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TECHNICAL PROCEDURE: GRAM STAINING

Procedure Title: Gram Staining of Bacterial Smears
Department: Microbiology
Procedure Type: Qualitative Differential Stain
Effective Date: _____
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PRINCIPLE

Gram staining is a **differential staining procedure** used to classify bacteria according to differences in their cell-wall properties.

The procedure uses four main reagents:

1. **Crystal violet** – primary stain
2. **Gram's iodine** – mordant
3. **Decolorizer** – removes the crystal violet-iodine complex from cells that do not retain it
4. **Safranin** – counterstain

Gram-positive bacteria generally retain the crystal violet-iodine complex and appear **purple to blue**, while Gram-negative bacteria are decolorized and subsequently take up the counterstain, appearing **pink to red**.

I. Clinical Reason for Performing the Test

Gram staining is often performed as an initial diagnostic test for suspected bacterial infections because it can quickly identify the presence and type of bacteria. Differentiating bacteria into Gram-positive and Gram-negative categories provides information that can help guide the initial selection of appropriate antibiotics. This rapid preliminary result is particularly useful in infections such as pneumonia, urinary tract infections, and meningitis.

SPECIMEN REQUIREMENTS

I. Collection

Specimens must be collected using aseptic techniques to minimize contamination.

- **Sputum:** Collect from a deep cough into a sterile container.
- **Blood:** Collect from two separate venipuncture sites and inoculate each into a culture bottle.
- **Cerebrospinal fluid (CSF):** Collect through lumbar puncture.
- **Ascitic, pleural, and synovial fluids:** Collect using sterile methods; these specimens may require centrifugation before staining.
- **Urine:** Midstream collection in a sterile container is preferred. For catheterized specimens, collect directly from the catheter port rather than the collection bag.
- **Swabs:** Specimens from the throat, nose, wounds, rectum, or cervix should be collected from the infected area while avoiding contamination of adjacent tissues.

All specimens must be clearly labeled and transported promptly to preserve bacterial integrity.

II. Labeling

Specimens must be clearly labeled to ensure positive patient identification and maintain specimen integrity. In accordance with CLIA requirements, the laboratory must have procedures for specimen labeling that include the patient name or a unique patient identifier and, when appropriate, the specimen source. The CDC further recommends using at least two patient identifiers and including relevant specimen information such as the collection site, collection date, and specimen type.

III. Rejection

Specimens should be rejected when they do not meet the laboratory's established acceptance criteria or when their condition may compromise the reliability of testing. Possible reasons for rejection include improper or delayed transport, mislabeling, inappropriate specimen type or container, specimen degradation, contamination, and insufficient specimen quantity. Rejected specimens should be documented and handled according to laboratory policy.

IV. Storage and Transport

Specimens should be stored and transported under conditions appropriate for the specific specimen and laboratory test. Proper handling, including appropriate collection and transportation, is important for maintaining specimen quality and obtaining reliable laboratory results. Specimens should be transported to the laboratory promptly and using appropriate containers and packaging to protect the specimen during transit. Storage and shipping conditions, including temperature requirements when applicable, should follow the requirements established for the specific test.

V. Procedures for Submission to Central Laboratories

Specimens requiring testing at a central or reference laboratory shall be referred according to the laboratory's established written procedures. The receiving laboratory must be CLIA-certified or meet equivalent requirements. The submitting laboratory shall follow the receiving laboratory's current specimen requirements, including those for specimen preparation, handling, transportation, and submission. Specimens shall be maintained under appropriate conditions and handled in a manner that preserves their integrity until transfer to the receiving laboratory. The date and time of specimen receipt shall be documented.

VI. Procedures for Microscopic Examination

After the staining procedure is completed and the slide has dried, examine the stained smear using a bright-field microscope. First, locate the stained cells using the 40× objective. Apply a drop of immersion oil to the smear and examine the preparation using the 100× oil-immersion objective. Examine multiple microscopic fields to evaluate the stained microorganisms.

During microscopic examination, observe and record the Gram reaction of the organisms, their color, shape, arrangement, and location on the smear. Gram-positive organisms appear purple, while Gram-negative organisms appear pink. Common bacterial shapes include cocci and bacilli.

REAGENTS OR MEDIA, SUPPLIES, EQUIPMENT

I. Reagents

The following reagents are required for Gram staining:

- Crystal violet
- Gram's iodine
- Decolorizer, such as 95% ethanol
- Safranin
- Distilled or tap water for rinsing

II. Supplies

- Clean glass microscope slides
- Slide rack
- Timer
- Absorbent or bibulous paper
- Pencil or laboratory marker
- Personal protective equipment (PPE)

III. Equipment

- Bright-field microscope with a 100× objective
- Immersion oil

IV. Preparation of Reagents

If reagents are prepared in the laboratory, preparation shall follow the laboratory's validated formulation.

For Hucker's crystal violet, FDA describes preparation using crystal violet and ethanol combined with an ammonium oxalate solution. The mixture is allowed to stand for 24 hours and then filtered before use.

Gram's iodine is prepared using iodine, potassium iodide, and distilled water.

For Hucker's counterstain, a safranin stock solution is prepared using safranin O and 95% ethanol. A working solution is then prepared by diluting the stock solution with distilled water.

The decolorizer may consist of 95% ethanol. Commercially prepared Gram stain reagents may also be used in accordance with the manufacturer's instructions.

V. Storage Requirements

Prepared reagents shall be stored in appropriately labeled containers under the conditions specified by the validated laboratory procedure or the reagent manufacturer.

Gram's iodine should be protected from light. When prepared in-house, it should be stored in a dark container to reduce degradation.

All reagents should be checked before use for proper labeling, expiration or preparation date, and signs of contamination or deterioration. Commercial reagents should be stored according to the manufacturer's instructions.

CALIBRATION

Not applicable to the Gram staining procedure.

Gram staining is a manual microscopic procedure and does not require calibration of the staining procedure itself. However, laboratory equipment used for the examination should be maintained and checked in accordance with the laboratory's established equipment maintenance procedures and applicable manufacturer requirements.

QUALITY CONTROL

I. Identify Control Materials to Use

Known Gram-positive and Gram-negative control organisms should be used to verify the performance of the Gram-staining reagents and staining procedure.

- **Gram-positive control**: *Staphylococcus aureus*
- **Gram-negative contro**l: *Escherichia coli*

These organisms are used as reference controls because their expected Gram reactions are known. Gram-stain QC slides containing S. aureus and E. coli are also commercially available for evaluating staining reagents and staining technique.

II. Preparation, Handling, and Storage

Control organisms or prepared control slides shall be handled and stored according to the laboratory's validated procedure and the manufacturer's instructions, when applicable. Control slides should be protected from contamination and damage and maintained under the storage conditions specified by the manufacturer. When control organisms are maintained as laboratory cultures, appropriate quality-control procedures for their preparation, maintenance, and storage shall be followed.

III. Frequency of Testing

Gram-stain reagents and staining performance should be checked using positive and negative reference organisms with each new batch of stains and at least weekly during patient testing. More frequent QC testing should be performed when required by the laboratory's validated procedure, reagent manufacturer's instructions, or applicable regulatory requirements.

IV. Expected Results

Control organism	**Expected result**
Staphylococcus aureus	Gram-positive; purple/violet cocci
Escherichia coli	Gram-negative; pink/red bacilli

V. Corrective Actions

When QC results are unacceptable, the affected patient results shall not be reported until the cause of the QC failure has been evaluated and acceptable QC results have been obtained. The laboratory should review the staining reagents, control material, and staining procedure to identify the cause of the failure. Corrective actions taken to resolve the QC failure shall be documented according to laboratory policy.

VI. Recording and Storage of QC Data

All QC procedures and results shall be documented according to laboratory policy. QC records should include the date of testing, control material used, observed reaction or result, and any corrective action taken. Records should also include applicable reagent information, such as lot numbers, preparation or opening dates, and expiration dates. QC records shall be stored according to the laboratory's record-retention policy and applicable regulatory requirements.

VII. Alternatives (If no QC materials are available)

If prepared QC organisms or commercial QC slides are not available, the laboratory should follow its validated alternative QC procedure. Patient results should not be reported using an unverified staining system when the laboratory's established QC requirements cannot be met.

STEP-BY-STEP INSTRUCTIONS

I. Quantitative Testing

Not applicable to the Gram staining procedure.

Results are interpreted based on the observed Gram reaction and the characteristics of the microorganisms, rather than on a numerical measurement.

II. Qualitative Testing

1. Position the slide rack over the sink and place the fixed slide on it.
2. Flood the slide with **crystal violet**. Allow it to remain on the slide for at least 15 seconds.
3. Gently rinse the slide with water, then tilt the slide to remove excess water.
4. Flood the slide with **Gram's iodine**. Allow it to remain for at least 15 seconds.
5. Gently rinse the slide with water, then tilt the slide to remove excess water.
6. Apply the **decolorizer** to the slide and rinse immediately with water to help prevent over-decolorization.
7. Tilt the slide to remove excess water.
8. Flood the slide with **safranin**. Allow it to remain for at least 15 seconds.
9. Gently rinse the slide with water, then tilt the slide to remove any excess water.
10. Gently blot the slide with bibulous paper, taking care not to wipe the smear from the slide.
11. Allow the stained slide to air dry.
12. Once the slide is dry, examine it using a bright-field microscope.
13. First locate the cells using the 40× objective.
14. Place a drop of immersion oil in the center of the smear and examine the slide using the 100× oil-immersion objective.
15. Record the observed Gram reaction. Gram-positive cells appear purple, while Gram-negative cells appear pink.

III. Interpretation

Interpret the stained smear according to the color, shape, and arrangement of the observed microorganisms.

- **Gram-positive organisms:** Appear purple or violet because they retain the primary crystal violet stain.
- **Gram-negative organisms:** Appear pink to red after losing the primary stain during decolorization and taking up the counterstain.
- **Cocci:** Round or spherical bacterial cells.
- **Bacilli:** Rod-shaped bacterial cells.

The Gram reaction and bacterial characteristics should be recorded according to the laboratory's approved reporting format. Gram staining provides preliminary information about the organisms present and does not by itself establish identification to the species level.

CALCULATIONS

Not applicable to the Gram staining procedure.

Gram staining is primarily a qualitative microscopic examination rather than a quantitative test.

REPORTING RESULTS

I. Reference Intervals

Not applicable to the Gram staining procedure.

Gram stain results are reported based on the observed Gram reaction and the characteristics of microorganisms, rather than on a numerical reference interval.

II. Procedures for reporting abnormal results

Gram stain findings shall be reported according to the laboratory's established procedures for entering and reporting patient results. Findings requiring urgent or alert reporting shall be communicated according to the laboratory's established protocol and applicable requirements. The laboratory shall maintain a defined procedure for reporting patient results and, when applicable, imminently life-threatening, panic, or alert values.

III. Reporting format

Gram stain results shall be reported using the laboratory's approved reporting format. The report should include the observed Gram reaction and, when identifiable, the shape and arrangement of the microorganisms. Relevant findings may be described using terms such as Gram-positive or Gram-negative cocci and Gram-positive or Gram-negative bacilli.

PROCEDURE NOTES

I. Special precautions

Handle all specimens and stained slides using appropriate laboratory safety practices. Use aseptic techniques when handling specimens to minimize contamination. During the decolorization step, rinse the slide promptly after applying the decolorizer to prevent over-decolorization. When blotting the stained slide, handle it carefully to avoid removing the smear. Use immersion oil only with the appropriate oil-immersion objective.

II. Possible sources of error

Several factors may affect the accuracy and interpretation of Gram-stained smears. Thick or clumped smears may make microscopic examination difficult and may interfere with proper decolorization. The decolorization step should be carefully controlled, as insufficient or excessive decolorization can result in inaccurate Gram reactions. Thicker smears may require additional decolorization time. Fresh cultures should be used whenever possible, since older cultures may lose cell wall integrity, causing Gram-positive organisms to appear Gram-negative or Gram-variable.

III. Answers to common problems

Common Problem	Possible Cause / Corrective Action
Smear does not adhere to the slide	The slide may require additional cleaning before preparing the smear.
Smear is too thick	Spread the specimen more thinly across the slide to allow proper staining and examination.
Cells appear distorted or fragmented	Avoid overheating the slide during fixation.
Smear washes off during staining	Ensure that the smear is properly fixed before staining.
Gram-positive organisms appear Gram-negative	Over-decolorization may have occurred. Review the decolorization step and ensure that the slide is not excessively decolorized.
Gram-negative organisms appear Gram-positive	Under-decolorization may have occurred. Review the decolorization step.
Staining results are unreliable	Check the staining reagents for expiration, deterioration, or contamination and verify their performance using appropriate QC materials.
Uneven staining occurs	Ensure that the staining rack is level so that the staining solution covers the slide evenly, and use gentle washing during the rinse steps.

LIMITATIONS OF METHODS

Gram staining provides rapid preliminary information about the presence and Gram reaction of bacteria but has limitations in identifying microorganisms precisely. Compared with molecular techniques and genetic sequencing, Gram staining is less specific and cannot provide the same level of information regarding bacterial species and antimicrobial resistance patterns. Therefore, Gram staining serves primarily as a preliminary diagnostic method and may require additional testing for more precise identification and characterization of microorganisms.

A TROUBLESHOOTING OR BACK-UP PLAN

If the Gram-staining procedure cannot be performed or the staining system becomes unsuitable for use, testing shall be discontinued until the problem has been evaluated and appropriate corrective action has been taken. The laboratory shall follow its established procedures for addressing test-system problems and shall document the corrective actions taken. If acceptable quality-control results cannot be obtained, affected patient results shall not be reported until the problem has been resolved and the accuracy and reliability of the results can be assured. When necessary, the laboratory shall follow its established procedure for using an appropriate backup testing system or referring the specimen for testing.

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