PROVIDENCE
Sacred Heart
Medical Center &
Children's Hospital

Gram Stain Procedure

Department of Microbiology

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1.0 Purpose or Principle

The Gram stain is used to classify bacteria on the basis of their cellular morphology and Gram reaction. Crystal violet serves as the primary stain that is bound to the bacterial cell wall after treatment with a weak solution of iodine. The iodine serves as a mordant to bind the dye. The slide is then flooded with an acetone-alcohol decolorizer. Gram-positive bacteria have a thick peptidoglycan 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 by age, antimicrobial agents, or other factors. Gram-negative organisms have a single peptidoglycan layer attached to an asymmetric lipopolysaccharide-pospholipid bilayer outer membrane interspersed with protein. The outer membrane is damaged by the alcohol decolorizer, allowing the crystal violet-iodine complex to leak out and be replaced by the safranin counterstain. Gram-negative organisms stain pink or red.

Interpretation of Gram-stained smears involves consideration of staining characteristics and cell size, shape, and arrangement. These characteristics may be influenced by many factors, including culture age, medium, incubation atmosphere, staining technique, and the presence of inhibitory substances. Similar considerations apply to interpretation of smears from clinical specimens, but additional factors include the presence of host cells and phagocytosis.

2.0 Clinical Significance

The Gram stain is used for the rapid presumptive diagnosis of infectious agents and to assess the quality of clinical specimens. Bacterial culture results should be correlated to the Gram stain smear results obtained from the original specimen.

3.0 Scope

This procedure is classified under CLIA as Highly Complex. It should be carried out by technical personnel familiarized and trained to interpret smears from all specimen types. Testing includes but is not limited to: basic troubleshooting, QC checks, and record keeping of technical proficiency in accordance with the department SOP. Records are to be kept within the employee's record in the department of continued competence and proficiency. Performance reviews of technical personnel are to be carried out annually.

4.0 Safety - Personal Protective Equipment

Performance of this procedure will expose testing personnel to biohazardous material and possible chemical hazards. All specimens must be handled as potentially infectious material as outlined in the Microbiology Biohazards and Safety document. The reagents and chemicals that are used in this procedure may be hazardous to your health if handled incorrectly. A brief listing of precautions for each chemical hazard is included in the reagent section of this procedure. More extensive information concerning the safe handling of the reagents and/or chemicals used in this procedure, as well as other important safety information may be obtained by consulting the

Material Safety Data Sheet (MSDS). Before performing any part of this procedure, the technologist must take any and all precautions and adhere to all prescribed policies.

This procedure may expose you to:

- Bloodborne and airborne pathogens
- Hazardous reagents

To perform this procedure, you must use:

- Gloves
- Laboratory Coat
- Biological safety cabinet

Disinfectant following procedure:

- Bleach dilution sprayers can be used for on demand disinfectant.

Reference for spill/decontamination

- MSDS
- Chemical hygiene plan

When discarding stained smears, handle as biological waste. Treat slides as sharps, since they may puncture biohazard bags. If cardboard boxes are used, seal with tape prior to disposal.

5.0 Specimen Requirements

Smears to be Gram stained may be prepared from clinical specimens, broth cultures, or colonies growing on solid media. Gram stains are of little value for direct smears of stool, throat samples, and urine. Stains are not part of standard protocols for evaluating catheter tip specimens.

6.0 Materials

6.1 Equipment

- Slide warmer for heat fixation
- Slide staining rack
- Slide dryer
- Light microscope with 10X and 100X objectives

6.2 Consumables

- Clean glass slides
- Microscope immersion oil

6.3 Reagents

- Gram Crystal Violet (BD Catalog Number 212525). Flammable and slightly toxic. Store at room temperature.
- Gram Iodine (BD Catalog Number 212530). Slightly toxic. Store at room temperature.
- Gram Decolorizer (1 part acetone + 1 part ethanol). Flammable and slightly toxic. Store at room temperature. Reagent expires 1 y from date of preparation.
- Gram Safranin (BD Catalog Number 212532). Flammable and slightly toxic. Store at room temperature.
- Methanol. Flammable and moderately toxic. Store at room temperature.

6.4 Control Materials and Usage

Quality control testing should be performed weekly and with each new lot of stain. Premade, heat-fixed slides may be used.

- *E. coli* ATCC 25922 – pink, Gram-negative rods
- *S. aureus* ATCC 25923 – deep violet, Gram-positive cocci

7.0 Smear Preparation and Staining

7.1 Smear Preparation

1. Place a small accession label on the frosted end of a glass slide. Place your initials in the blank area of the accession label. Be careful not to touch the clear glass portion of the slide where the specimen will be applied. Examine the slide to make sure it is not dirty or contaminated prior to specimen application.
2. Working within a biosafety cabinet, prepare a thin smear of the specimen on the clean, labeled glass slide. Smear material in a quarter-sized area in the center of the slide.
    a. Swab specimens: press and roll the swab against the glass slide to express material soaked into the swab. Always prepare the Gram stain and then sequentially inoculate culture plates so that the smear is representative of the original cellular and microbial composition of the clinical specimen.
    b. Specimens not received on swabs (aspirates, exudates, sputa, etc.): select purulent material with a sterile applicator and spread the sample on the slide. Material should be visible on the slide, but avoid making smears that are too thick.
    c. Body fluids and CSF: centrifuge or prepare a cytospin smear (refer to the Specimen Processing Procedures).
    d. Tissue biopsy specimens: prepare a touch preparation. Place tissue in a sterile petri dish, and mince with a sterile surgical scalpel. Touch the sides of one or more of the minced fragments to a clean glass slide.
    e. Urine: use a calibrated loop to place 0.01 mL of well-mixed specimen onto the glass slide. A pre-etched slide should be used for ease of locating the specimen microscopically.
    f. Broth cultures: use a sterile pipette (or a blunt venting needle for containers with septa) to transfer 1 or 2 drops to the slide. Spread the drop into an even film.
    g. Colonies from solid media: place a drop of sterile saline on slide. Transfer a small portion of colony with a wooden applicator or sterile loop. Gently mix to emulsify. The resulting smear should be slightly cloudy and homogenous.
3. Smear fixation:
    a. Air dry slides in a biosafety cabinet on a slide warmer until dry.
    b. Alternatively, fix with methanol to produce a cleaner background, and prevent specimens from washing off. Place a few drops of methanol on air-dried slide for 1 min, drain off remaining methanol without rinsing, and allow slide to air dry again. Do not use heat before staining.

7.2 Staining Procedure

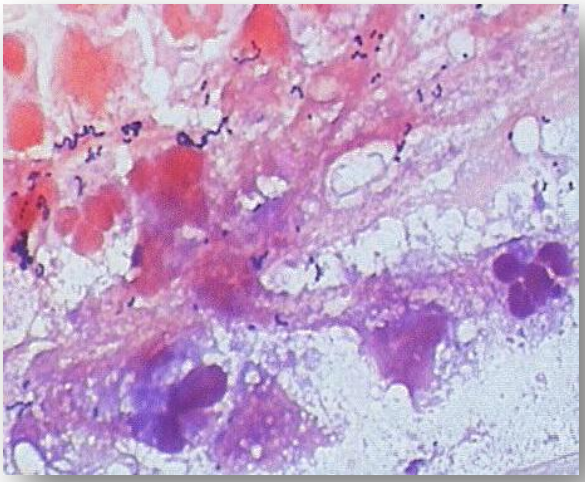
1. Place prepared smears on a staining rack over the sink.
2. Flood the slide with Crystal Violet for 30 s and rinse with tap water.
3. Flood the slide with Gram's Iodine for 30 s and rinse with tap water.
4. Hold the slide at an angle and decolorize with acetone-alcohol by letting the reagent flow over the smear until the purple stain no longer runs down the slide. Adjust decolorization time to thickness of the smear. Rinse with tap water.
5. Flood the slide with Safranin to counter stain for 30-60 s and rinse with tap water.
6. Dry smear in the slide dryer.

8.0 Interpretation of Results

8.1 Smear Quality Assessment and Examination

1. Perform the initial evaluation of the smear under low power (10X objective).
2. Evaluate the stain quality of the smear. If an excess of precipitated stain is observed, decolorize and restain the slide. Determine that the smear has been properly decolorized. Depending on the source of the specimen, the background should be generally clear or gram negative. If neutrophils are present, they should appear completely gram negative. If the slide appears over decolorized, completely decolorize and restain the smear.

Under-decolorized Smear



3. Determine that the thickness of the smear is appropriate. For proper interpretation, areas must be no more than one cell thick, with no overlapping of cells. Prepare a new slide if the smear is unreadable.
4. If no material is evident on the slide, retrieve the specimen and remake the smear.
5. For smear prepared from clinical specimens, examine several fields under low power for evidence of inflammation. Determine areas representative of inflammation or purulence and areas of apparent contamination with squamous epithelial cells. If no purulence is seen, choose areas of apparent necrosis, inflammatory cell debris, and mucus. If cells are present, determine the average count of neutrophils and epithelial cells in 20 to 40 representative fields that contain cells.

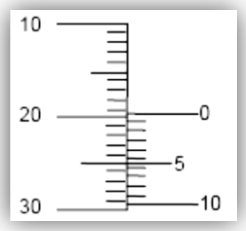
8.2 Microorganism Characterization

1. Observe microorganisms for characteristic staining and morphology under oil immersion.
2. Gram positive organisms are deep violet.
3. Gram negative organisms are pink or red.
4. Refer to the appropriate section of the Quality Evaluation Systems for the interpretation and reporting of Gram stains according to the specimen site.

9.0 Documenting Microscope Coordinates

NOTE: This procedure is used when there are low numbers of organisms present, and a supervisor is not available to review the smear. Documenting the location of the organism(s) in question enables review at a later time. Always record on which scope the coordinates were taken and the orientation of the etched area of the slide on the stage.

1. Place the organism or item of interest in the center of the field.
2. Record the coordinates. When interpreting the scale on the right side of the stage, the left side of the scale is read first. If the zero on the right side of the scale is between numbers, record the lower number on the left side. Then the right side of the scale is interpreted by selecting the number that aligns exactly with a line on the left side. Record this number after the first number. Do the same with the scale on the top of the stage. Interpret the bottom of the scale first, then the upper part of the scale.



Example: These coordinates would be recorded as 19.5

10.0 Specimen Quality Evaluation System Using Q-score

Lower respiratory and wound specimens should be evaluated for quality based on the quantity of neutrophils versus squamous epithelial cells present. Q-score is not used for tissue or fluid specimens.

1. Place one drop of immersion oil on the slide, and examine the specimen under low power (10X objective) magnification. Evaluate the neutrophils and squamous epithelial cells as follows:
   - No cells* = 0
   - 1-9 cells/lpf = 1 (few)
   - 10-24 cells/lpf = 2 (moderate)
   - $\geq$ 25 cells/lpf = 3 (abundant)

   *If only 1 or 2 squamous epithelial cells are seen on the smear, report as 0. Squamous epithelial cells must contain nuclei to be reported.

2. Determine the Q value of the specimen according to the following Q-score chart. Note that the Q score is always 3 when there are no squamous cells present.

Squamous Epithelial Cells (-)

Neutrophils (+)	0	1	2	3
0	3	0	0	0
1	3	0	0	0
2	3	1	0	0
3	3	2	1	0

11.0 Lower Respiratory Specimens

11.1 Specimens with a Q-Score of 0

Q-0 specimens are processed. However, the client should be notified regarding the poor quality of the specimen with a comment added to the report. Report the cells, mixed flora if present (do not list potential pathogens), and add the following comment:

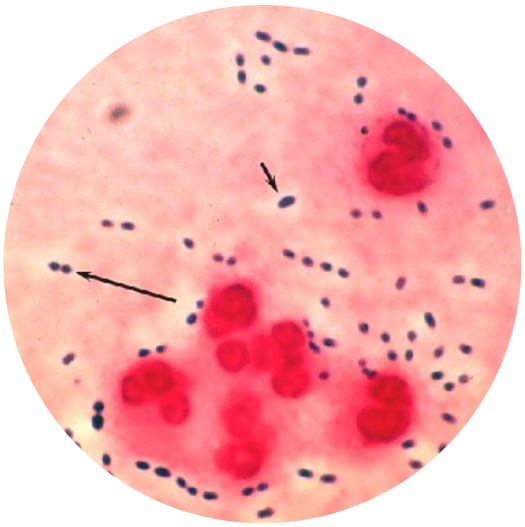
Squamous cells in the specimen indicate the presence of superficial material that may contain contaminating or colonizing bacteria unrelated to infection. Collection of another specimen is suggested, avoiding superficial sources of contamination. [Q0]

Exceptions:
1. If the smear has $\geq$ 25 cells/oil immersion field that resemble *S. pneumoniae*, leave smear for Rounds review.
2. Do not use Q0 comment for cystic fibrosis (CRCF) cultures.

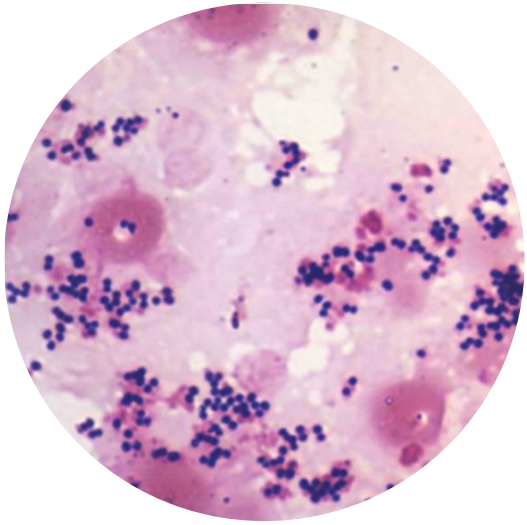
11.2 Specimens with Q-Score 1, 2, or 3

Report the quantity of cells observed on low power (10X objective). Evaluate and report microorganisms with oil immersion (100X objective) objective according to the following:

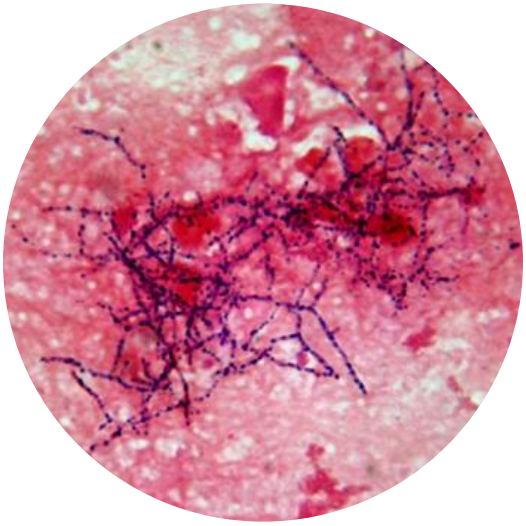
- **Mixed flora** = report, regardless of number, if Gram positive rods, Gram negative rods with pointed ends and cigar shaped rods with bars, strep, yeast without pseudohyphae, bacteria adhering to squamous cells, and potential pathogens less than the required number per oil immersion field are seen.



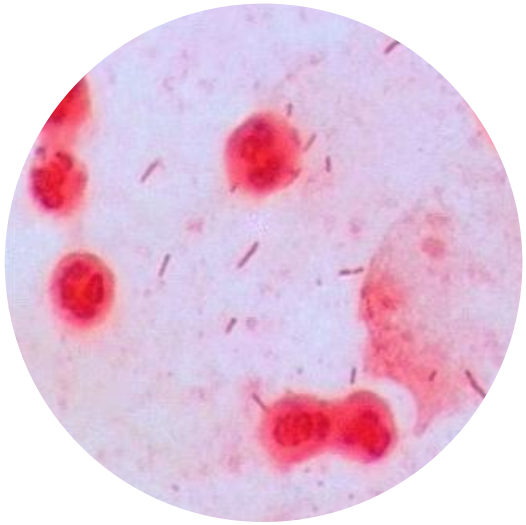
Gram-positive cocci resembling Strep pneumo: pairs of lancet-shaped diplococci, sometimes encapsulated, or with a "chewed up" appearance, report if ≥ 25 cocci/oif. Do not report "Strep." If it cannot be called Pneumo, report as "Mixed Flora".



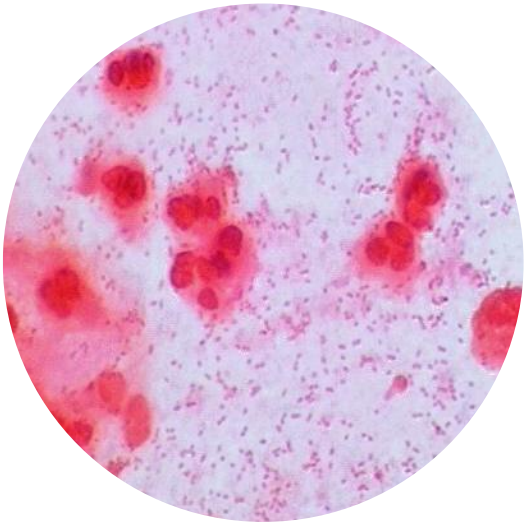
Gram-positive cocci resembling Staph: Gram-positive cocci in clusters, report if > 50 cocci/oif.



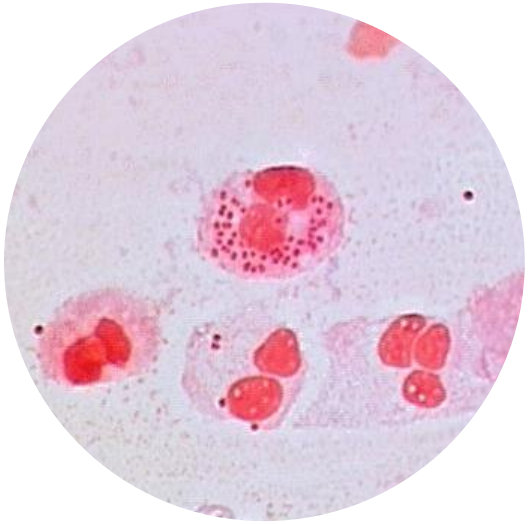
Gram-positive Branching Rods resembling Nocardia: beaded Gram positive rods that frequently appear as tangled masses of organisms, do not report; leave for Rounds to review.



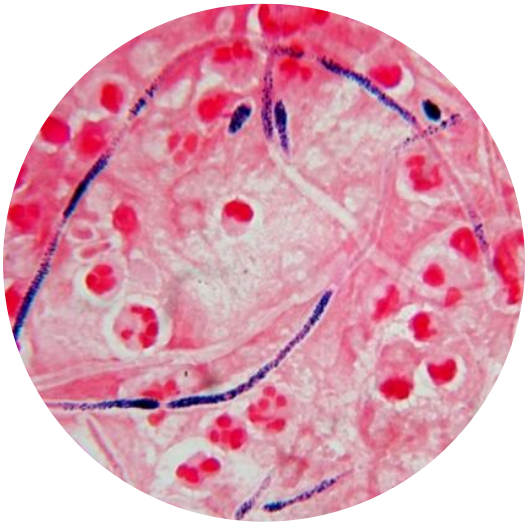
Gram-negative rods resembling Enterics/Pseudomonas = large rods with a uniform size and shape and sometimes bipolar staining or thin, faint staining rods of medium size, often arranged end to end, report if ≥ 10 organisms/oif.



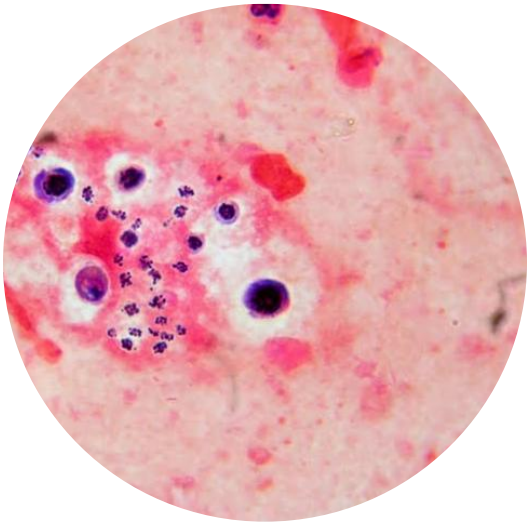
Gram-negative rods resembling *Bacteroides/Haemophilus*: small Gram-negative coccobacilli/ pleomorphic rods, report if ≥ 10 organisms/oif.



Gram-negative diplococci resembling *Moraxella, Acinetobacter,* or *Neisseria:* gram-negative diplococci, report if ≥ 25 organisms/oif.



Yeast: large, gram positive to gram variable cells, report if at least one yeast cell with pseudohyphae per oil immersion field.



Yeast: if yeast cells appear round and/or encapsulated, hold smear for Rounds review for possible *Cryptococcus*

Exceptions:

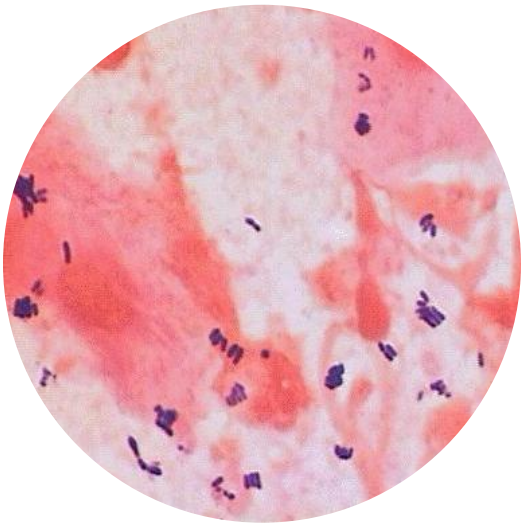
1. For a transtracheal aspirate or lung aspirate with no squamous cells, report any bacteria seen, regardless of their number.

11.3 Smears Suggestive of Aspiration Event

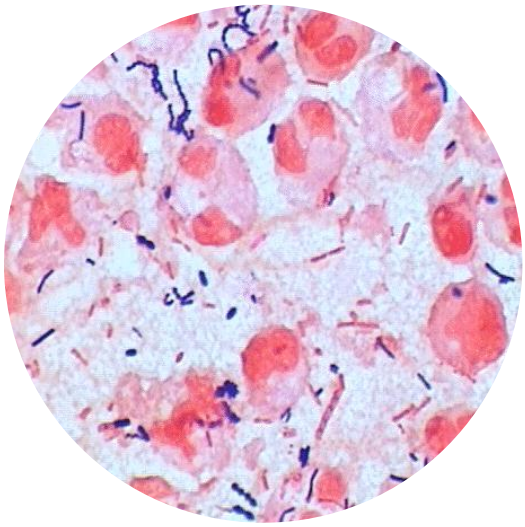
Report cells and bacteria according to lower respiratory protocol listed above. Hold smear for Rounds review if the following criteria are met:

1. No more than few squamous epithelial cells/lpf
2. Abundant neutrophils/lpf
3. Mixed gram-positive and gram-negative flora (> 50 organisms/oif) OR diphtheroids (> 50 organisms/oif)
4. Some of the organisms are seen intracellular in neutrophils

After Rounds confirmation, attach the following comment to the direct smear report:
Gram stain suggestive of an aspiration event. [ASPGR]



Suggestive of an Aspiration Event: diphtheroids > 50 organism/oif.



Suggestive of an Aspiration Event: polymicrobial, >50 organisms/oif.

12.0 Wounds, Tissues, and Body Fluids

Do not calculate Q-score for body fluids, tissues, or valve vegetations (i.e. sterile body sites). Do not quantitate cells for concentrated fluid specimens. For CSF specimens, add comment: **Specimen concentrated by Cytospin.** For other body fluids that are centrifuged add the comment: **Specimen centrifuged for direct smear and culture.**

12.1 Specimens with a Q-Score of 0

Q0 specimens are processed. However, the client should be notified regarding the poor quality of the specimen with a comment added to the report. Report the cells, mixed flora if present (do not list potential pathogens), and add the following comments:

1. **Specimen is unsuitable for anaerobic culture due to superficial contamination as indicated by the presence of squamous epithelial cells. [SPUNS]**
2. **Squamous cells in the specimen indicate the presence of superficial material that may contain contaminating or colonizing bacteria unrelated to infection. Collection of another specimen is suggested, avoiding superficial sources of contamination. [Q0]**

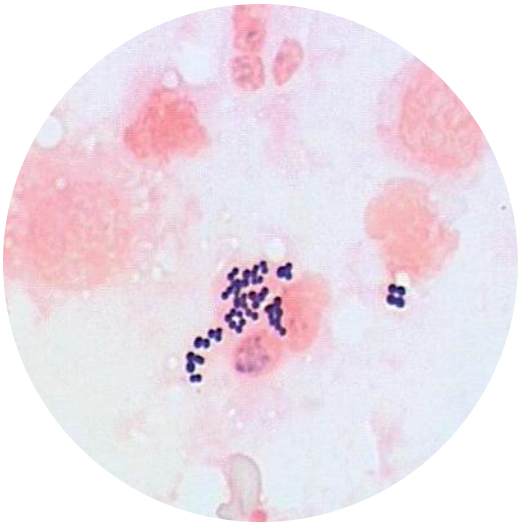
12.2 Specimens with a Q-Score of 1, 2, or 3 and Sterile Body Sites:

1. Report the quantity of cells observed on low power (10X objective). If the specimen has squamous epithelial cells, add the comment:

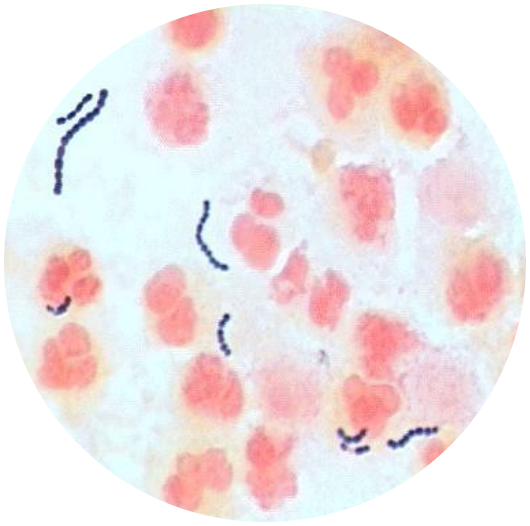
   Specimen is unsuitable for anaerobic culture due to superficial contamination as indicated by the presence of squamous epithelial cells. [SPUNS]

2. Evaluate and report microorganisms under oil immersion (100X objective) according to the following morphologic descriptions. Any number or type of microorganisms seen is significant.

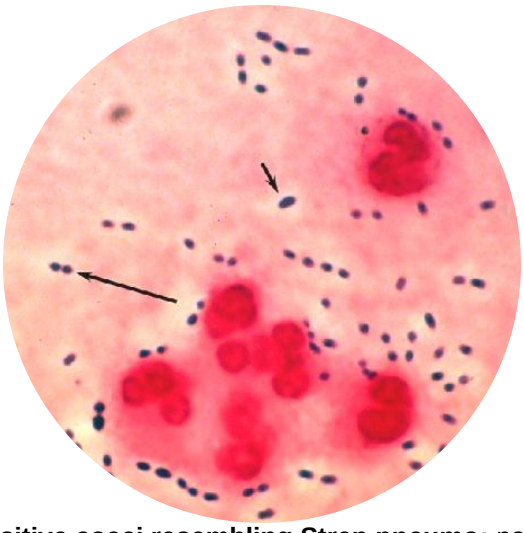
Critical value: When bacteria are seen in CSF, ventricular fluid, subdural fluid, or LP, notify the supervisor or charge technologist and handle the result as a critical value. Contact the unit if inpatient, or call the doctor for outpatients. Document phone calls in the computer. If *Neisseria* is suspected from an inpatient, notify Epidemiology immediately.



Gram positive cocci resembling Staph: gram-positive cocci in clusters.



Gram positive cocci resembling Streptococcus: gram-positive cocci in chains.



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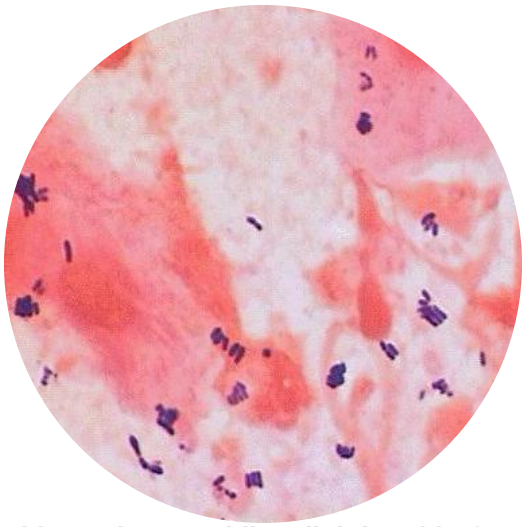
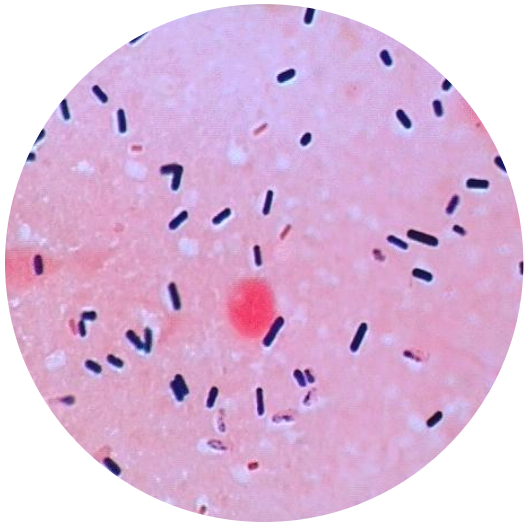
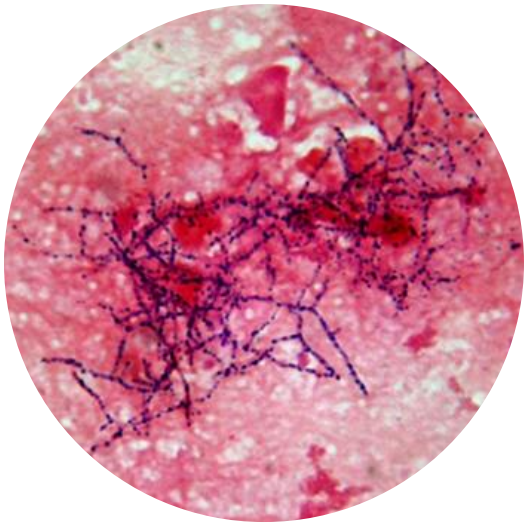


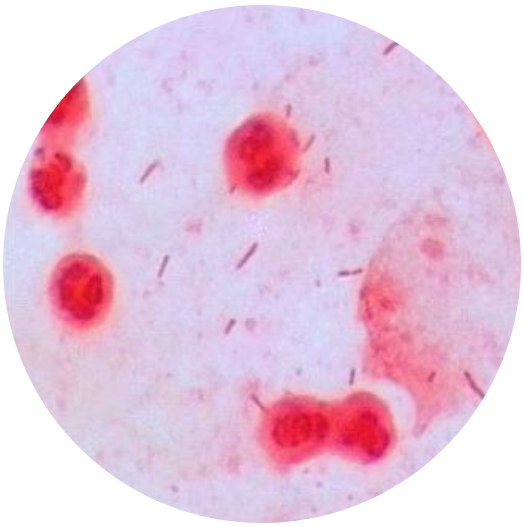
Gram-positive rods resembling diphtheroids: irregularly shaped, may be palisading gram-positive rods.



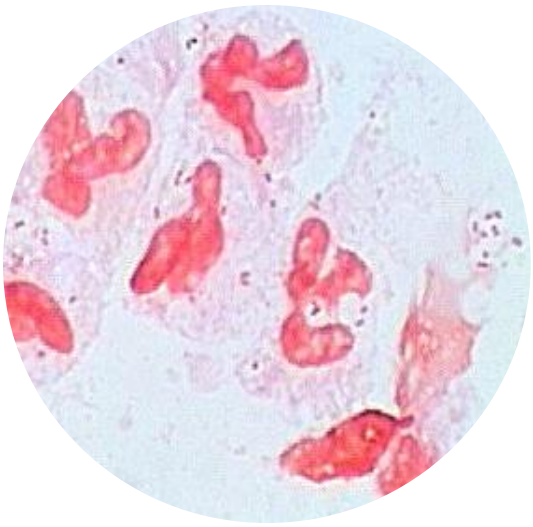
Gram positive rods resembling *Clostridium/Bacillus*: large, gram-positive bacilli, often with“box car” morphology.



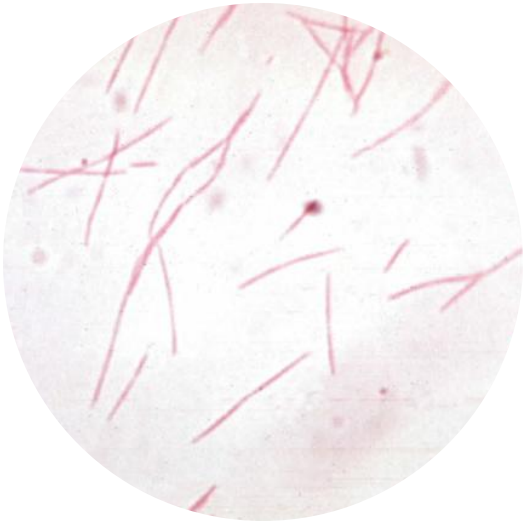
Gram-positive Branching Rods resembling Nocardia: beaded Gram positive rods that frequently appear as tangled masses of organisms, report IF Kinyoun modified acid-fast positive.



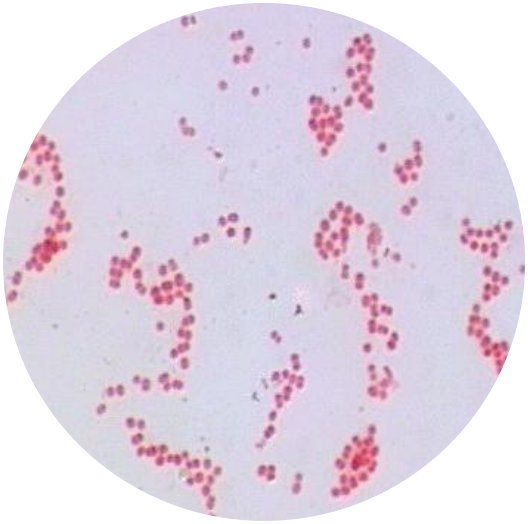
Gram negative rods resembling Enterics/Pseudomonas: large Gram negative rods with a uniform size and shape and sometimes bipolar staining or thin, faint staining Gram negative rods of medium size, often arranged end to end.



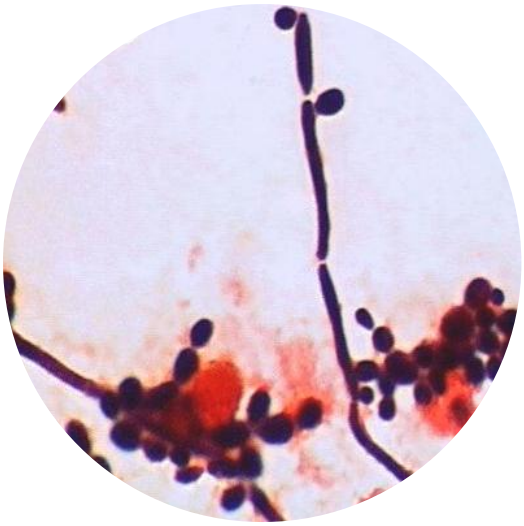
Gram-negative rods resembling *Bacteroides/ Haemophilus*: small Gram-negative coccobacilli/ pleomorphic rods



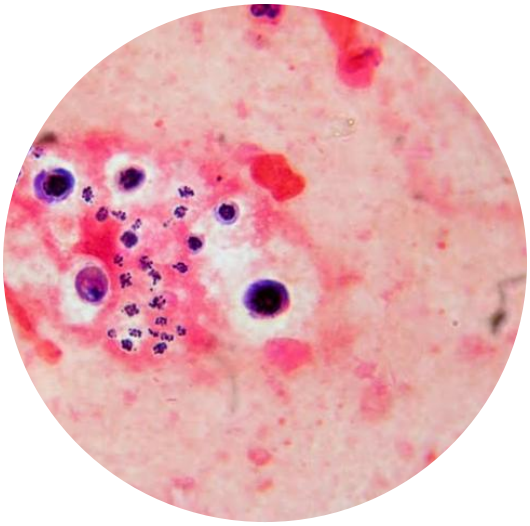
Gram negative rods resembling *Fusobacterium*: long, slender gram-negative rods with pointed ends, often faintly staining.



Gram negative diplococci resembling *Moraxella, Acinetobacter, Neisseria*: biconcave pairs of gram negative diplococci.



Yeast: large, gram positive to gram variable oval cells that may have pseudohyphae.



Yeast: if yeast cells appear round and/or encapsulated, hold smear for Rounds review for possible *Cryptococcus*

13.0 Genital Specimens

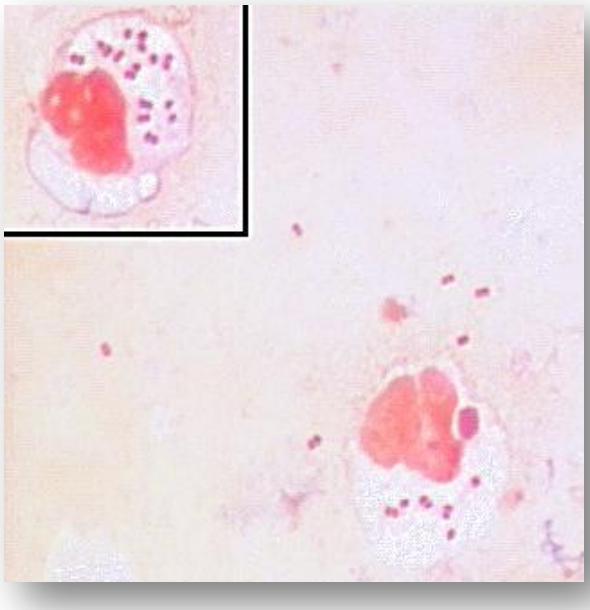
13.1 Genital specimens requesting or processed for *Neisseria gonorrhoeae*

1. Prepare a smear and Gram stain it.
2. Place one drop of immersion oil on the slide, and examine the specimen under low power (10X objective) magnification for neutrophils as follows:

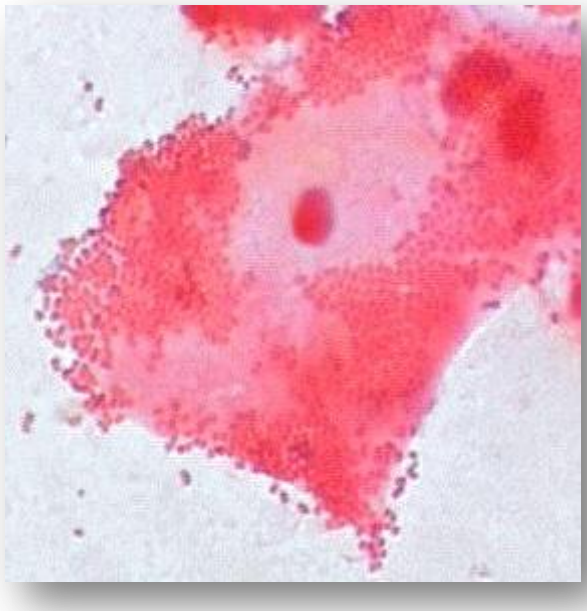
   - No cells
   - 1-9 cells/lpf = 1 (few)
   - 10-24 cells/lpf =2 (moderate)
   - ≥ 25 cells/lpf = 3 (many)

3. Examine the specimen under oil immersion (100X objective) for Gram-negative diplococci, and report as follows:
   - Do not report the presence or absence of *N. gonorrhoeae* in patients less than 13 years old. Report: **Gram stain smears not routinely performed on patients < 13 years old. [GEN13]**
   - Female genital sites:
     - Demonstrating no Gram negative diplococci **OR** with ONLY EXTRACELLULAR Gram negative diplococci, report: **Not suggestive of *Neisseria gonorrhoeae*, to be confirmed by culture. [NOTGC]**
     - Demonstrating 3 or more neutrophils with INTRACELLULAR Gram negative diplococci, report: **Organisms present that could represent *N. gonorrhoeae* as well as other species of bacteria. Diagnosis should be based on culture report. [MBGC]**
   - Male genital sites:
     - Demonstrating the absence of Gram negative diplococci, report: **Not suggestive of *Neisseria gonorrhoeae*; to be confirmed by culture. [NOTGC]**
     - Demonstrating Gram negative diplococci in the presence of neutrophils, report: **Gram-negative diplococci, suggestive of *Neisseria gonorrhoeae*. [GNDCGC]**
     - Demonstrating Gram negative diplococci in the absence of neutrophils: Notify Rounds.

N. gonorrhoeae in Male



N. gonorrhoeae in Female



13.2 Genital Specimens for Routine Culture

1. Examine the specimen under low power (10X objective) for neutrophils as follows:
   - No cells = 0
   - 1-9 cells/lpf = 1 (few)
   - 10-24 cells/lpf = 2 (moderate)
   - ≥ 25 cells/lpf = 3 (abundant)
2. Do not report the presence or absence of squamous epithelial cells. Examine the specimen under oil immersion (100X objective) for Gram-negative diplococci as outlined above in A.3.
3. Do not perform or interpret genital smears on females < 13 years old.
4. Place a drop of immersion oil on the slide. Examine the smear under oil immersion (100X objective) for evidence bacterial vaginosis.
   - Using the following criteria, quantify *Lactobacillus* (long thin Gram positive rods), *Gardnerella* (small Gram negative/Gram variable bacilli)/*Bacteroides* (small Gram negative coccobacilli/ pleomorphic rods), and *Mobiluncus* (curved Gram negative rods) as follows for bacterial vaginosis:

     0 = no organisms/oil immersion field

     1+ = < 1/oil immersion field (rare)

     2+ = 1-5/oil immersion field (few)

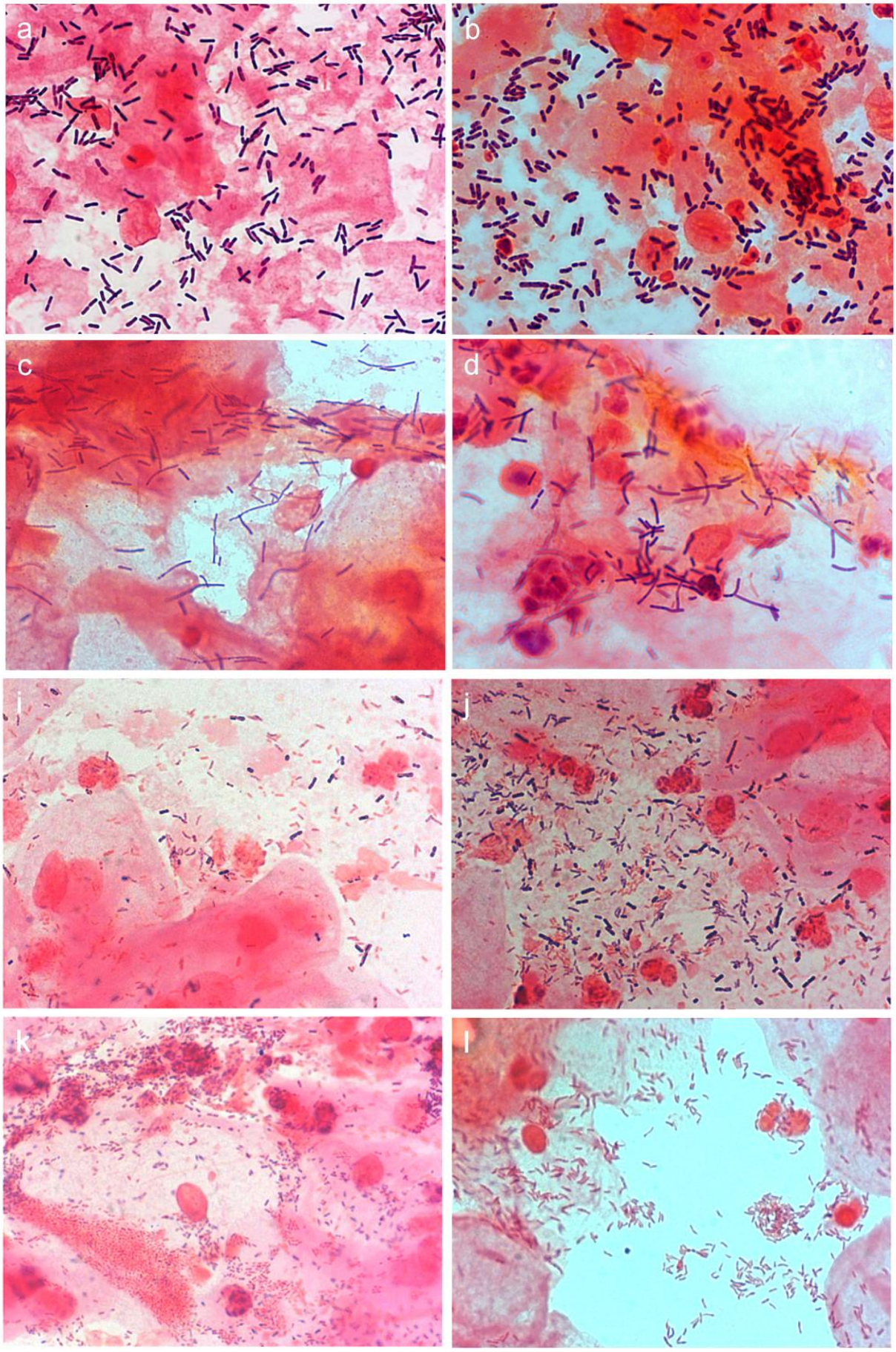
     3+ = 6-30/oil immersion field (moderate)

     4+ = > 30/oil immersion field (abundant)
   - Using the score chart for bacterial vaginosis, determine a value of 0 to 4+ for each of the 3 organism morphologies.
   - Add the 3 values to obtain the total score.

	+		+		+
Lacto	Score	*G. vag* / *Bacteroides*	Score	Curved GNR	Score
4+	0	4+	4	3 to 4+	2
3+	1	3+	3	1 to 2+	1
2+	2	2+	2	0	0
1+	3	1+	1		
0	4	0	0		

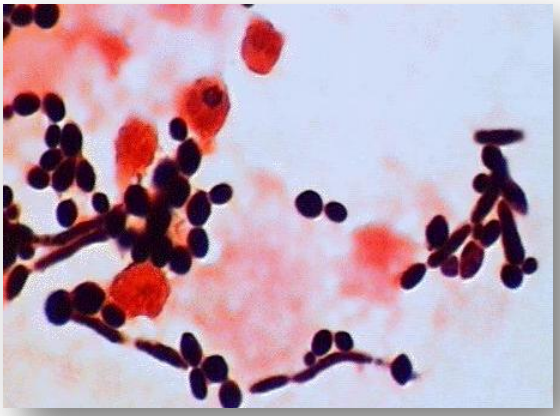
Quantitation	Total Score	Interpretation
4+ = >30 orgs/OIF 3+ = 6 to 30 orgs/OIF	0 to 3	Smear is not suggestive of bacterial vaginosis.
2+ = 1 to 5 orgs/OIF 1+ = < 1 orgs/OIF	4 to 6	Altered vaginal flora, hold smear for review.
0 = no orgs/OIF	7 to 10	Smear is suggestive of bacterial vaginosis.

If the score is 0 to 3, report: **Smear not suggestive of bacterial vaginosis. [BVNEG]**
If the score is 4 to 6, report: **Altered vaginal flora. [BVINT].**
If the score is 7 to 10, report: **Smear suggestive of bacterial vaginosis. [BVPOS]**



Figures a, b, c, d: *Lactobacillus* (not suggestive of BV). Figures i, j: mixture of *Lactobacillus* and *Gardnerella, Bacteroides-Prevotella* and *Mobiluncus* (altered flora). Figures k, l: bacterial vaginosis.

5. Also examine the smear under oil immersion (100X objective) and report the presence or absence of yeast.
   Report: **Yeast seen or No yeast seen**



13.3 Surgical Wound or Body Fluid Specimens from Genital Sites

Includes: tubo-ovarian abscess or ovarian abscess, pelvic abscess, uterine drainage or ruptured membranes, PID (pelvic inflammatory disease), IUD (intrauterine device), Fallopian tubes, and placenta (do not Q-score placentas).

1. Examine the smear for cells and organisms according to the Quality Evaluation System for Wounds and Body Fluids.
2. Check for *Neisseria gonorrhoeae*.

14.0 Fecal Specimens

1. Evaluate the specimen for neutrophils, using the oil immersion objective.
   - If no neutrophils are seen, report no neutrophils seen
   - $\underline{\leq}$ 1/oil immersion field, report few
   - 2/oil immersion field, report moderate
   - 3/oil immersion field, report abundant
2. Staph is reported only when virtually the entire oil immersion field is covered with Staph (Consult with Rounds before reporting; if a supervisor/director is unavailable, leave the smear for review).
3. Yeast - report its presence only when pseudohyphae are seen. (Consult with Rounds before reporting; if a supervisor/director is unavailable, leave the smear for review).
4. Mixed flora - report its presence or "No bacteria" if absent.
5. If curved Gram negative rods are seen, consult with the supervisor.

15.0 Urine Specimens

Gram stains are not routinely performed on urine specimens submitted for culture. However, a smear may be ordered separately.

Evaluate the specimen for cells according to the Quality Evaluation System for Wounds and Body Fluids. Evaluate for bacteria according to the following criteria. Do not evaluate bacteria present on squamous cells.

- If bacteria present are $\geq$1/oil immersion field, report the genus observed as $\geq 10^5$/mL
- If bacteria present are <1/oil immersion field, report the genus observed as $< 10^5$/mL
- **Gram positive cocci resembling Streptococcus:** gram-positive cocci in chains.
- **Gram positive cocci resembling Staph:** gram-positive cocci in clusters.

- **Yeast:** large, gram positive to gram variable oval cells that may have pseudohyphae.
- **Gram negative rods resembling Enterics/Pseudomonas:** large Gram negative rods with a uniform size and shape and sometimes bipolar staining or thin, faint staining Gram negative rods of medium size, often arranged end to end.
- **Gram-negative rods resembling *Bacteroides/Haemophilus*:** small Gram-negative coccobacilli/ pleomorphic rods.
- **Gram negative diplococci resembling *Neisseria/ Acinetobacter*:** biconcave pairs of gram negative diplococci.
- **Gram positive rods resembling *Clostridium/Bacillus*:** large, gram-positive bacilli, often with“box car” morphology.
- **Gram-positive rods resembling diphtheroids:** irregularly shaped, gram-positive rods.
- **Gram negative rods resembling *Fusobacterium*:** long, slender gram-negative rods with pointed ends, often faintly staining.
- **Gram positive rods resembling *Lactobacillus*:** long thin Gram-positive rods.
- **Gram variable rods resembling *Gardnerella*:** small Gram negative/Gram variable bacilli.

16.0 Quality Control & Quality Assurance

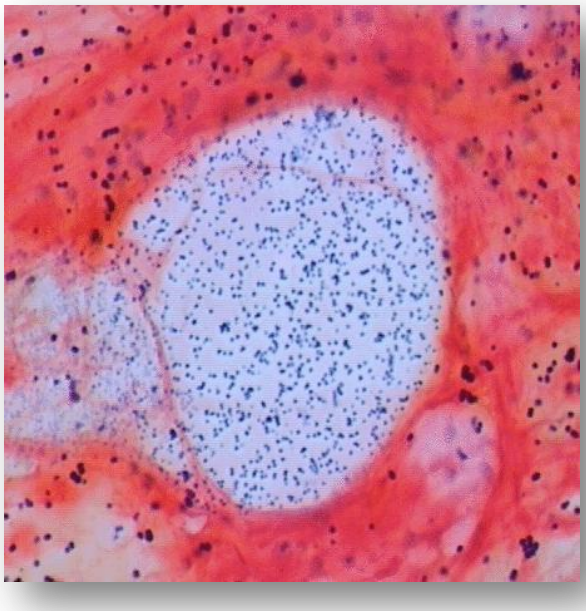
16.1 Quality Control

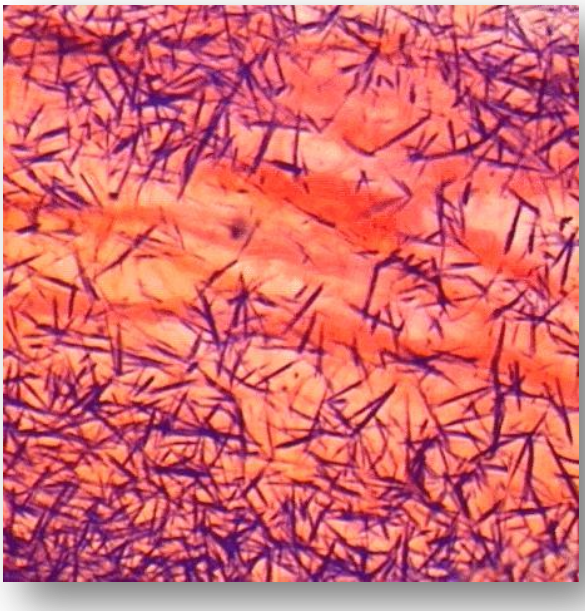
Quality control testing should be performed weekly and with each new lot of stain. Premade, heat-fixed slides may be used.

- *E. coli* ATCC 25922 – pink, Gram-negative rods
- *S. aureus* ATCC 25923 – deep violet, Gram-positive cocci

Common causes of poor Gram stain results include:

1. Use of dirty or contaminated slides – avoid touching the viewing area of the glass slide
2. Smear preparations that are too thick
3. Overheating smears when heat fixation is used
4. Excessive rinsing during the staining procedure, especially if the smear is not properly fixed
5. Stain precipitation – If an excess of precipitated stain is observed, decolorize and restain slide. If precipitate continues, use freshly filtered crystal violet or counterstain in a clean container. Precipitated gram positive stain generally appears as irregular coccoid shapes or needle-like crystals.





16.2 Quality Assurance

1. Each slide should be initialled by the person that prepares the smear so that retraining can occur if the smear preparation is inadequate.
2. Technical staff that interpret Gram stains should evaluate each smear for proper specimen placement, adequate specimen application, and proper staining and decolorization. If any there are any issues noted with the quality of the smear, it should be remade or restained.
3. When uncertain how to interpret or report a Gram stain, staff should consult with the Microbiology director, supervisor, technical specialist, or charge technologist. If the smear must be left for review, it should be documented on the Smear Review Log and left by the double-headed microscope.
4. Each week the Microbiology director and technical specialist randomly review six wound and six lower respiratory smears and cultures. Interpretations are compared to the results reported by technical staff in the LIS. If discrepancies are discovered, the reports are corrected or amended and the smear or culture is returned to the original reader for review.

17.0 Limitations

1. The sensitivity of the Gram stain is 10^5 cells/mL, or 10^4 cells/mL if the specimen has been prepared with the cytocentrifuge.
2. The application of too much or too little material to the glass slide can impact the interpretation of the smear and the work-up of culture results.
3. Background material and artifacts can interfere with interpretation.
4. Specimens collected from patients on antibiotics may present with unusual organism morphologies.
5. Gram-positive organisms may appear gram-negative due to cell death as a result of age or treatment with antimicrobial agents. The use of fresh culture is advisable for best staining results for bacterial isolates. Spore-formers, such as *Clostridium* and *Bacillus* may appear gram-variable or gram-negative, especially as the cells age.
6. Gram neutral organisms appear colorless against a generally gram-negative background and may be intracellular. This reaction has been seen in smears of clinical specimens when fungal elements or *Mycobacterium* spp. are present. If *Mycobacterium* is suspected, prepare another smear for acid fast staining and consult Rounds.
7. Be wary of interpretations made from observing very few organisms, especially in the absence of inflammation or if the organisms are unevenly distributed. Collection tubes, slides, and transport media may harbor nonviable bacteria. For critical specimens, such as CSF, where results will define an infectious process, prepare a second smear to confirm rare findings of microorganisms.

18.0 References

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3. Gram stain images from lower respiratory, wound, and genital specimens: MTS Lab Training and Competency Assessment. University of Washington, Department of Laboratory Medicine. http://www.medtraining.org/
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5. BD Package Insert: Gram Stain Kits and Reagents. Rev. 06/2008.

19.0 Document Control

- Medical Director Approval: Reviewed by Dr. Schappert 3/10/2010

- Microbiology Director Approval: Dr. Ann Robinson 5/10/2006, updates reviewed 3/2013
- Microbiology Supervisor Approval: Jerry Claridge 1/2007
- Reviews by Jerry Claridge: 9/2007, 9/2008, 9/2009, 3/2011, 3/2013
- Revisions & Updates: 10/15/2010 Added nuclei comment for identifying squamous epithelial cells. 1/19/2011 Added diphtheroids > 50 organisms/oif as criteria suggestive of an aspiration event. 3/12/13 Document updated for PPM standard format, added sections: Clinical Significance, Scope, Safety, Smear Preparation, Quality Assurance, and Limitations, and added photomicrographs.