## Clinical microbiology

It is a branch of medical science concerned with the prevention, diagnosis and treatment of infectious diseases. In addition, this field of science studies various clinical applications of microbes for the improvement of health. There are four kinds of microorganisms that cause infectiou disease: bacteria, fungi, parasites and viruses, and one type of infectious protein called prion.

However, bacteria are now also being classified according to their immunologic and genetic characteristics.

This lecture focuses on the Gram stain, bacterial morphology, and metabolic characteristics, all of which enable the clinician to rapidly determine the organism causing infection.

### *Demonstration of an infectious agent*

A- Direct smear (GRAM STAIN)

Because bacteria are colorless and usually invisible to light microscopy, colorful stains have been developed to visualize them. The most useful is the Gram stain, which separates organisms into 2 groups: gram-positive bugs and gram-negative bugs. This stain also allows the clinician to determine whether the organism is round or rod-shaped.

For any stain you must first smear the substance to be stained (sputum, pus, etc.) onto a slide and then heat it to fix the bacteria on the slide.

There are 4 steps to the Gram stain:

1) Pour on crystal violet stain (a blue dye) and wait 60 seconds.

2) Wash off with water and flood with iodine solution. Wait 60 seconds.

3) Wash off with water and then "decolorize" with 95% alcohol.

4) Finally, counter-stain with safranin (a red dye). Wait 30 seconds and wash off with water.

When the slide is studied microscopically, cells that absorb the crystal violet and hold onto it will appear blue. These are called gram-positive

organisms. However, if the crystal violet is washed off by the alcohol, these cells will absorb the safranin and appear red. These are called gram-negative organisms.

The different stains are the result of differences in the cell walls of gram-positive and gram-negative bacteria.

## STRUCTURE OF GRAM-NEGATIVE BACTERIA.

### A- Cell Wall

1- **outer membrane** serves as the primary permeability barrier of the cell and helps to retain proteins in the periplasmic space. this membrane.

2- **Porins** are water-filled channels in the outer membrane that facilitate transport of nutrients and low molecular weight substances, including antimicrobial agents, into the cell. Bacteria vary in the number and types of porins they contain.

3- **Lipopolysaccharides** are found on the surface of the cell and are the major component of endotoxin. They contribute to the bacterium's ability to cause disease and they give gram-negative bacteria their net negative charge.

4- **Lipoproteins** attach the outer membrane to the murein layer.

5- The **peptidoglycan layer** of gram-negative bacteria is a relatively thin polymer consisting of cross-linked N-acetylmuramic acid and N-acetylglucosamine. It is often referred to as the murein layer or cell wall and is responsible for maintaining the shape of the organism. It is located within the periplasmic space.

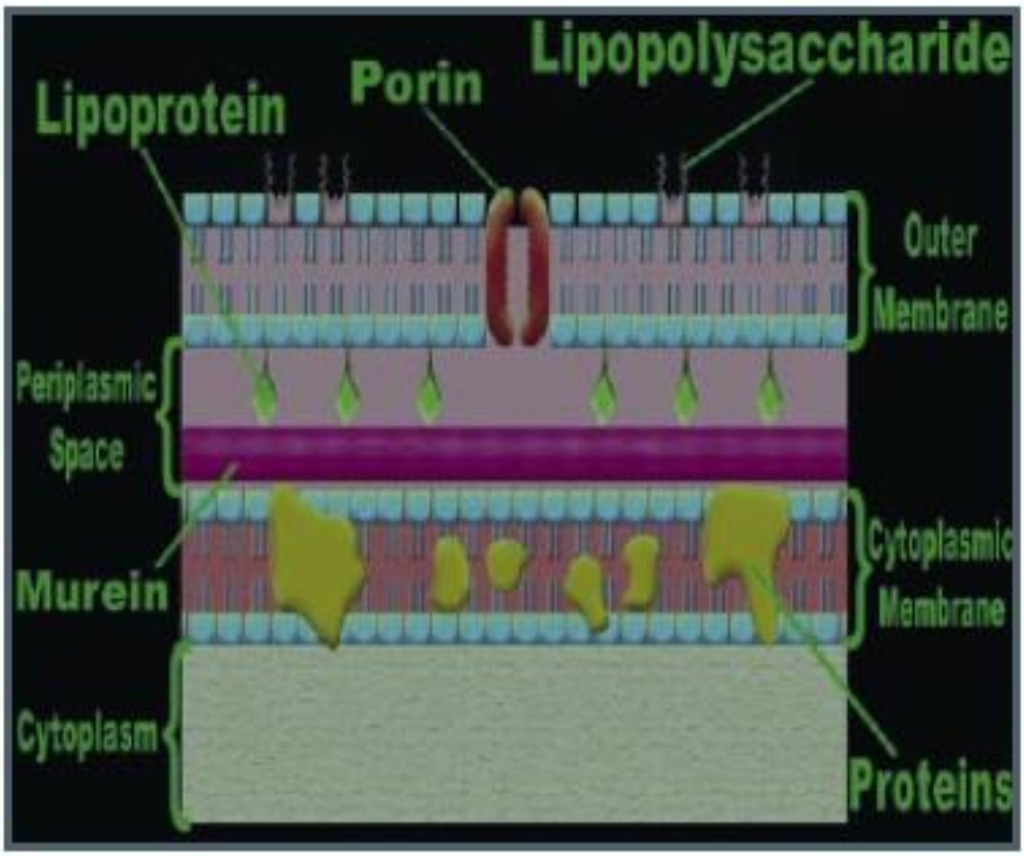
6- The **periplasmic space** lies between the outer membrane and the cytoplasmic membrane. Periplasmic proteins include binding proteins for specific substrates, hydrolytic enzymes and detoxifying enzymes.

**B- Cytoplasmic Membrane**

The cytoplasmic membrane surrounds the cytoplasm of the cell and contains proteins and phospholipids. Many of the proteins contained in the cell membrane are enzymes responsible for cellular metabolism. The cytoplasmic membrane also serves as a permeability barrier and a permeability link for substances entering the cell.

**C- Cytoplasm and Other Internal Components**

The cell cytoplasm contains the chromosome, ribosomes and other internal structures. The vast majority of bacteria have a single chromosome but a few, such as *Vibrio cholera*, have two chromosomes.




Gram Negative Bacteria

## STRUCTURE OF GRAM-POSITIVE BACTERIA

### Cell Wall

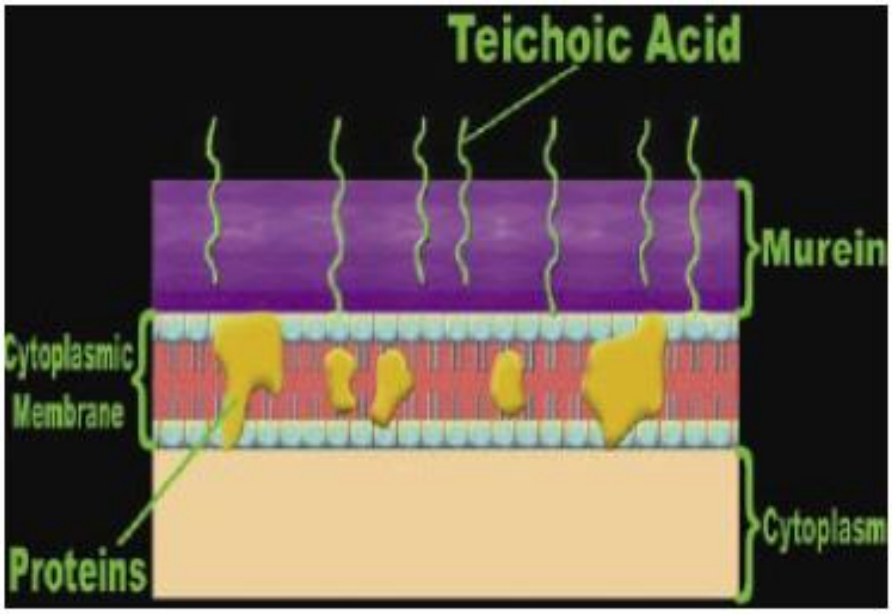
Since the gram-positive cell wall contains only two major components it is much less complicated than the gram-negative cell wall.

• **Teichoic acids** are polymers that are interwoven in the peptidoglycan layer and extend as hair-like projections beyond the surface of the gram-positive cell. They also are major surface antigens in those organisms that possess them.

• The **peptidoglycan layer,** or murein layer, of gram-positive bacteria is much thicker than that of gram-negative bacteria. It is responsible for maintaining the shape of the organism and often is referred to as the cell wall.

### The Cytoplasmic Membrane, Cytoplasm, and Other Internal Components

These structures are very similar in both gram-positive and gram-negative bacteria.




**Gram positive Bacteria**

It is present in both gram-positive and gram-negative organisms.

| Gram-Positive Cells | Gram- Negative cells |
|---|---|
| 2 Layers:<br>1. Inner cytoplasmic membrane<br>2. Outer thick peptidoglycan layer (60-100% peptidoglycan | 3 Layers:<br>1. Inner cytoplasmic membrane<br>2. Thin peptidoglycan layer (5-10% peptidoglycan)<br>3. Outer membrane with lipopolysaccharide (LPS) |
| Low lipid content | High lipid content |
| NO endotoxin (except *Listeria monocytogenes)* | Endotoxin (LPS) - lipid A |
| NO periplasmic space | Periplasmic space |
| NO porin channel | porin channel |
| Vulnerable to lysozyme and penicillin attack | Resistant to lysozyme and penicillin attack |

DIFFERENCES BETWEEN GRAM- **positive and gram negative cell**

**What does this mean clinically?**

The differences between gram-positive and gramnegative organisms result in varied interactions with the environment. The gram-positive thickly meshed peptidoglycan layer does not block diffusion of low molecular weight compounds, so substances that damage the cytoplasmic membrane (such as antibiotics, dyes, and detergents) can pass through. However, the gramnegative outer lipopolysaccharide-containing cell membrane blocks the passage of these substances to the peptidoglycan layer and sensitive inner cytoplasmic membrane. Therefore, antibiotics and chemicals that attempt to attack the peptidoglycan cell wall (such as penicillins and lysozyme) are unable to pass through.

Interestingly, the crystal violet stain used for Gram staining is a large dye complex that is trapped in the thick, cross-linked gram-positive cell wall, resulting in the gram-positive blue stain. The outer lipid-containing cell membrane of the gram-negative organisms is par-dally dissolved by alcohol, thus washing out the crystal violet and allowing the safranin counter stain to take.

BACTERIAL MORPHOLOGY

Bacteria have 4 major shapes:

1) **Cocci:** spherical.

2) **Bacilli:** rods. Short bacilli are called **coccobacilli.**

3) **Spiral forms:** comma-shaped, S-shaped, or spiral- shaped.

4) **Pleomorphic:** lacking a distinct shape (like jello).

The different shaped creatures organize together into more complex patterns, such as pairs (diplococci), clusters, strips, and single bacteria with flagella.

***B- Culture technique***

One of the most important reasons for culturing bacteria in vitro is its utility in diagnosing infectious diseases. Isolating a bacterium from sites in body normally known to be sterile is an indication of its role in the disease process. Culturing bacteria is also the initial step in studying its morphology and its identification. Bacteria have to be cultured in order to obtain antigens from developing serological assays or vaccines**.**

The specimens received in the laboratory are plated on the culture media. The appropriate culture media is selected depending upon the bacteria suspected. The following precautions need to be taken into consideration when the culture methods are processed

1- Optimal atmospheric conditions

2- Optimal temperature

3- Growth requirement of the bacteria

***A- Atmospheric conditions*:**

Colonies of bacteria are usually large enough to identify after 18–24 hours of incubation (usually at 37°C), but for some bacteria longer incubation times are required (from 2 days to several weeks). Microorganisms can be grouped on the basis of their need for oxygen to grow.

1- Facultatively anaerobic bacteria can grow in high oxygen or low oxygen content and are among the more versatile bacteria.
2- strictly anaerobic bacteria grow only in conditions where there is minimal or no oxygen present in the environment. Bacteria such as bacteroides found in the large bowel are examples of anaerobes.

3- Strict aerobes only grow in the presence of significant quantities of oxygen. *Pseudomonas aeruginosa*, an opportunistic pathogen, is an example of a strict aerobe.
4- Microaerophilic bacteria grow under conditions of reduced oxygen and sometimes also require increased levels of carbon dioxide. *Neisseria species* (*e.g.*, the cause of gonorrhea) are examples of micraerophilic bacteria

In case of Mycobacteria especially the scotochromogen the culture bottles are placed in dark or the bottles are covered with black paper and kept for incubation at 37°C.

*Temperature:*

Most of the bacteria requires a temperature of 37°C for optimal growth. This temperature is provided placing the inoculated culture plates in the incubator set at 37°C temperature.

**Growth requirement of the bacteria**

Different bacteria have different growth requirements. For eg Streptococcuspneumoniae requires factor V and factor X for its growth, which are found in chocolate agar. Thus for sample suspected of S. pneumoniae the samples are plated on chocolate agar. Similarly depending upon the growth requirements the appropriate culture media are used.

## *Classification of culture media used in Microbiology laboratory*

### A- *On the basis of consistency*

**1- Solid medium .**

solid medium contains agar at a concentration of 1.5-2.0% or some other. Solid medium is useful for **isolating bacteria** or for determining the colony characteristics of the isolate.

**2- Semisolid media .**

They are prepared with agar at concentrations of 0.5% or less. They have soft custard like consistency and are useful for the cultivation of **microaerophilic bacteria** or for **determination of bacterial motility.**

**3- Liquid (Broth) medium .**

These media contains specific amounts of nutrients but don't have trace of gelling agents such as gelatin or agar. Broth medium serves various purposes such as propagation of large number of organisms, fermentation studies, and various other tests. e.g. sugar fermentation tests**.**

### B- *Classification of culture media based on the basis of composition*

**1- Synthetic or chemically defined medium .**

A chemically defined medium is one prepared from purified ingredients and therefore whose exact composition is known.

**2- Non synthetic or chemically undefined medium**

Non-synthetic medium contains at least one component that is neither purified nor completely characterized nor even completely consistent from batch to batch. Often these are partially digested proteins from various organism sources. Nutrient broth, for example, is derived from cultures of yeasts.

C- ***Classification of Bacterial Culture Media based on the basis of purpose/ functional use/ application***

1- **General purpose media/ Basic media** .

Basal media are basically simple media that supports most non-fastidious bacteria. Peptone water, nutrient broth and nutrient agar are considered as basal medium. These media are generally used for the primary isolation of microorganisms**.**

**2- Enriched medium (Added growth factors)**

Addition of extra nutrients in the form of blood, serum, egg yolk etc, to basal medium makes them enriched media. Enriched media are used to grow nutritionally exacting (fastidious) bacteria. B**lood agar**, chocolate agar.

3- **Selective and enrichment media**.

**They** are designed to inhibit unwanted commensal or contaminating bacteria and help to recover pathogen from a mixture of bacteria. Various approaches to make a medium selective include **addition of antibiotics, dyes, chemicals, alteration of pH or a combination of these.** Example **MacConkey's Agar** used for **Enterobacteriaceae** members contains bile salt that inhibits most gram positive bacteria.

4- **Differential/ indicator medium: differential appearance:**

Certain media are designed in such a way that different bacteria can be recognized on the basis of their colony colour. Such as **Blood agar** (various kinds of hemolysis i.e. α, β and γ hemolysis).

5- **Transport media :**

Clinical specimens must be transported to the laboratory

immediately after collection to prevent overgrowth of contaminating organisms or commensals.

6- **Anaerobic media :**

Anaerobic bacteria need special media for growth because they need low oxygen content, reduced oxidation –reduction potential and extra nutrients.

7- **Assay media .**

These media are used for the assay of vitamins, amino acids and antibiotics. E.g. antibiotic assay media are used for determining antibiotic potency by the microbiological assay technique.

***Blood agar, hemolysis, and hemolytic reactions***

Blood agar is a solid growth medium that contains red blood cells. The medium is used to detect bacteria that produce enzymes to break apart the blood cells. This process is also termed hemolysis. The degree to which the blood cells are hemolyzed is used to distinguish bacteria from one another.

There are three types of hemolysis, designated alpha, beta and gamma.

1- Alpha hemolysis is a greenish discoloration that surrounds a bacterial **colony** growing on the agar. This type of hemolysis represents a partial decomposition of the hemoglobin of the red blood cells. Alpha hemolysis is characteristic of *Streptococcus pneumonia*.

2- Beta hemolysis represents a complete breakdown of the hemoglobin of the red blood cells in the vicinity of a bacterial colony. There is a clearing of the agar around a colony. Beta

hemolysis is characteristic of *Streptococcus pyogenes* and some strains of*Staphylococcus aureus*.

3- Gamma hemolysis is a lack of hemolysis in the area around a bacterial colony. A blood agar plate displaying gamma hemolysis actually appears brownish. This is a normal reaction of the blood to the growth conditions used (37° C in the presence of carbon dioxide).

**Mannitol Salt Agar (MSA):**

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

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, in turn, changes the indicator color from red to yellow.

This growth differentiates *S.aureus* from *S.epidermidis*, which forms colonies with red zones or both zones.

**MacConkey's Agar (MAC):**

MacConkey's Agar is both a selective and differential media; it is selective for Gram negative bacteria and can differentiate those bacteria that have the ability to ferment lactose.

By utilizing the available lactose in the medium, Lac+ (Lactose positive) bacteria such as *Escherichia coli, Enterobacter* and *Klebsiella* will produce acid in the medium, results in the appearance of red or pink colonies.

Non-lactose fermenting bacteria such as, *Proteus species,Salmonella*, *Pseudomonas aeruginosa* and *Shigella* cannot utilize lactose in the medium, and will use peptone instead. and leads to the formation of white or colorless colonies in the plate.

**Eosin Methylene Blue (EMB) Agar (Levine):**

Eosin methylene blue agar (EMB) is both a selective and differential medium used for the detection and isolation of Gram-negative intestinal pathogens.

Acid production from lactose fermentation causes precipitation of the dyes on the surface of the colony resulting in different colors.

- Large amounts of acid → green metallic sheen
- Small amounts of acid → pink
- No fermentation → colorless

*Enterobacter aerogenes* produces large colonies which are pink-to-buff around dark centers. *Escherichia coli* produce small, dark colonies with a green metallic sheen. *Pseudomonas, Proteus, Salmonella* and *Shigella sp* produces colorless colonies because it does not ferment lactose.

***Salmonella Shigella Agar (SS Agar).***

It is a selective and differential medium widely used in sanitary bacteriology to isolate Salmonella and Shigella from feces, urine, and fresh and canned foods. Inhibition of gram-positive microorganisms is obtained by the bile salts mixture.

1. **Lactose fermenter:** If lactose fermentation occurs, the medium will turn red due to the acidic pH. E.g. *Escherichia coli, Klebsiella pneumoniae* gives red colonies.
2. **Non-Lactose fermenter**: Salmonella, Shigella,and other non-lactose fermenters appear as transparent or translucent colorless colonies. Colonies of *Salmonella spp.* may appear with or without black centers (depending on the species isolated).

**Biochemical reactions:**

microbiology laboratories typically will identify a pathogen in a clinical sample, purify the microorganism by plating a single colony of the microorganism on a separate plate, and then perform a series of biochemical studies that will identify the bacterial species.

**Coagulase test**

used to distinguish between *Staphylococcus aureus* from coagulase negative *Staphylococcus* spp.

**Catalase test**

The catalse test is primarily used for gram positive bacteria and can for instance be utilized to distinguish *Staphylococcus* spp. and *Micrococcus* spp., which are catalase positive from *Streptococcus* spp. and *Enterococcus* spp.

**Citrate test**

used to distinguish between, among others *Citrobacter freundii* and *Escherichia coli*.

**Indole test**

Confirmation of suspected *E. coli*-strains.

**Oxidase test**

The oxidase test is used for identification of gram negative bacteria.

**Urease test**

The urease test can be used to distinguish between *E. coli*, which is urease negative, from *Proteus* spp., which are urease positive.

**Voges-Proskauer (VP) test**

*Klebsiella* spp. and *Enterobacter* spp. has the capacity to perform butanediole fermentation in contrast to *Escherichia coli*, *Salmonella* spp. and *Shigella* spp.

**Serologic systems:**

Selected antisera can be used to classify different bacterial species. This may be based on either carbohydrate or protein antigens from the bacterial cell wall or the capsular polysaccharide. **(**Group A streptococcal M proteins or O and H polysaccharide antigens of salmonella).