GRAM STAIN

I. PRINCIPLE

The Hacker modification provides greater stability and better differentiation of organisms. Bacteria either stain gram-positive or gram-negative on the basis of differences in their cell wall compositions. Gram-positive species have a thick peptido-glycan layer and large amounts of teichoic acids; they are unaffected by alcohol decolorization and retain the initial stain, appearing deep violet if their cell walls are undamaged. Gram-negative cell walls have a single peptidoglycan layer attached to an asymmetric lipopolysaccharide-phosolipid bilayer outer membrane interspersed with protein; the outer layer is damaged by alcohol decolorizer, allowing the crystal violet-iodine complex to leak out and be replaced by counterstain.

II. CLINICAL SIGNIFICANCE

The gram stain is used to classify bacteria on the basis of their forms, sizes, cellular morphologies, and Gram reactions; in a clinical microbiology lab it is additionally a critical test for the rapid presumptive diagnosis of infectious agents and serves to assess the quality of clinical specimens.

III. SPECIMEN

A. Smears for gram stain may be prepared from:
    1. clinical specimens

IV. REAGENT

A. Crystal violet stain-purchased commercially
B. Safranin stain-purchased commercially
C. Gram Iodine-purchased commercially, prepared by adding the iodine concentrate to the gram iodine diluent and mix well.
D. Decolorizer-prepared by mixing one part 95% ethyl alcohol with one part acetone.
E. Storage
    1. Reagents are stored at room temperature.
    2. Gram iodine solution is stable for 3 months once prepared.

V. INSTRUMENTATION/EQUIPMENT

A. Equipment:
    1. Slide warmer
B. Materials:
    1. 1" x 3" clean glass slides with frosted end
    2. NERL Water

3. sterile disposable pipets and applicator sticks
4. immersion oil

VI. CALIBRATION: N/A

VII. QUALITY CONTROL

A. All Gram stain reagents are quality controlled by lot # as they are received in the laboratory
B. Staff will perform a gram positive and gram negative (QC slide) control each day of testing.
C. To perform QC, stain a prepared smear of *Escherichia coli* , *Staphylococcus aureus.* The results of the staining procedure should show Gram negative bacilli (red-pink) and Gram positive cocci (blue-purple). The slide background serves as a negative control.
D. Gram stain QC results are logged on the appropriate daily/monthly QC chart at the workbench.
E. Do not report patient results if quality control is not acceptable.
F. Monthly proficiency testing is performed by all staff responsible for testing to assure consistent reporting.

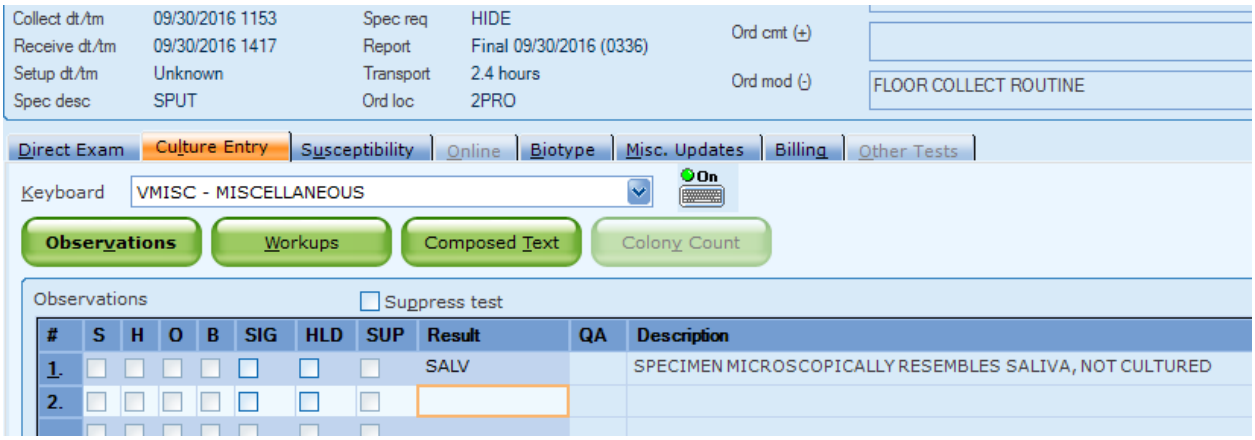
VIII. PROCEDURE:

A. Preparation:
    1. Smears should be prepared using a new 1" x 3" glass slide. Alcohol washed slides are essential if swabs are to be plated or placed in liquid media. Each slide will be labeled with patients last name, first name, accession number assigned to order, and specimen type.
    2. Prepare a thin film over a large area of the slide by using a sterile wire loop or applicator stick. If the swab is used, roll the swab across the slide to avoid destruction of cellular entities.
    3. Prepare smears from swabs containing dried material or small amounts of specimen by emulsification in a drop of normal saline on the slide.
    4. For smears of specimens such as sputum or feces, select any flecks of mucous or blood-tinged particles, since they are more likely to yield infectious agents.
    5. Prepare smears from concentrated centrifuged specimens by using a sterile disposable pipet to place a drop on the slide. Use the tip of the pipet to spread the drop to an even film.
    6. Allow the smears to dry on the slide warmer before staining.

B. Stepwise
    1. Flood the fixed smear with crystal violet stain. Allow the stain to remain for 15 seconds to 1 minute.
    2. Rinse the slide gently with tap water.

3. Flood the slide with working gram iodine solution and allow to stand for 15 seconds to 1 minutes.
4. Rinse the slide gently with tap water.
5. Rinse the slide gently with gram decolorizer. Decolorization is complete when the solution runs clear from the slide. (Depends on thickness of the smear).
6. Rinse the slide gently with tap water.
7. Flood the slide with safranin stain and allow to stand for 15-30 seconds.
8. Rinse the slide gently with tap water.
9. Drain the slide and air dry it in an upright position or blot dry.
10. Examine the slide microscopically.
11. Examine the smear microscopically
12. Using the 10X objective, quantitate the number of inflammatory and epithelial cells.
13. Quantitate and identify bacterial morphologies and Gram stain reactions using the 100X objective (oil immersion).

C. Screening Sputum's for acceptability:
   1. Gram stains of expectorated sputum specimens for routine culture are used to determine the acceptability of the specimen prior to culturing. Sputum gram stains are screened using low power. Specimens with greater than 10 squamous epithelial cells/low power field (10X objective) are rejected.
   2. Specimen is acceptable if columnar epithelial cells or macrophages are present. When a gram stain is performed to determine acceptability, send gram stain with specimen if deemed specimen is acceptable for culture.
   3. When specimen is not acceptable, notify the nurse regarding rejected sputum culture testing and that it needs to be reordered when the specimen is recollected
      a. Within Micro Result Entry in Sunquest:
      b. Look up patient by accession number.
      c. The screen that comes up is the Direct Exam page. To turn the micro keyboard off, press ";". Then type "HIDE".
      d. Then change to the tab, to Culture Entry. Turn the keyboard off by pressing the ";" key. Then type "SALV" = specimen microscopically resembles saliva, not cultured.



e. Tab to the next line, free text date, time and who was called for the specimen rejection.
f. Finalize the culture by pressing the "/" key and save.
g. Lastly, retain results but credit the test.

IX. RESULTS FOR CSF OR STAT SURGICAL SPECIMEN

A. To pull up the micro keyboard press "F8".
B. Quantify epithelial cells and white blood cells using 10x objective as follows:
    1. Few = 0-10/lpf
    2. Moderate = 10-25/lpf
    3. = >25/lpf
C. Quantitate and identify bacterial morphologies as follows:
    1. Few = 1-5 in every field
    2. Moderate = 6-30 organisms/oil field
    3. Many = >30 organisms/oil field
D. Observe gram reaction as either gram positive (deep violet) or gram negative (pink or red). Gram variable organisms may also be observed.
E. Staining characteristics: even, bipolar, beaded or irregular staining may occur.
F. Shape of organisms: may appear as:
    1. Coccus
    2. Coccobacillary
    3. Rod
    4. Filament or yeast-like or pleomorphic (variation in shape).
G. Characteristic arrangements may be noted:
    1. Singles
    2. Pairs
    3. Chains
    4. Tetrads

5. Clusters
6. Palisades or Chinese letters.

H. Document QC done with every patient on Gram Stain worksheet above gram stain area.

X. REPORTING RESULTS

A. All shifts report a preliminary gram stain report, the final observation should be "preliminary report; slide to be reviewed by the Microbiology Department at Methodist. The “I” button is the preliminary report key.

B. Quantify epithelial cells as follows:
   1. Few = 0-10/lpf
   2. Moderate = 10-25/lpf
   3. Many = >25/lpf

C. Quantitate and identify bacterial morphologies and Gram stain reactions using the 100 X objective (oil immersion) as follows:
   1. Few = 1-5 in every field
   2. Moderate = 6-30 organisms/hpf
   3. Many = >30 organisms/hpf

D. Record results in LIS.

XI. PROCEDURAL NOTES/PROBLEM SOLVING TIPS:

A. Gram stain observations should be consistent with culture results.

B. LIMITATIONS OF THE PROCEDURE:
   1. Use results of gram stains in conjunction with other clinical and laboratory findings. Use additional procedures (e.g. special stains, direct antigen tests, etc.) to confirm findings suggested by gram stained smears.
   2. False gram stain results may be related to inadequately collected specimens.

C. Excessive Decolorization
   1. Excessive heat during fixation: Heat fixing the cells, when done to excess, alters the cell morphology and makes the cells more easily decolorized.
   2. Low concentration of crystal violet: Concentrations of crystal violet up to 2% can be used successfully, however low concentrations result in stained cells that are easily decolorized. The standard 0.3% solution is good, if decolorization does not generally exceed 10 seconds.
   3. Excessive washing between steps: The crystal violet stain is susceptible to wash-out with water (but not the crystal violet – iodine complex). Do not use more than a 5 second water rinse at any stage of the procedure.
   4. Insufficient iodine exposure: The amount of the mordant available is important to the formation of the crystal violet – iodine complex. The lower the concentration, the easier to decolorize (0.33% - 1% commonly used). Also, QC of the reagent is important as exposure to air and elevated temperatures hasten the loss of Gram’s iodine solution. A closed bottle (0.33% starting concentration) at room temperature will lose >50% of available iodine in 30 days, an open bottle >90%. Loss of 60% iodine results

in erratic results.

5. Prolonged decolorization: 95% ethanol decolorizes more slowly, and may be recommended for inexperienced technicians while experienced workers can use the acetone-alcohol mix. Skill is needed to gauge when decolorization is complete.
6. Excessive counterstaining: As the counterstain is also a basic dye, it is possible to replace the crystal violet-iodine complex in gram positive cells with an over-exposure to the counterstain. The counterstain should not be left on the slide for more than 30 seconds.

D. Leukocytes
1. If properly decolorized, leukocytes will appear gram-negative (pink-to-red), due to the retention of the safranin counterstain.

E. Troubleshooting
1. If the smear is too thick, the cells can appear gram-positive in very thick area. You may see Gram-variability from the thick to the thin areas.
Solution: Try to prepare a single cell layer of organism.
2. If cells are prepared in hyper or hypotonic solutions, morphology may be disturbed. Solution: Smear the cells onto the slide dry with a sterile toothpick.
3. Over warming the smear (this happens most often when smears are warmed prior to being completely air dried, or when flaming too much to fix the slides) will cause all cells to appear Gram-negative.
Solution: Dry slide thoroughly prior to "heat fixing", be extremely careful when using flames.
4. Gram-variability, this can be due to the organism itself, and not to the staining method.
Solution: The vast majority of Gram-variable organisms are Gram-positive. Characteristically Gram-variable organisms (e.g. Corynebacterium variabilis) or those whose membrane alter with age and appear Gram-variable (e.g. Arthrobacterium spp.) are grouped with the Gram-positive organisms. Therefore, they are treated as Gram-positive organisms.

XII. REFERENCES

A. Clinical Microbiology Procedures Handbook, section 1.5 ASM, 1994.
B. Bailey and Scott's Diagnostic Microbiology 12th edition, 2007. Page 80-83.
C. The Microbiology Network, "The Gram Stain", Feb 2006. www.microiol.org. 04/2017.
D. CAP, 325 Waukegan Road, Northfield Illinois 60093-2750. Survey - Gram Stain D5-C 2016, slide D5-13 discussion.
E. Arrow Scientific. "Differentiating Gram-negative and Gram-positive Bacteria". 2013 www.arrowscientific.com. 04/2017

POLICY CREATION :		***Date***
Author:	***Angie Guppy, MLT (ASCP)***	***11/17/18***
Medical Director:	***Kathryn Kramer, MD***	***11/17/18***

MEDICAL DIRECTOR		
DATE	NAME	SIGNATURE
SECTION MEDICAL DIRECTOR		

REVISION HISTORY (began tracking 2011)			
Rev	Description of Change	Author	Effective Date

Reviewed by

Lead	**Date**	**Coordinator/ Manager**	**Date**	**Medical Director**	**Date**