PRINCIPLES AND TYPES OF STAINING

BY

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Why we must do staining??

Micro organisms are omnipresent but their visualization in living state is extremely difficult because of

- Minute
- Transparent/colourless
- Motile in aqueous medium which makes them invisible.
- To solve this problem a variety of staining procedures are been developed, and now these are the main tools in microbiological studies.

What are stains??

- A stain is any colouring organic compound that when combined with another sub, imparts colour to that substance.
- The terms “dyes” and “stains” are often useed interchangeably by biologists, but they are not same.
- Dyes- general colouring purpose.
- Stains-used only for biological analysis.
- Most of stains used for bacterial staining are derivatives of aniline ($C_6H_5NH_2$)
- Aniline dyes {crystal violet, methylene blue,basic fuchsin, safranin,eosin}

Purpose o staining:

1.To see organism better:

Staining helps to observe org in contarst to background.

2. To differentiate one org. from another: differential staining- Grams and acid fast

3. To determine particular structures: spores, cell wall, nuclei etc

Structural components of stains:

- A Stain have 3 constituents;

Benzene - org. colourless solvent

+

Chromophore- chem.grp that imparts colour to benzene.

+

Auxochrome - chem grp that intensifies the colour.

The ability of stain to bind macromolecular cellulr components such as nucleic acids depends on electric charge found on the chromogen portion as well as cellular component to be stained.

- Chromophore: Otto N.Witt {chroma= color; phorous=to bear}
- chromophore is a grp with multiple bonds that is associated with a compoud and produces colour in that compound.
- e.g nitroso(NO),nitro(NO2),azo(N=N),p-Quinoid etc
- Nitrobenzene -(pale green);azobenzene-(orange red);p-quinones-yellow;o-quinones-orange or red.

Auxochrome {auxein=to increase, chroma=colour}

- These intensifies the colour when present in a molecule together with a chromophore.
- The most effective auxochromes are : -OH,NH2,-NHR,-NR2,Cl, ad CO2H.

Classification of Stains

1. On the basis of origin, stains can be classified as natural and synthetic.

i. **Natural stains:**

These stains are obtained directly from natural products. For example, Haematoxylin is obtained from the heartwood of a tree (Haematoxylon campechianum).

The natural stains are used mainly for histological purposes.

ii**. Synthetic stains:**

These are artificially produced mainly from coal tar products and hence popularly called coal-tar dyes.

A majority of stains used in microbiology are the synthetic type and manufactured from Aniline.

For example, Crystal violet, Safranin, Methylene blue and Acid fuschin

2. On the basis of chemical behavior, dyes are classified as acidic, basic and neutral.

- **An acidic dye** is one in which the colour bearing ion, the chromophore, is an anion.

- **A basic dye** is one in which the colour bearing ion, the chromophore, is a cation.

- **A neutral dye** is a complex salt of a dye acid with a dye base.

- Acid dyes generally combine more strongly with cytoplasmic (basic) elements of the cell, and basic dyes combine best with nucleic acid (acidic) elements of the cell. Table 3.2 shows the chemical characteristics of a stain or dye.

Classification based on staining activity:

- **Nuclear stains**: These are caidic in nature and stain the chromtin material only.e.g: carmine,haematoxylin.
- **Cytoplasmic stains**: basic and stain cytoplasm and inclusion bodies.e.g : fast green,aniline blue,erythrosine,eosin,orange-G.
- **Histological stains**: tissues e.g : Safranin

SIMPLE STAINING

- The simple stain can be used as a quick and easy way to determine cell shape, size and arrangements of bacteria. True to its name, the simple stain is a very simple staining procedure involving single solution of stain. Any basic dye such as methylene blue, safranin, or crystal violet can be used to color the bacterial cells.

- These stains will readily give up a hydroxide ion or accept a hydrogen ion, which leaves the stain positively charged. Since the surface of most bacterial cells and cytoplasm is negatively charged, these positively charged stains adhere readily to the cell surface. After staining, bacterial cell morphology (shape and arrangements) can be appreciated.

- Preparation of a smear and heat fixing

- Using a sterilized inoculating loop, transfer loopful of liquid suspension containing bacteria to a slide (clean grease free microscopic slide) or transfer an isolated colony from a culture plate to a slide with a water drop.
- Disperse the bacteria on the loop in the drop of water on the slide and spread the drop over an area the size of a dime. It should be a thin, even smear.

- Allow the smear to dry thoroughly.
- Heat-fix the smear cautiously by passing the underside of the slide through the burner flame two or three times. It fixes the cell in the slide. Do not overheat the slide as it will distort the bacterial cells.

- Staining :
- Cover the smear with methylene blue and allow the dye to remain in the smear for approximately one minute (Staining time is not critical here; somewhere between 30 seconds to 2 minutes should give you an acceptable stain, the longer you leave the dye in it, the darker will be the stain).
- Using distilled water wash bottle, gently wash off the excess methylene blue from the slide by directing a gentle stream of water over the surface of the slide.

- Wash off any stain that got on the bottom of the slide as well.
- Saturate the smear again but this time with Iodine. Iodine will set the stain
- Wash of any excess iodine with gently running tap water. Rinse thoroughly. (You may not get mention about step 4 and 5 in some text books)
- Wipe the back of the slide and blot the stained surface with bibulous paper or with a paper towel.
- Place the stained smear on the microscope stage smear side up and focus the smear using the 10X objective.

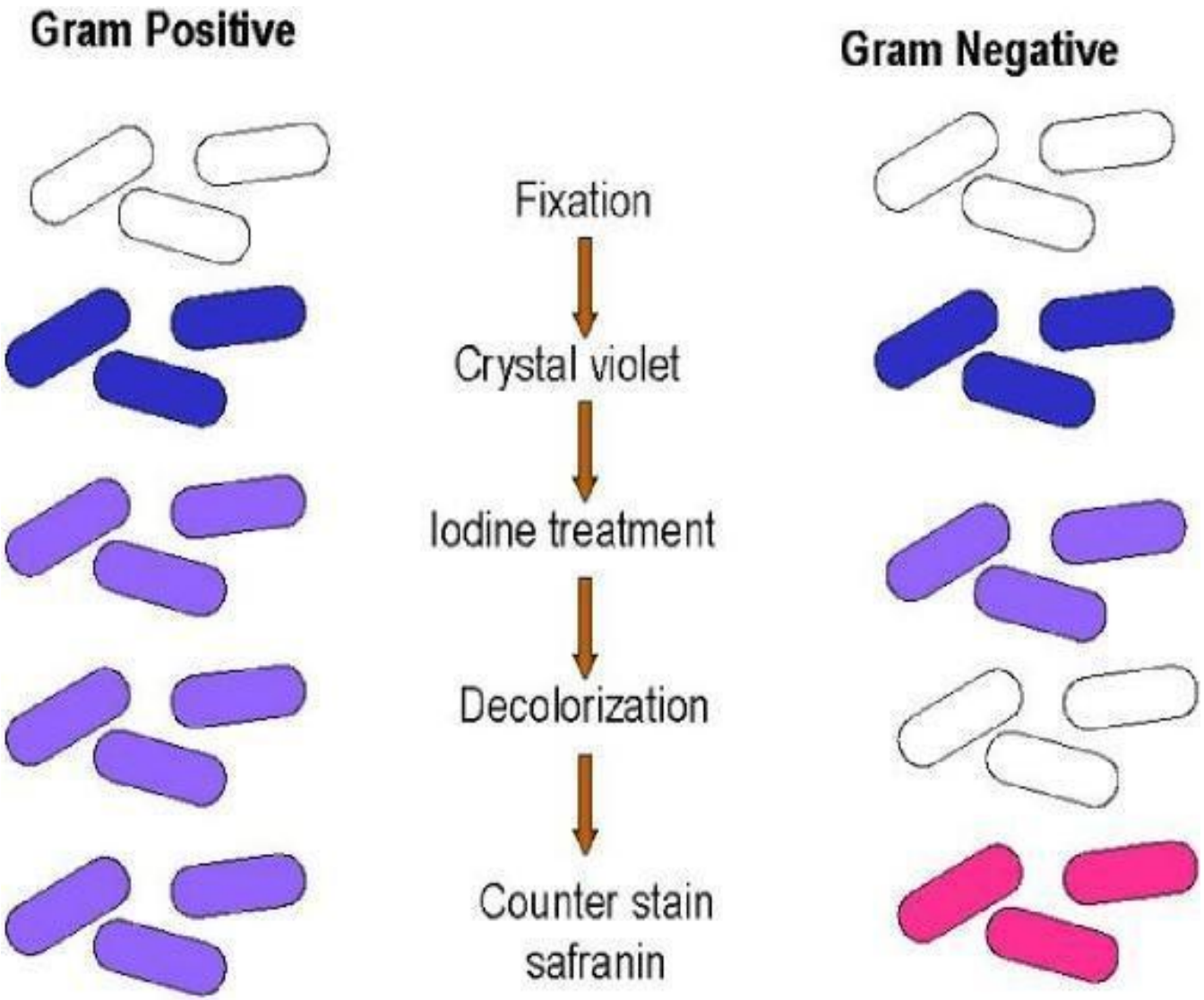
DIFFERENTIAL STAINING:

- When more than one staining reagents are used and specific objects(e.g specific m.orgs/particular structure of m.org) exhibit different staining reactions readily distinguishable, the procedure is called differential staining.
- Since the orgs not only differ from their environment but also from one another chemically, diff orgs reactdifferently to a given stain based on their chemical nature.
- This is the fundamental principle of differential staining.

- Differential staining may be defined as technique of chemically distinguishing between the different types of m.orgs.
- The most widely used differential staining in M.b are gram-staining and acid fast staining.
- The gram staining-to diiferentiate G+ and G-ve bacteria.
- The acid fast- use to identify Mycobacterium tuberculosis, causative agent for tuberculosis.

GRAM STAINING:

- Gram staining method, the most important procedure in Microbiology, was developed by Danish physician Hans Christian Gram in 1884. Gram staining is still the cornerstone of bacterial identification and taxonomic division.
- This differential staining procedure separates most bacteria into two groups on the basis of cell wall composition:
- Gram-positive bacteria (thick layer of peptidoglycan-90% of cell wall)- stains purple
- Gram-negative bacteria (thin layer of peptidoglycan-10% of cell wall and high lipid content.


Gram Positive
Gram Negative
Fixation
Crystal violet
Iodine treatment
Decolorization
Counter stain
safranin

Mechanism:

- The differences in staining responses to the gram stain can be related to chemical and physical diff in their cell walls.
- The -ve bacterial cell wall is thin,complex,multilayered structure and contains relatively a high lipi contents in addition to protein and mucopeptides.
- The higher amount of lipid is readily dissolved by alcohol resulting in the formation of large pores in the cell wall which doesnt close on dehydration of cell wall proteins,thus facilitating the leakage of crystal violet-iodine comple(CV-I) complex and resulting in the discolourizingof bacterium which later takes the counter stain and appears red.
- In contrast, the g+ve cell walls are thick and chemically simple, composed mainly of protein and cross-linked mucopeptides.
- When treated with the alcohol, it causes dehydration and closure of cell wall pores, there by not allowing to loss of CV-I complex and cells remain purple.

STRUCTURAL STAINING

- SPORE STAINING:

1. Bacteria in the genera Bacillus and Clostridium form an exceptionally resistant structure capable of surviving for longer periods in an unfavourable environment.
2. This dormant structure is called endospore, since it developes inside the cell.
3. Endospore are not easily stained by most of the dyes. Considerable amount of heating is required in order to make the stain penetrate the spore coat, a thick wall primarily responsible for endospore resistance.
4. But once stained, they strongly resist the decolourization. This property is the basis of enospore staining techq.
5. Two methods in spore staining- Schaeffer fulton and Dorner method

Procedure

penetrate the spore-coat of an endospore.

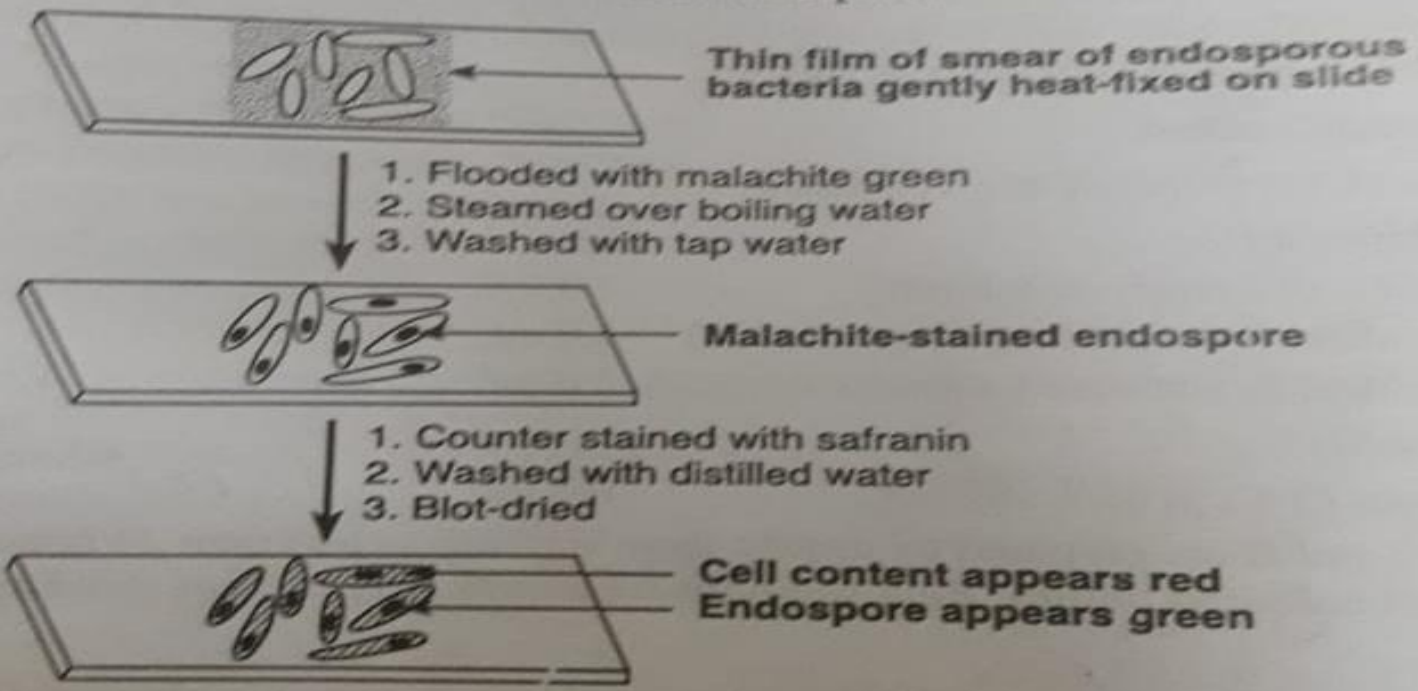


Fig. 5.9. The endospore staining procedure.

The slide is kept on a suitable stand and heated with steam from below for 5 mi
If the stain dries up during heating, more stain is added to the smear from ti
time as per requirement.

Mechanism:

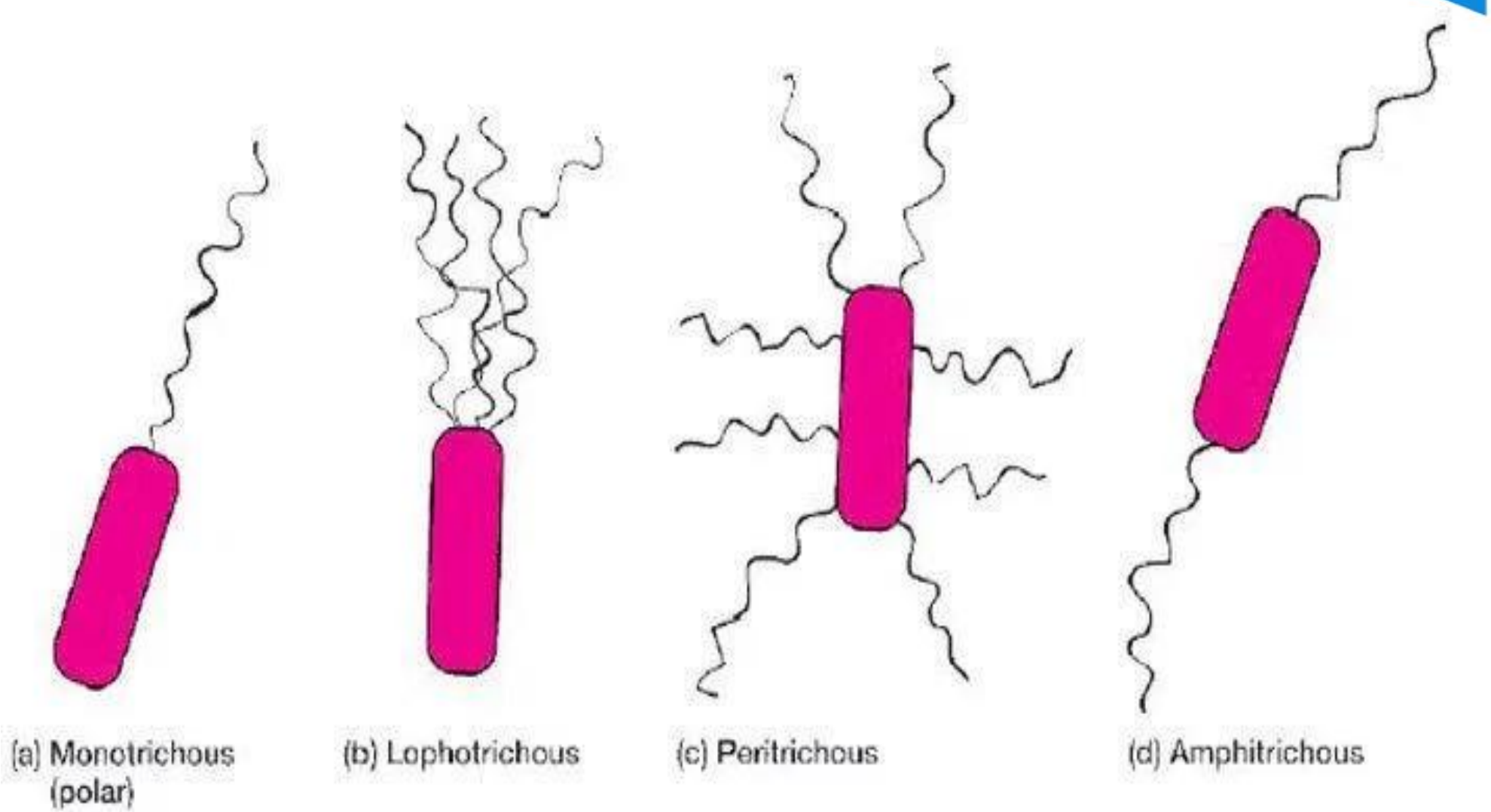
- Endospores are extremely resistant due to their wal and sporecoat.The spore coat doesnt take stain eaisly.
- Malachite green however penetrates spore coat of endospore after considerable heating.
- Once stained, the endospore doesnt decolourize easily hence appears green even after washing.
- In contrast, the counter stain fails entering the endospores but stains rst of the cell cpntent that appears red.

Flagellar staining:

- Most motile bacteria possess flagella, the shape, number and position of which are important characteristics in the differentiation of genera and species identification
- Staining bacterial flagella differs from staining other bacterial structures because it usually requires extraordinary care for the slides, stain, and cells.
- Flagellar stains are painstakingly prepared to coat the surface of the flagella with dye or a metal such as silver.

- The number and arrangements of flagella are critical in identifying species of motile bacteria.
- Two techniques for staining flagella are in use:
- A wet-mount procedure (Ryu method)
- Dried-smear preparation (Leifson staining technique)
- A wet-mount technique for staining bacterial flagella is highly successful when a stable stain and regular slides and cover slips are used. This technique is simple for routine use when the number and arrangement of flagella are critical in identifying species of motile bacteria.
- The preparations are not permanent because the stain precipitates as the wet mount dries.

- **Observe** the slide and note the following:
- Presence or absence of flagella
- Number of flagella per cell
- Location of flagella per cellflagellar arrangement of bacteria
- **Quality contro**l:
- Peritrichous: Escherichia coli
- Polar: Pseudomonas aeruginosa
- Negative: Klebsiella pneumonia


(a) Monotrichous (polar)
(b) Lophotrichous
(c) Peritrichous
(d) Amphitrichous

Capsule staining:

- The main purpose of capsule stain is to distinguish capsular material from the bacterial cell.
- A capsule is a gelatinous outer layer secreted by bacterial cell and that surrounds and adheres to the cell wall.
- Most capsules are composed of polysaccharides, but some are composed of polypeptides.
- The capsule differs from the slime layer that most bacterial cells produce in that it is a thick, detectable, discrete layer outside the cell wall.
- The capsule stain employs an acidic stain and a basic stain to detect capsule production.

Principle of Capsule Staining:

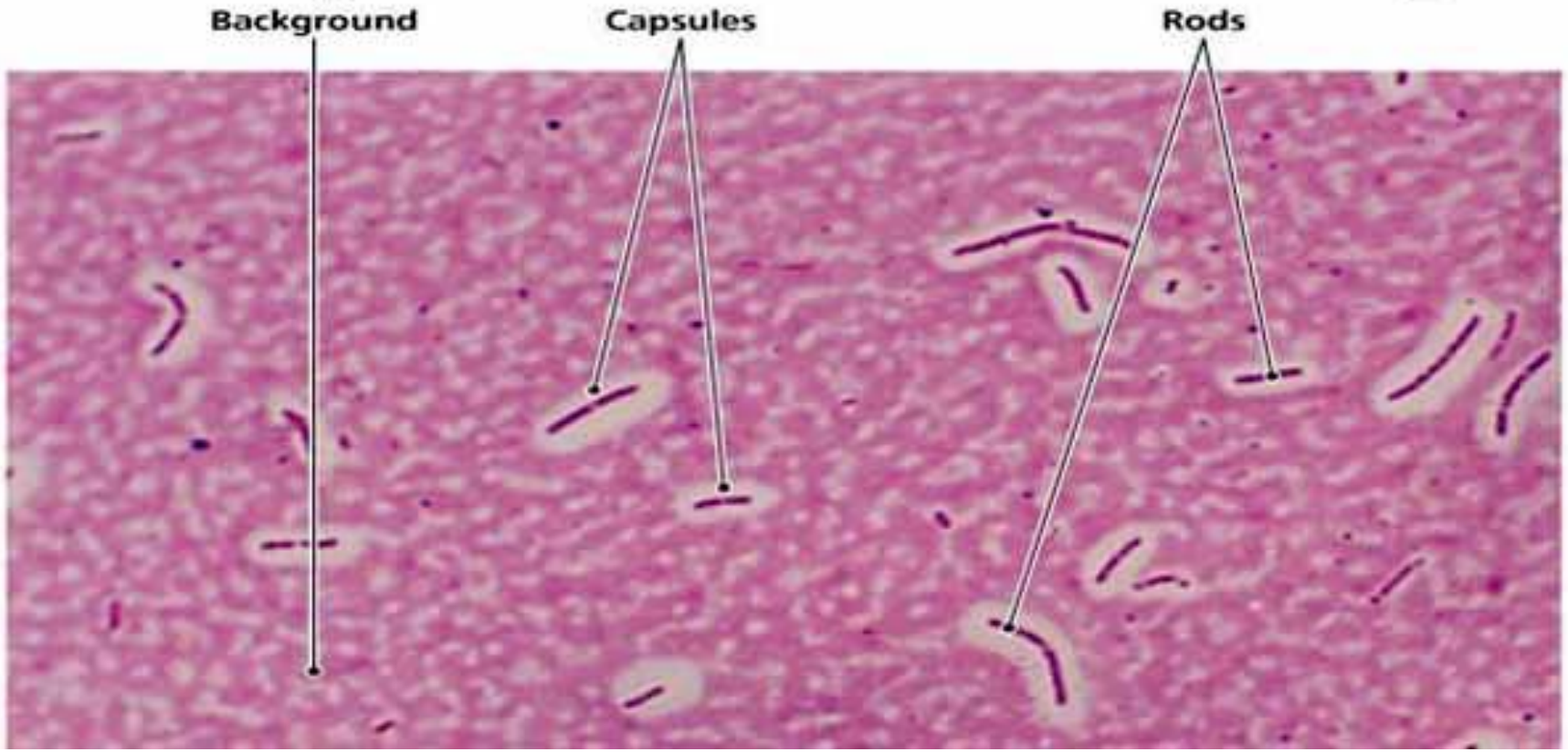
- Capsules stain very poorly with reagents used in simple staining and a capsule stain can be, depending on the method, a misnomer because the capsule may or may not be stained.
- Negative staining methods contrast a translucent, darker colored, background with stained cells but an unstained capsule.
- The background is formed with india ink or nigrosin or congo red. India ink is difficult to obtain nowadays; however, nigrosin is easily acquired.
- A positive capsule stain requires a mordant that precipitates the capsule. By counterstaining with dyes like crystal violet or methylene blue, bacterial cell wall takes up the dye. Capsules appear colourless with stained cells against dark background.

- Capsules are fragile and can be diminished, desiccated, distorted, or destroyed by heating. A drop of serum can be used during smearing to enhance the size of the capsule and make it more easily observed with a typical compound light microscope.

Reagents used for Capsule Staining:

- Crystal Violet (1%)
- Crystal Violet (85% dye content) = 1 gm
- Distilled Water = 100 ml
- Nigrosin
- Nigrosine, water soluble = 10 gm
- Distilled Water = 100 ml

Capsule Staining



- Capsule: Clear halos zone against dark background
- No Capsule: No Clear halos zone

Positive:

- Bacillus anthracis, Klebsiella pneumoniae, Streptococcus pneumonia Neisseria meningitidis Clostridium spp, Rhizaobium spp, etc.

Bacterial mobility- hanging drop method:

- **Purpose:**
- Motility means ability of movement by own power. Based on motility, bacteria can be divided into two groups as follows.
- 1) Motile Bacteria:
- A bacteria, which has the intrinsic ability of movement in the surrounding medium, in which it remains suspended, is a motile bacteria.

(2) Non-motile Bacteria

- A bacteria, which does not have the intrinsic ability of movement in the surrounding medium, in which it remains suspended, is a non-motile bacteria.
- Non-motile bacteria may show apparent motility, resulting from their brownian movement caused by the bombardment of the water molecules in the surrounding medium, on the bacteria cells.
- In wet mount, though the shape and size of bacteria can be observed, motility may be hampered, as the suspension is pressed between the slide and the cover slip.
- That is why; hanging drop preparation or motility test is performed for clear observation of the motility of bacteria, besides their shape and size. It is useful in the identification of bacteria.

- **Principle:**
- A very small drop of bacteria suspension is hung from the center of a cover slip into the cavity of a cavity slide.
- The hanging drop is observed under a microscope using oil-immersion objective.
- If the bacteria are motile, its cells can be seen to have erratic movement in the surrounding medium.
- In contrast, if it is non-motile, its cells remain static in the medium without any movement or may show brownian movement resulting from the bombardment by the water molecules in the medium, on the bacteria cells.

Materials Required:

- Cavity slide, cover slip, petroleum jelly or Vaseline, immersion oil, 24-hour old broth culture of bacteria, loop and microscope (compound, dark-field or phase-contrast).

Procedure:

- 1. A cavity slide is cleaned properly under tap water, such that water does not remain as drops on its surface.
- A cavity slide is a glass slide with a small round depression at the center, into which a small drop of bacteria suspension can hang

- 2. The slide is dried by wiping with bibulous paper and subsequently, moving it over flame or keeping it in the sun
- .3. A ring of petroleum jelly (or vaseline) is applied around the cavity.
- 4. A loop is sterilised over flame and cooled. A lapful of bacteria suspension is taken from the 24-hour old broth culture aseptically. A small drop of the suspension is placed at the center of a cover slip. The broth culture should not be more than 24 hours old, because bacteria may lose their motility, as they grow older.

- 5. The cavity slide is inverted and placed on the cover slip, in such a way that, the cavity covers the drop.
- 6. The slide and cover slip are pressed together gently, so that the cavity is sealed. Care should be taken to see that no part of the cavity touches the drop.
- 7. The slide is inverted quickly, such that the drop hangs into the cavity without touching it.
- 8. The slide is clipped to stage of the microscope.
- 9. The edge of the drop is focused under low power objective.

1. **Motility:**

Motile or non-motile

2. **Shape of bacteria**:

- Spherical (coccus)
- Rod-shaped (bacilli)
- Comma-like (vibrio)
- Spiral (spirochetes)

3. Arrangement of bacteria:

- Pairs (diplobacillus/diplococcus)
- In fours (tetrads)
- In chains (streptococcus/streptobacillus)
- Grape-like clusters (staphylococcus)