LAB 3:

BACTERIAL SAINTS

Stains (Dye) is any colored, organic compound, that used to stain tissues, cells, cell components, cell contents, or microorganisms. Stains are an important tool for biological researchers, particularly microbiologists. The stain may be natural or synthetic. The object stained is called the substrate. The small size and transparency of microorganisms and cells make them difficult to see even with the aid of a high-power microscope. Staining facilitates the observation of a substrate by introducing differences in optical density or in light absorption between the substrate and its surroundings or between different parts of the same substrate.

Bacterial smear:

The preparation of a smear is required for many laboratory procedures, including the staining. A smear can be prepared from a solid or broth medium.

Bacterial smear is a thin layer of bacteria placed on a slide for staining. Preparing the smear requires attention to several details that help to prevent contamination of the culture and ensure safety to the preparer.

Bacterial smear preparation:

1- Handle a clean slide from the edge, label the slid at the bottom part (use wax or diamond pencil)

2- Hold the loop as you would hold a pencil, and place it nearly vertically into the blue tip portion of a Bunsen burner flame for a few seconds or until it glows red hot. Allow the loop to cool several seconds.

3- Bacterial sample:

a- From broth culture

shake the tube well to mix the culture, remove the cap and place the tip of the tube in the flame for a few seconds, this will burn off any dust kill any airborne organisms that might happen to fall into the tube.

Dip the loop carefully into the liquid, remove the loop, re-flame the tip of the tube briefly, then replace the cap, transfer the loopful of broth to the center of the slide and spread over the target circle.

b- From solid culture

Place loopful of water on the slide then transfer inoculums to the water and homogenize the smear.

*Note: It is preferred that the microbial culture used in the staining process is a recent one, since the old one gives false results.

4-Sterile the loop.

5-Leave the smear to dry at room temperature (by air).

6-Heat fixation: After drying, Pass the slide over the flame to fix the smear (avoid over heating of the slide). This procedure will kill any bacteria that may still be alive, facilitates stain penetration, and fixes cells to the slide and prevent removal of these cells during washing between two sequent steps of staining.

Types of stains:

Stains are selected for staining based on the chemical properties of the dye and the specimen being observed, which determine how the dye will interact with the specimen. stain consisting of a colored ion (a chromophore) and a counter ion to balance the charge. Attachment of the chromophore part of the stain complex to a cellular component represents the staining reaction.

Depending upon the stain, the chromophore can be either positively charged (cationic) and have an affinity for negative ions or negatively charged (anionic) with an affinity for positive ions.

Thus, stains are divided into three groups according to the ions charge which carry the color of the stain.
These two groups are:

1-Basic (cationic) stain: these stain act well on bacteria because they have color bearing ions that are positively charged. These ions will attract to the bacterial cell wall which is negatively charged, combine with it and stain the cell. e.g. methylene blue, safranin, crystal violet, malachite green.

2-Acidic (anionic) stain: these stain that are negatively charged (have anionic chromophore) do not attract to the bacterial cell wall (which is negatively charged}, therefore they do not stain the cell (do not penetrate the bacterial cell but they make the background area around a cell opaque or dark) and this process is called "negative staining. e.g. Eosin, Nigrosine, India ink, eosin, rose Bengal (These stains show or clarify the shape and size of the cell and extracellular structure such as capsules).

3-Neutral stain:

In neutral stain, both cation and anion are colored, such that net charge is neutral. Neutral stain is a salt of acidic and basic stain. e.g. giemsa stain (combining the basic dye methylene blue and the acid dye eosin). Giemsa stain when it is combined with Wright stain form Wright-Giemsa stain. It can be used to study the adherence of pathogenic bacteria to human cells.

Classification of stains according to the purpose of use:

1-Simple stains:

Simple staining implies the use of only a single stain, which is usually enough to reveal the morphological features of most bacterial cells, including relative size, shape, and characteristic arrangements for groups of cells. e.g. crystal violet, methylene blue and carbolfuchsin.

1- Apply a couple drops from crystal violet to a fixed smear.
2- Let it for 1 min.
3- Washed off with tap water gently.
4- Let the slide to dry at room temperature.
5- Examine under $100X$ (oil immersion lens).

2-Differential stains:

Stains that react differently with different cell types are known as differential stains. In the differential stain more than one stains are involved in the staining proses.

The most important and widely used differential stain for bacteria is the Gram stain (named according to the name of its discoverer scientist "Hans Christian Gram" in 1884). Based on their reaction to the Gram stain, bacteria can be divided into two groups: Gram positive and Gram negative. The differential response to the Gram stain is based on fundamental differences in the cell wall structure and composition of cells. In a manner quite like the Gram stain, the Acid-Fast stain differentiates an important group of bacteria, the mycobacteria, based on lipid content of their cell wall.

Gram staining:

1- apply crystal violet (primary stain) to heat fix bacterial smear for 1 min.
   *Crystal violet will stain all vegetative cells with purple color.
2- Wash off gently the stain by distal water.
3- apply Gram's Iodine solution for 1 min.
   *Gram's Iodine as "mordant solution" that form an insoluble complex by binding to primary stain. The resultant crystal violet-iodine (CV-I) complex which cannot be removed easily after treating with alcohol in decolorization step form G +ve bacteria due to composition of their cell wall, in contrast this complex is washed out from G -ve bacteria. The color of all cells will appear purple-black at this point.
4- Wash off gently the stain by distal water.

5- Cover the smear with 95% alcohol by a couple drops and let it stand for 30 sec.

*Ethyl alcohol, 95%: an organic solvent serves a dual function as a lipid solvent and as a protein dehydrating agent. Its action is determined by the lipid concentration of the bacterial cell wall. it removes or decolorizes the purple color of the primary stain from G -ve cells but not from G +ve cells, this process is called "decolorization" and ethanol is called decolorizer agent. Acetone can be used as alternative decolorizer.

7- Wash off gently by distal water.

8- Cover the smear with Safranin for 30 sec.

* Safranin stain is the counter stain "secondary stain" that used to stain the cells that lost the primary stain after treating with alcohol. These cells are colored with red color.

9- Wash off gently by distal water

10- Let it dry at room temperature.

Factors affecting the efficiency of Gram staining process

1- Bacterial smear thickness: a smear with an appropriate amount must be prepared, because thick smear does not allow to determine the cells shape, arrangement and other details. Furthermore, the thick smear cannot be removed by washing. On the other hand, the thin smear may lose stains easily, therefore it may lead to false results.

2- Smear fixation: if the smear overheated during heat fixing, the cell walls will be ruptured and that may lead to fails results.

3- Concentration and freshness of reagents may affect the quality of the stain: these solutions should be fresh (recently prepared) or must be filtered before using.

4- The nature and age of the bacterial culture: Gram stain is reliable only on cells from cultures that are in the exponential phase of growth. Older cultures contain more ruptured and dead cells. So, cells from old cultures may stain Gram negative even if the bacteria are Gram positive.

5- The washing water: since the slide must be washed during subsequent steps of staining, it must be take attention to avoid excessive washing because water that left on the slide will dilute reagents, particularly Gram's iodine.

*NOTE: False results that may resulted from staining are: -
1- False positive staining where the G -ve may appear as G +ve
Because ethanol is used less than 30 sec and the (CV-I) complex has not been removed.
2- False negative staining G+ ve may appear as G-ve.
Because iodine solution may be used less than 1min and the (CV-I) complex has not been formed perfectly and not contacted to the cell wall for that the crystal violet has been easily removed by alcohol and the cells stained with safranin and appear as G- ve.

Theories explaining the Gram staining principles:
Gram positive cells take up the crystal violet, which is then fixed in the cell with the iodine mordant. This forms a crystal-violet iodine complex which remains in the cell even after decolorizing. It is thought that this happens because the cell walls of G +ve organisms include a thick layer of protein-sugar complexes called peptidoglycans. This layer makes up 60-90% of the G +ve cell wall. Decolorizing the cell causes this thick cell wall to dehydrate and shrink, which closes the pores in the cell wall and prevents the stain from exiting the cell. At the end of the gram staining procedure, gram positive cells will be stained a purplish-blue color.

Gram negative: cells also take up crystal violet, and the iodine forms a crystal violet-iodine complex in the cells as it did in the G +ve cells. However, the cell walls of G -ve organisms do not retain this complex when decolorized. Peptidoglycans are present in the cell walls of G -ve organisms, but they only comprise 10-20% of the cell wall. Gram negative cells also have an outer layer which gram

positive organisms do not have; this layer is made up of lipids, polysaccharides, and proteins. Exposing gram negative cells to the decolorizer dissolves the lipids in the cell walls, which allows the crystal violet-iodine complex to leach out of the cells. This allows the cells to subsequently be stained with safranin. At the end of the gram staining procedure, gram negative cells will be stained a reddish-pink color.

Acid Fast Stain:

The acid-fast stain is another differential stain that used to identify the acid-fast organisms such as members of the genus *Mycobacterium*.

Acid-fast organisms are characterized by wax-like, nearly impermeable cell walls; they contain mycolic acid and large amounts of fatty acids, waxes, and complex lipids. Acid-fast organisms are highly resistant to disinfectants and dry conditions.

Because the cell wall is so resistant to most compounds, acid-fast organisms require a special staining technique. The primary stain used in acid-fast staining, carbolfuchsin, is lipid-soluble and contains phenol, which helps the stain penetrate the cell wall. This is further assisted by the addition of heat. The smear is then rinsed with a very strong decolorizer, which strips the stain from all non-acid-fast cells but does not permeate the cell wall of acid-fast organisms. The decolorized non-acid-fast cells then take up the counterstain.

1- Cover the smear with carbol fuchsin and steam it over a boiling water bath for 8 minutes. Add additional stain if stain boils off.

2- Cooldown the slide then decolorizes with acid alcohol for 15 to 20 sec.

3- Stop decolorization action of acid-rinsing briefly with water.

4- Apply the counter stain (methylene blue) for 30 sec.

5- Rinse briefly with water to remove excess methylene blue.

6- Blot dry with bibulous paper. Examine under oil immersion.

3- Special stains:

special stains are used to stain specific structure of bacteria, such as spore or flagella and helping to identify the presence of capsule. The three most common special stain are negative staining for Capsule, spore staining, and flagella stain.

1- Negative stain:

like nigrosine, India ink, eosin, Rose Bengal do not penetrate the bacterial cell but they make the background area around a cell opaque or dark. So, these stains show or clarify the shape and size of the cell and extracellular structure such as: capsules.

The acidic dye nigrosine will be used to visualize the capsular or sheath that surrounds some bacteria. In general, the size and shape of microorganisms is often less distorted with indirect staining procedures, especially when sampled from a broth culture. Therefore, negative staining is useful whenever their need for morphology of individual bacteria is in question.

1- Preparing a clean, greaseless slide, put a small drop of nigrosine and mix it with a small drop from a broth culture or part of colony from solid culture.

2- Spread the drop across the slide using the edge of another slide as a spreader.

3- After air drying, the smear is observed using the high power lens 40*x*, or oil immersion if desired. The back ground should be blue - gray and the bacteria will be evident by the absence of any color.

2- Spore stain (Schaeffer-Fulton Stain):

Endospores are formed by a few genera of bacteria, such as *Bacillus* and *Clostridium*. By forming spores, bacteria can survive in hostile conditions. Spores are resistant to heat, dryness,

chemicals agent, and radiation. Bacteria can form endospores in approximately 6 to 8 hours after being exposed to adverse conditions.

Because of their tough protein coats made of keratin, spores are highly resistant to normal staining procedures. The primary stain in the endospore stain procedure is malachite green, is driven into the cells with aid of heating. Since malachite green is water-soluble and does not adhere well to the vegetative which disrupted by heat, the malachite green rinses easily from the vegetative cells, allowing them to readily take up the counterstain.

1- Primary stain: malachite green (steam over boiling water for 5 mints) heating allow malachite green to enter spore coat (like acid -fast cell walls are resistant to most staining reagents). Vegetative cells take up malachite green as well.

2- Rinse gently with water.

3- Spore coats of endospores retain stain (endospores remain green). Meanwhile water washes malachite green from vegetative cells (become clear).

5- Add counter stain (safranin) for 20 sec.

6- Rinse the slide gently with water.

7- Carefully blot the slide dry.

8- Observe the slide under the microscope, using proper microscope technique. Endospores will stain green. Vegetative cells will stain red.

3-Flagella stain (Leifson's stain):

The flagella stain allows observation of bacterial flagella under the light microscope. Bacterial flagella are normally too thin to be seen under such conditions. The flagella stains employ a mordant to coat the flagella with stain until they are thick enough to be seen. These staining techniques are typically very difficult.

1- First of all, take two hours old flagellated cell culture slant and

add two to three drops of sterile distill water in the slant with the help of sterile pipette.
*Note that the distill water is added slowly without disturbing the growth of cells.
2- After addition of distill water incubated the slant for 20 minutes.
Then take a drop of suspension from the slant and place the drop
3- on a clean slide which is kept in slanting position.
The drop should flow slowly from one end of slide to other end to avoid folding of flagella on cell.
4- Allow smear to air dry here we don't use heat fixation treatment.
5- After air drying the slide is flooded with Leifson's stain till a thin film of shinny surface appear.
6- After this give a gentle stream of water wash treatment to a slide.
7- Now treat the slide with 1% methylene blue treatment for 1 minute.
8- Give the slide water wash treatment, air dry and observe under oil immersion lens.

Mechanism:

1- First of all, in this procedure thickness of flagella is increase so it can be visible.
2- The Leifson's stain is made up of tannic acid, basic fuschin stain prepared in alcohol base. When we treat Leifson's stain with cell the tannic acid get attach to the flagella and alcohol get evaporated.
3- After evaporation of alcohol the thickness of flagella is increased due to deposition of tannic acid. Whereas Basic-fuschin stain the Flagella.
After Leifson's stain treatment cells are treated with Methylene blue stain. This Methylene blue stains the cell.