis**. All strains produce the identical toxin and are capable of colonizing the throat. The differences in virulence between the three strains can be explained by their differing abilities to produce the toxin in rate and quantity, and by their differing growth rates. The gravis strain has a generation time (in vitro) of 60 minutes; the intermedius strain has a generation time of about 100 minutes; and the mitis stain has a generation time of about 180 minutes. The faster growing strains typically produce a larger colony on most growth media. In the throat (in vivo), a faster growth rate may allow the organism to deplete the local iron supply more rapidly in the invaded tissues, thereby allowing earlier or greater production of the diphtheria toxin. Also, if the kinetics of toxin production follow the kinetics of bacterial growth, the faster growing variety would achieve an effective level of toxin before the slow growing varieties.

## 7.5 CORYNEBACTERIA – CULTIVATION ON TELLURITE AGAR AND TINSDALE AGAR

**Tellurite Blood Agar** – in which 0.4% tellurite inhibits other bacteria, diphtheria bacilli reduce tellurite to metallic tellurium which is incorporated in the colonies giving them a grey or black colour (Fig. 73).

**Tinsdale agar** (**TIN**) is used for the primary isolation and identification of *Corynebacterium diphtheriae*. The medium differentiates between *C. diphtheriae* and diphtheroids found in the upper respiratory tract. It contains L-cysteine and sodium thiosulfate that are $H_2S$ indicators.

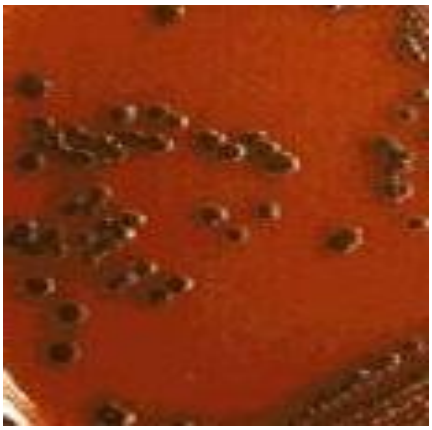

**Fig. 73 *C. diphteriae* - Tellurite agar, strain gravis.**

Potassium tellurite is the selective agent (inhibits most of the upper respiratory tract normal flora) that turns the media brown-black as a result from the reduction of potassium tellurite to metallic tellurite. This differentiation is based on the ability of *C. diphtheriae* to produce black (or brown) colonies, surrounded by a brown/black halo (Fig. 74). The dark halo is due to the production of $H_2S$ from cystine, interacting with the tellurite salt (**cystinase activity**).

**Fig. 74 *C. diphteriae* – Tinsdale agar. Small, brownish-black colonies surrounded by brown halo.**

## 7.6 ELEK TEST

The **Elek culture plate precipitin test** is routinely used for the detection of exotoxin from toxigenic strains of *Corynebacterium diphtheriae*. The test for toxigenicity, which detects the potent exotoxin, a phage-encoded protein, is the most important test and should be done without delay on any suspect isolate that is found by routine screening or while investigating a possible case of diphtheria. The toxigenic species *C. diphtheriae* acquire this characteristic when infected by the family of β-phages or other families of corynephages. The Elek test was

first described in 1949 and replaced the in vivo virulence test in guinea pigs, a test that was used by many countries at that time.

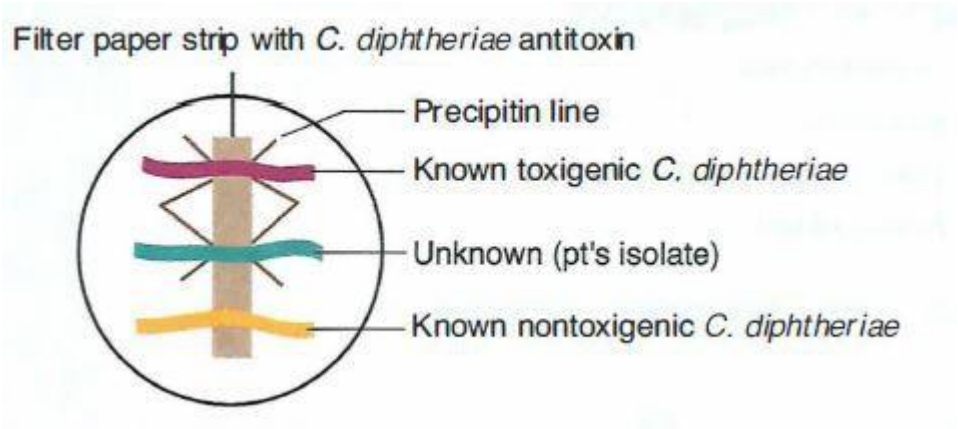


**Fig. 75 Elek´s Test.**

### The Elek Test Principle

A filter paper strip impregnated with diphtheria antitoxin is buried just beneath the surface of a special agar plate before the agar hardens. Strains to be tested, known positive and negative toxigenic strains are streaked on the agar's surface in a line across the plate, and at a right angle to the antitoxin paper strip. After 24 hours of incubation at 37° C, plates are observed for the presence of fine precipitin lines at a 45-degree angle to the streaks (Fig. 75). The presence of precipitin lines indicated that the strain produced toxin that react with the antitoxin.

### Diphteria Toxin – Mode of Action

Diphtheria toxin (DT) is an extracellular protein of *Corynebacterium diphtheriae* that inhibits protein synthesis and kills susceptible cells. Diphtheria toxin is a single polypeptide chain consisting of two subunits linked by disulfide bridges, known as an A-B toxin. Binding to the cell surface of the B subunit (the less stable of the two subunits) allows the A subunit (the more stable part of the protein) to penetrate the host cell. The diphtheria toxin catalyzes the transfer of $NAD^+$ to a diphthamide residue in eukaryotic elongation factor-2 (eEF2), inactivating this protein. It does so by ADP-ribosylating the unusual amino acid diphthamide. In this way, it acts as a RNA translational inhibitor. The acceptor is diphthamide, a unique modification of a histidine residue in the elongation factor found in archaebacteria and all eukaryotes, but not in eubacteria.

The catalysed reaction is as follows:

**$NAD^+$ + peptide diphthamide $\rightleftharpoons$ nicotinamide + peptide N-(ADP-D-ribosyl)diphthamide**

Diphtheria toxin has also been associated with the development of myocarditis. Myocarditis secondary to diphtheria toxin is considered one of the biggest risks to non-immunized children.

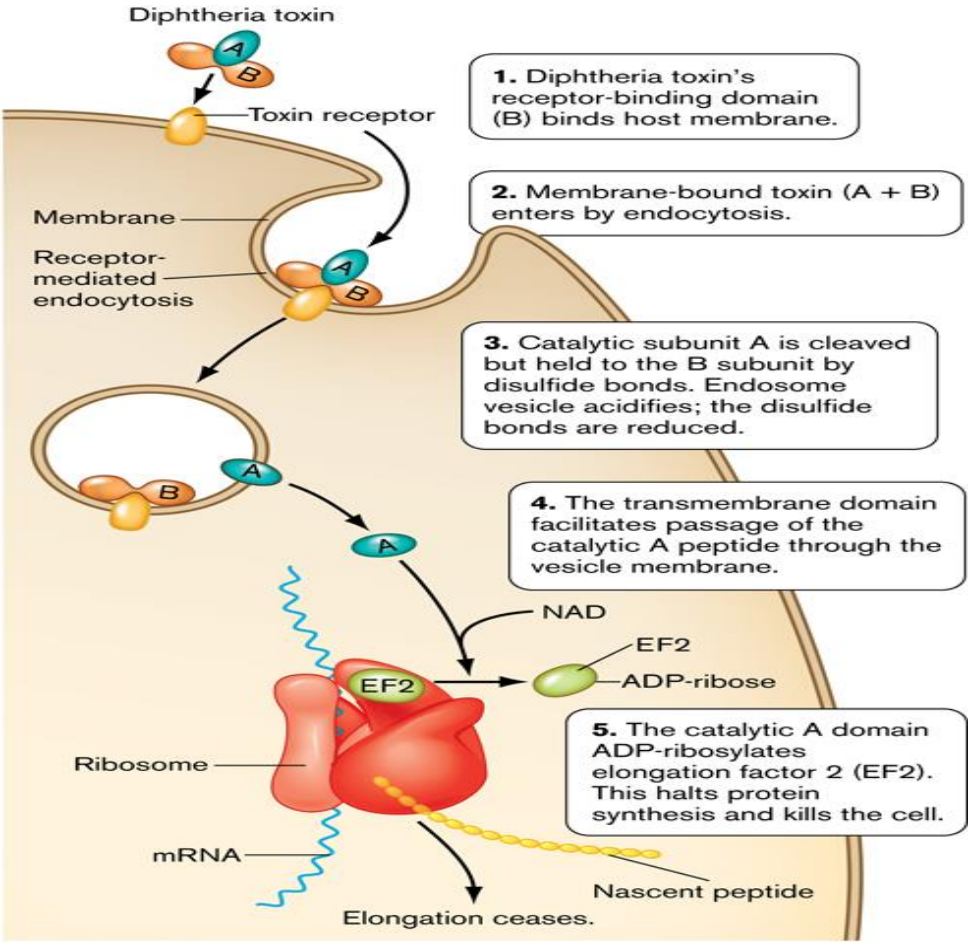


**Fig. 76 Diphteria toxin - mode of action.**

Diphtheria toxin is extraordinarily potent. The lethal dose for humans is about 0.1 μg of toxin per kilogram of bodyweight. A massive release of toxin into the body will likely cause lethal necrosis of the heart and liver. The toxin is labile, prolonged storage, incubation at 37° C for 4-6 weeks, treatment with 0.2- 0.4% formalin, acid pH converts it to **toxoid**.

### Schick skin test

In 1913, Schick designed a skin test as a means of determining susceptibility or immunity to diphtheria in humans. Diphtheria toxin will cause an inflammatory reaction when very small amounts are injected intracutaneously. The Schick Test involves injecting a very small dose of the toxin under the skin of the forearm and evaluating the injection site after 48 hours. A positive test (inflammatory reaction) indicates susceptibility (nonimmunity). A negative test (no reaction) indicates immunity (antibody neutralizes toxin).

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# 8 BORDETELLA

The *Bordetellae* are small, Gram-negative, aerobic coccobacilli. The genus *Bordetella* contains the species [17]*B. pertussis* and *B. parapertussis*, which cause pertussis in humans. Other members of the genus are *B. bronchiseptica*, causing respiratory disease in various animals and occasionally in humans, and *B. avium* as well as *B. hinzii*, which cause respiratory disease in poultry and are very rarely found in humans. Closely related organism, *B. parapertussis* can cause a milder form of bronchitis. *B. parapertussis* causes similar, but often milder type of cough with posttussive vomiting. *B. bronchiseptica* is the causative agent of respiratory diseases in cats and swine, but can cause broncho-pulmonary symptoms in severely immunosupressed individuals.

## 8.1 BORDETELLA PERTUSSIS

*Bordetella pertussis* is the organism of major clinical significance within this genus, it causes whooping cough in infants and young children. There does not appear to be a zoonotic reservoir for *B. pertussis* - **humans are its only host**. *B. pertussis* is an extremely small, strictly aerobic, Gram-negative, non-motile cocobacillus (short rod). Compared to other *Bortdetella* species, *B. pertussis* does not grow on common laboratory media and can be distinguished from *B. parapertussis* in that *B. pertussis* is oxidase positive but urease negative, while *B. parapertussis* is oxidase negative and urease positive. *B. bronchiseptica* is positive for both enzymes.

### Whooping Cough (Pertussis)

Whooping cough (pertussis) is a highly contagious respiratory tract infection. In many people, it's marked by a severe hacking cough followed by a high-pitched intake of breath that sounds like "whoop."

Before the vaccine was developed, whooping cough was considered a childhood disease. Now whooping cough primarily affects children too young to have completed the full course of vaccinations and teenagers and adults whose immunity is weakened.

After an incubation period of 1 to 2 weeks, whooping cough begins with the catarrhal phase. The subsequent paroxysmal phase, lasting 2 to 4 weeks, is characterized by severe and spasmodic cough episodes.

At the end of the catarrhal phase, a leukocytosis with an absolute and relative lymphocytosis frequently begins, reaching its peak at the height of the paroxysmal stage. At this time, the total blood leukocyte levels may resemble those of leukemia ($\geq$ 100,000/mm$^3$), with 60 to 80 percent being lymphocytes.

The convalescent phase, lasting 1 to 3 weeks, is characterized by a continuous decline of the cough.

[17] B. - Bordetella

**Pertussis toxin**

Pertussis toxin is an oligopeptide AB-type exotoxin that is the major cause of pertussis. Following binding of the B component to host cells, the A subunit is inserted through the membrane and released into the cytoplasm in a mechanism of direct entry. The A subunit gains enzymatic activity, the membrane-bound regulatory protein $G_i$ that normally inhibits the eukaryotic adenylate cyclase. The $G_i$ protein is inactivated and cannot perform its normal function to inhibit adenylate cyclase ($G_i$ protein is a G protein subunit that inhibits the production of cAMP from ATP). The conversion of ATP to cyclic AMP cannot be stopped and intracellular levels of cAMP increase. This results leads to increased mucus secretion and interferes with many cellular functions (disruption of cellular functions, decrease phagocytic activities such as chemotaxis, engulfment, the oxidative burst, and bacteridcidal killing). Pertussis toxin causes T cell lymphocytosis, hypoglycemia, increased [18]IgE synthesis, and increased histamine and endotoxin sensitivity.

**Adenylate cyclase toxin**

This exotoxin penetrates the host cells, is activated by calmodulin and catalyzes the conversion of ATP to cAMP. Like pertussigen, it also inhibits phagocyte and NK cell functions. However, in contrast with pertussigen, the cAMP increase caused by this toxin is short-lived.

**Tracheal cytotoxin**

This is a peptidoglycan-like molecule (monomer) which binds to ciliated epithelial cells, thus interfering with ciliary movement. In higher concentrations, it causes ciliated epithelial cell extrusion and destruction. The destruction of these cells contributes to pertussis.

**Dermonecrotic (heat-labile) toxin**

Dermonecrotic toxin is a very strong vaso-constrictor and causes ischemia and extravasation of leukocytes and, in association with tracheal cytotoxin, causes necrosis of the tracheal tissue.

**Filamentous haemagglutinins (agglutinogens)**

These are not exotoxins but are filament-associated lipo-oligo-saccharides which are implicated in the binding of the organism to ciliated epithelial cells. Antibodies against these molecules are protective, probably by preventing bacterial attachment.

## 8.2 PERTUSSIS - SAMPLING

Laboratory diagnosis of pertussis is highly important. Culture has remained the gold standard of diagnosis. The specimens should be taken from the posterior nasopharynx by intranasal aspiration or by a swab (Fig. 77). Calcium alginate swabs are better than dacron, rayon or cotton wool swabs. The specimens should be plated immediately onto selective media, and

[18] IgE – Immunoglobulin E

the charcoal supplemented with 10% horse blood and 40 mg/l cephalexin is currently the medium of choice.

**Fig. 77 *B. pertussis* - sampling.**

The cough plate is the best means of early diagnosis. The exposed plate is held 4-5 inches from the patient's mouth during several expulsive coughs (Fig. 77).

## 8.3 BORDETELLA PERTUSSIS – GRAM STAIN

*B. pertussis* is an extremely small, strictly aerobic, Gram-negative cocobacillus, that is encapsulated and does not produce spores. It is arranged singly, in pairs or in small groups (Fig. 78) and is not easily distinguished from *Haemophilus* species. *B. pertussis* and *B. parapertussis* are nonmotile.

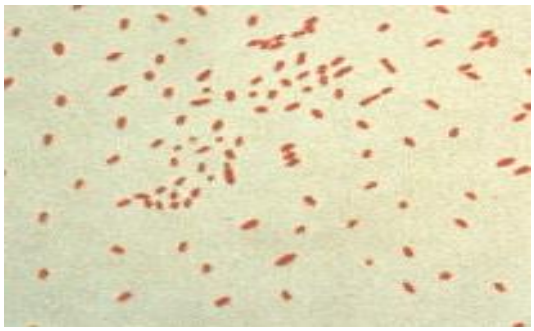

**Fig. 78 *B. pertussis* - Gram stain.**

## 8.4 BORDETELLA PERTUSSIS – CULTIVATION (BORDET GENGOU BLOOD AGAR, CHARCOAL HORSE BLOOD AGAR)

*Bordetella pertussis* is a slow-growing organism that requires specialized conditions for growth. It is the most fastidious species within the genus. The culture positivity is often highest during the first 2 weeks of illness, and cultures are seldom positive if cough has lasted more than 4 weeks.

**Bordet Gengou Blood Agar** is a solid medium recommended for use in qualitative

procedures for the isolation of *Bordetella* pertussis and *Bordetella parapertussis*. Also used for the "cough plate" method in case of whooping cough. Bordet Gengou Blood Agar contains potato infusion and glycerol to supply the nutrients necessary to support the growth of *B. pertussis*. Defibrinated animal blood supplies additional nutrients and enables the detection of hemolytic reactions, which aid in the identification of *B. pertussis*.

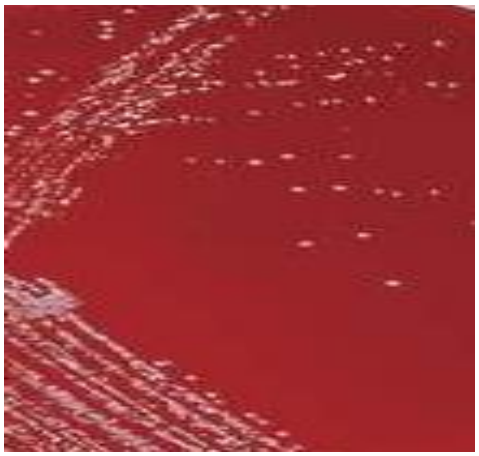

**Fig. 79 *Bordetella pertussis* - Bordet Gengou Blood Agar.**

*Bordetella pertussis* produces small, domed, glistening colonies that resemble bisected pearls (Fig. 79). The colonies are usually surrounded by a zone of hemolysis; however, some strains of *B. pertussis* are not hemolytic. *B. parapertussis* grows faster than *B. pertussis* and is oxidase negative.

**Charcoal Horse Blood Agar** with cefalexin, beef extract, peptone, and nicotinic acid provide essential nutrients for the growth of *Bordetella* spp. *Bordetella pertussis* in particular, requires the addition of charcoal and horse blood (10%) to neutralize the growth-inhibiting effects. Plates are incubated in air without elevated carbon dioxide at 35°C for a minimum of 7 days before being reported as negative (most isolates are detected in 3 to 4 days).

**Fig. 80 *Bordetella pertussis* on Charcoal Horse Blood Agar with cefalexin.**

Colonies are small, shiny and round (Fig. 80). With age they become whitish grey. Repeated subculture of *B.pertussis* leads to loss of fastidiousness and laboratory adaptation to a variety of media

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# 9 ANAEROBIC BACTERIA

The oxygen requirement of bacteria reflects the mechanism used by those particular bacteria to satisfy their energy needs. Obligate anaerobes do not carry out oxidative phosphorylation. Furthermore, they are killed by oxygen, they lack enzymes such as catalase [which breaks down hydrogen peroxide ($H_2O_2$ ) to water and oxygen], peroxidase [by which [19]NADH + $H_2O_2$ are converted to [20]NAD and $O_2$] and superoxide dismutase [by which superoxide, $O_2^{\cdot}$, is converted to $H_2O_2$]. These enzymes detoxify peroxide and oxygen free radicals produced during metabolism in the presence of oxygen. Anaerobic respiration includes glycolysis and fermentation. During the latter stages of this process NADH (generated during glycolysis) is converted back to NAD by losing a hydrogen. The hydrogen is added to pyruvate and, depending on the bacterial species, a variety of metabolic end-products are produced.

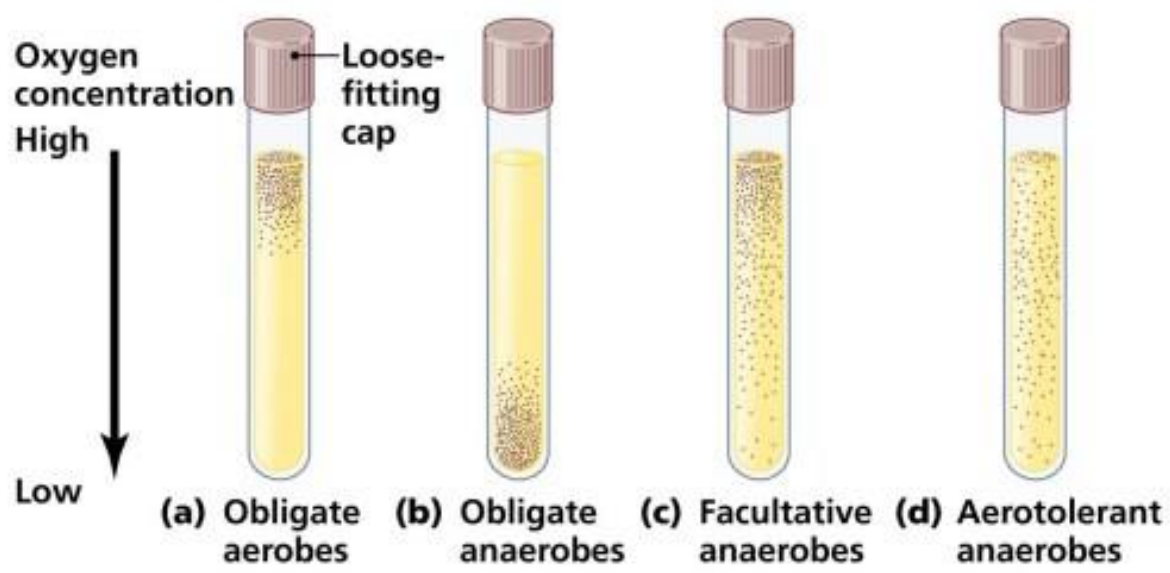


**Fig. 81 Different categories of bacteria - on the basis of oxygen requirements.**

On the basis of oxygen requirements, bacteria can be divided into following different categories (Fig. 81):

1. **Aerobes -** grow in ambient air, which contains 21% oxygen and small amount of (0,03%) of carbondioxide (*Bacillus cereus*).
2. **Obligate aerobes -** they have absolute requirement for oxygen in order to grow (*Psuedomonas aeruginosa*, *Mycobacterium tuberculosis*).
3. **Obligate anaerobes** - these bacteria grow only under condition of high reducing intensity and for which oxygen is toxic (*Clostridium perfringens, Clostridium botulinum*).
4. **Facultative anaerobes** - they are capable of growh under both aerobic and anaerobic conditions (Enterobacteriaceae group, *Staphylococcus aureus*).

[19] NADH - the reduced form of nicotinamide-adenine dinucleotide
[20] NAD - Nicotinamide adenine dinucleotide

5. **Aerotolerant anaerobes** - are anaerobic bacteria that are not killed by exposure to oxygen.
6. **Capnophiles** - bacteria require increased concentration of carbondioxide (5% to 10%) and approximately 15% oxygen. This condition can be achieved by a candle jar (3% carbondioxide) or carbondioxide incubator, jar or bags (*Haemophilus influenzae*, *Neisseria gonorrhoeae*).
7. **Microaerophiles** - are those groups of bacteria that can grow under reduced oxygen (5% to 10%) and increased carbondioxide (8% to 10%). Higher oxygen tensions may be inhibitory to them. This environment can be obtained in specially designed jars or bags (*Campylobacter jejuni*, *Helicobacter pylori*).

Aerobes can survive in the presence of oxygen only by virtue of an elaborate system of defenses. Without these defenses key enzyme systems in the organisms fail to function and the organisms die. Obligate anaerobes, which live only in the absence of oxygen, do not possess the defenses that make aerobic life possible and therefore can not survive in air. The tolerance to oxygen is related to the ability of the bacterium to detoxify superoxide and Hydrogen peroxide, produced as byproduct of aerobic respiration.

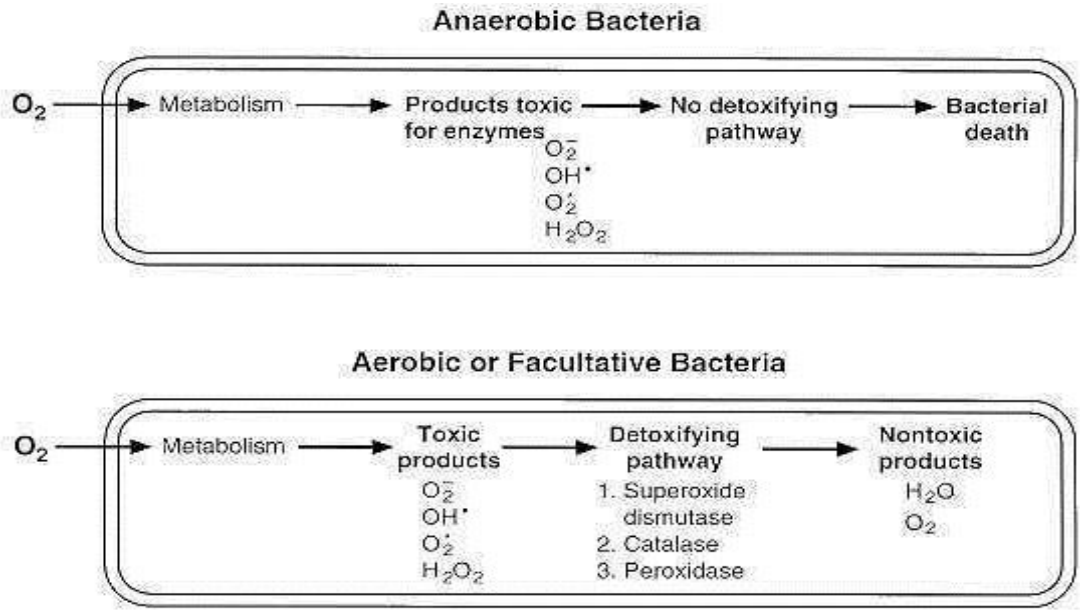


**Fig. 82 Metabolism of Anaerobic and Aerobic or Facultative bacteria.**

The assimilation of glucose in aerobic condition results in the terminal generation of free radical superoxide ($O_2^-$). The superoxide is reduced by the enzyme superoxide dismutase to oxygen gas and Hydrogen peroxide ($H_2O_2$).
Subsequently, the toxic hydrogen peroxide generated in this reaction is converted to water and oxygen by the enzyme catalase, which is found in aerobic and facultative anaerobic bacteria, or by various peroxidases which are found in several aerotolerant anaerobes.

| .ANAEROBIC NON - SPORE - FORMERS | |
|---|---|
| **Gram-negative rods** | *Bacteroides*<br>*Fusobacterium* |
| **Gram-positive rods** | *Actinomyces*<br>*Eubacterium*<br>*Bifidobacterium*<br>*Lactobacillus*<br>*Propionibacterium* |
| **Gram-positive cocci** | *Peptostreptococcus*<br>*Peptococcus* |
| **Gram-negative cocci** | *Veillonella*<br>*Acidominococcus* |
| **ANAEROBIC SPORE - FORMERS** | |
| **Gram-positive rods** | *Clostridium tetani*<br>*Clostridium perfringens*<br>*Clostridium botulinum* |

**Table 5 Anaerobic bacteria - non-spore-formers and spore-formers.**

# 9.1 CLOSTRIDIA

### *Clostridium tetani* (TETANUS)

*Clostridium tetani*, a Gram-positive rod that forms a terminal spore is commonly found in the soil, dust and animal feces. Contamination of wounds, which provide anaerobic conditions, can lead to spore germination and tetanus, a relatively rare, but frequently fatal disease. Tetanus is also know as lockjaw because of the patient's inability to open the mouth as a result of muscle paralysis.

Infection usually occurs when spores (in dirt, feces or saliva) enter wounds and scratches where they germinate and produce tetanus toxin. The organism is non-invasive and thus remains in the local wound.

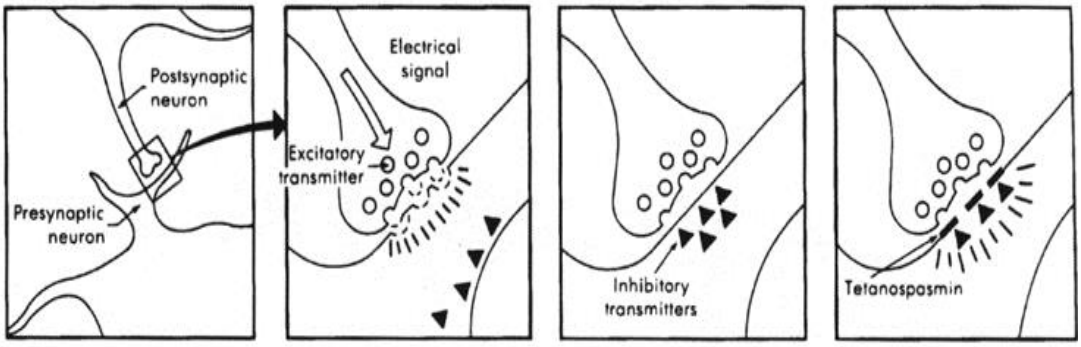


**Fig. 83 Tetanospasmin – mode of action.**

The exotoxin (tetanospasmin) binds to ganglioside receptors on inhibitory neurones in central nervous system. The effect of the toxin - to block the release of inhibitory neurotransmitters (glycine and gamma-amino butyric acid) - it produces the generalized muscular spasms characteristic of tetanus. This stops nerve impulse transmission to muscle leading to spastic paralysis. The toxin can act at peripheral motor nerve end plates, the brain, spinal cord and also in the sympathetic nervous system. It is transported within the axon and across synaptic junctions until it reaches the central nervous system. Because inhibitory neurons are involved, the result is unopposed muscle contraction.
In generalized tetanus, the most common form, the patient typically experiences lockjaw (trismus). This is a stiffness of the jaw muscles that results in inability to open the mouth or swallow leading to the appearance of a sardonic smile (*risus sardonicus*). Cephalic tetanus is a rare infection involving the middle ear. It can affect cranial nerves. Local tetanus is also rare and manifests itself as localized muscle contractions in the area of infection.

### *Clostridium perfringens* (GAS GANGRENE)

*Clostridium perfringens*, a Gram-positive rod, causes wound colonization (gas gangrene) after soil, and to a lesser extent intestinal tract, contamination.
The organism produces several tissue degrading enzymes (including lecithinase [alpha toxin], proteolytic and saccharolytic enzymes).

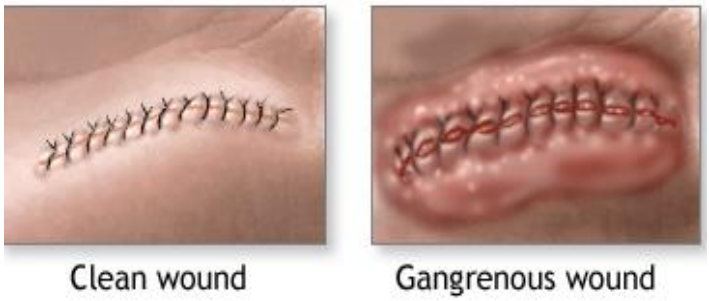


**Fig. 84 Gas gangrene.**

Necrosis and destruction of blood vessels and the surrounding tissue, especially muscle, result (myonecrosis is a condition of necrotic damage, specific to muscle tissue) (Fig. 84). This creates an anaerobic environment in adjacent tissue and the organism spreads systemically.

### *Clostridium botulinum (BOTULISM)*

It is a serious paralytic illness caused by *Clostridium botulinum.* The toxin (only types A, B, E and F cause illness in humans) binds to receptors on peripheral nerves, where acetylcholine is the neurotransmitter and inhibits nerve impulses (Fig. 85). Flaccid paralysis and often death (from respiratory and/or cardiac failure) ensue.
The organism does not grow in the gut, but pre-formed exotoxin from prior germination of spores may be present in inadequately autoclaved canned food (usually at home). The toxin is heat labile and can be destroyed if heated at 80°C for 10 minutes or longer.

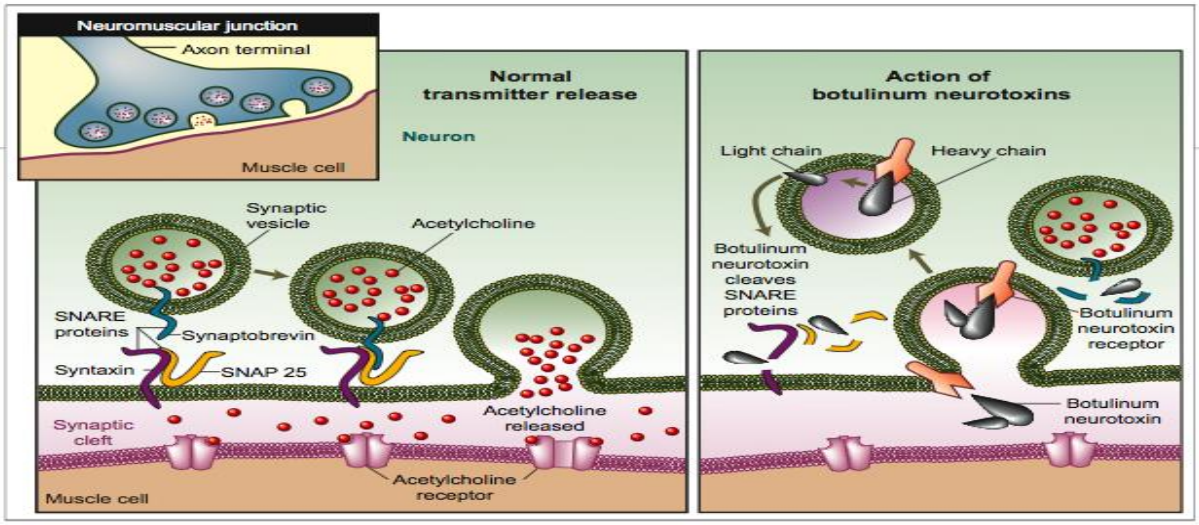


**Fig. 845 Botulotoxin – mode of action.**

The incidence of the disease is low, but the disease is of considerable concern because of its high mortality rate if not treated immediately and properly. Botulism can be prevented by using food preservation methods that are designed to inhibit the growth of *C. botulinum*. For example, low acid (pH > 4.4) canned foods are heat treated to 121°C for 3 min (known as the "botulism cook") or equivalent.

### *Clostridium difficile* (PSEUDOMEMBRANOUS COLITIS)

*Clostridium difficile* causes antibiotic-associated diarrhea (AAD) and more serious intestinal conditions such as **colitis** and **pseudomembranous colitis** in humans. These conditions generally result from overgrowth of *Clostridium difficile* in the colon, usually after the normal intestinal microbiota flora has been disturbed by antimicrobial chemotherapy. People in good health usually do not get *C. difficile* disease. Individuals who have other conditions that require prolonged use of antibiotics and the elderly are at greatest risk. Also, individuals who have recently undergone gastrointestinal surgery, or have a serious underlying illness, or who are immunocompromised, are at risk. *C. difficile* produces two toxins. **Toxin A** is referred to as an enterotoxin because it causes fluid accumulation in the bowel. **Toxin B** is an extremely lethal (cytopathic) toxin.

## 9.2 LABORATORY DIAGNOSIS OF ANAEROBIC BACTERIA

Anaerobes are normally found within certain areas of the body but result in serious infection when they have access to a normally sterile body fluid or deep tissue that is poorly oxygenated. Some anaerobes normally live in the crevices of the skin, in the nose, mouth, throat, intestine, and vagina. Injury to these tissues (cuts, puncture wounds, or trauma) especially at or adjacent to the mucous membranes allows anaerobes entry into otherwise

sterile areas of the body and is the primary cause of anaerobic infection. A second source of anaerobic infection occurs from the introduction of spores into a normally sterile site. Spore-producing anaerobes live in the soil and water, and spores may be introduced via wounds, especially punctures. Anaerobic infections are most likely to be found in persons who are immunosuppressed, those treated recently with broad-spectrum antibiotics , and persons who have a decaying tissue injury on or near a mucous membrane, especially if the site is foul-smelling. The identification of anaerobes is highly complex, and laboratories may use different identification systems. Organisms are identified by their:

- colonial and microscopic morphology,
- growth on selective media,
- oxygen tolerance,
- biochemical characteristics (these include sugar fermentation, bile solubility, esculin, starch, and gelatin hydrolysis, casein and gelatin digestion, catalase, lipase, lecithinase, and indole production, nitrate reduction, volatile fatty acids as determined by gas chromatography)
- susceptibility to antibiotics (by the microtube broth dilution method).

## 9.3 ANAEROBIC INFECTIONS – SPECIMEN COLLECTION

The keys to effective anaerobic bacteria cultures include collecting a contamination-free specimen and protecting it from oxygen exposure. Anaerobic bacteria cultures should be obtained from an appropriate site without the health care professional contaminating the sample with bacteria from the adjacent skin, mucus membrane, or tissue. Swabs should be avoided when collecting specimens for anaerobic culture because cotton fibers may be detrimental to anaerobes. Abscesses or fluids can be aspirated using a sterile syringe that is then tightly capped to prevent entry of air. Tissue samples should be placed into a degassed bag and sealed, or into a gassed out screw top vial that may contain oxygen-free prereduced culture medium and tightly capped. The specimens should be plated as rapidly as possible.

## 9.4 ANAEROBIC BACTERIA - GRAM STAIN

### Gram-positive anaerobes

**Gram-positive** anaerobes include the following:

- *Actinomyces* (head, neck, pelvic infections; aspiration pneumonia)
- *Bifidobacterium* (ear infections, abdominal infections)
- *Clostridium* (gas, gangrene, food poisoning, tetanus, pseudomembranous colitis)

- *Peptostreptococcus* (oral, respiratory, and intra-abdominal infections)
- *Propionibacterium* (shunt infections)

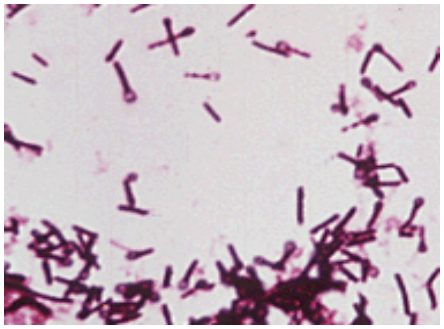

**Fig. 856 *Clostridium tetani* - Gram stain.**

*Clostridium tetani* is Gram-positive, spore producing, motile bacterium. The organism produces terminal spores within a swollen sporangium giving it a distinctive drumstick appearance (Fig. 86). Although the bacterium has a typical Gram-positive cell wall, it may stain Gram-negative or Gram-variable, especially in older cells.

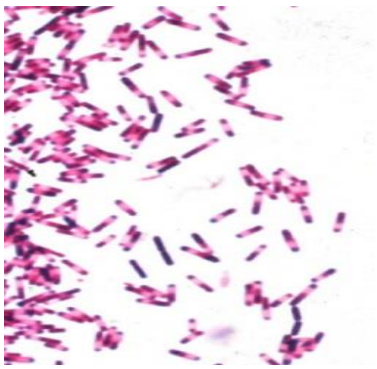

**Fig. 867 *Clostridium botulinum* - Gram stain.**

*Clostridium botulinum* is a large, Gram-positive, spore-forming, rod-shaped motile anaerobic bacterium

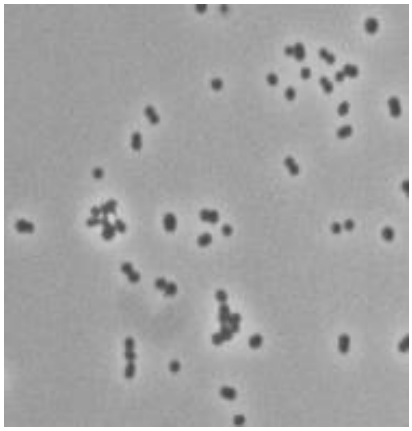

**Fig. 878 *Peptostreptococcus* sp. - Gram stain.**

*Peptostreptococci* are anaerobic, non-spore-forming, non-motile, Gram-positive cocci that occur singly, in pair, tetrads, short chains or clusters (Fig. 88).

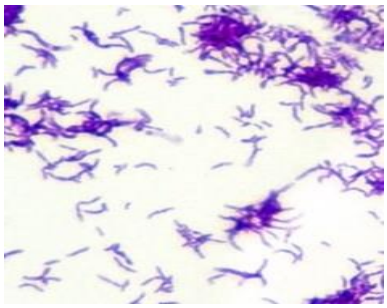

**Fig. 88 *Propionibacterium acnes* - Gram stain.**

*Propionibacterium acnes* are small Gram-positive, non-spore-forming, pleomorphic bacilli.

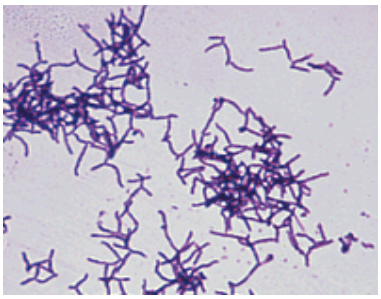

**Fig. 90 *Actinomycetes* - Gram stain.**

Actinomycetes are Gram-positive obligate anaerobes, non-spore-forming, fungus-like bacteria that form filamentous branches (known to reside in the mouth and in the intestinal tract).

### Gram-negative anaerobes

**Gram-positive** anaerobes include the following:

- *Bacteroides* (the most commonly found anaerobes in cultures; intra-abdominal infections, rectal abscesses, soft tissue infections, liver infection)
- *Fusobacterium* (abscesses, wound infections, pulmonary and intracranial infections)
- *Porphyromonas* (aspiration pneumonia, periodontitis)
- *Prevotella* (intra-abdominal infections, soft tissue infections)

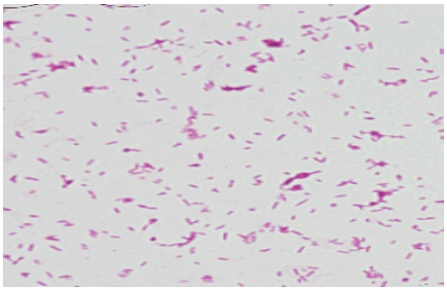

**Fig. 91 *Bacteroides* - Gram stain.**

*Bacteroides* sp. are Gram-negative rods, non-spore-forming, they do produce a very large capsule.

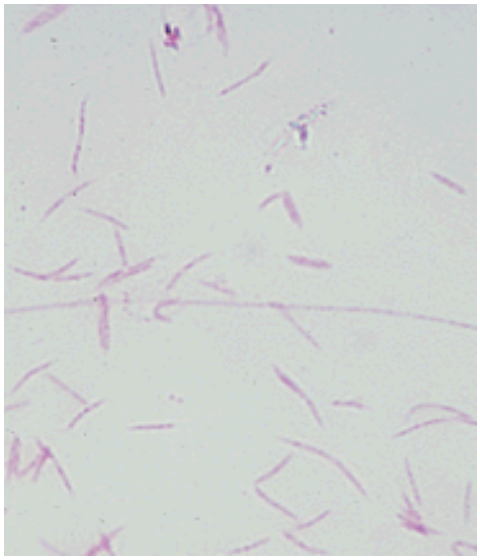

**Fig. 882 *Fusobacterium* - Gram stain.**

*Fusobacterium:* Gram-negative bacilli, spindle-shaped cells with sharp ends.

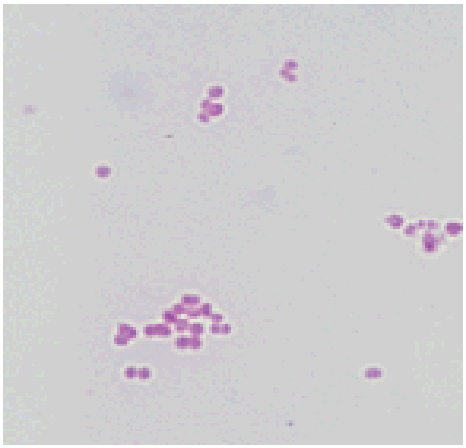

**Fig. 893 *Veillonella* - Gram stain.**

*Veillonella* are Gram-negative non-motile diplococci, normal flora of the mouth.

## 9.5 ANAEROBIC BACTERIA – WIRTZ-CONKLIN STAIN

Endospores produced by genus *Clostridium* do not stain easily. Endospores are stained by *Wirtz-Conklin* method where malachite green is used for staining and heat is used to

penetrate stain. The rest of the cell is then decolorized and counterstained a light red with carbolfuchsin.

**Fig. 94 *C. botulinutm*, Wirtz-Conklin stain.**

*C. botulinum*: these gram-positive bacilli have subterminal spores (no terminal spores).

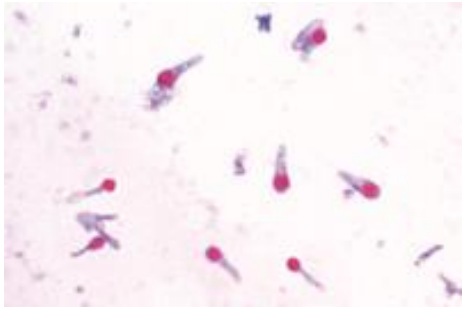

**Fig. 905 *C. tetani*, Wirtz-Conklin stain.**

*C. tetani* produces terminal spores with drum stick appearance.

## 9.6 ANAEROBIC BACTERIA - CULTIVATION

An anaerobic bacteria culture is a method used to grow anaerobes from a clinical specimen. Obligate anaerobes are bacteria that can live only in the absence of oxygen. Obligate anaerobes are destroyed when exposed to the atmosphere for as briefly as 10 minutes. Some anaerobes are tolerant to small amounts of oxygen. Facultative anaerobes are those organisms that will grow with or without oxygen. The methods of obtaining specimens for anaerobic culture and the culturing procedure are performed to ensure that the organisms are protected from oxygen. It is crucial that the health care provider obtain the sample for culture via aseptic technique. Anaerobes are commonly found on mucous membranes and other sites such as the vagina and oral cavity. Therefore, specimens likely to be contaminated with these organisms should not be submitted for culture (throat or vaginal swab). Some types of specimens should always be cultured for anaerobes if an infection is suspected. These include abscesses, bites, blood, cerebrospinal fluid and exudative body fluids, deep wounds, and dead tissues. The specimen must be protected from oxygen during collection and transport and must be transported to the laboratory immediately. **Cultures should be placed in an environment that is free of oxygen, at 35°C for at least 48 hours before the plates are examined for growth.**

## Anaerobic Growth Media

Most strict anaerobes require not only the absence of oxygen to initiate growth, but also a redox potential below -300mV, which can be only achieved by the supplementation of media with reducing agents.

**Thioglycolate broth** is a multi-purpose, enriched differential medium used primarily to determine the oxygen requirements of microorganisms. Sodium thioglycolate in the medium consumes oxygen and permits the growth of obligate anaerobes. This, combined with the diffusion of oxygen from the top of the broth produces a range of oxygen concentrations in the media along its depth. The oxygen concentration at a given level is indicated by a redox sensitive dye like resazurine that turns pink in the presence of oxygen.

**Fig. 916 Thioglycolate broth.**

Reducing media chemically remove molecular oxygen ($O_2$) that might interfere with the growth of anaerobes. Thioglycolate combines with dissolved $O_2$ to deplete in media. The primary plating media for inoculating anaerobic specimen includes a nonselective blood agar and one or all of the following mentioned selective media.

**Non selective media used in anaerobic bacteriology:**

1. Anaerobic blood agar: It is a nonselective medium for isolation of anaerobes and facultative anaerobes.
2. Egg-yolk agar (EYA): Nonselective for determination of lecithinase and lipase production by clostridia and fusobacteria.
3. Cooked meat broth: Nonselective for cultivation of anaerobic organisms; with addition of glucose, can be used for gas-liquid chromatography.
4. Peptone-yeast extract glucose broth (PYG): Nonselective for cultivation of anaerobic bacteria for gas-liquid chromatography.

**Selective and differential media used in anaerobic bacteriology:**

1. Bacterioides bile esculin agar (BBE): It is selective and differential for *Bacteriodes fragilis* group and good for presumptive identification.
2. Laked Kanamycin-vancomycin blood agar (LKV): It is selective for isolation of *Prevotella* and *Bacteriodes* spp.
3. Anaerobic phenylethyl alcohol agar (PEA): Selective for inhibition of gram negative rods and swarming by some clostridia.

4. Cycloserine cefoxitin fructose agar (CCFA): selective for clostridium difficile.
5. Thioglycollate broth: Non selective for cultivation of anaerobes; as well as facultative anaerobes and aerobes.

## Special Culture Techniques for Anaerobic Bacteria

### Candle jar

A microaerophile is a microorganism that requires oxygen to survive, but requires environments containing lower levels of oxygen than are present in the atmosphere (20% concentration). Many microphiles are also capnophiles, as they require an elevated concentration of carbon dioxide. In the laboratory they can be easily cultivated in a candle jar. A candle jar is a container into which a lit candle is introduced before sealing the container's airtight lid . The candle's flame burns until extinguished by oxygen deprivation, which creates a carbon dioxide-rich, oxygen-poor atmosphere in the jar. Many labs also have access directly to carbon dioxide and can add the desired carbon dioxide levels directly to incubators where they want to grow microaerophiles. Candle jars are used to grow bacteria requiring an increased $CO_2$ concentration (capnophiles). Candle jars increase $CO_2$ concentrations and still leave some $O_2$ for aerobic capnophiles.

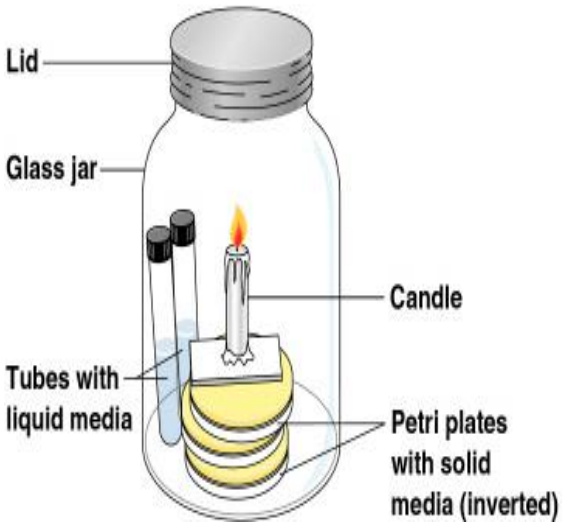


**Fig. 927 Candle jar.**

### Gas pack

Gas packs can generate $CO_2$ also and are generally used in place of candle jars. The packet consist of a bag containing a Petri plate and $CO_2$ gas generator. The gas generator is crushed to mix the chemicals it contains and start the reaction that produces $CO_2$. This gas reduces the oxygen concentration in the bag to about 5% and provides $CO_2$ concentration of about 10%.

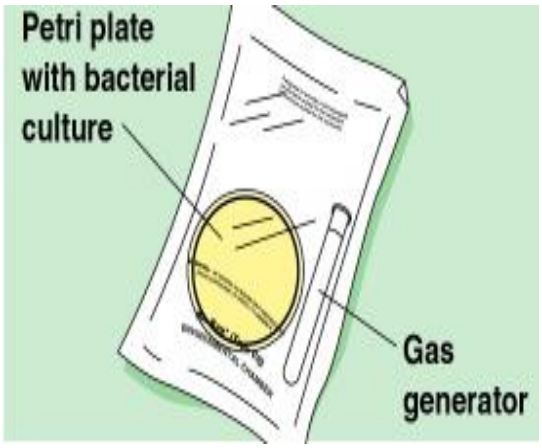


**Fig. 938 Gas pack.**

**Anaerobic jar**

Petri plates can be incubated in an anaerobic jar or anaerobic chamber. Sodium bicarbonate and sodium borohydride are mixed with a small amount of water to produce $CO_2$ and $H^+$. A palladium catalyst in the jar combines with the $O_2$ in the jar and the $H^+$ to remove $O_2$.

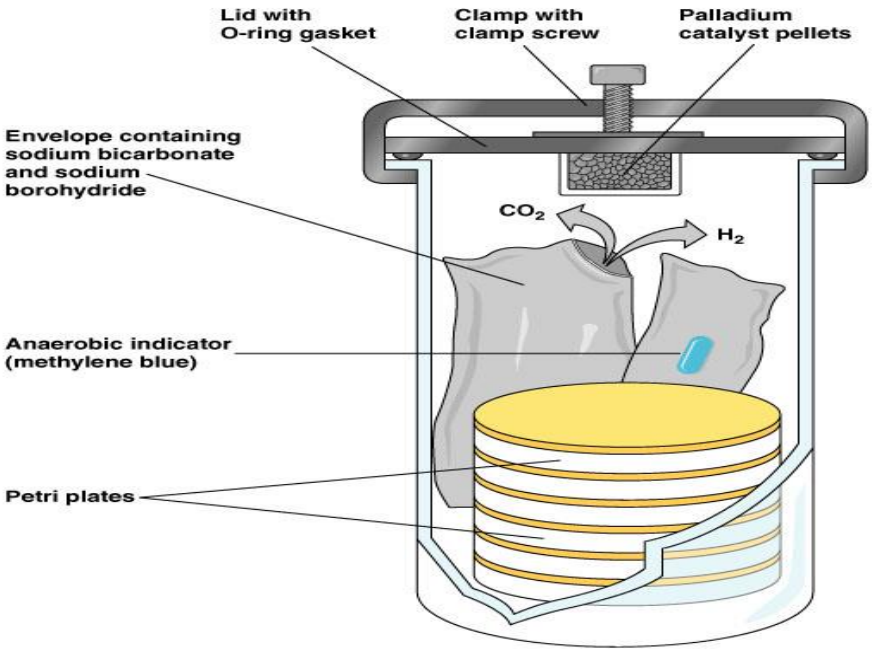


**Fig. 949 Anaerobic jar.**

**Biological method**

Biological method can be use to establish anaerobic conditions. One half of the solid medium in the Petri´s dish is innoculated with the tested sample, the second half is inoculated with *Serratia marcescens* - aerobic bacteria able to produce anaerobic environment by the consumption of oxygen. Petri dish is sealed with the wax or parafin and cultured in aerobic environment.

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# 10 SPIROCHETES

**Spirochaetes** (also spelled **spirochetes**) belong to a phylum of distinctive diderm (double-membrane) bacteria, most of which have long, helically coiled (corkscrew-shaped) cells. Spirochaetes are chemoheterotrophic in nature, with lengths between 5 and 250 µm and diameters around 0.1–0.6 µm. Spirochaetes are distinguished from other bacterial phyla by the location of their flagella, sometimes called axial filaments, which run lengthwise between the bacterial inner membrane and outer membrane in periplasmic space (Fig. 100). These cause a twisting motion which allows the spirochaete to move about.

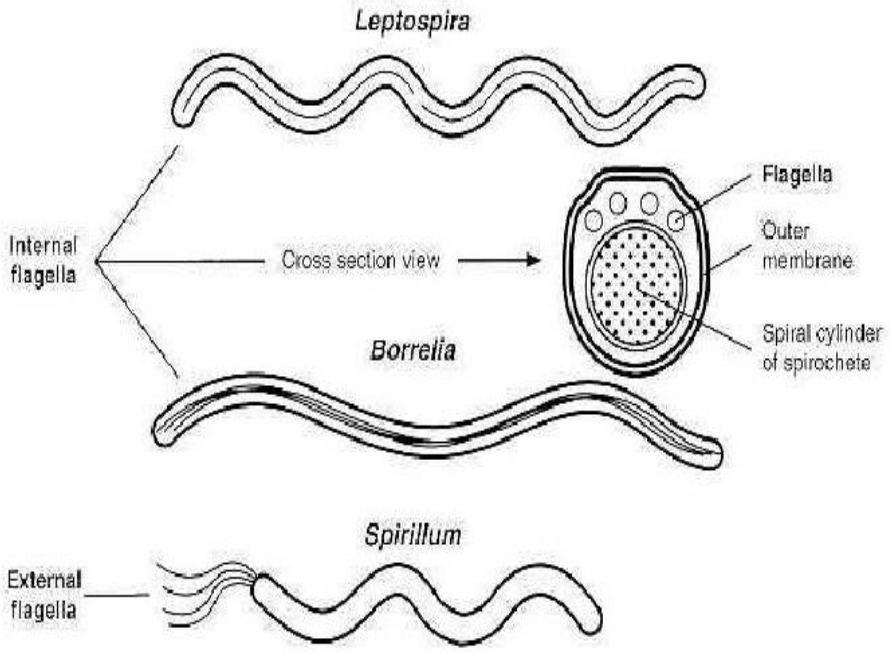


**Fig. 10095 Spirochetes - structure.**

When reproducing, a spirochaete will undergo asexual transverse binary fission. In addition, the spirochetes are microaerophilic or anaerobic and are extremely sensitive to oxygen toxicity. The complete genome sequence has revealed there are no genes for catalase or superoxide dismutase.

The order of Spirochaetales is divided into two families:

1. Spirochaetaceae
2. Leptospiraceae

Two of the four genera of Spirochaetaceae, *Treponema* and *Borrelia*, include species that are pathogenic to man. Among Leptospiraceae, only one genus, *Leptospira*, has pathogenic species.

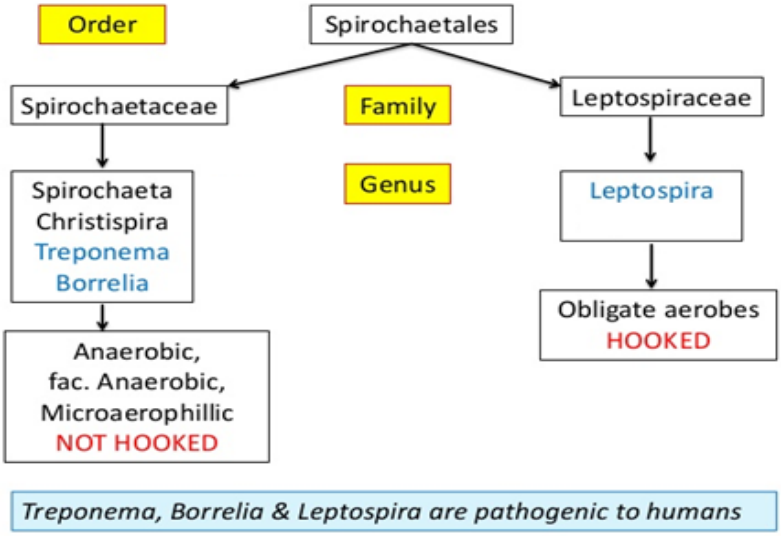


**Fig. 101 The order Spirochaetales.**

Disease-causing members are *Leptospira* species, *Borrelia burgdorferi*, *B. garinii*, *B. afzelii*, *Borrelia recurrentis*, *Treponema pallidum* subspecies, *Brachyspira pilosicoli* and *Brachyspira aalborgi* (Table 6).

| Disease-causing members | Disease |
|---|---|
| ***Leptospira* species** | **leptospirosis** |
| ***Borrelia burgdorferi*** | **Lyme disease** |
| ***B. garinii*** | |
| ***B. afzelii*** | |
| ***Borrelia recurrentis*** | **relapsing fever** |
| ***Treponema* species** | **treponematoses** |
| ***Brachyspira pilosicoli*** | **intestinal spirochaetosis** |
| ***Brachyspira aalborgi*** | |

**Table 6 Disease - causing members of Spirochetes.**

## 10.1 TREPONEMA PALLIDUM

*Treponema pallidum* is a spirochaete bacterium with subspecies that cause treponemal diseases such as syphilis, bejel, pinta, and yaws. Classification of the pathogenic treponemes is based primarily upon the clinical manifestations of the respective diseases they cause (Table 7).

| Subspecies | Disease |
|---|---|
| ***T. pallidum* subsp. *pallidum*** | **Venereal syphilis** |
| ***T. pallidum* subsp. *pertenue*** | **Yaws** |
| ***T. pallidum* subsp. *endemicum*** | **Endemic syphilis** |
| ***T. carateum*** | **Pinta** |

**Table 7 Classification of the pathogenic treponemes.**

## 10.2 SYPHILIS

The clinical course of syphilis evolves through three phases. The initial or **primary phase (primary syphilis)** is characterized by one or more skin lesions **(chancres)** at the site where the spirochete penetrated. Although spirochetes are disseminated in the blood soon after infection, the chancre represents the primary site of initial replication. Histologic examination of the lesion reveals endarteritis and periarteritis (characteristic of syphilitic lesions at all stages) and infiltration of the ulcer with polymorphonuclear leukocytes and macrophages. Phagocytic cells ingest spirochetes, but the organisms often survive. Clinical manifestations of the primary stage include regional lymphadenopathy. In the **secondary phase (secondary syphilis),** the clinical signs of disseminated disease appear, with prominent skin lesions dispersed over the entire body surface. Spontaneous remission may occur after the primary or secondary stages, or the disease may progress to the **late phase (tertiary syphilis)** of disease, in which virtually all tissues may be involved. Tertiary syphilis may include gummatous syphillis where gummatous lesions may form on any organ or tissue, cardiovascular syphilis, which usually manifests as aortic disease and neurosyphillis. Neurosyphilis can manifest as acute syphilitic meningitis, meningovascular syphilis or as paresis or tabes dorsalis. It usually arises in tertiary syphilis, but can occur as early as 3 months post infection. Over 40% of patients with secondary syphilis experience some central nervous system involvement Each stage represents localized multiplication of the spirochete and tissue destruction. Although replication is slow, numerous organisms are present in the initial chancre, as well as in the secondary lesions, making the patient highly infectious at these stages.

### Congenital Syphilis

In utero infections can lead to serious fetal disease, resulting in latent infections, multiorgan malformations, or death of the fetus. Most infected infants are born without clinical evidence of the disease, but rhinitis then develops and is followed by a widespread desquamating maculopapular rash. Teeth and bone malformation, blindness, deafness, and cardiovascular syphilis are common in untreated infants who survive the initial phase of disease.

## 10.3 SYPHILIS – LABORATORY TESTING

Syphilis has several clinical manifestations, making laboratory testing a very important aspect of diagnosis. The etiological agent, *Treponema pallidum*, cannot be cultured, and there is no single optimal alternative test. Serological testing is the most frequently used approach in the laboratory diagnosis of syphilis. Syphilis has diverse clinical manifestations and shares many clinical features with other treponemal and nontreponemal diseases. Therefore, it is mandatory that the clinical diagnosis is always supported by appropriate laboratory tests and that the test results are interpreted with reference to the patient's history and physical examination findings.

Although [21]*T.pallidum* cannot be grown in culture, there are many tests for the direct and indirect diagnosis of syphilis. Direct diagnostic methods include the detection of *T. pallidum* by microscopic examination of fluid or smears from lesions, histological examination of tissues or nucleic acid amplification methods such as polymerase chain reaction (PCR). Indirect diagnosis is based on serological tests for the detection of antibodies.

## 10.4 TREPONEMA PALLIDUM - CULTIVATION

*T. pallidum* is an obligate human parasite, which does not survive outside its mammalian host and cannot be cultivated continuously under in vitro conditions. Optimal conditions for time-limited cultivation in tissue culture consisted of temperature between 33°C-35°C, atmospheric oxygen concentration in the 1,5% - 5% range, 20% fetal bovine serum in the culture medium and the testes extract. Stable propagation of *T. pallidum* strains can only be achieved in mammalian hosts, usually rabbits.

## 10.5 TREPONEMA PALLIDUM - MICROSCOPY

Because *T. pallidum* is too thin to be seen by light microscopy, **darkfield microscopy** or **special fluorescent stains** must be used. The diagnosis of primary, secondary, or congenital syphilis can be made rapidly by darkfield examination of the exudate from skin lesions (Fig. 102).

**Fig. 102 *T. pallidum*- darkfield microscopy.**

[21] T. - Treponema

Darkfield microscopy method may be used in the early stages of syphilis when a suspected syphilis sore (chancre) is present. It involves obtaining a scraping of the sore, placing it on a slide, and examining it with a special dark-field microscope. Material collected from oral and rectal lesions should not be examined because nonpathogenic spirochetes can contaminate the specimen. Because of the limitations of darkfield microscopy, a more useful test for detecting *T. pallidum* is the **direct fluorescent antibody test (**uses a labeled antibody to directly react with the antigen).
Fluorescein-labeled antitreponemal antibodies are used to stain the bacteria. A monoclonal antibody reagent is available that is specific for pathogenic treponemes, so oral and rectal specimens can be examined.

## 10.6 TREPONEMA PALLIDUM - SEROLOGICAL TESTS (NONTREPONEMAL AND TREPONEMAL TESTS)

Serological tests fall into two categories: nontreponemal tests for screening, and treponemal tests for confirmation.

### Nontreponemal Tests

**Nontreponemal tests** measure immunoglobulin G (IgG) and IgM antibodies (also called **reagins**) developed against lipids released from damaged cells during the early stage of disease and that appear on the cell surface of treponemes. The antigen used for the nontreponemal tests is **cardiolipin,** which is derived from beef heart.

| | Test |
|---|---|
| VDRL | Venereal Disease Research Laboratory |
| RPR | Rapid Plasma Reagin |
| USR | Unheated Serum Reagin ( modification of the VDRL test) |

**Table 8 Nontreponemal tests.**

The two tests used most commonly are the **Venereal Disease Research Laboratory (VDRL) test** and the **rapid plasma reagin (RPR) test.** Both tests measure the flocculation of cardiolipin antigen by the patient's serum, both tests can be performed rapidly. Only the VDRL test should be used to test CSF from patients with suspected neurosyphilis. Other nontreponemal tests in use include the unheated serum reagin (USR) test and the toluidine red unheated serum test (TRUST). All nontreponemal tests have essentially the same sensitivity (70% to 85% for primary disease, 100% for secondary disease, 70% to 75% for late syphilis) and specificity (98% to 99%).

### VDRL test

**Venereal Disease Research Laboratory** test utilizes an antigen which consists of cardiolipin, cholesterol and lecithin. The antigen particles appear as short rod forms at magnification of about 100x. Aggregation of these particles into large or small clumps is interpreted as positivity (reactive) (Fig. 103).

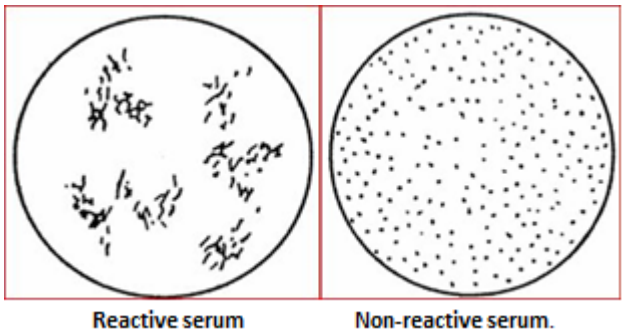


**Fig. 103 Venereal Disease Research Laboratory test.**

### RPR test

**Rapid plasma reagin (RPR) test** is the most commonly used non-treponemal test for the diagnosis of syphilis. The test takes the form of a flocculation assay in which a cardiolipin antigen and the patient's anti-cardiolipin antibodies form an antigen–antibody lattice, which can be visualised when carbon particles are trapped within it.

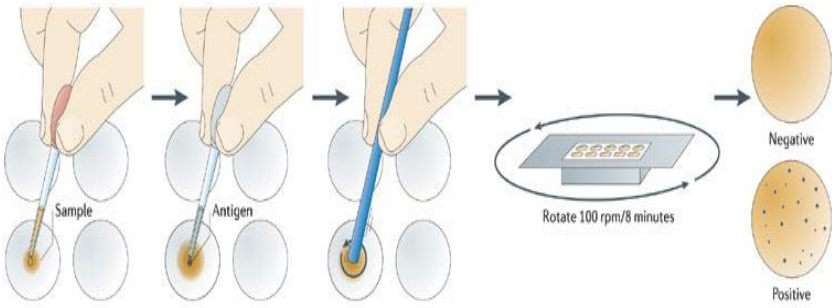


**Fig. 104 Rapid plasma reagin test.**

### Treponemal Tests

Treponemal tests use *T. pallidum* as the antigen and detect specific anti - *T. pallidum* antibodies. The treponemal test results can be positive before the nontreponemal test results become positive in early syphilis, and they can remain positive when the nonspecific test results revert to negative in some patients who have late syphilis.

| Test | |
|---|---|
| **FTA-ABS** | **Fluorescent Treponemal Antibody-Absorption** |
| **MHA-TP** | **Microhemagglutination *Treponema pallidum*** |
| **TP-PA** | ***T. pallidum* particle agglutination** |

**Table 9 Treponemal tests.**

Historically, the most commonly used treponemal test was the **fluorescent treponemal antibody-absorption (FTA-ABS) test.**

**FTA-ABS test**

FTA-ABS (**Fluorescent Treponemal Antibody-Absorption)** test is an indirect fluorescent antibody test. The patient's serum, which has been diluted 1:5 in sorbent (an extract from cultures of *Treponema phagedenis*, Reiter treponeme), is layered on a microscope slide to which *T. pallidum* subspecies *pallidum* has been fixed. If the patient's serum contains antibody, the antibody will coat the treponeme. Next, fluorescein isothiocyanate (FITC)-labeled antihuman immunoglobulin is added; this combines with the patient's IgG and IgM antibodies that are adhering to *T. pallidum*, and results in a visible test reaction when examined by fluorescence microscopy.

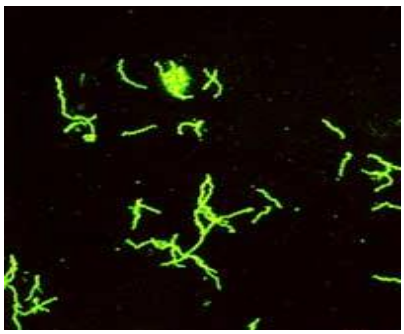

**Fig. 105 Fluorescent Treponemal Antibody-Absorption Test - visible test reaction.**

**TP-PA test**

***Treponema pallidum* Particle Agglutination (TP-PA) test** or one of a number of specific **enzyme immunoassays (EIAs).** The TP-PA test is a microtiter agglutination test, based on the agglutination of colored gelatin particle carriers sensitized with *T. pallidum* antigen. Patient sera are incubated with sensitized particles in microtiter wells and unsensitized gelatin particles in control wells. Patient sera containing specific antibodies will react only with the

antigen sensitized colored gelatin particles to form a smooth mat of agglutinated particles in the microtitration tray (+ or ++). A compact button formed by the settling of the non-agglutinated particles in the microtiter wells containing sensitized particles indicates lack of specific antibody in patient sera (-). A variety of specific EIAs have been developed and appear to have sensitivities (80% to 95% for primary disease, 100% for secondary and late syphilis) and specificities (96% to 99%) similar to the FTA-ABS and TP-PA tests.

**Fig. 106 *Treponema pallidum* Particle Agglutination Test.**

**MHA-TP test**

**Microhemagglutination *Treponema pallidum*** test is based on the principle of agglutination and pattern recognition. The test uses fixed chicken erythrocytes sensitized with components of the pathogenic *T. pallidum* (Nichols Strain). Hemagglutination occurs in the presence of *Treponema pallidum* antibodies in specimens.

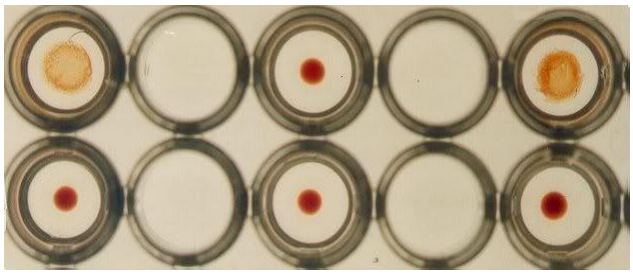

**Fig. 107 Microhemagglutination *Treponema pallidum* test.**

The upper, left-hand well contains a positive control test. The red cells have had treponemal antigens attached and antibodies in the serum have caused these cells to agglutinate and form a mat across the bottom of the well. These antibodies can be presumed to be specific for treponemes, since identical red cells that have not had the treponemal antigens attached do not cause haemagglutination, as seen in the bottom, left-hand well. A negative serum test is shown in the centre well, where no agglutination is observed. On the upper, right-hand of the

well is a patient's sample. The agglutination formed by the patient's serum support positive syphillis infection.

### Interpretation of the Results of Serological Tests

Because positive reactions with the nontreponemal tests develop late during the first phase of disease, the serologic findings are negative in many patients who initially have chancres. However, serologic results are positive within 3 months in all patients and remain positive in untreated patients with secondary syphilis. The antibody titers decrease slowly in patients with untreated syphilis, and serologic results are negative in approximately 25% to 30% of patients with late syphilis. Thus the limitation of the nontreponemal tests is reduced sensitivity in early primary disease and late syphilis. Although the results of treponemal tests generally remain positive for the life of the person who has syphilis, a negative test is unreliable in patients with AIDS.
Successful treatment of primary or secondary syphilis and, to a lesser extent, late syphilis, leads to reduced titers measured in the VDRL and RPR tests. Thus these tests can be used to monitor the effectiveness of therapy, although seroreversion is slowed in patients in an advanced stage of disease, those with high initial titers, and those who have previously had syphilis. The treponemal tests are influenced less by therapy than are the VDRL and RPR tests, with seroreversion observed in less than 25% of patients successfully treated during the primary stage of the disease.

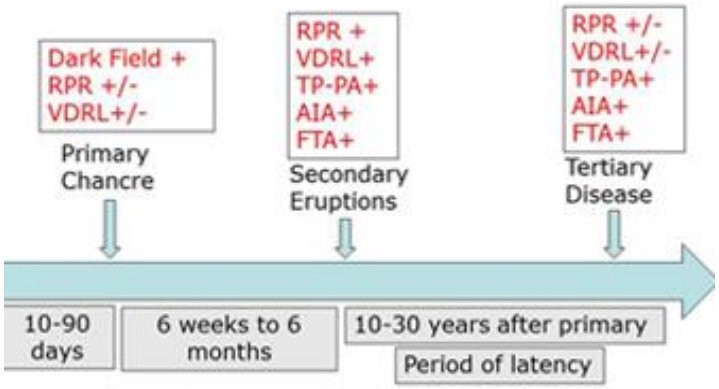


**Fig. 108 Syphilis - stages and possible results.**

### Diagnosis of Neurosyphilis and Congenital Syphilis

Diagnosis of neurosyphilis and congenital syphilis can be problematic. The diagnosis of neurosyphilis is based on clinical symptoms and laboratory findings.
A VDRL test on cerebrospinal fluid (CSF) is highly specific but not sensitive. Thus a positive VDRL confirms the diagnosis, but a negative test does not rule out neurosyphilis. In contrast, the FTA-ABS CSF test has high sensitivity but low specificity because of passive transfer of antitreponemal antibodies from blood to CSF. In this case, a positive FTA-ABS CSF test is

consistent with neurosyphilis but is not diagnostic, and a negative test would essentially rule out the diagnosis.

Positive serologic test results in infants of infected mothers can represent a passive transfer of antibodies or a specific immunologic response to a congenital infection. These two possibilities are distinguished by measuring the antibody titers in the sera of the infant during a 6-month period. The antibody titers in noninfected infants decrease to undetectable levels within 3 months of birth but remain elevated in infants who have congenital syphilis.

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# 11 MYCOBACTERIA

Mycobacteria are widespread organisms, typically living in and food sources. Tuberculosis and the leprosy organisms are obligate parasites and are not found as free-living members of the genus. Mycobacteria are aerobic and nonmotile bacteria (except for the species *Mycobacterium marinum*, which has been shown to be motile within macrophages) that are characteristically acid fast. Mycobacteria have an outer membrane. They do not have capsules, and most do not form endospores. The distinguishing characteristic of all *Mycobacterium* species is that the cell wall is thicker than in many other bacteria, which is hydrophobic, waxy, and rich in mycolic acids/mycolates. The cell wall consists of the hydrophobic mycolate layer and a peptidoglycan layer held together by a polysaccharide, arabinogalactan. The cell wall makes a substantial contribution to the hardiness of this genus. The biosynthetic pathways of cell wall components are potential targets for new drugs for tuberculosis.

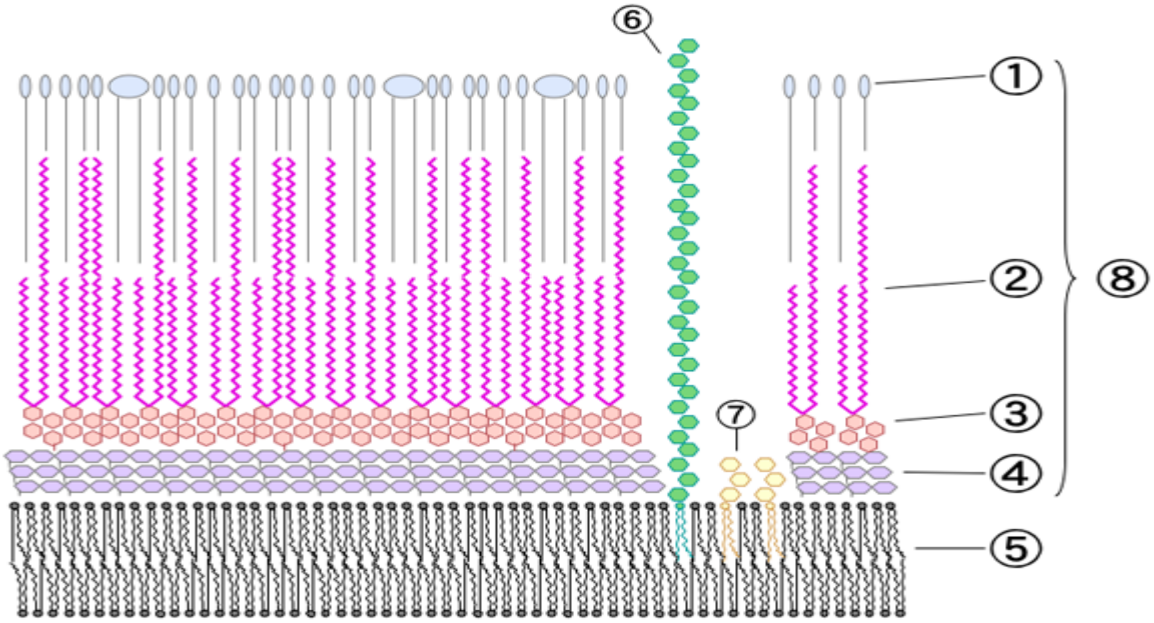



**Fig. 109 Mycobacterial cell wall:** 1-outer lipids, 2-mycolic acid, 3-polysaccharides (arabinogalactan), 4-peptidoglycan, 5-plasma membrane, 6-lipoarabinomannan (LAM), 7-phosphatidylinositol mannoside, 8-cell wall skeleton.

## 11.1 NONTUBERCULOUS MYCOBACTERIA – RUNYON CLASSIFICATION

Runyon classification is a system of identifying mycobacteria on the basis of pigmentation and growth condition of the organisms. The **Runyon classification** of nontuberculous mycobacteria based on the rate of growth, production of yellow pigment and whether this pigment was produced in the dark or only after exposure to light. It was introduced by Ernest

Runyon in 1959 (Fig. 110). On these bases, the nontuberculous mycobacteria are divided into four groups:

**Photochromogens (Group I)** - produce nonpigmented colonies when grown in the dark and pigmented colonies only after exposure to light and reincubation ([22]*M. kansasii*, *M. marinum*, *M. simiae).*

**Scotochromogens (Group II)** - produce deep yellow to orange colonies when grown in the presence of either the light or the dark (*M. scrofulaceum*, *M. gordonae*, *M. xenopi*, *M. szulgai).*

**Non-chromogens (Groups III & IV)** - nonpigmented in the light and dark or have only a pale yellow, buff or tan pigment that does not intensify after light exposure (*M. tuberculosis*, *M. avium-intra-cellulare*, *M. bovis*, *M. ulcerans*, *M. fortuitum*, *M. chelonae*).

| Group | Growth | Pigment | Examples | Disease |
|---|---|---|---|---|
| I | slow | yellow-orange on light (photochromogen) | 1. *M. kansasii* 2. *M. marinum* | 1. similar to TB 2. swimming pool granuloma |
| II | slow | yellow-orange in light or dark (scotochromogen) | M. scrofulaceum | cervical adenitis |
| III | slow | no pigment | M. avium intracellulare complex (MAC) | similar to TB, esp. in AIDS |
| IV | rapid (5 days) | no pigment | M. fortuitum M. cheilonae | soft tissue, lung, bone, CNS, eye infections |

**Fig. 110 Runyon classification.**

## 11.2 MYCOBACTERIUM TUBERCULOSIS (TUBERCULOSIS)

Tuberculosis (TB) is caused by the infectious agent known as *Mycobacterium tuberculosis* (MTB). This rod-shaped bacterium, also called Koch's bacillus, was discovered by Dr. Robert Koch in 1882. MTB is a small, slow-growing bacterium that can live only in people. It is not found in other animals, insects, soil, or other nonliving things. MTB is an aerobic bacterium, meaning it needs oxygen to survive. For this reason, during active tuberculous disease, MTB complexes are always found in the upper air sacs of the lungs. The bacterium is a facultative

[22] M. - Mycobacterium

intracellular parasite, usually of macrophages, and has a slow generation time, 15-20 hours, a physiological characteristic that may contribute to its virulence. The bacteria usually attack the lungs, but MTB bacteria can attack any part of the body such as the kidney, spine, and brain. If not treated properly, disease can be fatal. It is transmitted from person to person via droplets from the throat and lungs of people with the active respiratory disease.

### Latent tuberculosis infection (LTBI)

Latent tuberculosis infection (LTBI) is a state of persistent immune response to stimulation by *Mycobacterium tuberculosis* antigens without evidence of clinically manifested active tuberculosis. The lifetime risk of reactivation for a person with documented LTBI is estimated to be 5–10%, with the majority developing TB disease within the first five years after initial infection.

### Active tuberculosis (TB disease)

In some people, MTB bacteria overcome the defenses of the immune system and begin to multiply, resulting in the progression from latent tuberculosis infection to TB disease. Some people develop TB disease soon after infection, while others develop TB disease later when their immune system becomes weak.

| A Person with Latent TB Infection | A Person with TB Disease |
|---|---|
| Has no symptoms | Has symptoms that may include:<br><br>a bad cough that lasts 3 weeks or longer<br>pain in the chest<br>coughing up blood or sputum<br>weakness or fatigue<br>weight loss<br>no appetite<br>chills, fever, sweating at night |
| Does not feel sick | Usually feels sick |
| Cannot spread TB bacteria to others | May spread TB bacteria to others |
| Usually has a skin test or blood test result indicating TB infection | Usually has a skin test or blood test result indicating TB infection |
| Has a normal chest x-ray and a negative sputum smear | May have an abnormal chest x-ray, or positive sputum smear or culture |
| Needs treatment for latent TB infection to prevent TB disease | Needs treatment to treat TB disease |

**Table 10 Clinical symptoms in latent TB and active TB disease.**

### Extrapulmonary Tuberculosis

Extrapulmonary tuberculosis is the infection of any organ in the body other than the lungs by Mycobacterium tuberculosis is called extrapulmonary tuberculosis. It is most commonly a sequel of lung infection by the same organism. The most common sites of extrapulmonary tuberculosis are lymph nodes, pleura, abdomen, bone and joint, spinal cord and the brain and its coverings (Fig. 111).

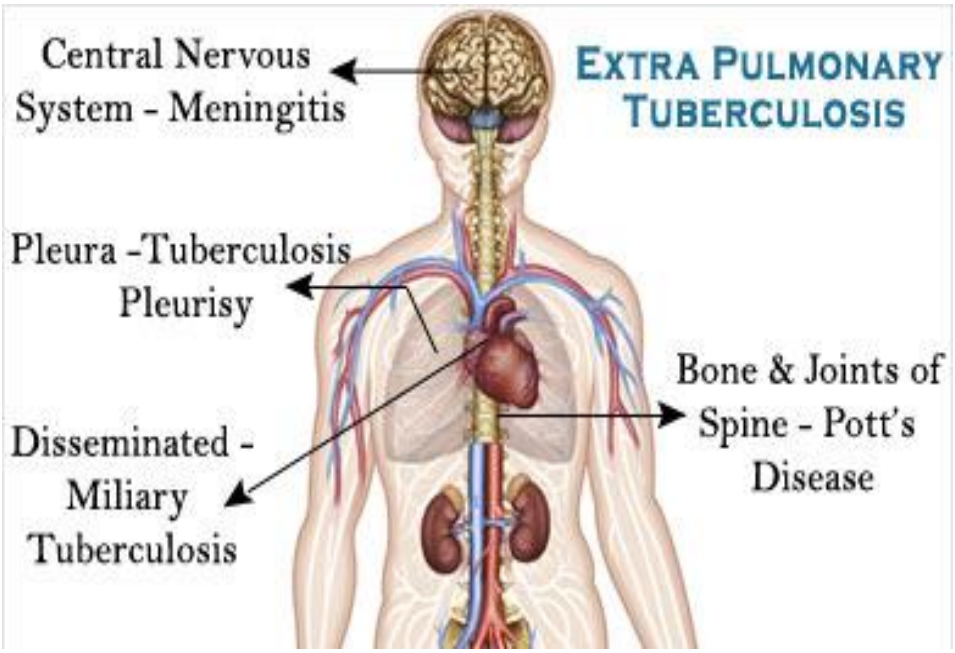



**Fig. 111 Extrapulmonary tuberculosis.**

## 11.3 MYCOBACTERIUM TUBERCULOSIS – SAMPLE COLLECTION

### Sputum

The large majority of specimens received for diagnosis are sputum samples. If good specimens are to be obtained, patients must be instructed in how to produce sputum. Specimens should be collected in a separate, ventilated room or preferably outdoors. Keeping both hands on hips, cough forcibly and collect sputum in the mouth; spit the sputum carefully into a wide-mouthed, unbreakable, leakproof container and close the lid tightly. Ideally, a sputum specimen should be 3–5ml in volume, although smaller quantities are acceptable if the quality is satisfactory.

Sputa should be transported to the laboratory as soon as possible. If a delay of a few days cannot be avoided, keep specimens cool (refrigerated but *not frozen*) Up to a week in cold conditions will not significantly affect the positivity rate of smear microscopy, however, the

additional growth of contaminants will result in an increased contamination rate on culture media.

### Laryngeal Swab

Laryngeal swabs may be useful in children and patients who cannot produce sputum or may swallow it. Collect laryngeal swabs in the early morning, before patients eat or drink anything. Use a sterile absorbent cotton swab for collection.
Transport each specimen in a container with a few drops of sterile 0.9% saline solution in order to keep the swab wet.

### Other Respiratory Specimens

Bronchial secretion (2–5 ml) and [23]BAL (20–40 ml).
Pleural effusions (20–50 ml).
Transbronchial and other biopsies taken under sterile conditions should be kept wet during transportation by adding few drops of sterile 0,9% saline to the tissue.

### Gastric Lavage

Gastric lavages often contain [24]MOTT and are therefore rarely used for adults, they are indicated for children, however, who produce almost no sputum. Make the collection early in the morning, when the patient has an empty stomach. Neutralize the specimen by adding 100 mg of sodium bicarbonate to the gastric aspirate and transport it immediately to the laboratory.

### Extrapulmonary Specimens

The laboratory may receive a variety of specimens for diagnosis of extrapulmonary TB – body fluids, tissues, urine etc. All liquid specimens should be collected in sterile glass containers without using any preservative. Specimens can be inoculated directly into liquid vials and transported to the laboratory for culture. Specimens must be transported to the laboratory immediately; they should be processed as soon as possible or kept at 2–6 ºC. The optimal volumes are at least 3 ml of cerebrospinal fluid and 5–10 ml of blood, collected in citrate blood tubes.

## 11.4 MYCOBACTERIUM TUBERCULOSIS ZIEHL-NEELSEN STAIN

*M. tuberculosis* does not retain any common bacteriological stain due to high lipid content in its wall, and thus is neither Gram-positive nor Gram-negative, hence Ziehl-Neelsen staining,

[23] BAL - bronchoalveolar lavage
[24] MOTT - mycobacteria other than tuberculosis

or acid-fast staining, is used. While *Mycobacteria* do not retain the crystal violet stain, they are classified as acid-fast Gram-positive bacteria due to their lack of an outer cell membrane.

### Ziehl-Neelsen Staining Procedure

In the 'hot' Ziehl-Neelsen technique, the phenol-carbol fuchsin stain is heated to enable the dye to penetrate the waxy mycobacterial cell wall. The stain binds to the mycolic acid in the mycobacterial cell wall. After staining, an acid decolorizing solution is applied. This removes the red dye from the background cells, tissue fibres, and any organisms in the smear except mycobacteria which retain (hold fast to) the dye and are therefore referred to as acid fast bacilli (AFB). Following decolorization, sputum smear is counterstained with malachite green (or methylene blue) which stains the background material, providing a contrast colour against which the red AFB can be seen. Among the *Mycobacterium* species, *M. tuberculosis* and *M. ulcerans* are strongly acid fast.

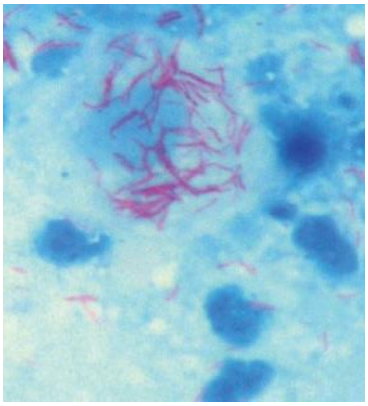


**Fig. 962 Ziehl-Neelsen stain - Acid fast bacilli (AFB).**

### Reporting of sputum smear

1. When no [25]AFB are seen after examining 300 fields, report the smear as 'No AFB seen'.
2. When very few AFB are seen i.e. when only 1 or 2 AFB are seen after examining 100 fields, request a further specimen to examine (Those AFB might have came from tap water (saprophytic mycobacteria), or it may be scratch of glass slide or by the use of same piece of blotting paper while drying.
3. When red bacilli are seen, report the smear as 'AFB positive' and give an indication of the number of bacteria present as follows:
    1. More than 10 AFB/field at least in 20 fields: report as + + +
    2. 1-10 AFB/field at least in 50 fields: report as + +
    3. 10-99 AFB/ 100 fields: report as +
    4. 1-9 AFB/100 fields: **report the exact number**

[25] AFB - acid fast bacilli

# 11.5 MYCOBACTERIUM TUBERCULOSIS - CULTIVATION

*M. tuberculosis* requires oxygen to grow. *M. tuberculosis* divides every 15-20 hours, which is extremely slow compared to other bacteria, which tend to have division times measured in minutes (*Escherichia coli* can divide roughly every 20 minutes).
*M. tuberculosis* is grown on a selective medium known as Löwenstein-Jensen medium. This method is quite slow, as this organism requires 6-8 weeks to grow, which delays reporting of results. A faster result can now be obtained using Middlebrook medium or BACTEC.

### Löwenstein-Jensen medium

The usual composition applicable to *Mycobacterium tuberculosis* is:

- Malachite green (inhibits most other bacteria)
- Glycerol (enhances the growth of *Mycobacterium tuberculosis*)
- Asparagine
- Potato starch
- Coagulated eggs
- Mineral salt solution (Potassium dihydrogen phosphate, Magnesium sulfate, Sodium citrate)
- Low levels of penicillin and nalidixic acid (to inhibit growth of gram positive and gram negative bacteria)

Löwenstein-Jensen medium doesn't contain any agar, solid consistence is attained by heat coagulation of the egg albumin.

**Fig. 973 *Mycobacterium tuberculosis* (Löwenstein-Jensen medium).**

*M. tuberculosis* on Löwenstein-Jensen medium after 6 weeks of cultivation, 37°C form typical nonpigmented, rough, dry colonies. The green color of the medium is due to the presence of malachite green which is one of the selective agents to prevent growth of most other contaminants.

# 11.6 MANTOUX TUBERCULIN SKIN TEST

The Mantoux Tuberculin Skin Test (TST) is the standard method of determining whether a person is infected with *Mycobacterium tuberculosis*. **Tuberculin** is purified protein derivative

(PPD), an extract of *Mycobacterium tuberculosis*, *M. bovis*, or *M. avium* that is used in skin testing in animals and humans to identify a tuberculosis infection. PPD is a poorly defined, complex mixture of antigens.
Tests based upon PPD are relatively unspecific since many of its proteins are found in different mycobacterial species. The tuberculin skin test is based on the fact that infection with *M. tuberculosis* bacterium produces a delayed-type hypersensitivity skin reaction. The components of the organism are contained in extracts of culture filtrates and are the core elements of the classic tuberculin PPD, that is used for skin testing for tuberculosis. Reaction in the skin to tuberculin PPD begins when T- cells, which have been sensitized by prior infection, are recruited to the skin site where they release lymphokines. These lymphokines induce induration (a hard, raised area with clearly defined margins at and around the injection site) through local vasodilation leading to fluid deposition known as edema, fibrin deposition, and recruitment of other types of inflammatory cells to the area.

### Tuberculin Skin Test - Administration

The TST is performed by injecting 0.1 ml of tuberculin- purified protein derivative (PPD) into the inner surface of the forearm. The injection should be made with a tuberculin syringe, with the needle bevel facing upward. The TST is an intradermal injection. When placed correctly, the injection should produce a pale elevation of the skin (a wheal) 6 to 10 mm in diameter.

### Tuberculin Skin Test - Reading

The skin test reaction should be read between 48 and 72 hours after administration. A patient who does not return within 72 hours will need to be rescheduled for another skin test. The reaction should be measured in millimeters of the induration (palpable, raised, hardened area or swelling, **Fig. 114**).
The reader should not measure erythema (redness). The diameter of the indurated area should be measured across the forearm (perpendicular to the long axis).

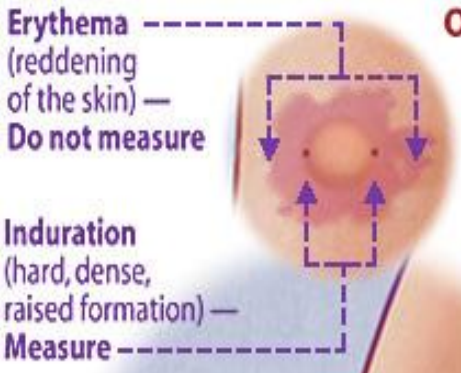
### Tuberculin Skin Test - Interpretation

Skin test interpretation depends on the measurement in millimeters (mm) of the induration and the person's risk of being infected with TB and/or progression to disease if infected. The following three cut points (**see Fig. 115**) should be used to determine whether the skin test reaction is *positive*. A measurement of 0 mm or anything below the defined cut point for each category is considered *negative*.

### Tuberculin Skin Test - False-positive Reaction

Some persons may react to the TST even though they are not infected with *M. tuberculosis*. The causes of these false-positive reactions may include, but are not limited to, the following: Infection with nontuberculosis mycobacteria, previous BCG vaccination, incorrect method of TST administration, incorrect interpretation of reaction and incorrect bottle of antigen used.

**The reaction to the Tuberculin Skin Test should be read by a trained health professional 48 to 72 hours after the injection. The reaction should be measured in millimeters.**

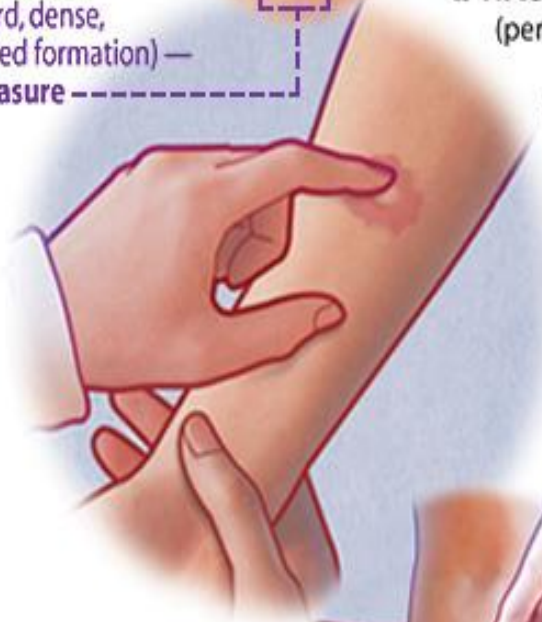



**Only the induration (palpable, raised and hardened area) should be measured for interpretation.**

**The reader should not measure erythema (redness).**

**1. WASH –** Wash hands or use hand sanitizer (per facility protocol).

**2. INSPECT SITE –** Locate the area where the skin test was administered — inspect the arm in good light and on a firm surface.

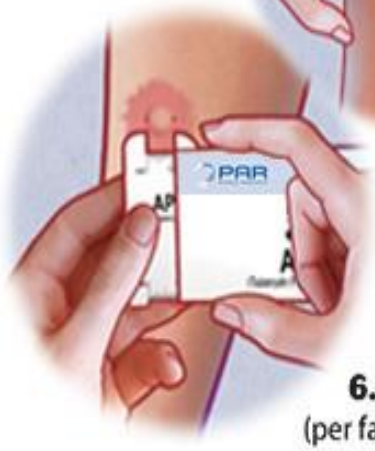


**3. FEEL INDURATION –** Lightly palpate the area with the pads of your fingertips to determine if there is an induration and to locate the margins or edges of the induration.

**4. MARK EDGES –** Measure the diameter of the indurated area across the forearm (perpendicular to the long axis) at the widest width of the induration. Using a ballpoint pen, mark lightly one edge of the induration with a fine dot and then repeat on the other edge.

**5. MEASURE –** Use a millimeter ruler or caliper. Gently lay a ruler on the skin, placing the first mark at zero (first line on the ruler).

The second mark will be the measurement reading. If the measurement falls within two divisions on the millimeter scale, record the lower mark. If there is no induration, the reading is measured as 0 millimeters.



**6. WASH AGAIN –** Wash hands or use hand sanitizer (per facility protocol).

**7. DOCUMENT –** Record the reading on the appropriate form using only millimeters. Do not simply record as "negative" or "positive."

Include the date and time the test was read, the name and signature of the person who read the skin test, and the presence or absence of adverse effects.

**Fig. 984 The reaction to Tuberculin Skin Test - results reading.**

**Interpretation of the Tuberculin Skin Test reading:**

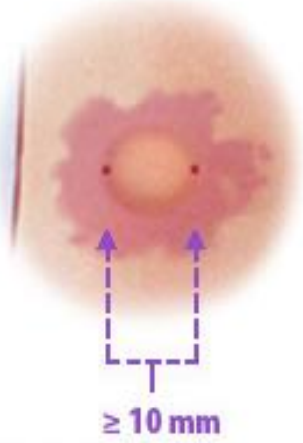
Skin test interpretation depends on two factors:

- Measurement of the induration in millimeters
- Person's risk of being infected with TB and of progression to disease if infected

**An induration of 5 or more millimeters is considered positive in:**

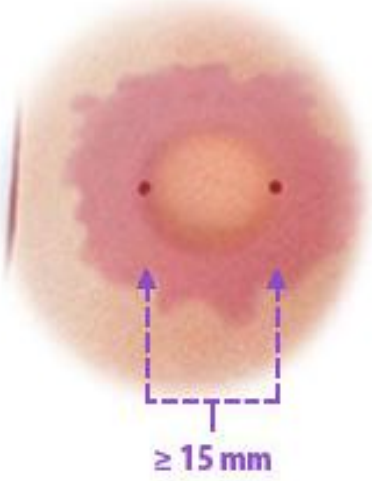

- HIV-infected persons
- Persons who have had a recent contact with another person with TB disease
- Persons with fibrotic changes on chest radiograph consistent with prior TB
- Patients with organ transplants
- Persons who are immunosuppressed for other reasons (e.g., taking the equivalent of ≥15 mg/day of prednisone for 1 month or longer.)

**An induration of 10 or more millimeters is considered positive in:**




- Recent immigrants (within the last 5 years) from high prevalence countries
- Injection drug users
- Residents and employees of high-risk congregate settings*
- Mycobacteriology laboratory personnel
- Persons with clinical conditions that place them at high risk
  Children < 4 years of age, or infants, children, and adolescents exposed to adults at high risk.

**An induration of 15 or more millimeters is considered positive in:**




- Persons with no known risk factors for TB.

**Fig. 995 Interpretation of the Tuberculin skin test results.**

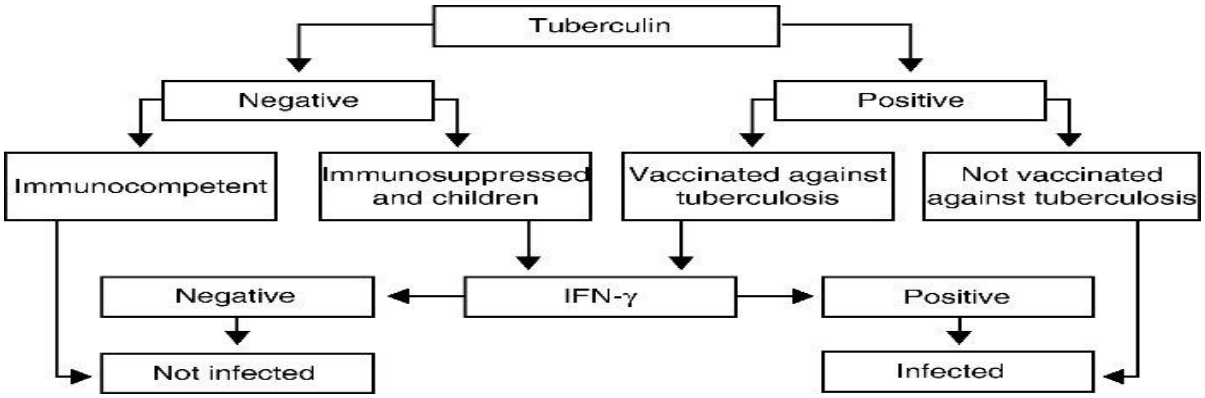



**Fig. 1006 Tuberculosis - algorythm of laboratory testing.**

## 11.7 INTERFERON-GAMMA RELEASE ASSAY (IGRA)

Interferon-Gamma Release Assays (IGRAs) are whole-blood tests that can aid in diagnosing *Mycobacterium tuberculosis* infection. They do not help differentiate latent tuberculosis infection (LTBI) from tuberculosis disease. Two IGRAs are commercially available:

- QuantiFERON®-TB Gold In-Tube test (QFT-GIT)
- T-SPOT®.*TB* test (T-Spot)

IGRAs measure a person's immune reactivity to *M. tuberculosis*. White blood cells from most persons that have been infected with *M. tuberculosis* will release interferon-gamma (IFN-γ) when mixed with antigens (substances that can produce an immune response) derived from *M. tuberculosis*. To conduct the tests, fresh blood samples are mixed with antigens and controls.

**Positive IGRA:** This means that the person has been infected with M. tuberculosis. Additional tests are needed to determine if the person has latent TB infection or TB disease. A health care worker will then provide treatment as needed.

**Negative IGRA:** This means that the person's blood did not react to the test and that latent TB infection or TB disease is not likely.

IGRAs are the preferred method of TB infection testing for the following:

- People who have received bacille Calmette–Guérin (BCG). (BCG is a vaccine for TB disease).
- People who have a difficult time returning for a second appointment to look for a reaction to the TST.

### QuantiFERON®-TB Gold In-Tube test

The QuantiFERON®-TB Gold In-Tube (QFT-G) is a blood test for use as an aid in diagnosing *Mycobacterium tuberculosis* infection (both latent tuberculosis infection and active tuberculosis disease).

The QFT-G is an indirect test for *M. tuberculosis* infection that is based on measurement of a cell-mediated immune response. A cocktail of 3 mycobacterial proteins ([26]ESAT-6, [27]CFP-10, and TB 7,7) stimulate the patient's T-cells *in vitro* to release interferon-gamma, which is then measured using [28]ELISA technology. The test detects infections produced by the *M. tuberculosis* complex (including *M. tuberculosis*, *M. bovis*, and *M. africanum* infections). BCG strains and the majority of other non-tuberculosis mycobacteria do not harbor ESAT-6, CFP-10, and TB 7,7 proteins, thus, patients either vaccinated with BCG or infected with most environmental mycobacteria should test negative. Results should always be interpreted in conjunction with other clinical and laboratory findings.

### *T-SPOT®.TB test*

The T-SPOT®.*TB* test is a unique, single-visit blood test, also known as an interferon-gamma release assay (IGRA) for TB infection. The T-SPOT.*TB* test does not cross react with the bacille Calmette-Guerin (BCG) vaccine and there is no association between T-SPOT.*TB* test results and immunocompromised status. According to the CDC Guidelines, IGRAs (e.g. the T-SPOT.*TB* test) may be used in place of a TST in most situations and are preferred for BCG vaccinated individuals. The T-SPOT.*TB* test enumerates the response of effector T-cells that have been sensitized to *Mycobacterium tuberculosis.* Interferon-gamma is captured and presented as spots from T cells sensitized to TB infection. (Fig. 117).

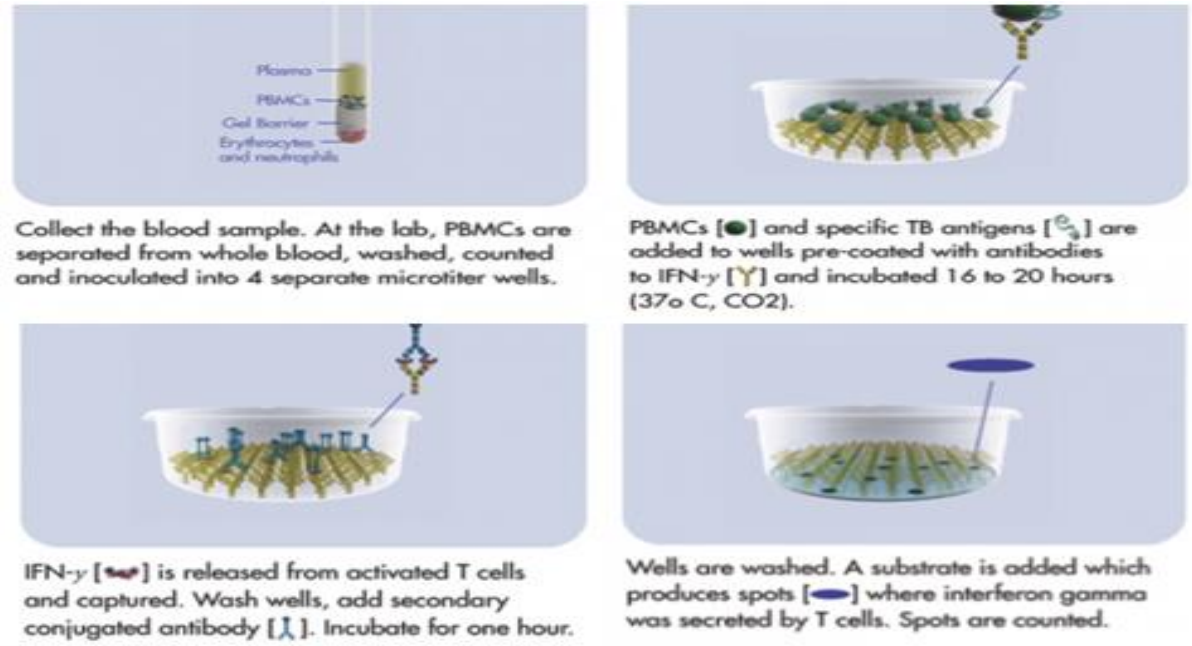



**Fig. 1017 T-SPOT®.TB test - principle.**

Results of T-SPOT®.*TB* test are interpreted by subtracting the spot count in the negative (NIL) control from the spot count in Panels A and B.

[26] ESAT-6 - early secreted antigenic target protein 6
[27] CFP-10 - culture filtrate protein-10 kDa
[28] ELISA - enzyme-linked immunosorbent assay

- Positive: > 8 spots
- Negative: < 4 spots
- Borderline: 5, 6, or 7 spots

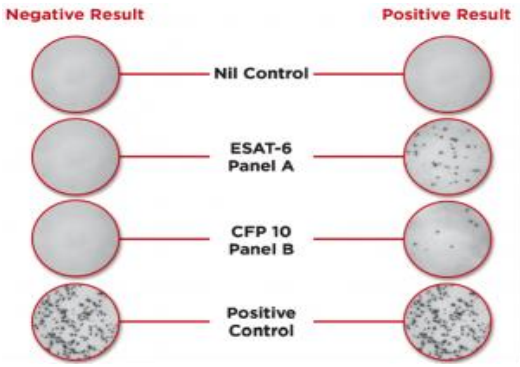



**Fig. 1028 Results of T-SPOT®.TB.**

## 11.8 PCR-BASED TB DIAGNOSTIC TEST

The new PCR-based TB diagnostic test called **Xpert MTB/RIF** is fast, sensitive, and automated. An accurate diagnosis can be obtained in less than 2 hours by adding a reagent to a sputum sample and, 15 minutes later, pipeting it into a cartridge that is inserted into the diagnostic instrument for 1–2 minutes (Fig. 119).

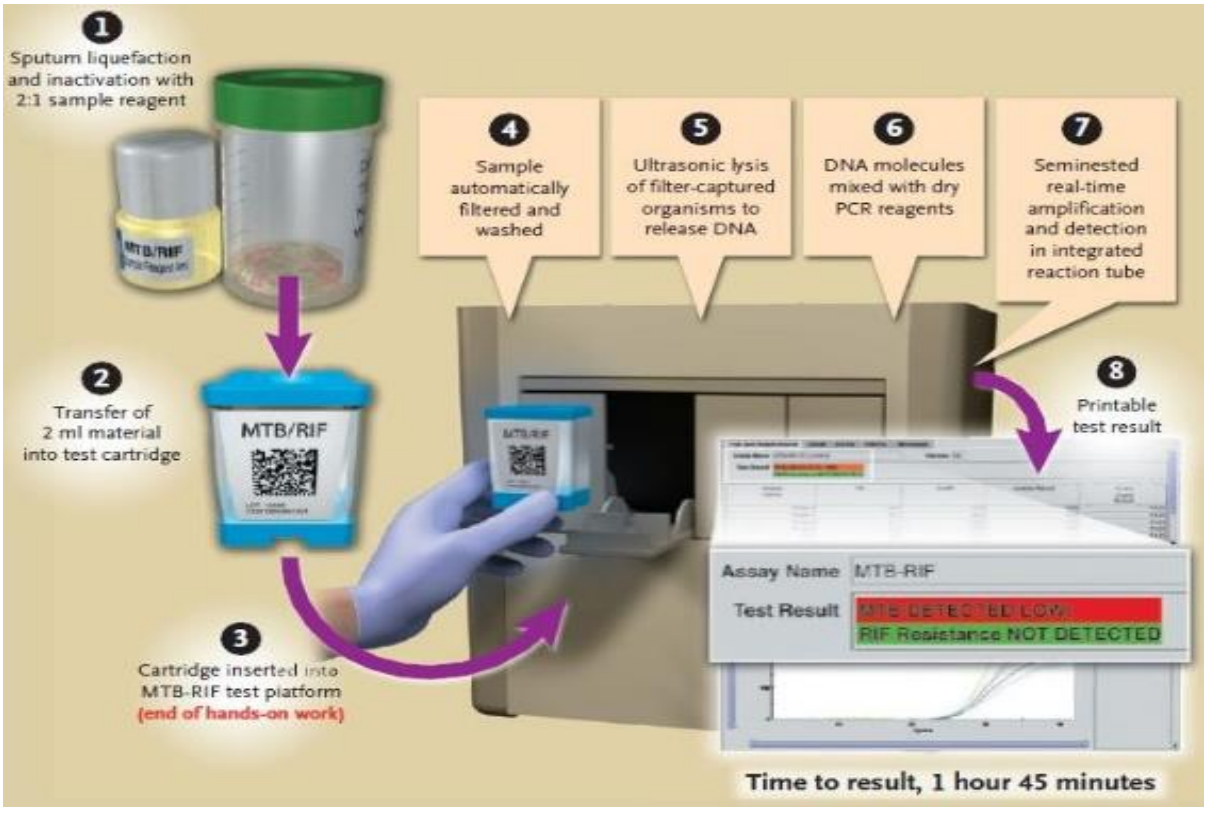



**Fig. 1039 PCR-based TB diagnostic test - Xpert MTB/RIF.**

## 11.9 BCG VACCINE

BCG is a vaccine for tuberculosis (TB) disease. BCG stands for 'Bacillus Calmette-Guérin', and is named after the two French scientists who developed the first TB vaccine – Albert Calmette and Camille Guérin. BCG vaccine for percutaneous use contains a live strain of a bacterium closely related to the one that causes TB in humans. The bacterium is an attenuated, live culture preparation of the Bacillus of Calmette and Guerin (BCG) strain of *Mycobacterium bovis*. It stimulates the immune system but does not cause disease. The vaccine is given intradermally, usually in the left upper arm. This is the recommended site, so that small scar left after vaccination can be easily found in the future as evidence of previous vaccination.

## 11.10 MYCOBACTERIUM LEPRAE - LEPROSY

Leprosy is a chronic infection caused by *Mycobacterium leprae*. Leprosy is also known as Hansen disease, named after G.A. Hansen, who is credited with the 1873 discovery of *M. leprae*. The incubation period of *M. leprae* can range between nine months and twenty years. Leprosy primarily affect superficial tissues, especially the skin and peripheral nerves. Initially, a mycobacterial infection causes a wide array of cellular immune responses. These immunologic events then elicit the second part of the disease, a peripheral neuropathy with potentially long-term consequences.

Individuals who have a vigorous cellular immune response to *M. leprae* have the tuberculoid form of the disease that usually involves the skin and peripheral nerves. This form of the disease is also referred to as paucibacillary leprosy because of the low number of bacteria in the skin lesions (< 5 skin lesions, with absence of organisms on smear). Results of skin tests with antigen from killed organisms are positive in these individuals.

Individuals with minimal cellular immune response have the lepromatous form of the disease, which is characterized by extensive skin involvement. The organism grows best at 27-30 °C, therefore, skin lesions tend to develop in the cooler areas of the body, with sparing of the groin, axilla, and scalp. This form of the disease is also referred to as multibacillary leprosy because of the large number of bacteria found in the lesions (>6 lesions, with possible visualization of bacilli on smear). Results of skin tests with antigen from killed organisms are nonreactive.

## 11.11 MYCOBACTERIUM LEPRAE ZIEHL-NEELSEN STAIN

*Mycobacterium leprae* is a strongly acid-fast rod-shaped organism with parallel sides and rounded ends. In size and shape it closely resembles the tubercle bacillus. It occurs in large numbers in the lesions of lepromatous leprosy, chiefly in masses within the lepra cells, often grouped together like bundles of cigars or arranged in a palisade. Chains are never seen.

Optical microscopy shows *M. leprae* in clumps, rounded masses, or in groups of bacilli side by side, and ranging from 1–8 μm in length and 0.2–0.5 μm in diameter. - the bacilli are densely clustered within the cytoplasmic vacuoles of foamy histiocytes. This unique structure called "globi" is demonstrated by the Ziehl-Neelsen stain (Fig. 120 ).

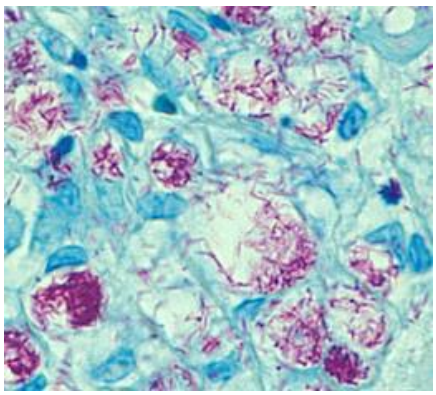


**Fig. 10420 *Mycobacterium leprae* - Ziehl-Neelsen stain.**

## 11.12 MYCOBACTERIUM LEPRAE – CULTIVATION

The organism has never been successfully grown on an artificial cell culture medium. Instead, it has been grown in mouse foot pads and more recently in nine-banded armadillos because they, like humans, are susceptible to leprosy. This can be used as a diagnostic test for the presence of bacilli in body lesions of suspected leprosy patients. The difficulty in culturing the organism appears to be because it is an obligate intracellular parasite that lacks many necessary genes for independent survival.

## 11.13 MYCOBACTERIUM LEPRAE - LEPROMIN SKIN TEST

Lepromin skin test - although not diagnostic of exposure to or infection with *M. leprae*, this test assesses a patient's ability to mount a granulomatous response against a skin injection of killed *M. leprae*. A sample of inactivated leprosy-causing bacteria is injected just under the skin, usually on the forearm, so that a small lump pushes the skin up. The lump indicates that the antigen has been injected at the correct depth. The injection site is labeled and examined 3 days, and again 28 days, later to see if there is a reaction. Patients with tuberculoid leprosy or borderline lepromatous leprosy typically have a positive response (>5 mm). Patients with lepromatous leprosy typically have no response. People who don't have leprosy will have little or no skin reaction to the antigen.

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