Antiseptic

Sunday, 15 March 2023 6:43 PM

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Experiment 4
Bacterial Identification
Simple staining, Gram staining and differential staining

- **Introduction**

Bacteria have nearly the same refractive index as water so they are opaque or nearly opaque and difficult to see even when observed under a microscope. To make them and their internal structures more visible under the light microscope, various staining methods are used.

1. **Simple Staining**

Aim: Simple staining.

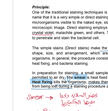
To compare the morphological shapes and arrangements of bacterial cells

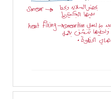
Principle:

One of the traditional staining techniques is simple staining of bacteria. It is clear from the name that it is a very simple or direct staining method that uses only one stain. To make the bacteria more visible to the naked eye, staining is used, which causes a divergence in a microscopic image. Direct staining employs basic dyes such as methylene blue, safranin, or crystal violet, malachite green, and others. The basic stains have different exposure times to penetrate and stain the bacterial cell.

The simple stains (Direct stains) make the organism visible and allow us to examine its shape and arrangement. Differential staining is utilized to distinguish a specific group of organisms. In general, the process consists of three sequential steps: smear preparation, heat fixing, and bacteria staining.

In preparation for staining, a small sample of microorganisms is placed on a slide and permitted to air dry. The **smear** is then heat fixed by quickly passing it over a flame. **Heat fixing** kills the microorganisms, makes them adhere to the slide, and prevent the samples from being washed out during the procedure and permits the bacteria to absorb the stain.





2. **Differential staining**

Differential staining requires the use of at least 2 chemical reagents that are applied to a specimen in sequence. The first reagent is a primary stain that binds to the cells and is used to establish a color contrast, the second reagent used is the decolorizing agent, which alters the chemical composition of cellular components the decolorizing agent may or may not remove the primary stain from the cells, depending on the cell type. The last reagent is the counter stain. Following decolorization, if the primary stain is not washed out, the counter stain cannot be absorbed and neither the cell nor its components will retain the color of the primary stain. If the primary stain is removed, the decolorized cellular components will accept and assume the contrasting color of the counter stain. In this way cell type or their structure can be differentiated based on their ability to retain the primary stain or to retained the most important differential stain used in bacteriology is the **Gram stain**.

Gram-Staining

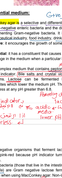
Aim:

To differentiate two principal groups of bacteria.

Principle:

The Gram staining method is one of the most important staining techniques in microbiology. It is almost always the first test performed for identification and classification of bacteria. It divides bacteria into two groups designated Gram-positive and Gram-negative according to their reaction to a staining procedure developed in 1884 by Christian Gram.

Gram staining is a common technique used to differentiate two large groups of bacteria based on their cell wall composition. The cell wall of Gram-positive bacteria contains thick layers of peptidoglycan and Gram-negative groups by coloring their cells red or violet. Gram staining is based on the ability of bacteria cell wall to retaining the crystal violet dye during solvent treatment. Gram positive bacteria stain violet due to the presence of a thick layer of peptidoglycan in their cell walls, which retains the crystal violet these cells are stained with. Alternatively, Gram negative bacteria have cell wall, which is attributed to a thinner peptidoglycan wall, which does not retain the crystal violet during the decolorizing process.



- **Differential medium:**

MacConkey agar is a selective and differential culture medium that is used for the isolation of Gram-negative enteric bacteria and the differentiation of lactose fermenting from lactose non-fermenting Gram-negative bacteria. It is one of the most commonly used media in pharmaceutical, dairy, food industry, drinking water control & clinical laboratories.

Selective: It encourages the growth of some microorganisms but suppresses the growth of others.

Differential: It has a constituent that causes an observable change such as color change or pH change in the medium when a particular microorganism reacts or not.

It is a complex medium that contains peptone, bile salts, crystal violet, lactose and Neutral red as pH indicator. It is a selective medium because bile salts and crystal violet inhibit certain organisms. Lactose can be fermented by some microorganisms producing acidic metabolites which lower the medium pH. The pH indicator turns red at a pH below 6.8 and is colorless at any pH greater than 6.8.

Gram negative organisms that ferment lactose will produce an acidic environment and appear pink-red because pH indicator turns red. Non-fermenters will produce colorless colonies.

Enterics bacteria (those that live in the intestines such as Escherichia coli, Enterobacter and Klebsiella) ferment lactose and produce pink-red colonies. Salmonella and Shigella produce colorless colonies when using MacConkey agar. Non-lactose fermenting Gram-negative bacteria such as, Proteus species, Salmonella, Pseudomonas, Neisseria and Shigella cannot utilize lactose as a carbon source, and will use peptone instead. This results in the formation of ammonia, which raises the pH of the agar, and leads to the formation of white or colorless colonies in the plate.

- **Bacterial morphology and arrangement:**

There are three basic shapes of bacteria:

1. **Cocci** (**plural: Cocci**): Spherical bacteria that may occur in:
   - Single (coccus)
   - Pairs (diplococci)
   - Groups of four (tetracocci)
   - Grape like clusters (staphylococci),
   - Chains (streptococci)
   - Cuticular arrangements of eight or more (sarcina)

2. **Bacillus** (**plural: Bacilli**): Rod- shaped bacteria that:
   - Generally, occur singly (bacillus)
   - Pairs (diplobacilli or streptobacilli)
   - Or in chains (streptobacilli).

3. **Spiral shape**: come in one of three forms.
   - Vibrio: curved or comma- shaped
   - Spirillum: rigid spiral rods
   - Spirochaete species: a thin, flexible spiral

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Practical part

- **Part 1: Smear preparation and heat fixing**

1. Obtain a clean microscope slide.
2. Label the frosted edge appropriately with the sample identification.
3. Transfer specimen or culture to the center of the slide.
4. Place a small drop of water on the microscope slide, or one drop of sterile saline or sterile water to the center of the microscope slide. Aseptically pick a small amount of an isolated colony with a loop and gently mix into the drop of sterile saline or water to prepare a smooth, even suspension of bacteria.
5. Allow the smear to air dry completely.
6. Fix the smear to the slide using heat fixation.
7. Allow the slide to cool to room temperature or air dry.

- **Part 2: Gram staining**

To a bacterial smear the following chemicals are applied to make a Gram-stain:

1. Gram's Crystal Violet Stain (primary stain)

The smear is flooded with Crystal Violet for **60 seconds**. Crystal violet is a purple chemical that sticks to the peptidoglycan layer of the bacterial cell wall. After 60 seconds, crystal violet is rinsed off using **distilled water**.

Explanation: All cells are **purple** after this step. Stopping here would be a simple stain.

2. Iodine (mordant)

Flood the smear with iodine for 60 seconds then wash immediately with distilled water. This causes crystal violet to stick to peptidoglycan like mortar causes bricks to stick together.

Explanation: Gram's iodine forms a complex with crystal violet. All cells are **purple** after this step.

3. 95% Ethanol/ acetone (decolorizing)

95% ethanol is added drop by drop for 5-10 seconds then washed immediately with water. The Acetone/Alcohol washes crystal violet out of the Gram-negative cell wall. The Gram-positive cell wall retains crystal violet as long as the acetone/alcohol wash lasts, not more than a few seconds. The acetone/alcohol wash is the **differential step** in the Gram-stain process.

Explanation: Gram-positive cells retain crystal violet and remain **purple**. Gram-negative cells lose crystal violet and are now **colorless**. Water rinse stops the decolorization process.

4. Safranin Stain (secondary or counter stain)

The smear is flooded with Safranin for 60 seconds then wash immediately with water. Safranin is a stuck to cytoplasmic component of the cell. All cells become stained with safranin.

Explanation: Safranin is a pinkred dye. After this step Gram positive cells remain **purple**, gram negative cells are now **pinkred**.

5. Drying the slide

Dry the smear by blotting it After paper. Then add one drop of oil on the smear to observe it under the microscope using oil-immersion objective lens (100x). Note the Gram-stain results then start shape.

- **Part 3: Use of the microscope**

- **General points:**
  Treat the instrument with care, clean the lenses and eyepieces with lens tissue, and remove any water or oil with lens tissue before leaving the laboratory.

- **To examine your slide:**

1. Focus on a specimen with the 40x lens
2. Close the field diaphragm down completely
3. Open the field diaphragm until it just appears in the image of the field diaphragm. This is not easy as the image can be hazy.
4. Open the field diaphragm until it just disappears from the field of view.
5. Close the field diaphragm and adjust the condenser aperture until you can just see the edge of it about the opening.
6. Switch the 100x objective lens and with oil on the specimen repeat the process. The microscope is now ready for use.
7. Repeat these steps every time you use your microscope.

- **Interpretation of results:**

1. **Color:** either gram positive or gram negative
2. **Shape:** cocci or bacilli
3. Older cells might have been dead or with damaged cell wall, giving false results. Gram-positive cells would have mixed results (purple and red) because not all cells might be damaged.
4. Excessive decolorization (> 10 seconds) would affect Gram-positive cells.
5. No proper decolorization (< 5 seconds) would affect Gram-negative cells.

- **Part 4: Streaking a MacConkey agar plate**

1. Take MacConkey agar plate & on the base draw lines to divide it into three sections, one for the control organisms (E.coli) and two for Gram and G-ve.
2. Don't touch the negative control section.
3. At the G-ve section streak the plate with E. coli
4. At the G+ve section streak the plate with the Gram-positive bacteria provided (Staphylococcus aureus)
5. Incubate the plate at 37°C

Please watch these short videos:

Smear preparation:
https://www.youtube.com/watch?v=nGFwpOoq8ukjkmPLJ-&t=21_hqbLdTP3WWQ__2Wc3kwqQ2GBbnwm1&b=1s

Gram staining:
https://www.youtube.com/watch?v=mnKbOomgQKk&t=17s

Oil immersion:
https://www.youtube.com/watch?v=_DDKqPZTJ1&t=7s

https://www.youtube.com/watch?v=jqwmmqbwsc0

How to Interpret MacConkey's Agar Bacterial Growth Medium
https://www.youtube.com/watch?v=0KqKdKvNaM4&t=4s