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Advanced Laboratory Techniques

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Lecture / 6

Staining used in diagnosis microorganisms (Bacteria, parasites and fungi)/ part 1

Staining used in diagnosis microorganisms // part 1

❖ **Staining related to diagnosis of bacteria:**

- Staining techniques are commonly employed to visualize bacterial cells under a microscope and aid in their identification.
- there are some of the main types of bacterial staining techniques:

1. Gram Staining:

- Developed by Hans Christian Gram, this technique classifies bacteria into Gram-positive and Gram-negative based on differences in their cell wall structure.
- Gram-positive bacteria retain the crystal violet stain and appear purple, while Gram-negative bacteria take up the counterstain (Safranin) and appear pink.
- (Procedure discuss previously).

2. Acid-Fast Staining:

- Acid-fast staining is used for the identification of acid-fast bacteria, particularly the genus *Mycobacterium*.
- These bacteria resist decolorization with acid-alcohol due to the presence of mycolic acids in their cell walls.
- They retain the primary stain (Carbol fuchsin) and appear red.

Procedure:

- Prepare a thin and even smear of the sample (such as sputum, tissue, or culture) on a clean microscope slide. Allow the smear to air-dry completely.
- Heat-fix the smear by passing it through the flame of a Bunsen burner or a slide warmer. This helps the cells adhere to the slide.
- **Primary Stain (Carbol fuchsin):** Flood the smear with Carbol fuchsin, a red-colored stain containing phenol. Heat the slide gently using a Bunsen burner or slide warmer for about 5 minutes. Do not allow the slide to dry out. The heat helps the stain penetrate the waxy cell wall of acid-fast bacteria.
- **Decolorization:** Rinse the slide with acid-alcohol (a mixture of hydrochloric acid and ethanol) to remove the stain from non-acid-fast bacteria. Rinse until the runoff is colorless.
- **Counterstain (Methylene Blue):** Counterstain the smear with methylene blue, which will stain non-acid-fast bacteria. Allow the methylene blue to act for about 1-2 minutes.
- **Washing and Drying:** Rinse the slide gently with water to remove excess stain. Blot the slide dry with bibulous paper or allow it to air-dry.
- Examine the slide under a light microscope using oil immersion at 1000x magnification. Acid-fast bacteria will appear red/pink, while non-acid-fast bacteria will appear blue.

Notes:
The acid-fast property of certain bacteria is due to the presence of mycolic acids in their cell walls, which resist decolorization by acid-alcohol.
The use of phenol in the Carbol fuchsin stain enhances the penetration of the dye into the bacterial cells.
Specialized staining procedures, such as the Ziehl-Neelsen method or the Kinyoun method, are commonly employed for acid-fast staining.

3. Endospore Staining:

- This technique is used to visualize endospores, which are resistant, dormant structures produced by certain bacteria.
- Malachite green is used to stain endospores, and Safranin is used as a counterstain.

Procedure:

- Prepare a thin and even smear of the bacterial sample on a clean microscope slide. Allow the smear to air-dry completely.
- **Primary Stain (Malachite Green):** Flood the smear with malachite green, a green-colored stain. Heat the slide gently using a Bunsen burner or slide warmer for about 5 minutes. Do not allow the slide to dry out. The heat helps the malachite green penetrate the endospores.
- **Cooling and Rinsing:** Allow the slide to cool. Rinse the slide with water to remove excess stain.
- **Counterstain (Safranin):** Counterstain the smear with safranin, a red-colored stain. Allow the safranin to act for about 1-2 minutes.
- **Washing and Drying:** Rinse the slide gently with water to remove excess stain. Blot the slide dry with bibulous paper or allow it to air-dry.
- Examine the slide under a light microscope using oil immersion at 1000x magnification. Endospores will appear green, while the rest of the cell will appear red.

Notes:

- Malachite green is a water-insoluble dye, and the heating step is crucial for driving the stain into the endospores.
- The counterstaining with Safranin helps visualize the vegetative cells, which do not retain the malachite green stain.

4. Capsule Staining:

- Capsule staining is used to highlight the presence of capsules, which are protective layers around some bacteria.
- Capsules often appear as clear halos around the stained bacterial cells.

Procedure:

- Prepare a thin smear of the bacterial culture on a clean microscope slide. Allow the smear to air-dry.
- **Negative Stain (India ink or Congo red):** Flood the smear with a negative stain, such as India ink or Congo red. Negative stains stain the background but do not stain the bacterial cells. Allow the negative stain to act for 1-2 minutes
- **Washing:** Rinse the slide gently with water to remove excess stain. Do not heat-fix the smear.
- **Counterstain (Safranin or Crystal Violet):** Apply a counterstain, such as safranin or crystal violet, to the smear. Allow the counterstain to act for about 1-2 minutes.
- **Washing and Drying:** Rinse the slide gently with water to remove excess counterstain. Blot the slide dry with bibulous paper or allow it to air-dry.
- Examine the slide under a light microscope using oil immersion at 100x magnification.
- Bacterial cells will appear stained with the counterstain, while the capsules will appear as clear halos around the cells against the stained background.

Notes:

- Capsules are often not visible with standard staining methods because they do not readily take up most stains. Negative staining, which stains the background rather than the cells, highlights the capsules as clear areas around the stained bacterial cells.
- India ink and Congo red are commonly used negative stains for capsule staining.
- The choice of counterstain may vary; Safranin and crystal violet are commonly used options.

5. Flagella Staining:

- Flagella staining involves coating the flagella (hair-like appendages used for movement) of bacteria with a dye, making them visible under the microscope.

Procedure:

- Prepare a thin smear of the bacterial culture on a clean microscope slide. Allow the smear to air-dry.
- **Mordant (Tannic Acid or Potassium Alum):** Flood the smear with a mordant, such as tannic acid or potassium alum. Allow the mordant to act for about 1-2 minutes.

- **Washing:** Rinse the slide gently with water to remove excess mordant.
- **Primary Stain (Basic Fuchsin or Carbol Fuchsin):** Flood the smear with a primary stain, such as basic fuchsin or carbol fuchsin. Heat the slide gently using a Bunsen burner or slide warmer for about 1-2 minutes. Do not allow the slide to dry out. The heat helps in penetrating the flagella.
- **Washing:** Rinse the slide gently with water to remove excess stain.
- **Drying:** Allow the slide to air-dry.
- Examine the slide under a light microscope. Flagella will appear as thin, stained structures extending from the bacterial cells.

Notes:

- The mordant helps in fixing the flagella to the bacterial cells and enhances the staining process.
- Basic fuchsin or carbol fuchsin is commonly used as a primary stain for flagella.
- The choice of mordant and staining time may vary based on the specific protocol or staining method used.

6. Negative Staining:

- Negative staining uses acidic dyes that are repelled by bacterial cells, leaving the background stained.
- This technique is useful for observing the morphology and arrangement of bacteria without distorting their shape.

Procedure:

- **Preparation of Slide:** Place a clean, grease-free microscope slide on the bench.
- **Application of Bacterial Culture:** Place a small drop of the bacterial culture on the slide.
- **Addition of Negative Stain:** Add a small drop of the negative stain (India ink or Congo red) to the bacterial drop on the slide. Mix the bacterial culture and stain gently using a loop or a sterilized applicator stick.
- **Spread the Mixture:** Use a second slide at a 45-degree angle to spread the mixture thinly across the surface of the slide.
- **Air-Drying:** Allow the smear to air-dry. Do not heat-fix.
- Examine the slide under a light microscope. Bacterial cells will appear colorless or very lightly stained against a dark background.

Notes:

- Negative staining is particularly useful for visualizing capsules, flagella, or other structures that may be difficult to stain using conventional methods.

- India ink and Congo red are commonly used negative stains. India ink creates a dark background, while Congo red may produce a reddish background.

7. Silver Staining:

- Silver stains, such as the Warthin-Starry stain, can be used to visualize bacteria in tissues.
- These stains are particularly helpful for visualizing the presence of bacteria in pathological samples.

Materials and Reagents:

- **Fixation Solution:** Typically containing methanol and acetic acid.
- **Sensitizing Solution:** typically containing sodium thiosulfate.
- **Silver Staining Solution:** Comprising silver nitrate and a reducing agent (e.g., formaldehyde).
- **Developing Solution:** Containing citric acid or other acidic solutions.
- **Stop Solution:** Typically containing acetic acid.
- **Destaining Solution:** Usually water.
- **Polyacrylamide Gel:** The gel containing separated proteins.

Procedure:

- **Fixation:** Immerse the gel in a fixation solution for a sufficient time (usually 30 minutes to 1 hour). This step helps to immobilize proteins in the gel.
- **Washing:** Wash the gel with water to remove excess fixative.
- **Sensitization:** Immerse the gel in a sensitizing solution (e.g., sodium thiosulfate) for a short period, typically a few minutes.
- **Rinsing:** Rinse the gel with water to remove excess sensitizing solution.
- **Silver Staining:** Incubate the gel in a silver staining solution containing silver nitrate and a reducing agent. The duration of staining may vary but is typically around 30 minutes to 1 hour.
- **Rinsing:** Rinse the gel with water to remove excess silver staining solution.
- **Developing:** Immerse the gel in a developing solution (e.g., citric acid). Bands containing proteins will become visible during this step.
- **Stop Solution:** Transfer the gel to a stop solution (e.g., acetic acid) to halt the development process.
- **Distaining:** If necessary, immerse the gel in a distaining solution (usually water) to remove background staining and improve contrast.
- Document the stained gel using a gel documentation system or by other means.

Notes:

- Silver staining is sensitive and can detect low amounts of proteins, making it useful for applications like 2D gel electrophoresis.

- Care should be taken to avoid contamination during the staining procedure.
- Specific formulations and timings may vary based on the silver staining kit or protocol used.

8. Methylene Blue Staining:

- Methylene blue is a simple stain that can be used for basic staining of bacterial cells.
- It stains both Gram-positive and Gram-negative bacteria.

Procedure:

- **Fixation:** If working with bacterial smears, fix the smear by passing the slide through a flame or using a fixative (e.g., heat fixation).
- **Application of Methylene Blue:** Flood the bacterial smear or tissue section with the methylene blue solution. Ensure the entire surface is covered.
- **Incubation:** Allow the methylene blue solution to act for about 1-2 minutes.
- **Rinsing:** Rinse the slide gently with water to remove excess stain.
- **Blotting:** Blot the slide dry with bibulous paper or allow it to air-dry.
- Examine the slide under a light microscope. Methylene blue stains the cells and cellular components, making them visible under magnification.

Notes:

- Methylene blue is a basic dye that binds to acidic components in cells, such as nucleic acids and certain proteins.
- This staining method is commonly used for general observations of cell morphology and structure.
- The intensity of staining and staining time can be adjusted based on the specific requirements of the experiment.