MLAB 2434: Clinical Microbiology
Laboratory: Clinical Specimen Preparation, Gram Staining and Evaluation
14 points

Objectives:
At the end of this activity, the student will be able to:

1. Prepare gram stain smears from primary/clinical specimens.
2. Correctly evaluate and record gram stains on primary/clinical specimens.
3. Evaluate specimen quality based on specimen types and gram-stained smear results.
4. Discuss the clinical applications of gram-stained smears.
5. Identify the characteristics of a quality gram stain.
6. Recognize key microscopic characteristics of bacteria on gram stained smears.
7. Distinguish significant findings from artifact on a gram-stained smear.

Materials:

2 Clinical Specimen Swabs with requisitions
2 microscope slides
Inoculating equipment:
- Bacti-Incinerator
- Inoculating loop

Staining equipment:
- Stain rack
- Forceps
- Wash bottle
- Slide drying rack
- Bibulous paper

Gram Stain Reagents:
- Crystal violet
- Gram's iodine
- Acetone-alcohol
- Gram's safranin

Microscope
Immersion oil
Lens cleaner
Bibulous paper

References:

1. Mahon and Manuselis, Textbook of Diagnostic Microbiology, Fourth Edition, Chapter 6
2. Bartlett, Diagnostic Bacteriology, Chapter 18.
3. Gram Stain Tutor software, http:www.medtraining.org
4. American Society for Microbiology, (1992). *Clinical Microbiology Procedures Handbook: Volume 1*. Washington, DC: American Society for Microbiology.

Principle:

Direct gram stains from clinical specimens can provide the physician with valuable information to begin treating patients with antibiotics long before culture results are obtained. In addition, the results of the gram stain can confirm specimen acceptability, identify specific infectious agents, and determine the probability of infection by observing indicators of inflammation. Of course, clinical gram stain results are preliminary only, and the physician should evaluate antibiotic therapy when culture and antibiotic sensitivity results are available.

Gram stains on certain specimens such as urine, stools, throats, and vaginas may not be routinely ordered and performed due to low potential for recovery of organisms or the presence of normal flora.

General Reporting Guidelines on Gram Stains from direct or clinical gram stains:

- Presence or absence of polymorphonuclear leukocytes (PMNs) or WBCs
- Presence or absence of microorganisms
- Presence or absence or epithelial cells **IF** indicated by the source.

1. **General Considerations in the Preparation of Gram-Stained Smears**
    a. **Select appropriate material:** Pus, blood or mucus should be selected because the infectious agent or agents are likely to be present in these substances.
    b. **Media contamination:**
        i. Microscope slides are not sterile. Sterile slides are commercially available but it is not common practice in the clinical setting to procure or utilize these. This insures we minimize the potential for contamination from a non-sterile slide to the media.
        ii. If two (2) swabs are submitted, use a dedicated swab to inoculate the gram stain slide. It is preferred that the swab is removed from the cap prior to inoculation. The second swab would then be used to inoculate media.
        iii. If a single swab is submitted, first inoculate solid media, second inoculate liquid media (if dictated by setup), then use the wet swab to prepare the slide.
    c. **Specimen smears**
        i. **Fluids:** Laboratories often use specimen sediments to prepare smears, or cytospins.

1. **Sediment smears:** A pipet is used to place a drop of the sediment, following centrifugation, onto the slide. The drop is not spread over the surface.
2. **Cytospin smears:** A cytocentrifuge is used to make smears. This centrifuge concentrates the specimen material onto a small space of the slide. These are primarily used for non-bloody body fluids. A note should be added to the patient report indicating the specimen was centrifuged.

ii. **Tissues:** Tissue is cut and then touched to the slide. This preserves host cell morphology and bacterial cell patterns. Tissue grinders can also be utilized.

iii. **Swabs:** Swabs should be rolled over the surface of the slide, preserving host cell integrity and bacterial cell arrangements.

Procedure:

2. **Preparation of Smears:**
   a. The instructor will provide each student two (2) clinical specimen swabs with requisitions.
   b. Label the slide appropriately. The label must include patient name, MR number or DOB, source, and date of service. Handle the slides by the edges **only**. Use a pencil. **DO NOT** use a Sharpie-type marker. Sharpie-type marker will dissolve during the staining process.
   c. Prepare a smear from the swab by rolling the swab back and forth across the slide.
   d. Allow the smear to air dry and then **heat fix** by holding the slide, smear side out, against the opening of the Bacti-Incinerator for about 3 seconds. Hold the slide with forceps to prevent burning of your fingers. Check to make sure the slide has been heated sufficiently by gently placing the slide, smeared surface up, against the back of the hand; the slide should feel moderately warm. After cooling, the slide is ready for staining.

3. **Gram Stain**
   a. Place the slides, smear surface up, on the staining rack over the sink or a staining dish or rack.
   b. Flood the slide with **crystal violet** and allow to react for thirty (30) seconds.
   c. Handling the slide with forceps, tilt it to about 45º angle to drain the dye off.
   d. Continue to hold the slide at an angle and immediately rinse it thoroughly with a **gentle** stream of water from the wash bottle or water faucet.
   e. Replace the slide on the rack and flood it with **iodine**. Allow it to react for thirty (30) seconds.
   f. With forceps, tilt the slide and allow to drain.
   g. Immediately rinse the slide thoroughly with water from the wash bottle or water faucet.
   h. Replace the slide on the rack and flood it with **decolorizer** for **ONLY 3 seconds**. Stop

rinsing as soon as the run-off becomes clear. Adjust decolorization time according to the thickness of the smear.

i. Rinse **immediately** with water. This stops the decolorizing process.
j. Replace the slide on the staining rack and flood it with **safranin**. Allow to stand for 30-45 seconds.
k. Drain the slide and wash thoroughly with water.
l. Allow the slide to dry in a rack or **blot** (not rub) carefully with bibulous paper.
m. When slides are dried, observe under low power and oil immersion and report results in report sheet.

4. **Evaluation of Clinical Specimen Gram Stains**
    1. Evaluate the smear under low power (10x) to:
        a. Determine if the smear was decolorized properly. If a slide is stained correctly, the WBC's, RBC's and epithelial cells will look pink.
        b. Determine if the thickness of the smear is appropriate. The ideal thickness is one cell thick with no overlapping of cells.
        c. Detect any large elements, such as yeast, fungal elements or parasites.
        d. Examine smears prepared from clinical specimens to observe evidence of inflammation. A clinical specimen is from the patient, not from the media.
            i. Note:
                1. Relative amounts of PMNs and RBCs (may see necrotic debris or protein in the background)
                    a. Presence or absence of PMNs will be quantitated on high power.
                2. Relative amounts of squamous epithelial cells, bacteria consistent with normal flora, or food debris (may indicate an improperly collected specimen)
                    a. If the specimen originates from the respiratory system (tracheal aspirate, sputum, etc.), squamous epithelial cells should be quantitated on low power to determine if the sample is a representative sample.
                    b. Squamous epithelial cells are not reported in non-respiratory specimens.
                3. Location and orientation of microorganims
    2. Switch to **oil immersion(100x)** and scan under oil. If the smear is small, the entire smear should be searched. If the smear is rather large, a minimum of 20 oil immersion fields from different parts of the smear should be searched. On high power, both PMNs and microorganisms will be evaluated.
        a. If no organisms are seen, report "no organisms seen". The shorthand "NOS" can also be used.
        b. If microorganism(s) are seen, describe the gram reaction and morphology. Gram reaction and morphology should be described for EACH type of organism seen.
            i. Gram reaction

1. Gram positive
2. Gram negative
3. Gram variable- partially gram-positive cells with some gram-negative cells
4. Note staining characteristics such as bipolar or beaded

ii. Predominant shapes of microorganisms
1. Overall shapes- coccus, coccobacillary, rods, filamentous
2. Appearance of ends- rounded, clubbed, tapered
3. Appearance of sides- parallel, ovoid
4. Pleomorphism- variations in shape
5. Branching

iii. Characteristics of arrangements
1. Singles, pairs, chains, tetrads, clusters, palisading, Chinese letters

iv. Record morphology of observed bacteria. Descriptions which can be used include:
1. Gram positive cocci in pairs and/or chains and or clusters
2. Gram negative bacilli (rods) large or small, no need to state whether appearance is in chains, clusters, or pairs.
3. Gram negative coccobacilli
4. Gram negative diplococcic, intracellular and/ or extracellular
5. Gram positive rods, large or small
6. Budding yeast
7. Fungal elements

c. Recording/Quantitating of results
i. For most sample types either the absence or presence of PMNs and microorganisms are always reported on clinical gram stains. Epithelial cells are only recorded for specific sample types, such as sputum.
ii. In addition to noting the morphology of the microorganism(s), relative numbers are reported.
1. Smears of clinical specimens
a. Many (>10 per oil immersion field)
b. Moderate (5 to 10 per oil immersion field)
c. Few (1 to 5 per oil immersion field)
d. Rare (<1 per oil immersion field)
2. Smears of broth cultures
a. Specify gram stain reaction and morphology
b. Quantification may not be included because this is an enrichment media.

iii. If multiple types of organisms are observed, each type of organism should be listed in order of predominating quantity from highest number to lowest number. For example, if you saw many gram-negative rods and few gram-positive rods, the gram-negative rods would be listed first.
iv. On the patient report, it is important to denote each type or organism

using the terminology "isolate" or shorthand I1, I2. In the example above- this would be written:

1. I1 Many gram-negative rods
2. I2 Few Gram positive rods

5. **Reporting of Smears From Urine**
    a. Although not routinely done, physicians can request a gram stain from urine.
        i. Report the average number per oil immersion field of each bacterial or cellular observation.
        ii. Report quantities of more than 10 as "greater than 10 organisms and/or PMNs per oil immersion field".
        iii. The following are general guidelines for what the urine culture may be expected to yield.

OBSERVATION	**RESULT**
No organisms or PMNs seen	Negative for bacteriuria- no significant growth
<10 organisms on entire slide	Borderline for bacteriuria
0-1 organisms/PMNs per oil field	Probable positive bacteriuria ≤ 100,000/ mL
≥1 organism or PMN per oil field	Positive for significant bacteriuria ≥ 100,000/mL

6. **Reporting of Smears From Sputum and Tracheal Aspirates- Screening for Acceptability**
    a. Because the lower respiratory tract specimens are difficult to collect without contamination by the normal flora of the upper respiratory tract, a Gram stain of each sputum or tracheal aspirate is prepared and **screened** microscopically on low power (10X). If a gram stain is not ordered by the physician, the microbiology department will still perform a Gram stain. Oral contamination is reflected by the presence of many squamous epithelial cells.
    b. The sputum is considered "Unacceptable for culture" if there are more than 25 squamous epithelial cells per low power field. No further examination of the Gram stain is made. Another specimen is requested, and the gram stain screen report is finalized.
    c. The sputum is reported as "Borderline acceptable" if it has 10-25 squamous epithelial cells per low power field. The gram stain is then examined under oil immersion. Report the number of PMNs and epithelial cells seen per oil immersion field. Report the number of microorganism(s) seen per oil immersion field, listing each morphotype in order of predominance. Proceed with culturing specimen.
    d. The specimen is considered "Acceptable" for culture if it has <10 squamous epithelial cells per low power field. Examine the gram stain under oil immersion. Report the number of PMNs and squamous epithelial cells seen per oil immersion field. Report the number of microorganism(s) seen per oil immersion field, listing each morphotype in order of predominance. Proceed with culturing specimen.
    e. PMNs and microorganisms are quantitated on sputum Gram stains as follows:
        a. Many (>10 per oil immersion field)

b. Moderate (5 to 10 per oil immersion field)
c. Few (1 to 5 per oil immersion field)
d. Rare (<1 per oil immersion field)

7. **Quality Control**
    a. Daily
        i. Check appearance of reagents for precipitate or crystal sediment.
        ii. Prepare a smear of *Escherichia coli (*ATCC 25922*) and Staphylococcus epidermidis (*ATCC 12228*) or Staphylococcus aureus (*ATCC 25923*).* Fix and stain. Expected results:
            1. *Escherichia coli:* pink, gram negative rods
            2. *Staphylococcus epidermidis* or *aureus:* deep blue, gram positive cocci

Gram Stains
Points =14

Name_____ Date_____

The student must have an "A=acceptable" rating from the instructor PRIOR to moving forward.

Patient Name & Source	Gram Stain Result	Instructor Approval (A)/(U)
Example Report: William Moore Right hand abscess	Moderate PMNs seen **I1:** Many gram-positive cocci in clusters **I2:** Few gram-negative rods	
Example Report: Diana Nelson Sputum	Rare epithelial's (EPIs) seen Moderate PMNs seen **I1:** Moderate gram-positive cocci in pairs **I2:** Rare gram-negative diplococci	
1.		
2.		

Grading Rubric:
Each item listed is worth = 0.5 points:

- Patient name/ source
- Organism quantity/ gram reaction/ morphology/arrangement
- Presence/absence of PMNs
- Presence/absence of EPIs (as indicated)

Each item listed is worth= 1 point:
Instructor Approval

Name:_____
Date:_____

Laboratory: Preparation of Smears and Gram Staining
Study Questions
Points= 8

1. What elements are always reported (presence/absence) on clinical specimen gram stains? (3 pts)

2. Would a sputum with 16 epithelials/ lpf be acceptable? (1 pt)

3. One swab is submitted for gram stain and culture. What is the appropriate order of inoculation of the slide and media? (3 pts)

4. To check for specimen acceptability for sputums and tracheal aspirates on a gram stain smear, epithelial cells are screened with the _____ objective. (1 pt)