# Gram-Stain

Mohammad Rahbar

# PRINCIPLE:

- The Gram's stain is used to classify bacteria on the basis of their forms, sizes, cellular morphologies, and gram reaction. It is a critical test for rapid presumptive diagnosis. This procedure is based upon the Hucker modification of the original Gram's stain method Gram-positive and gram-negative bacteria are both stained by the crystal violet (primary) stain. Addition of the iodine leads to the formation of a crystal violet-iodine complex within the cell wall .

# PRINCIPLE:

- The decolorizer extracts lipid from the cell wall of gram-negative bacteria, thereby increasing the crystal violet-iodine complex to diffuse from the cell. At the same time, gram-positive bacteria are dehydrated, causing a decrease in cell wall porosity and trapping the crystal violet-iodine complex within the cell. Due to the increase in porosity, safranin (counterstain) is able to permeate the cell wall of gram negative bacteria.

# SPECIMEN:

- Smears may be prepared from
- Clinical specimens,
- Broth cultures

  Colonies.

# SUPPLIES AND EQUIPMENT

**A- Gram's stain kit**

- 1) Crystal violet solution – crystal violet, 2.3%; ammonium oxalate, 0.1%; 20% ethanol
- 2) Safranin O solution – safranin, 0.6% in 20% ethanol
- 3) Gram's iodine solution – iodine, 0.33%; potassium iodide, 0.66%
- 4) Decolorizer solution – isopropanol, 75%; acetone, 25% .

# Gram Stain

- B. Kit storage and preparation
- 1) Store kit components at room temperature until the expiration date.
- 2) All kit components are supplied ready to use.

# C. Other supplies

- 1) Disposable plastic loops
- 2) Glass microscope slides
- 3) Slide warmer, dry heat block, flame, or absolute methanol.

# 5. QUALITY CONTROL

- The Gram's stain reagents should be tested using known gram-positive and gram-negative organisms each day of use.
- 1) Gram-positive control – *Staphylococcus aureus* ATCC 25923
- 2) Gram-negative control – *Escherichia coli* ATCC 25922.
- B Do not interpret test unless the controls yield expected results.

# 6. **PROCEDURE:**

- A. Smear preparation

A. Smear preparation

) b-While working in a biological safety cabinet, apply clinical material to the slide

# 1] *Liquid specimens, =2 ml*

- b] Remove all but a small portion of the supernatant.
- c] Gently re-suspend the pellet using a sterile transfer pipette.
- d] Place one drop of the re-suspended pellet onto the clean slide. Smear the drop over a small area of the slide using the tip of the transfer pipette.

# Continue..

- 2] *Liquid specimens, <2 ml sample received*
- place one drop of the re-suspended pellet onto the clean slide. Smear the drop over a small area of the slide using the tip of the transfer pipette

# *Highly viscous or purulent samples*

- a] Dilute with one drop of sterile saline on the slide

b] Spread smear over an area the size of a quarter .

# *Swab samples*

- a] If two swabs are submitted, use one to inoculate media and the other to prepare the smear.
- b] If only one swab is received, inoculate the solid media first, then prepare the smear and place the swab tip into liquid media (if applicable).

# Swab

- c] Roll the swab gently across the slide surface, covering and area the size of a quarter
- d] Alternatively, place the swab in a sterile tube with a small amount of sterile saline. Cap the tube and vortex the swab. Wring the swab against the side of the tube and use the expressed liquid to prepare the smear and inoculate media.

# 4] *Tissue*

- a] Transfer sample to a sterile Petri dish lid
- b] Mince tissue with a sterile scalpel, selecting purulent, necrotic, or bloody portions
- c] Touch several pieces of tissue to the slide (impression smears)
- d] If available, grind portions of the minced tissue and a small quantity of culture broth with a sterile tissue grinder. Prepare a thin smear of the grindings the size of a quarter.

# *Urine*

- a] Place one drop of urine on a microscope slide
- b] Do not spread drop .

# Culture colonies

- a) Label a glass microscope slide with the laboratory accession number and isolate number.
- b) Place one drop of sterile water or saline on the labeled slide.
- c) Transfer a small amount of an isolated colony to the slide with a disposable loop.
- d) Emulsify the growth into the water.

# 3) **Culture broth**

- ) Using a sterile plastic transfer pipette, aspirate all zones of the broth exhibiting growth.
- b) Place one drop of the broth onto a labeled glass slide and spread the broth to create a smear the size of a quarter.
- 4) Air dry smears at room temperature or place on a slide warmer or heat block (approximately 60C).

# B. Smear fixation

- 1) **Heat fixation**
- **a) Pass air-dried smears through a flame two or three times. Do not overheat.**
- **b) Allow slide to cool before staining.**

# 2) **Methanol fixation**

- a) Place air-dried smears in a coplin jar with methanol for one minute.
  Alternatively, flood smear with methanol for 1 minute.
- b) Drain slides and allow to dry before staining.

# C. Gram's stain procedure

- 1) Flood the prepared slide with crystal violet for one minute.
- 2) Rinse the slide gently with tap water.
- 3) Flood the slide with Gram's iodine for one minute.
- 4) Rinse the slide gently with tap water.
- 5) Working with one slide at a time, flood the slide with decolorizer for 5 seconds and rinse with tap water. Repeat decolorization step for thick smears such as sputum.

# Gram's stain procedure

- 6) Flood the slide with safranin for 30 second .
- 7) Rinse the slide gently with tap water.
- 8) Drain the slide in an upright position. Blot the back of the slide and place on a slide warmer or heating block to completely dry.

# D. Smear observation

- 1) **Clinical specimens**
- a) Scan 20-40 fields using both low power and oil immersion.
- b) When using the low power objective, look for:
- 1] SEC's (squamous epithelial cells)
- 2] WBC's
- 3] RBC's
- 4] Fungal elements

# Smear observation

- When using the oil immersion objective, examine only those areas that both contain inflammatory cells and that are appropriately gram-stained .

- ] Thick smears almost always contain some areas that are too thick and areas that are too thin.
- 2] The areas that are too thick are typically under-decolorized while areas that are too thin may be over-decolorized.

# 2) **Culture colonies and broth**

- 2) **Culture colonies and broth –** examine stained smear using oil immersion.

# 7. **INTERPRETATION:**

- A. Gram-positive bacteria and yeast will stain blue to purple.
- B. Gram-negative bacteria will stain pink to red.

# C. Clinical specimens

- ) Quantitate WBC's, RBC's, and epithelial cells
- a) Rare: <1 per low-power field
- b) Few: 1-9 per low-power field
- c) Moderate: 10-25 per low-power field
- d) Many: >25 per low-power field

# Quantitate bacteria and yeasts

- a) Rare: <1 per oil immersion field
- b) Few: 1-5 per oil immersion field
- c) Moderate: 6-30 per oil immersion field
- d) Many: >30 per oil immersion field

# 3) Urine

- a) Determine the average number of organisms per oil immersion field
- b) Each bacterial cell seen corresponds to 100,000 organisms/ml of urine
- c) The presence of many SEC's and/or multiple bacterial morphotypes suggests contamination.

# 9. **REPORTING RESULTS**

- A. Clinical specimens – see Screening Sputum and Tracheal Aspirates for Acceptability for Culture SOP for details on this sample type.
- 1) **WBC's, RBC's, and SEC's**
- a) Report quantitation and the cell type
- 1] Example 1: Few SEC's
- 2] Example 2: Moderate RBC's

# Reporting

- b) **Always** report the presence **OR** absence of WBC's
- 1] Example 1: No WBC's seen
- 2] Example 2: Many WBC's seen

# Reporting

- 2) **Bacteria and fungal elements**
- a) Report quantitation and the morphology
  b) Example 1: Many gram-positive cocci
- c) Example 2: Few gram-negative rods

# Reporting

- 3) **Urine**
- Report “X number of organisms seen per oil immersion field corresponding to X cfu/ml of urine”
- b) Example: 1 gram-negative rod per oil immersion field corresponding to 100,000 cfu/ml of urine.

# Reporting

- 4) Issue a written preliminary report with the Gram's stain results
- 5) **Telephonically notify the ordering provider or ward of any significant direct smear findings (e.g. any organisms seen on CSF Gram's stain). Document the date, time, and to whom you reported the results.**

# 10. **PROCEDURE NOTES:**

- A. Relatively large numbers of microorganisms (100,000 cells/ml) must be in a clinical sample to be observed on the direct smear – approximately the equivalent of moderate growth in culture.
- B. Recovery of organisms not observed on direct Gram's stains should prompt a review of both the smear and the culture.

# PROCEDURE NOTES:

- C. Morphologies noted on the direct Gram's stain should usually be recovered in the culture. Some times we observe microorganism in direct smear but no growth in culture. Possible explanations for this occurrence:
- 1) Organisms that are dead or dying are
- visualized on the smear but are not viable and therefore do grow in culture
- 2) Residual effects of antimicrobial agents in the culture prevent growth of the organism
- 3) Microscope slide or Gram's stain reagents are contaminated
- 4) The organism observed requires special incubation conditions to grow (anaerobic atmosphere, special media, prolonged incubation, etc.)

# Notes

- D. Proteinaceous samples will stain with a pink background. It is important to “look beyond” the background as gram-negative rods may be missed.
- E. Great care must be taken when examining smears since some organisms may be few in number.
- F. Smear preparations that are too thick may be difficult to interpret

# 11. **LIMITATIONS OF THE PROCEDURE:**

- A. Application of excessive heat during fixation of smear may affect the morphologic appearance of host cells and microorganisms.
- B. Treatment with antimicrobial agents may cause gram-positive bacteria to appear gram-negative.