Module 3 - Gram Stain/Clinical Significance of Isolates

Objectives:
Upon completion of MM550 lectures, required reading, on-line material and laboratory exercises, the learner will:

1. Explain the principle of the Gram-stain procedure.

2. Interpret Gram-stained smears.
   - Gram reaction
   - Organism morphology
   - Organism arrangement
   - Cellular elements

3. Explain how Gram-stain results are affected by:
   - Age of bacterial cells
   - Antibiotic therapy

4. Discuss the purpose of performing Gram stains on:
   - Bacterial/yeast isolates
   - Specimens

5. Perform Gram stains.

6. Evaluate the clinical significance of microbial isolates based upon the organism’s propensity for causing disease
   - Normal flora
   - Potential/Opportunistic Pathogen
   - Strict Pathogen

7. Evaluate the clinical significance of microbial isolates based upon:
   - Body site
     1) Strict pathogens
     2) Opportunistic pathogens
     3) Normal flora (commensal, indigenous, colonization)
     4) Potential environmental contaminants
   - Host
     1) Age
     2) Immune status
     3) Location
     4) Geographic
     5) Inpatient vs. outpatient
     6) Nosocomial infection

I. INTRODUCTION – Gram Stain

Bacterial cells are stained with dyes to increase the contrast of the cells (to increase the difference between the cell and its surroundings). Different dyes have different affinities to various parts of the cell based largely on the chemical composition of both the dye and the cellular component. A differential stain employs two dyes resulting in one part of a cell or an entire cell staining one color and the other parts or another cell a different color. Two examples are:

**Gram stain:** one type of cell is purple (gram-positive), while another type is pink (gram-negative)

**Acid-fast stain:** organisms which are resistant to acid decolorization are pink, non-acid fast cells are blue.
II. **GRAM STAIN**

A. Cellular characteristics.

1. Gram reaction.
   
   The Gram reaction is either
   
   - **gram-positive** = purple
   - **gram-negative** = pink
   
   Principle: all cells take up the crystal violet. The Gram’s iodine (mordant) complexes with the crystal violet to make a larger molecule. Acetone-alcohol (decolorizer) dehydrates the lipids in the cell wall creating “holes”. Gram-negative cell walls have more lipids than gram-positive cells. Therefore, the dehydration process creates more “holes” in gram-negative cells. The crystal violet-iodine complex is washed out of the gram-negative cells. The gram-negative cells are now able to take up the safranin (reddish-pink) counterstain.

2. Cellular morphology.
   
   The cellular morphology is the shape of the individual bacterium. In general, bacterial cells have three shapes:
   
   - **Cocci**
   - **Rods/Bacilli**
   - **Spiral**

   The cellular morphology can vary greatly in size. There are large cocci and tiny cocci, as well as very short rods (which are often difficult to distinguish from cocci) and extremely long rods. Spiral-shaped bacteria are only rarely observed in the laboratory and generally do not stain with the Gram stain.
3. Cellular arrangement.

The cellular arrangement or grouping is based on the plane in which the cells divide. Some examples of arrangements/morphologies are:

**Cocci are round or slightly oval:**
- Single
- Tetrad
- Cluster
- Pair or diplococci (gram-positive)
- Chain
- Kidney bean diplococci (gram-negative)

**Bacilli are rod-shaped:**
- Rods or bacilli
- Rods or bacilli with spores (will appear as clear areas within the cell)
- Fusiform: cells are thin with pointed ends
- Coccobacilli: cells are very short resembling cocci
- Palisade/Palisading arrangement: cells parallel to each other
- “Chinese letter” arrangement: cells form V or Y configurations and/or clumps that resemble “Chinese letters”

**Arrangement is always noted with gram-positive cocci.** Gram-positive cocci in clusters is indicative of *Staphylococcus* sp., while gram-positive cocci in pairs or chains is indicative of *Streptococcus* sp. or *Enterococcus* sp.

**Pleomorphic** refers to cells that vary in size and shape.

4. Presence of spores

**Spores** are formed inside the bacterial vegetative cell and are very resistant structures. Spores are formed in two genera of gram-positive rods: *Bacillus* sp. and *Clostridium* sp. Spores do not stain with the Gram stain and appear clear (sometimes they will appear very light pink). Therefore, a spore will be a clear area inside a purple rod-shaped cell.
B. Staining Procedure

1. **Smear Preparation:** Fundamental to a good stain is a good smear!
   
   1) Label slide (multiple smears can be done on a single slide as long as the slide is divided into sections with a wax marking pencil. Care must be taken that no overflow or splattering of material occurs.
   
   2) Obtain organisms:

<table>
<thead>
<tr>
<th>Broth Culture</th>
<th>Colony on Agar</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Mix broth by gently shaking tube</td>
<td>a) Drop (small) saline on slide</td>
</tr>
<tr>
<td>b) Sterilize loop &amp; allow loop to cool</td>
<td>b) Sterilize loop &amp; allow loop to cool</td>
</tr>
<tr>
<td>c) Remove tube cap</td>
<td>c) Touch growth (pick up very minute amount of organism from isolated colony on the agar medium)</td>
</tr>
<tr>
<td>d) With loop, pick up loopful of broth</td>
<td>d) Gently mix organism in saline on slide</td>
</tr>
<tr>
<td>e) Recap broth</td>
<td></td>
</tr>
<tr>
<td>f) Touch loop to slide</td>
<td></td>
</tr>
<tr>
<td>g) If broth is not turbid, repeat b-f. Place organism on same area of the slide as the first loopful</td>
<td></td>
</tr>
</tbody>
</table>

3) Flame loop to sterilize.

4) Allow smear to air dry or place on 60°C slide warmer

5) Heat fix dried smear by placing on 60 °C slide warmer for approximately 10 minutes.
   This kills the organism and causes the cells to adhere to the slide.

2. Gram-Stain Technique

   a. Prepare & fix smear
   
   b. Place the fixed slide on the staining rack over a sink.
   
   c. Flood the slide with crystal violet and allow this to stand for one minute; rinse with tap water.
      *gram-positive and gram-negative bacteria are now both stained violet*

   d. Flood the slide with Gram's iodine (mordant) and allow this to stand for one minute;
   
   e. Rinse with tap water.
      *gram-positive and gram-negative bacteria are now blue-black*

   f. Decolorize the slide by holding the slide at a slight angle and allow about five drops of acetone-alcohol to flow across the slide until no more color is readily removed (this takes about two or three seconds). RINSE WITH WATER IMMEDIATELY.
      *gram-positive bacteria are now blue-black
gram-negative bacteria are colorless*

   g. Flood the smear with safranin (counterstain) and leave for one minute
   
   h. Rinse with tap water.
      *gram-positive bacteria are now purple and
gram-negative bacteria are now red*

   i. Dry the smear by blotting (using paper towels). Do not rub the smear!
   
   j. Examine with the microscope.
     *Examine first with low-power, high-power and then the oil-immersion objective*
3. Gram stain inconsistencies:

a. Over-decolorization - a critical step and takes practice to master. If too much acetone-alcohol is used, the crystal violet-iodine complex will be washed out of the gram-positive cells and they will appear to be gram-negative.

b. Under-decolorization - occurs when the decolorizer is not left on long enough. Human cells stain purple (they should stain pink) and gram-negative organisms stain purple.

c. Improper reagent use – omitting a reagent or using reagents in the wrong order or with incorrect timing produces erroneous results.

d. Excessive rinsing and prolonged counterstaining (safranin) - may remove crystal violet or crystal violet-iodine complex from gram-positive cells so they appear gram-negative.

e. Smear preparation too thick – unable to differentiate bacteria, cells.

f. Smear preparation too thin – unable to view cells/bacteria in specimen

g. Using an organism that is >24 hours old - is not reliable as dead gram-positive cells do NOT retain crystal violet. The result is gram-positive cells appear to be gram-negative.

h. Antibiotics - can alter cell wall integrity, which can alter the gram reaction as well as organism morphology. Often, gram-positive cells will stain gram-negative.
III. Determining the Clinical Significance of an Isolate

The isolation of bacteria and fungi from patient specimens does not mean the isolate(s) is (are) causing clinical disease. Determining the significance of an isolate is based upon many factors. This is a difficult task and necessary to provide quality patient care while avoiding the use of unnecessary antimicrobial therapy. Often, this determination is made on a case-by-case basis. However, there are a few general guidelines.

Terminology

**Normal (Microbial) Flora:** exists in a symbiotic relationship with the host. These organisms are isolated from the host in the absence of disease and is referred to as colonization. The normal flora plays an important role in protecting the host from infection with pathogenic microorganisms by competing for the same nutrients and receptor sites on host cells, and by producing bacteriocins, bacterial products that are toxic to other organisms. Under normal conditions, a balance is maintained that limits the quantity or dominance of any one organism.

**Pathogen:** any microorganism causing disease, usually requiring treatment

**Possible Pathogen/Oppportunistic Pathogen:** microorganism that can be a pathogen in the correct clinical setting or situation

Considerations When Determining Clinical Significance of the Isolate

**The isolate**

1) Is it a potential pathogen based on this body site? For example: Enteric organisms from respiratory specimens  
*Staphylococcus aureus* from a swabbed skin site, respiratory specimen, GI specimen

2) Is it considered a primary pathogen based on this body site? For example: *Legionella pneumophila* from respiratory specimen  
*Salmonella* sp. from GI tract  
*Neisseria gonorrhoeae* from any source

**The source/specimen**

1) Does the specimen come from a sterile body site (i.e., no normal flora)? For example: Cerebral spinal fluid (CSF)  
Pleural, Peritoneal, Synovial

2) Does the specimen come from a sterile body site, but can become contaminated with normal flora via the collection method? For example: Sputum (contaminated with normal oral flora)  
Urine (contaminated with genital/GI/skin flora)  
Blood (contaminated with skin flora with improper collection...usually number of positive bottles is very low, i.e., 1 out of 4 bottles)

3) Can you determine the quality of the specimen via results of a direct specimen examination? Sputum Gram stain (# squamous epithelial cells/#PMNs)  
Wound Gram stain (# squamous epithelial cells/#PMNs)
Considerations (cont.)

The patient

1) Is the patient immunosuppressed?

2) Is the patient's normal flora out of balance? For example:
   - On antibiotic therapy
     - Hospitalization (patients often become colonized with Enterobacteriaceae endemic to the hospital.... *Serratia* sp., *Enterobacter* sp.)

3) Is the patient in a “group” that is particularly susceptible to this pathogen? For example:
   - Unvaccinated children <5 years of age & *Haemophilus influenzae* type b
   - Pregnant women & *Listeria monocytogenes*

4) What is the geographic location of the patient?
   - Is the patient an inpatient or outpatient?
   - Can this be a nosocomial infection?

Additional References:
III. LABORATORY WORK

I. Perform Gram Stains

A. Supplies

1. Per table
   a. Plate of Neisseria sp.
   b. Plate of Bacillus sp.
   c. Plate of Corynebacterium sp.
   c. Plate of E. coli
   d. Broth of Staphylococcus sp
   e. Broth of Streptococcus sp.

   Bacti-incinerator (used to sterilize loops)
   Glass slides (in Micro drawer at end of bench)
   Saline bottle (in Micro drawer at end of bench)
   Wax pencil (in Micro drawer at end of bench)

2. Per student:
   Microscope
   Loop

B. Gram Stain

1. Located on each side of the table are equipment and reagents necessary for performing the Gram stain.

2. Gram stain each of the following:

   Neisseria sp. (rec’d on plate)
   Bacillus sp. (rec’d on plate)
   Corynebacterium sp. (rec’d on plate)
   E. coli (rec’d on plate)
   Staphylococcus sp. (rec’d in broth)
   Streptococcus sp. (rec’d in broth)

   See Module 3, page 4 for slide preparation/Gram stain procedure

Hint: you may use a wax pencil (in micro drawers) to section one slide into 4 sections which will allow you to stain 4 different organisms on one slide. Make sure to label (with pencil...pen will be washed off) placement of organisms on the frosted-end of the slide.

   After preparing slide, you may place it on the slide warmer while it is still wet. Allow it to remain on the slide warmer for a minimum of 10 minutes after drying to ensure proper fixation of organism to slide.

3. Evaluate your Gram stains and record results on the next page.
Module 3 Gram Stain: Laboratory Record Sheet

Note:  1. Gram reaction and morphology are noted on all Gram stains (i.e., gram-positive rod)

2. Arrangement is always noted for gram-positive cocci. More than one arrangement type can be noted (i.e., gram-positive cocci in pairs, gram-positive cocci in pairs and chains)

3. For other organisms, arrangement is only noted if there is a consistent pattern. For example:
   - gram-negative diplococci (many are arranged in pairs)
   - gram-positive rods palisading (many are arranged like Chinese letters)

<table>
<thead>
<tr>
<th>Organism</th>
<th>Gram Reaction</th>
<th>Morphology</th>
<th>Arrangement</th>
<th>Notes (i.e. spores, etc.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus sp.</td>
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<tr>
<td>Streptococcus sp.</td>
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<tr>
<td>Bacillus sp.</td>
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<tr>
<td>E. coli</td>
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<tr>
<td>Corynebacterium sp.</td>
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<tr>
<td>Neisseria sp.</td>
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</tbody>
</table>

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