- **Dyes, Stains**
- **Classification**
- **Staining methods**

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Definition:-

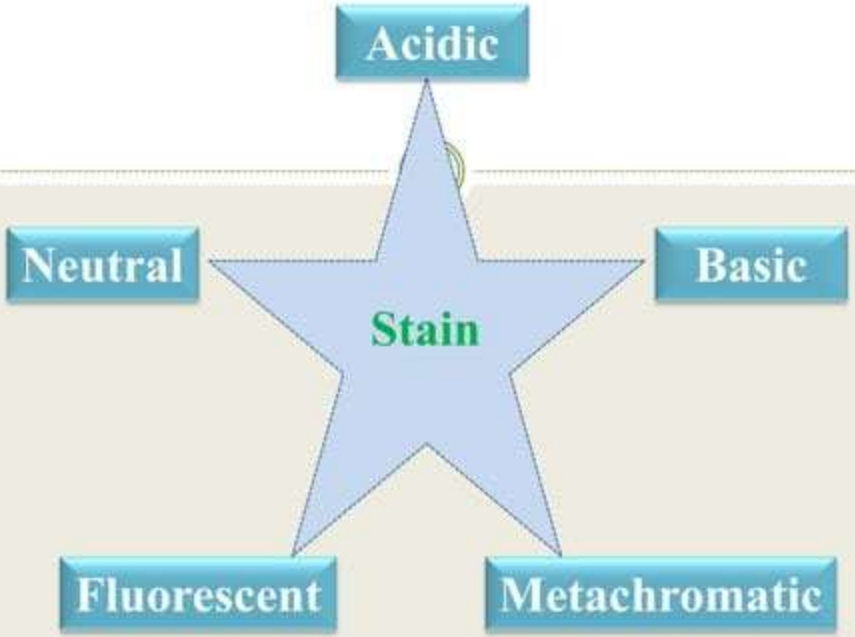
Stain - Artificial coloration of a substance to facilitate examination of tissues, microorganisms, or other cells under the microscope.

Dye - A stain or colouring matter; a compound consisting of chromophore and auxochrome groups attached to one or more benzene rings, its colour being due to the chromophore and its dyeing affinities to the auxochrome.

Chromophore- It is the part of a molecule responsible for its color. The color that is seen by our eyes is the one not absorbed within a certain wavelength spectrum of visible light.

Auxochrome - It is a functional group of atoms attached to the **chromophore** which modifies the ability of the **chromophore** to absorb light, altering the wavelength or intensity of the absorption.

Classification of Biological Stains



Acidic Stain- Those stain in which the charge present on chromophore group is negative, after its electrolytic dissociation or ionization.

e.g. Sodium + eosinate (Eosin stain), Picric acid.

Basic Stain - Those stain in which the charge present on chromophore group is positive, after its electrolytic dissociation or ionization.

e.g. Methylene blue+ chloride- (Methylene blue stain)

Neutral Stain- Those stain in which the charge present on chromophore group are both positively charged and negatively charged chromophore group.

e.g. Giemsa's stain (eosinate of methylene blue)

Metachromatic stain - In this stain multiple colors with one **dye**.

e.g. Toluidine blue

Fluorescent stain - The stain can fluoresce when exposed to ultraviolet light.

e.g. Fluorescein

Staining Methods

- Simple Staining
- Negative Staining
- Gram's Staining
- Acid-fast Staining
- Cell Wall Staining
- Capsule Staining

Simple Staining

Objective

To perform a simple staining procedure.

To compare the morphological shapes and arrangements of bacterial cells.

Principle

The bacterial smear is stained with a single reagent, which produces a distinctive contrast between the organism and its background. Basic stains with a positively charged chromogen are preferred because bacterial nucleic acids and certain cell wall components carry a negative charge that strongly attracts and binds to the cationic chromogen. The purpose of simple staining is to elucidate the morphology and arrangement of bacterial cells. The most commonly used basic stains are methylene blue, crystal violet, and carbol fuchsin.

Requirements

Methylene blue, crystal violet, and carbol fuchsin, Microincinerator or Bunsen burner, inoculating loop, staining tray, microscope, lens paper, bibulous (highly absorbent) paper, and glass slides

Simple Staining

Procedure

1. Take a clean grease free slide.
2. Prepare a smear on the slide by removing a loopfull suspension with a sterile wireloop. Allow it to dry.
3. Heat fix the smear by passing the slide rapidly through flame for three to four times.
4. Place the slide on staining rack and flood the smear with solution (e.g. methylene blue). Then allow it to react for 2-3 minutes.
5. Wash the slide with running water. Air dry or blot dry the slide.
6. Observe the slide under oil immersion lens

Mechanism

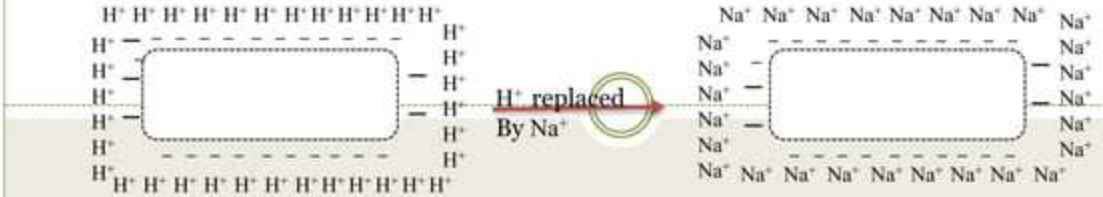
The bacterial cell surface has overall negative charges. It is due to presence of large number of carboxylic groups (-COOH group) of acidic amino acids, located at the cell surface.

When ionization of carboxylic group occurs, it give negative charges to cell surface as per following equation

$$COOH \xrightarrow{\text{ionization}} COO^{-} + H^{+}$$

Simple Staining

In the nature H^+ ion is replaced by another positively charged Na^+ , K^+. The unstained cell surface will appears as shown below-



While for simple staining basic dyes are used, e.g. Methylene blue .

Methylene blue stain is present as its salt methylene blue chloride. When MBCl undergoes ionization, it results in formation of positively charged chromophore group and negatively charged chloride ions

$$MBCl \xrightarrow{\text{Ionization}} MB^+ + Cl^-$$

Methylene blue chloride — + ve MB — - ve Chloride

Simple Staining

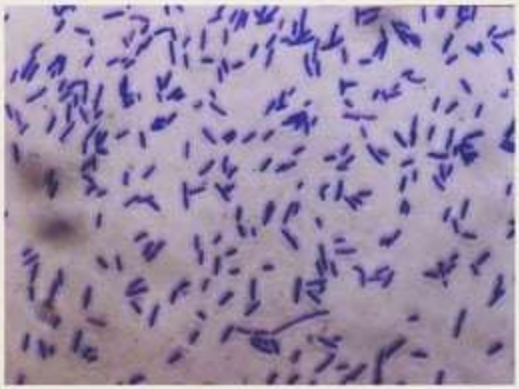
When methylene blue stain is applied on the cell surfaces, the + vely charged methylene blue ions replace the Na^+ ions present at the cell surface. Thus ionic bond formation takes place between –vely charged cell surface and +vely charged MB^+ chromophore groups. It results in staining of cell as

Result Interpretation of Simple Staining

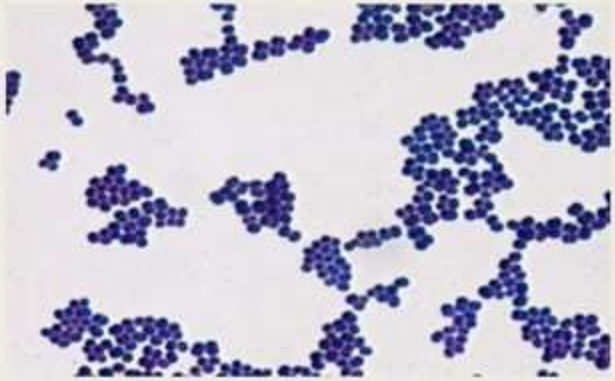
Bacilli and diplobacilli: Rod-shaped bacteria, purple

Spirilla: spiral-shaped bacteria, purple

Cocci: spherical-shaped, bacteria, purple



Bacilli sp.



Staphylococcus sp.

Negative Staining

Objective

To perform a negative staining procedure.

To understand the benefit obtained from visualizing unstained microorganisms.

Principle

Some microorganisms having the outer layer is slimy or non- ionic, therefore stain can not penetrate the cell easily, at that time they made visible by negative staining. Actually organisms are not stained therefore this is called negative staining. It also known as **Relief staining**.

Acidic stain like 5% or 10% Nigrosin, 2% Congo red, Eosin, India ink can be used.

Burri's India ink method or Fleming's Nigrosin method are used in negative staining.

Requirements

Microscopic glass slide, Inoculating loop, Staining Rack, Wash bottle India Ink / Nigrosine, Microscope (with 100X objective lens) etc.

Negative Staining

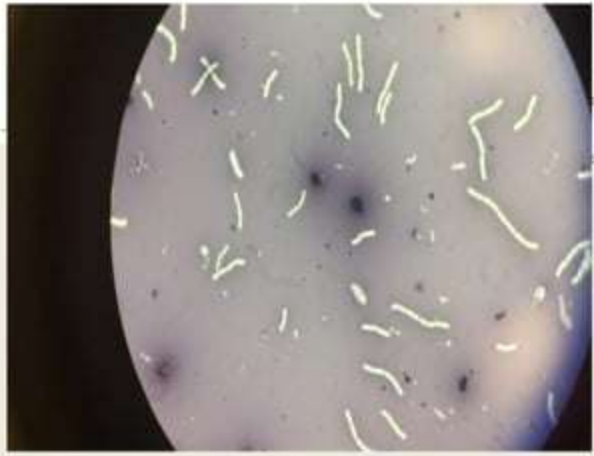
Procedure

1. Prepare a smear on a clean grease free slide with sterile nichrome wireloop.
2. Allow it to air dry and do not heat fix it.
3. Take small drop of 10% Nigrosin or India ink at on end of the smear.
4. Prepare a thin film of a stain over a dried smear using the edge of another slide.
5. Allow the film to air dry and then observe under oil immersion lens.

Mechanism

In a simple staining the bacterial cell surface has negative charges. The stain used for this method is an acidic stain with negative charge on its chromophore group. Thus negatively charged cell surface will repel acidic stain, therefor can not combine or bind with negatively charged cell surface. Instead of it, it will get deposited around the cell, making the coloured background and bacteria remains colourless.

Negative Staining



Gram's Staining

Objective

To differentiate between the two major categories of bacteria: **Gram** positive and **Gram** negative.

To understand how the **Gram stain** reaction affects **Gram** positive and **Gram** negative bacteria based on the biochemical and structural differences of their cell walls.

Principle

This test differentiates the bacteria into Gram-Positive and Gram-Negative Bacteria, depending upon the property whether they retain or loose the primary stain, when subjected to the treatment of decolorizing agent. In the original Gram staining , the smear was stained with aniline gentian violet, treated with Lugol's iodine, decolorize with absolute alcohol counter stain with Bismarck brown.

Original method was modified by several workers. One of the several modifications is Huckers and Conn method . It gives better results.

Gram's Staining

Requirements

Microscopic glass slide, Inoculating loop, Staining Rack, Crystal violet, Gram's iodine, ethyl alcohol 70% to 95%, Safranin, Microscope (with 100X objective lens) etc.

Procedure

1. Prepare a smear on a clean grease free slide. Allow to air dry and heat fix.
2. Flood the smear with Hucker's crystal violet and allow to react for 1 minute. Then wash the slide with water.
3. Apply Gram's iodine and allow to react for 1 minute. Then wash the slide with water.
4. Apply 70% to 95% alcohol on the smear until no more color appear to flow from the preparation. Then wash the slide with water.
5. Finally, apply Safranin for 1 minute and then wash it with water.
6. Air dry or blot dry with paper.
7. Observe the slide under oil immersion oil.

Gram's Staining

Observation

Gram positive bacteria are stained violet in colour (Bacillus, Clostridium, Staphylococcus, Streptococcus, etc.)

Gram negative bacteria are stained pink in colour (Pseudomonas, Xanthomonas, Azotobacter, Salmonella, Shigella, Neisseria etc.)

Mechanism

Mechanism of Gram's staining having four different theories.

Peptidoglycan theory---

In the cell wall of Gram negative bacteria, very small amount of peptidoglycan is present. It is approximately 5 to 10% of dry weight of cell. While in case of Gram positive bacteria it is about 40 to 90% of dry weight of the cell.

In case of Gram negative bacteria only one sheet of peptidoglycan is present, while in Gram positive bacteria about 40 sheets of peptidoglycan is present.

Gram's Staining

Peptidoglycan theory---

The peptidoglycan of cell wall of Gram positive bacteria is extensively cross-linked, therefore the CV-I complex is trapped in such a tough network.

Thus they can not be decolorized by alcohol treatment and remain violet in color. But in case of Gram negative bacteria percentage of cross-linkages in the peptidoglycan of cell wall is very less, therefore network or meshwork is loose and pores are large enough through which CV-I complex can be extracted easily during alcohol treatment. These cells subsequently take colour of counter stain and appear pink in color.

Lipid content theory---

Magnesium ribonucleate theory---

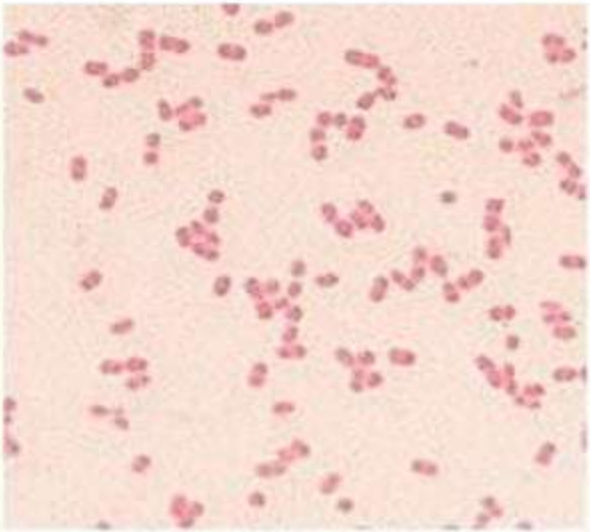
Stearn and Stearn theory---

Gram's Staining

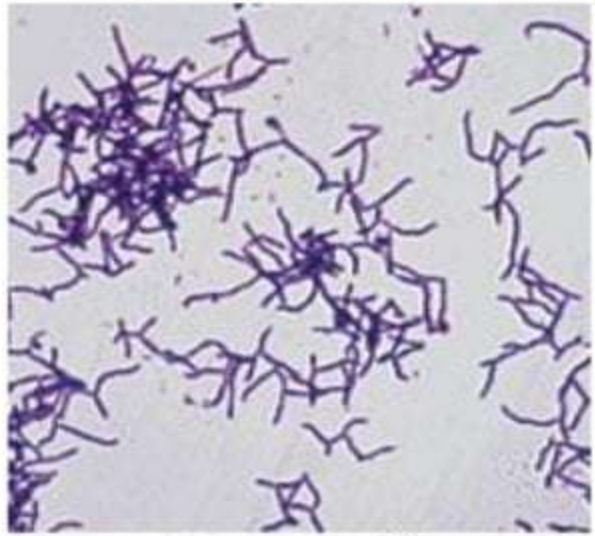
Result and interpretation

Gram-positive: Blue/Purple Color

Gram-Negative: Red/Pink Color



Gram Negative



Gram Positive

Acid Fast Staining

Objective

To differentiate between acid-fast bacilli and non-acid-fast bacilli.

To stain *Mycobacterium* species.

Principle

This test widely used a differential staining technique in bacteriology, developed by Poul Ehrlich in 1883 during his work on tuberculosis. By this method, organism are grouped into two groups as acid-fast organism and non-acid fast organisms.

Some bacterias which can not be stained by usual staining procedures and if any how they are stained, can't be decolorized even by strong decolourizing agent like acid. Therefore such organism are called as acid-fast organisms. While all other micro-organism are easily decolourize by acid. Therefore they are called as non-acid fast organisms. The acid fastness property is observed in *Mycobacterium tuberculosis, Mycobacterium leprae* and also in Nocordia species . The acid fastness is due to presence of covering made up of waxy substances.

Acid Fast Staining

Reagents and requirements

- ✓ Carbol- Fuschin (Primary dye)
- ✓ 20% Sulphuric acid or acid-alcohol (Decolorizer)
- ✓ Methylene Blue dye (counterstain) or malachite green
- ✓ Microscopic slide, Inoculating loop, Staining Rack, etc.

Preparation of reagents

Carbol fuschin

Distilled water- 100ml, Basic fuschin - 1g ,

Ethyl alcohol (100% ethanol)- 10ml, Phenol crystals- 5ml

Acid alcohol (3% hydrochloric acid in 95% ethyl alcohol)

Ethyl alcohol- 95 ml, Distilled water- 2 ml, Concentrated HCl - 3 ml

0.3% methylene blue in 1% acetic acid or 1% Malachite green

Methylene blue- 0.3g, Distilled water- 99ml, Acetic acid- 1ml

Acid-Fast Staining

Procedure

1. Prepare a smear on a clean grease free slide. Allow to air dry and heat fix.
2. Flood the smear with ZNCF i.e. Zeihl Neelson carbol fuchsin stain. Then place the slide on water bath and steaming is done for 3 to 5 minutes. Apply the stain repeatedly during steaming to avoid evaporation and drying.
3. Wash the slide with water.
4. Decolourize the smear with acid alcohol until no more stain appears in washing.
5. Wash the slide properly with water.
6. Flood the smear with counter stain as 0.3% methylene blue or 1% malachite green. Allow to react for 1 minute.
7. Wash the slide with water.
8. Blot dry the slide and observe under oil immersion objectives.

Acid-Fast Staining

Observation

Acid fast: Bright red to intensive purple, Red, straight or slightly curved rods, occurring singly or in small groups, may appear beaded.

(*Mycobacterium tuberculosis, Mycobacterium smegmatis*)

Non-acid fast: Blue color; In addition, background material should stain blue.

(*Nocardia*)

Mechanism

The acid fastness of *Mycobacterium tuberculosis* and *Nocordia* is due to the presence of high lipid content of the cell wall (May be upto 60% w/w). The major lipid includes mycolic acid and glycolipids. It is also called as **cord factor**. Due to high lipid content in cell wall, the cells are less permeable to stain. The penetration of primary stain can be increased by two ways

1. Chemical way- use phenolic stain with high affinity for waxy covering of organisms.
2. Physical way- use of steaming during staining.

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2. Physical way- use of steaming during staining.

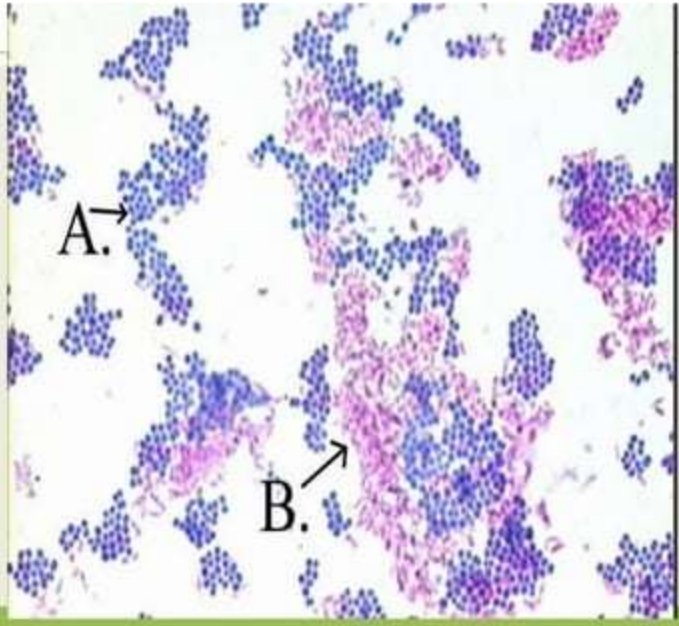
The real mechanism of acid fast staining can be explained on the basis of relative solubilities. The primary stain fuchsin is more soluble in phenol than in water and acid-alcohol. While the phenol is more soluble in the waxy layers of cell wall of organisms. Therefor when phenol containing acid fuschin is applied as primary stain , then it enters the lipid of cell wall. Heating or steaming is allowed the easy penetration of stain by softening the waxy material. Now once the waxy layer is stained, it will not be decolorized by strong decolourizing agent like acid – alcohol. The reason is that the phenolic stain are more soluble in lipids than acid-alcohol. Thus acid fast organism retains the primary stain and appear pink in colour. But non-acid fast organisms are easily decolorized by acid-alcohol. Such organisms are stained by counter stain appear blue or green.

Acid fast Staining

Result and interpretation

Acid-fast Bacteria appear Red in color.

Non- Acid fast Bacteria appear blue in color



Acid fast: Bright red to intensive purple (B), Red, straight or slightly curved rods, occurring singly or in small groups, may appear beaded

Non-acid fast: Blue color (A)

Cell wall Staining

(Chance's Method)

Objective

To perform a cell wall staining procedure.

To better visualize cells and cell components under a microscope.

To stain certain cell components, such as a nucleus or a cell wall, or the entire cell.

Principle

Cell wall staining technique is a special staining technique in which especially stain the cell wall of the bacterial cell. Cell wall is the outer most rigid covering of cell and depending upon the structure of cell wall bacterial cell are classified in Gram positive and Gram negative cells. Staining of bacterial cell wall helps us in a demonstration of the cell wall. There are various staining techniques used to stain cell wall like Chances method, Ringer's method and Dyar's method.

Requirements

A Clean grease free slide, Fresh cell suspension, 0.5 % New fuchsin solution,

0.5 % Congo red solution, inoculating loop, staining tray, microscope etc.

Cell wall Staining

Procedure

1. A clean grease free slide is taken.
2. The smear is made on a slide by using a sterile wire loop.
3. The slide is air dried and not heat fixed.
4. After air-drying the slide is flooded with 0.5 % New fuchsin solution and allowed to react for 3 minutes.
5. After 3 minutes excess stain is drained out and the slide is flooded with 0.5 % Congo red solution and kept for 4 minutes.
6. Further, the slide is gently washed with water.
7. Air dried and observed under oil immersion lens.

Cell wall Staining

Mechanism

The bacterial cell wall is acidic in nature as well as the cytoplasm is also acidic but the cell wall is more acidic than cytoplasm. When the first stain i.e. new fuchsin is applied to smear, it stains both, the cell wall and cytoplasm because the stain is a basic dye.

While when 0.5% congo red is applied, which is a selective decolourizing agents, it is selectively decolorizes only the less acidic cytoplasm, leaving behind coloured cell wall.

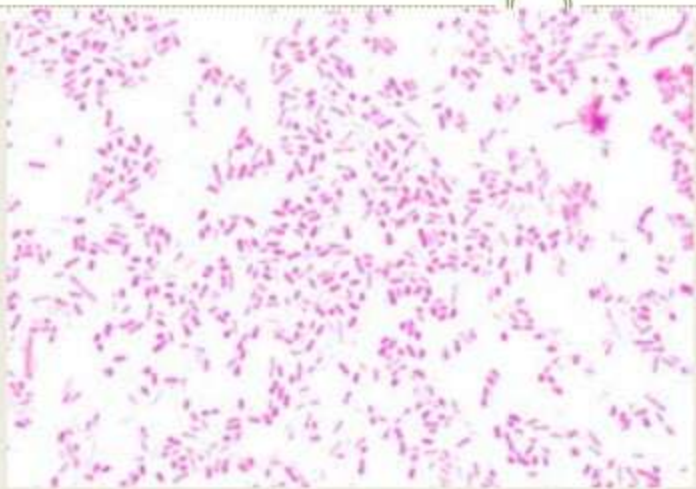
Observation-

Microscopic field after Chance's method of staining shows the pink coloured cell wall and colourless cytoplasm .

Cell wall Staining

Result and interpretation

Pink coloured cell wall and colourless cytoplasm appears .



Capsule Staining
(Maneval's Method)

Objective

To perform a capsule staining procedure.

To Identify capsules around bacteria.

To distinguish capsular material from the bacterial cell.

Principle

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.

Capsule Staining

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.

Capsule Staining

Requirements

Staining tray, Staining rack, Slide holder, Slide, Disposable gloves.

Congo red (1% aqueous solution)

Maneval solution-

0.05 g of fuchsin, 3.0 g of ferric chloride, 5 ml of acetic acid (glacial),

3.9 ml of phenol (liquified), 95 ml of distilled water, Capsulated cell culture

Procedure

Take a loopful of capsulated cell suspension on a clean grease free slide.

Add a drop of 1 % congo red solution in the suspension and then spread it gently on the slide to form a smear.

Allow the suspension to air dry and do not heat fix it.

Flood the slid with Maneval's stain and keep it for 2 minutes.

After 2 minutes discard excess stain and do not water wash the slide.

Allow the slide to air dry and observe under oil immersion objective.

Capsule Staining

Mechanism

Capsules are non-ionic in nature so they do not get stain easily.

By using the negative staining method demonstration of capsule becomes easy.

So in this method first we use congo red stain as it is acidic stain it stains the background in red colour.

Further, we use Maneval's stain this stain has four important components and they are-

10 % $FeCl_3$ – It acts as a chemical fixative.

5 % phenol – It increases penetration power of the stain.

Acid fuchsin – It is a basic dye it stains the cell.

Acetic acid – It decreases the pH of smear to acidic side.

Due to acetic acid pH is shifted to the acidic side and due to which congo red stain turns red to blue in colour that is why the colour of background changes from red to blue in colour.

Due to acid fuchsin, the cell gets stained in pink colour.

It creates a clear contrast in between cell, capsule and background and due to which capsule can be detected easily.

Capsule Staining

Observation-

Pink colour bacterial cell at the centre surrounded by colourless capsule against a dark blue colour background observed.

Result and interpretation

- ❑ The capsule is seen as a clear halo around the rod-shaped bacterium. Fig-1
- ❑ There is no halo surrounding the cocci-shaped cells. Fig-2

Fig-1 Encapsulated *Bacillus* sp. stained using Maneval's capsule staining method.

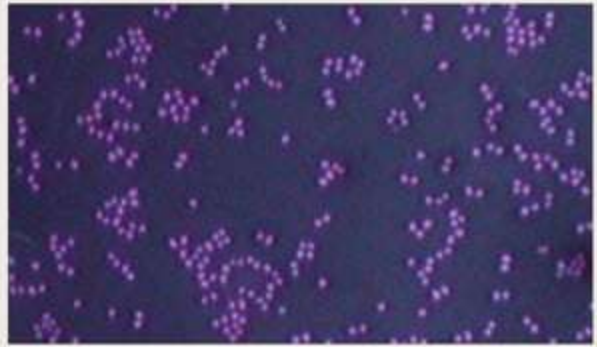


Fig-2 Staphylococcus epidermis stained using Maneval's capsule staining method.


Thank you