

The Gram-stain

Course

Medical
Microbiology

Unit VII

Laboratory
Investigations
and
Identification of
Causative
Agents

Essential Question

Why is the
Gram-stain one
of the most
important
microbial stains?

TEKS

130.207 (c)
1A, 1B, 2A-H,
3D, 4C,E,F

Prior Student Learning

Basic
understanding of
microbial
infectious
diseases

Estimated time

5-7 hours

Rationale

One of the first procedures performed by the medical microbiologist for the identification of bacteria is the Gram-stain.

Objectives

Upon completion of this lesson, the student will be able to

- List basic differences between Gram-negative and Gram-positive bacteria
- Perform a Gram-stain

Engage

Review the Sample Gram-stain Images with students. Discuss.

Key Points

- I. Bacteria (Microorganisms)
 - A. A pathogen is microorganism that causes disease
 - B. Nonpathogenic – microorganisms that do not cause disease
 1. microorganisms that live within the body and do not cause disease are called normal flora
 - C. Infection is caused by microorganisms entering the host and multiplying
 - D. Toxins – poisonous substance produced by a cell
 1. exotoxins
 2. endotoxins
 - E. Bacteria damage the host with exotoxins: intracellular toxin released into the environment (the human body) by living gram-positive bacteria; endotoxins are phospholipopolysaccharide macromolecules that comprise the gram-negative bacterial cell wall and are released only when the cell is lysed as it dies
 - F. Types of bacteria
 1. bacilli (rod-shaped)
 2. cocci (round shaped)
 3. spirilli (spiral shaped)
 - G. Koch's postulates - Robert Koch, a German microbiologist who identified four conditions that must be satisfied to prove that an agent causes disease:
 1. the organism should be found in all cases of the disease, and in the locations affected by the disease

2. The organism should be grown in pure culture
3. The organism should reproduce the disease when introduced into another susceptible host
4. The same organism should be re-isolated from the intentionally infected individual

II. Differentiation

A. Gram-stain

1. Discovered by Hans Christian Gram in the 1800's
 - a. He was originally looking for a stain for all bacteria to make them more easily visible in a microscope field; in the process of looking for a universal stain, he stumbled upon a difference between two distantly related groups of bacteria - those that retained the stain (Gram-positive) and those that did not (Gram-negative)
 - b. The Gram-stain is one of the first steps in identifying an unknown bacterial culture
 - c. Gram reaction is important in medicine because some antibiotics are effective against only Gram-negative bacteria (e.g., erythromycin) and some against only Gram-positive (e.g., penicillin).
2. Bacterial cell walls contain a complex polymer, peptidoglycan
 - a. Gram-positive cell walls consist of several layers of peptidoglycan (cross-linked by teichoic acid and lipoteichoic acid)
 - b. Gram-negative cell walls have one layer of peptidoglycan surrounded by a lipid-based outer membrane
3. Procedure
 - a. Prepare a thin smear of the culture or specimen to be examined; allow the slide to air dry and heat fix; place smear on the staining rack
 - b. Flood the slide with crystal violet for 30 seconds to one minute
 - c. Rinse with distilled water, tilting the slide to remove excess water
 - d. Flood the slide with Gram's iodine for one minute
 - e. Rinse with distilled water, tilting the slide to remove excess water
 - f. Hold the slide by the short edge using forceps or clothespin; flood the slide with ethyl alcohol or acetone (Gram decolorizer) for about 15 seconds
 - g. Rinse with distilled water, tilting the slide to remove excess water
 - h. Counterstain the smear by flooding the slide with Safranin for 30 seconds
 - i. Rinse with distilled water
 - j. Blot dry with bibulous paper or air dry

- k. Examine the slide under oil immersion objective for Gram-positive or Gram-negative bacteria
- l. Record findings
4. Reagents
 - a. Crystal violet -- primary stain: stains all bacteria blue to purple
 - b. Gram's iodine -- a mordant that enhances reaction between the cell wall and the primary stain
 - c. Ethyl alcohol or acetone (Gram decolorizer) -- Gram-positive bacteria retain the primary stain (crystal violet) because of the makeup of their cell wall; Gram-negative bacteria do not retain the primary stain during the decolorizing process because of the large amount of lipopolysaccharide in the cell wall
 - d. Safranin -- this is the counterstain, and it will have no effect on the Gram-positive bacteria, but stains the Gram-negative bacteria pink to red
5. Results
 - a. Gram-positive bacteria -- stain dark blue to purple
 - b. Gram-negative bacteria -- stain pink to red
 - c. Note: Do not use cultures older than 48 hours because Gram-positive may stain as Gram-negative bacteria

<i>Gram-Negative Bacteria</i>	<i>Gram-Positive Bacteria</i>
more complex cell wall	simple cell wall
thin peptidoglycan cell wall layer	thick peptidoglycan cell wall layer
outer lipopolysaccharide wall layer	no outer lipopolysaccharide wall layer
retain safranin	retain crystal violet/iodine
appear pink/red	appear blue/purple

III. Pathogens

A. Gram-positive pathogens

1. *Staphylococcus aureus* (cocci in clusters, skin infections)
2. *Streptococcus pyogenes* (cocci in chains, strep-throat)
3. *Clostridium tetani* (rods, tetanus)

B. Gram-negative pathogens

1. *Neisseria gonorrhea* (cocci in pairs called diplococci, gonorrhea)
2. *Pseudomonas aeruginosa* (rod, opportunistic infection microorganism)
3. *Vibrio cholera* (rod, cholera)

Activity

- I. Complete the Gram-stain Laboratory Investigation
- II. Complete the Gram-stain Worksheet

Assessment

Laboratory Investigation Rubric

Materials

Gram-stain Procedure Handout

Prepared slides of Gram-negative and Gram-positive bacteria

Gram stain supplies:

(Hint: Number the bottles 1 - 4 for student use)

- Crystal Violet
- Gram's Iodine
- Acetone or Ethyl Alcohol (Gram decolorizer)
- Safranin

Bacterial cultures of Gram-positive and Gram-negative bacilli and cocci

Microscope slides

Microscopes

Sinks

Gloves

Inoculating Loops

Bunsen Burners

Oil for oil immersion lens

Timer

Biohazard container

Staining racks

Goggles

Bilbous paper

Disinfecting solution

Paper towels

Burton, Gwendolyn R. W. and Engelkirk, Paul G. (2000). Microbiology for the Health Sciences, 6th ed. Baltimore: Lippincott Williams & Wilkins.

Delost, Maria Dannessa, Introduction to Diagnostic Microbiology, 1997, ISBN 0-8016-7853-6

Hunt, Madelyn D. and Lyons, Lisa C. (1996). Exploring the Unseen World of Microbes. New York: McGraw-Hill Publishing Co.

Accommodations for Learning Differences

For reinforcement, the student will review the steps of the Gram-stain procedure and repeat the laboratory investigation.

For enrichment, the student will choose a microorganism and research the characteristics of growth and the diseases it may cause and the consequences of an infection. Design a multimedia presentation.

National and State Education Standards

National Health Science Cluster Standards

HLC10.01 Health care workers will apply technical skill required for all career specialties. They will demonstrate skills and knowledge as appropriate.

TEKS

130.207(c)(1)(A) demonstrate safe practices during laboratory and field investigations;

130.207(c)(1)(B) demonstrate an understanding of the use and conservation of resources and the proper disposal or recycling of materials;

130.207(c)(2)(A) know the definition of science and understand that it has limitations, as specified in subsection (b)(2) of this section;

130.207(c)(2)(B) know that hypotheses are tentative and testable statements that must be capable of being supported or not supported by observational evidence. Hypotheses of durable explanatory power which have been tested over a wide variety of conditions are incorporated into theories;

130.207(c)(2)(C) know scientific theories are based on natural and physical phenomena and are capable of being tested by multiple independent researchers. Unlike hypotheses, scientific theories are well-established and highly-reliable explanations, but they may be subject to change as new areas of science and new technologies are developed;

130.207(c)(2)(D) distinguish between scientific hypotheses and scientific theories;

130.207(c)(2)(E) plan and implement descriptive, comparative, and experimental investigations, including asking questions, formulating testable hypotheses, and selecting equipment and technology;

130.207(c)(2)(F) collect and organize qualitative and quantitative data and make measurements with accuracy and precision using tools such as calculators, spreadsheet software, data-collecting probes, computers, standard laboratory glassware, microscopes, various prepared slides, stereoscopes, metric rulers, electronic balances, hand lenses, Celsius thermometers, hot plates, lab notebooks or journals, timing devices, Petri dishes, lab incubators, dissection equipment, meter sticks, and models, diagrams, or samples of biological specimens or structures;

130.207(c)(2)(G) analyze, evaluate, make inferences, and predict trends from data;

130.207(c)(2)(H) communicate valid conclusions supported by the data through methods such as lab reports, labeled drawings, graphic organizers, journals, summaries, oral reports, and technology-based reports;

130.207(c)(3)(D) evaluate the impact of scientific research on society and the environment;

130.207(c)(4)(C) recognize the factors required for microbial reproduction and growth;

130.207(c)(4)(E) describe the morphology and characteristics of

microorganisms using a variety of microbiological techniques;
130.207(c)(4)(F) discuss the results of laboratory procedures that are used to identify microorganisms.

Texas College and Career Readiness Standards

Mathematical Standards

IV. Measurement Reasoning D. Measurement involving statistics and probability 1. Compute and use measures of center and spread to describe data. 2. Apply probabilistic measures to practical situations to make an informed decision.

VI. Statistical Reasoning A. Data collection B. Describe data C. Read, analyze, interpret, and draw conclusions from data 1. Make predictions and draw inferences using summary statistics.

Science Standard

I. Nature of Science: Scientific Ways of Learning and Thinking A. Cognitive skills in science 4. Rely on reproducible observations of empirical evidence when constructing, analyzing, and evaluating explanations of natural events and processes. C. Collaborative and safe working practices 2. Understand and apply safe procedures in the laboratory and field, including chemical, electrical, and fire safety and safe handling of live or preserved organisms. 3. Demonstrate skill in the safe use of a variety of apparatuses, equipment, techniques and procedures.

Gram-stain Procedure

1. Prepare a thin smear of the culture or specimen to be examined.
Allow the slide to air dry and heat fix. Place smear on the staining rack.
2. Flood the slide with crystal violet for 30 seconds to one minute.
3. Rinse with distilled water. Tilt the slide to remove excess water.
4. Flood the slide with Gram's iodine for one minute.
5. Rinse with distilled water. Tilt the slide to remove excess water.
6. Hold the slide by the short edge using forceps or clothespin.
Flood the slide with ethyl alcohol or acetone (Gram decolorizer) for about 15 seconds.
7. Rinse with distilled water. Tilt the slide to remove excess water.
8. Counterstain the smear by flooding the slide with Safranin for 30 seconds.
9. Rinse with distilled water.
10. Blot dry with bibulous paper or air dry.
11. Examine the slide under oil immersion objective for Gram-positive or Gram-negative bacteria.
12. Record findings.

The Gram-stain Laboratory Investigation

PURPOSE:

In this laboratory investigation, the student will learn the procedure for performing the Gram-stain.

BACKGROUND INFORMATION:

The Gram stain separates bacteria into one of two groups:

1. Gram-positive bacteria
These retain the color of the first stain used (Crystal Violet).
2. Gram-negative bacteria
These assume the color of a second stain (Safranin).

Bacteria stain differently because of differences in the structure and chemical composition of their cell walls.

MATERIALS:

Prepared slides of Gram-negative and Gram-positive bacteria

Gram stain supplies:

(Hint: Number the bottles 1 - 4 for student use)

Crystal Violet

Gram's Iodine

Acetone or Ethyl Alcohol (Gram decolorizer)

Safranin

Bacterial cultures of Gram-positive and Gram-negative bacilli and cocci

Microscope slides

Microscopes

Sinks

Gloves

Inoculating Loops

Bunsen Burners

Oil for oil immersion lens

Timer

Biohazard container

Staining racks

Goggles

Bibbous paper

Disinfecting solution

Paper towels

PROCEDURE:

1. Wash hands and put on gloves.
2. Assemble equipment and supplies.
3. Prepare work area: place one or two towels on the counter; pour surface disinfectant on them until just wet.
4. Prepare a thin smear of the culture or specimen to be examined. Allow the slide to air dry and heat fix. Place smear on the staining rack.
5. Flood the slide with crystal violet for 30 seconds to one minute.
6. Rinse with distilled water. Tilt the slide to remove excess water.
7. Flood the slide with Gram's iodine for one minute.
8. Rinse with distilled water. Tilt the slide to remove excess water.
9. Hold the slide by the short edge using forceps or clothespin. Flood the slide with ethyl alcohol or acetone (Gram decolorizer) for about 15 seconds.
10. Rinse with distilled water. Tilt the slide to remove excess water.
11. Counterstain the smear by flooding the slide with Safranin for 30 seconds.
12. Rinse with distilled water.
13. Blot dry with bibulous paper or air dry.
14. Examine the slide under oil immersion objective for Gram-positive or Gram-negative bacteria.
15. Record findings.
16. Dispose of materials properly.
17. Clean work surface with disinfectant
18. Remove and discard gloves and wash hands with disinfectant.

DATA:

Record the results of the Gram-stain:

1. Slide #1

2. Slide #2

3. Slide #3

CONCLUSION:

Please answer the following questions.

1. What is the color seen of Gram-positive bacteria?
2. What is the color seen of Gram-negative bacteria?
3. Describe the three types of bacteria. Draw pictures of their shapes.
4. What is the purpose of Gram's iodine?
5. The oil immersion objective lens provides a **total** magnification of _____.
6. A 16-year old high school student presents with sores on his legs that keep spreading. He states he works out every day in the gym and shares towels with other students. The physician cultures the sores and sends two swabs to the laboratory for identification and antibiotic sensitivity. The laboratory technologist performs a Gram-stain on one of the swabs. Results of the Gram-stain are: Gram-positive cocci in clusters and many white blood cells. What is the suspected bacteria causing the sores (infection of the skin)?

Laboratory Investigation Rubric

Student: _____ Date: _____

Scoring Criteria	4	3	2	1	N/A
	Excellent	Good	Needs Some Improvement	Needs Much Improvement	
Problem is appropriately identified.					
Problem is precise, clear, and relevant.					
Association between the problem and the predicted results is direct and relevant.					
All variables are clearly operationalized.					
Demonstrates comprehension of the use of scientific concepts and vocabulary.					
All significant data is measured.					
Data is recorded effectively and efficiently.					
Data table is well designed to the task requirements.					
All graphs are appropriate.					
All data accurately plotted.					

Graph visually compelling; highlights conclusions of the study.					
Conclusion relates directly to the hypothesis.					
Conclusion has relevancy in resolution of the original problem.					
Conclusion relates the study to general interest.					

Gram Stain Worksheet

Complete the following table.

Organism	Gram (+ or -)	Shape	Sketch	Disease/ Condition	General Information
<i>Bacillus anthracis</i>					
<i>Bacillus influenza</i>					
<i>Candida albicans</i>					
<i>Clostridium tetani</i>					
<i>Enterococcus</i>					
<i>Escherichia coli</i>					
<i>Lactobacillus</i>					

<i>Mycobacterium tuberculosis</i>					
<i>Neisseria gonorrhoeae</i>					
<i>Pseudomonas aeruginosa</i>					
<i>Staphylococcus aureus</i>					
<i>Staphylococcus pneumonia</i>					
<i>Streptococcus pyogenes</i>					
<i>Vibrio cholera</i>					

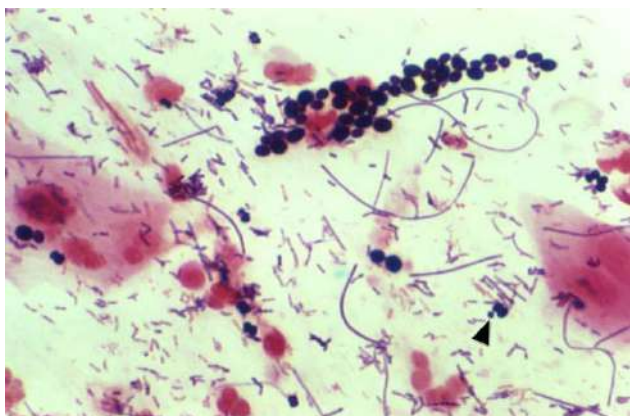
Sample Gram Stain Images



<http://phil.cdc.gov/phil/details.asp>

CDC/ Dr. W.A. Clark

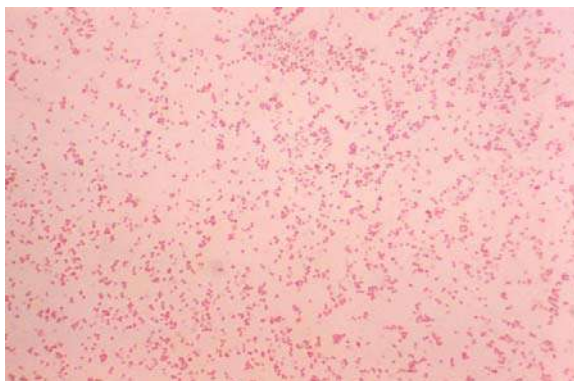
This Gram-stained photomicrograph revealed the presence of numerous Gram-positive, *Oerskovia turbata* bacteria, which can be seen arranged in small clumps.



<http://phil.cdc.gov/phil/details.asp>

CDC/ Joe Miller

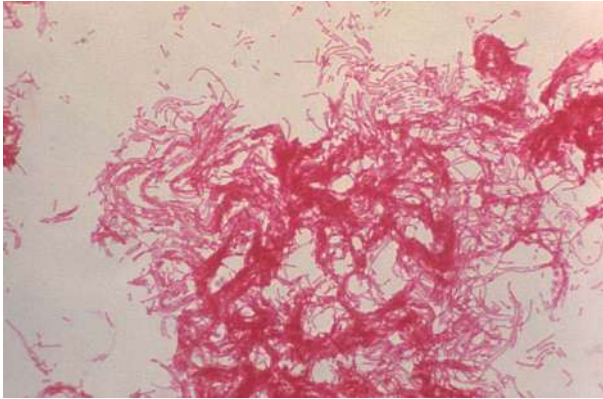
This is a Gram-stained photomicrograph of a vaginal discharge specimen shows an overgrowth of *Candida albicans* organisms.



<http://phil.cdc.gov/phil/details.asp>

CDC/ Dr. W.A. Clark

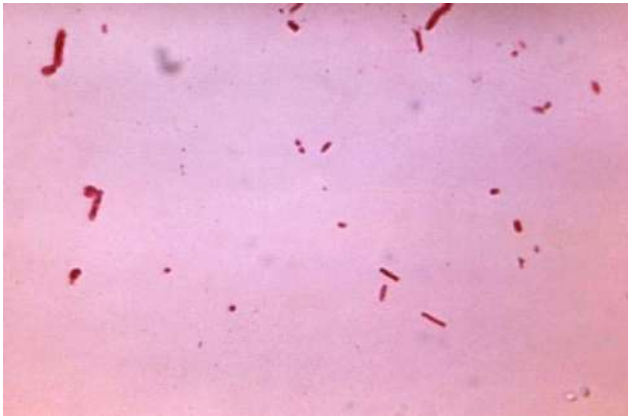
This Gram-stained photomicrograph revealed the presence of numerous Gram-negative *Neisseria lactamica* diplococcal bacteria.



<http://phil.cdc.gov/phil/details.asp>

CDC/ Dr. Mike Miller

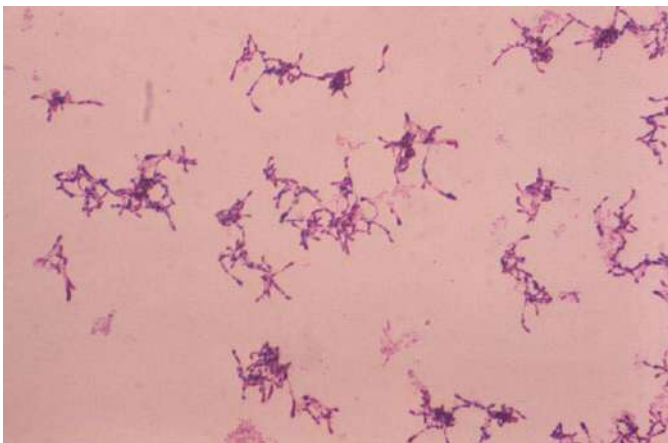
Under a magnification of 1200X, this Gram-stained photomicrograph revealed the presence of numerous Gram-negative, *Haemophilus ducreyi* bacteria that were in a bacterial culture.



<http://phil.cdc.gov/phil/details.asp>

CDC/ Dr. Holdeman

Under a magnification of 956X, this Gram-stained photomicrograph depicts numbers of Gram-negative *Bacteroides variabilis* bacteria.



<http://phil.cdc.gov/phil/details.asp>

CDC/ Dr. W.A. Clark

Normally found in the human mouth and respiratory tract, this Gram-stained photomicrograph revealed numerous Gram-positive *Rothia dentocariosa* bacteria.