

TECHNIQUES MANUAL

THE GRAM STAIN

A NEW LOOK AT AN OLD TOOL

Gilda L. Jones
Stanley M. Dever

Bacteriology Training Section
Division of Laboratory Training and Consultation
Laboratory Program office

Reprinted October 1985

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service
Centers for Disease Control
Atlanta, Georgia 30333

LIBRARY
CENTERS FOR DISEASE CONTROL
ATLANTA, GEORGIA 30333

QW25
J77g
1984

99447

QW25 J77g 1984
Jones, Gilda L.
The gram stain

099447

086385

086380

Preface

The Gram stain, developed over a century ago, has many important uses. It can provide the clinician with valuable information within minutes of obtaining the specimen. When properly used and interpreted, the Gram stain can reveal the adequacy of the patient specimen. Knowledge of the numbers of gram-negative or gram-positive morphotypes, or both, can guide the clinician in the selection of appropriate therapy. Selection of adequate culture media should be guided by the results of the Gram stain. The stain results can also provide a quality control mechanism for determining that all of the bacteria seen on the smear were isolated.

This manual is the result of an effort to assemble information on the use and interpretation of this vastly underused and sometimes misused tool.

099447

~~086385~~

11-27-65 pub. State Library

ACKNOWLEDGMENTS

We gratefully acknowledge the efforts of Ray Simons, scientific photographer, whose expertise made possible the photographs used in this publication.

We thank Dr. Pegi Brooks, educational consultant, whose assistance and constructive critique were invaluable. Special thanks also are due Miriam Dugger who helped with the manuscript.

We are further indebted to Dr. John H. Krickel, Director, Division of Laboratory Training and Consultation, Laboratory Program Office, whose support and encouragement made this production a reality.

CONTENTS

Preface -----	ii
Acknowledgments -----	iii
I. Introduction -----	1
II. Clinical Uses of Bacterial Stains -----	2
III. Composition of Staining Solutions -----	4
IV. Gram Stain Technique -----	7
A. Preparation of the smear (thin film) -----	7
B. Fixing the smear -----	10
C. Staining procedure -----	11
D. Helpful hints -----	11
E. Quality control -----	13
V. Bacterial Morphology -----	14
VI. Gram Stains of Clinical Specimens -----	23
VII. Avoiding Problems With the Gram Stain -----	27
VIII. Appendix -----	30
IX. References -----	34

THE GRAM STAIN: A NEW LOOK AT AN OLD TOOL

I. Introduction

Hans Christian Gram (1853-1938), a Danish physician working in Berlin, immortalized himself at the age of 31 by developing the differential staining technique that is the basis of bacterial taxonomy. Christian Gram, working in the municipal hospital of Berlin, attempted to develop a procedure that would differentially stain schizomycetes from tissue cells. He began his work on pneumococci with lungs from human pneumonia victims and with lung tissue from experimental animals.

Gram's discovery was a mixture of accident and shrewd application of a chance observation. He attempted to obtain blue nuclei and brown cytoplasm in kidney sections by first staining with gentian violet and then with iodine-potassium iodide solution. This application of iodine, instead of the intended result, made destaining and clearing of the gentian violet, ordinarily very difficult, quite easy with the next alcohol step. In Gram's words, "The experiments resulted from the accidental observation that aniline-gentian violet preparations of tissues, after treatment with iodine-potassium iodide, are completely and rapidly decolorized in alcohol." (14) During this work, Gram noticed the outstanding resistance of some bacterial cells to decolorization. He published his staining method in 1884 (14). Although Gram did not present his procedure in the exact form in which it is used today, its four fundamental steps are identical.

The staining procedure as originally presented by Gram used Ehrlich's aniline gentian violet, an aqueous solution of iodine-potassium iodide, absolute alcohol as a decolorizer, and sometimes Bismarck brown as a counterstain (5). Although the method is now fundamentally the same, a series of important modifications has resulted in procedures that produce more reliable results. Three of the procedures that gave superior results were Hucker's modification (18), Burke's modification (7), and the Kopeloff-Beerman modification (22).

The procedures used in the United States today usually have the following features. The primary dye is crystal violet, since it is a more reproducible substance than the old dye mixtures called gentian violet. The primary dye is a stable solution, and it may contain a mordant such as ammonium oxalate for constancy of action, or sodium bicarbonate, added just before use, to intensify the uptake of color. The staining step and the iodine step may be carried out at an alkaline pH by adding sodium bicarbonate or sodium hydroxide to the dye, to the iodine solution, or to both. This avoids poor results, which are sometimes due to the acid state of the organisms, their suspension medium, or the reagents (7, 22). Alcohol (95%) is most commonly used as a decolorizer, although acetone and acetone-alcohol mixtures are sometimes used and are excellent (22). The dilution of the alcohol with water, by repeated use or by water remaining from a preceding wash step, is to be avoided. Safranin is the most popular of the possible counterstains. There is no Gram procedure that can be referred to as best for all laboratories and for all situations. The worker should adopt at

least two of the well-accepted methods, practice them until familiar with their characteristics, and use controls of organisms with known Gram reactions. The use of this plan will give much better results than constantly changing methods in the hope of finding one that is foolproof.

II. Clinical Uses of Bacterial Stains

The Gram stain is one of the most helpful procedures for diagnosing infectious diseases. It provides evidence of the quality of a specimen and lets physicians know something about the agent(s) responsible for infection, thus guiding them to a more rational selection of preliminary antibiotic therapy, pending culture results. Unfortunately, the Gram stain is grossly underused, as shown by Edwards and associates who reported that immediate Gram stains were made of only 36 of 1018 specimens (3.5%) (11). Gram stains are also frequently misinterpreted because smears have been inadequately decolorized. Because the uses to be made of bacterial stains are much too numerous to mention here, the following discussion is restricted to situations in which microscopic examination of stained smears has proved to be of greatest value.

A. Blood specimens

Microscopic examination of whole blood usually is unrewarding. The number of organisms in the bloodstream of bacteremic patients is generally fewer than 1 organism per milliliter. The examination of buffy coat smears from peripheral blood, however, has been used with modest success. Brooks et al. (6) observed gram-positive bacteria in buffy coat smears from 3 of 11 patients with positive blood cultures, and Fadden (12) reported bacteria seen in the smears from 7 of 10 infants with clinically significant bacteremia. In each of these studies, positive smears were only observed in patients with overwhelming bacteremias; e.g., more than 50-100 bacteria per milliliter of blood. A Gram stain should be made on positive culture broths. Stains should also be used to presumptively identify a blood culture isolate. As an example, Agger and Maki (1) reported that a predominance of clusters of gram positive cocci was 98% sensitive and 100% specific for identification of *Staphylococcus* and *Peptococcus*. A predominance of pairs or chains of gram-positive cocci was 100% sensitive and 98% specific for identifying *Streptococcus*.

B. Cerebrospinal fluid specimens

Because relatively few types of microorganisms commonly cause meningitis, the Gram stain is indispensable in providing rapid, presumptive identification of the etiologic agent.

The frequency of positive Gram stains in patients with meningitis is directly related to the number of bacteria present in the cerebrospinal fluid. The number of bacteria in turn is a function of the organism responsible for the meningitis, the patient's antibacterial response, the duration of infection (early versus late and uncontrolled), and prior antimicrobial therapy.

Feldman (13) determined the mean number of bacteria that were recovered in CSF specimens from meningitis patients as follows: *Haemophilus influenzae* (2×10^7 colony forming units [CFU] per milliliter), group B. *Streptococcus* (1×10^7 CFU/ml), *Streptococcus pneumoniae* (3.9×10^5 CFU/ml), and *Neisseria meningitidis* (1.3×10^5 CFU/ml).

Feldman also noted that if there were fewer than 10^7 CFU/ml, then there was generally less than one organism seen per microscopic field. The frequency of positive CSF Gram stain varies from about 66% to 82% (20, 29), and this frequency decreases if the patients have been treated previously with antibiotics (10, 17).

C. Respiratory specimens

To interpret the culture results of sputum, it is necessary to determine the magnitude of oral contamination of the specimen. An objective system was devised (28) on the basis of cellular contents. The presence of squamous epithelial cells is considered presumptive evidence of oral-pharyngeal contamination. Gram-stained smears, examined under 100X magnification, are scored on the basis of the number of squamous epithelial cells and leukocytes observed. Generally, specimens containing fewer than 10 squamous epithelial cells and greater than 25 leukocytes are acceptable for culture and diagnostic interpretation. If the specimen does not meet these criteria, another should be collected. When the sputum specimen is examined, ciliated epithelial cells and alveolar macrophages from the lower respiratory tract can readily be differentiated from the squamous epithelial cells and, therefore, do not confuse interpretation.

Heineman and coworkers (16) observed that, unless sputum specimens are routinely examined microscopically, the culture results from half of the specimens would be misleading. Ries, et al. (31) reported that the reliability of the Gram stain examination was directly related to the quality of the specimen. There was a high incidence of false-positive sputum smears with specimens contaminated with saliva. Many microorganisms were seen on Gram stain and cultured from the specimen that were not responsible for the pleuropulmonary infection. However, the Gram stain of properly collected, uncontaminated specimens, was reliable.

D. Transtracheal aspiration

Immediate diagnosis can be accurately made from the Gram stain of transtracheal aspiration (TTA) in more than 90% of the instances (31). The results can prompt the initiation of therapy against *Haemophilus influenzae*, *Staphylococcus aureus*, or a gram-negative bacillary pneumonia. Otherwise, those bacteria would not have been treated optimally because the same organisms in sputum may have been considered contaminants from the oral-pharyngeal cavity.

E. Urine specimens

The microscopic examination of urine is a sensitive and specific test for determining the likelihood of a urinary tract infection, and the morphology of the bacteria can be used to guide initial antimicrobial therapy. Significant infections are commonly defined as bacteriuria of at least 10^5 CFU/ml of urine. This correlates with the presence of one or more bacteria per oil immersion microscopic field in a Gram-stained smear of well-mixed, uncentrifuged urine (21). The sensitivity of this procedure in experienced hands is between 80% and 90% (24) and represents the most rapid screening test for bacteriuria that has been developed to date.

Uninfected specimens containing less than 10^4 bacteria per milliliter generally reveal no bacteria or leukocytes on Gram stain. The presence of many squamous epithelial cells and mixed vaginal type flora indicate contamination and the need for a repeat specimen regardless of the total number of bacteria. Microscopic examination also serves as a quality control measure, since a positive Gram stain and negative culture alert the microbiologist to the possibility of a slow-growing or fastidious aerobe or an anaerobe.

F. Urethral discharge

Microscopic examination of a Gram-stained smear of urethral discharge for the presence of typical gram-negative intracellular diplococci is a sensitive and specific diagnostic test of gonorrhea (30). The accuracy of this test, as compared to a culture, is highest in symptomatic males (95%) and slightly lower (70%) in the asymptomatic patient (15). The presence of a gram-negative diplococci in a urethral smear necessitates immediate treatment for gonorrhea, as outlined by the Centers for Disease Control (8).

If the original microscopic smear of the urethral discharge is negative, a culture should be done. The absence of diplococci on the Gram-stained smear is presumptive evidence of nongonococcal urethritis and therapy for this condition should be started immediately.

III. Composition of Staining Solutions

A. The primary stain

A difficulty of the original Gram method was the lack of stability of the Ehrlich's aniline gentian violet solution. Although a large number of dyes could be substituted for gentian violet, a study of 73 other dyes showed that no dye was superior

or even equal to crystal violet (2). Hucker and Conn demonstrated that ammonium oxalate in the formula gave better results than when the crystal violet was used alone (19). Burke (7) and Kopeloff and Beerman (22) added nothing to their stock solution of crystal violet but added sodium bicarbonate to small quantities of working solutions just before use, or even on the slide itself.

The resulting alkaline pH for staining helps to give more clear-cut results in differentiating between gram positive and gram negative. Therefore, crystal violet, while not specific for the Gram stain, is as good or better than any other dye yet tested.

B. The mordant

The term mordant is used to describe iodine-potassium iodide solution or a substitute for this solution as used in the gram procedure. Gram's choice of iodine, which he expected to form a background color in his sections, was fortunate. No other reagent can be substituted for the best and most reliable results (27). Sheppe and Constable (32) demonstrated that, on storage, the iodine solution develops sufficient acidity through oxidation to cause errors in Gram differentiation. Therefore, many formulas for the iodine solution contain an alkalizing agent such as sodium bicarbonate or sodium hydroxide.

C. The decolorizer

The application of the decolorizer is the most critical stage of the Gram procedure. There is danger of incorrect results from both over and under decolorization; therefore, the decolorizer and the technique should be as carefully standardized as possible. Ethyl alcohol has been one of the most popular decolorizers. Addition of water to the alcohol increases its rate of decolorization up to a dilution of 40% alcohol (5). A dilution of 60% alcohol is too rapid a decolorizer. Hucker and Conn (19) found little practical difference between the decolorization effect of 95% and absolute alcohol. These, however, gave more dependable results than alcohol solutions containing more water. Thus, it is necessary to conduct the procedure in such a way that the alcohol does not change its dilution. Remove as much water from the slide as possible without blotting.

The most often suggested substitutes for ethyl alcohol are acetone (22, 26), a mixture of acetone and ether (7), or a mixture of acetone and ethyl alcohol (23). Acetone is a more rapid decolorizer than alcohol (23) and must be used with some care, but its use in the Gram stain has been demonstrated by Lillie (26) and others. The choice of alcohol, acetone, or mixtures of these two is almost universally used. The strict standardization of the method is more important than the choice of method.

D. The counterstain

The influence of the concentration of the counterstain on Gram differentiation was shown by Lasseur and Schmitt (25) who reported that 0.031% and 0.5% solutions of basic fuchsin gave very different results.

The 0.5% solution resulted in many gram-positive cells appearing red since it was strong enough to replace the primary dye remaining after decolorization (25). Also, if no counterstain is used, gram-negative cells may appear gram-positive (3, 4, 27). The counterstain is, therefore, a definite part of the gram procedure. It is usually a weak solution of about 0.25% dye and its time of application should be carefully controlled.

Extensive studies by Hucker and Conn (19) clearly demonstrate that not all basic dyes can be used as a counterstain. Some counterstains are so powerful in their action that they tend to decolorize some of the gram-positive organisms. Their experiments on the reliability of various counterstains indicated that "pyronin and Bismarck brown are the best counterstains, while eosin and safranin are fair substitutes." Because good contrast is important in practical work, they chose safranin as a personal preference. This choice is confirmed by the experience of most workers.

E. Other factors

Although many have observed that the culture medium can influence the degree of Gram positivity shown by an organism, the effect has not been studied sufficiently to allow an exact statement to be made. Most of the recorded variations have been observed on unusual media, such as that using various types of inhibitors (antibiotics, dyes, high salt content, or other chemical inhibitors).

All investigators agree that the age of the culture influences the degree of Gram positivity of the culture. According to Lasseur and Schmitt (25), there is a stage, which differs for each species of organism, in which the gram-positive characteristic is most marked. Differences between young, 24-hour cultures and cultures several days old can be easily demonstrated. The old cultures are nearly always less gram-positive (23). Hucker and Conn (19) state that adult cells of approximately 48 hours of age are sometimes more gram-positive than younger cells. Kopeloff and Cohen (23) state that 24-hour cells are more gram positive than 48-hour cultures. The evidence shows that the custom of using only an 18- to 24-hour culture for the determination of Gram characteristics is unwise. Although serious errors due to use of a 12- or 48-hour culture probably would occur only in special

instances, it would obviously be better to use at least three different ages of an unknown culture to determine its Gram character, as urged by Hucker and Conn (19) after extensive studies of this question.

Prolonged heat fixation can cause gram-positive cells to stain gram-negatively. However, small differences in the heat fixing step will not have a major effect on the results obtained.

Lasseur a Schmitt (25) showed that thick smears of *Vibrio cholerae* took almost four times as long to be decolorized as did thin smears. For best results, standard suspensions of organisms should be spread over an area of the slide, so that individual cells are separated from each other. In all preparations, large masses of grouped organisms should be avoided.

IV. Gram-Stain Technique

A. Preparation of the smear (thin film)

The Gram stain is one of the most important tools a microbiologist can use to aid in the identification of pathogenic bacteria. No automated technique yet developed can identify cellular components of a specimen or distinguish between gram-positive and gram-negative bacteria. A Gram stain performed on a specimen immediately after its arrival in the laboratory may give the clinician a clue to the identity of the organism causing the infection.

1. Labeling

The first step in making smears from any source, is to label the slide with the patient's name or the specimen number. This is most easily done when frosted-end slides are available. Use a lead pencil to write on the frosted end. However, if plain slides are used, a glass etcher (diamond point pen) may be used to mark the slide. Avoid wax pencils or ink marking pens, as these may wash off during the staining.

2. Thin smears

In order for a Gram stain to be interpreted correctly, the smear must be made properly. Thin smears are preferred, since there will be less tendency for cells and bacteria to overlap. The smear will stain more evenly and be easier to interpret.

a. Swab specimens

Thin smears can be obtained from specimens by rolling the swab lightly over the surface of the slide (see figures 1 & 2). Never rub, as rubbing may cause disruption of the cells.

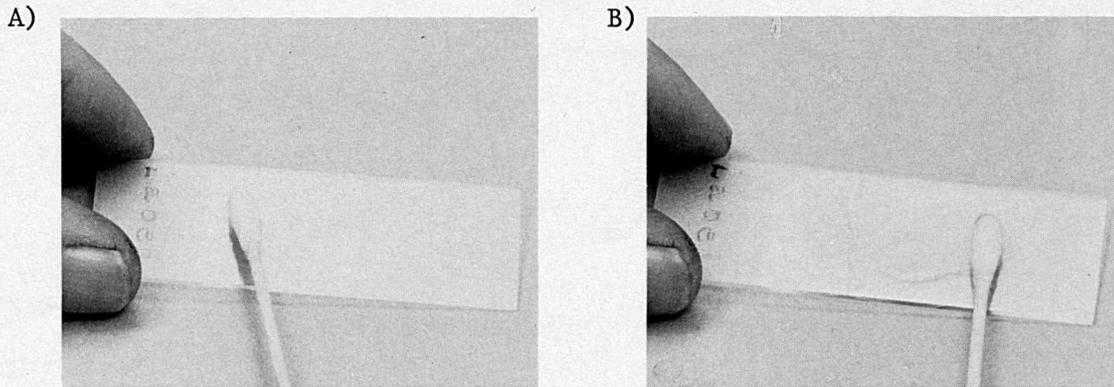
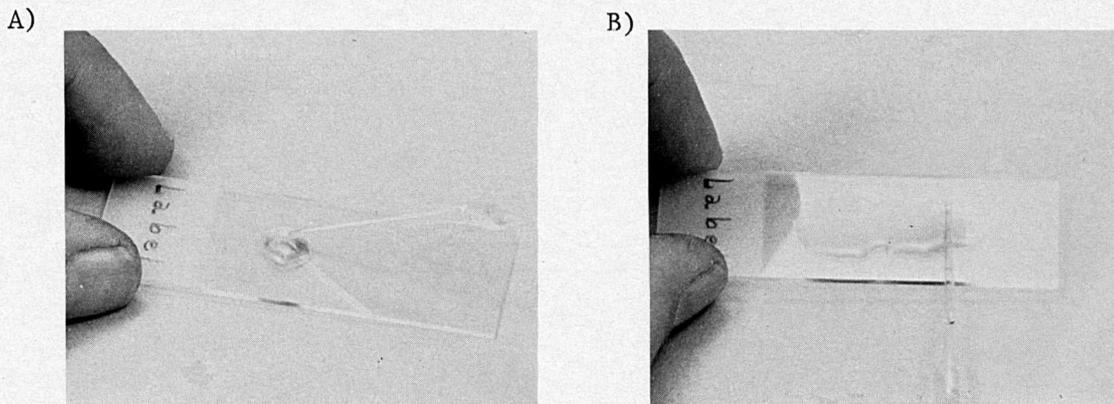


Figure 1. Making a thin smear with swab.

b. Liquid specimens

All liquid specimens should be mixed before the smear is made. Do this gently to avoid aerosols and destruction of cells. Use a sterile Pasteur pipette to place a small drop of the fluid on the slide, and with the exception of urines and spinal fluids, smear the drop with the side of the pipette or with a sterile swab. This technique is illustrated below. Spreading the liquid prevents it from washing off during staining, or from being too thick to read when stained.



Gentle rolling motion

Figure 2. Applying a drop and spreading with pipette.

Urines and spinal fluids, on the other hand, are not usually mucoid and need not be smeared out. Since these two fluids may not contain any organisms or cells to stain, it can be difficult to find the smear and focus on it with a microscope

For this reason it is a good idea to etch a circle or two parallel lines on the slide with a diamond point pen, and place the drop within the markings.

Sometimes a fluid specimen will be so mucoid that making a thin smear using a pipette will be almost impossible. In that case, a sterile swab may be used to sample the fluid, and smears may be made by rolling swab over the surface of the slide. The smear may still be somewhat thick, but it should be acceptable for staining.

All swabs and pipettes are discarded into disinfectant and the smears are either air-dried or placed on a commercial slide warmer to dry before staining.

Spinal fluids require the use of sterile slides. Immediately before placing any fluid on the slide, it should be dipped in alcohol and then flamed.

c. Smears from culture media

Gram stains of colonies from culture plates are usually done as the first step in identifying an organism. The smear should be thin. Making heavy suspensions seems to be a common problem in making smears from culture. A good way to avoid this tendency is to use only part of one colony for the smear. It is important that the colony to be stained is well isolated. Staining from an area of heavy growth may result in a smear of more than one organism. Determining the Gram stain reaction of the colony in question from such a smear is impossible.

(1) Procedure

- (a) Place a small drop of sterile water or saline on a clean slide.
- (b) Touch the top of the isolated colony to be stained with a flamed and cooled loop or straight wire.
- (c) Make an emulsion on the slide; however, avoid harsh mixing as this may distort the organism or cause the formation of aerosols. It may be helpful to place the slide on a dark background when making the suspension in order to judge the thickness. The resultant emulsion should be thin enough that newsprint could be read through it.

(2) Possible problems

- (a) More than one colony type in the smear makes the stain useless for identifying the unknown bacteria.
- (b) Harsh mixing when making the emulsion leads to aerosols and bacterial distortions.
- (c) Thick smears lead to variable Gram staining, which makes interpretation of the Gram stain very difficult.

B. Fixing the smear

The bacterial suspension should be allowed to dry completely before fixation.

1. Methanol-fixing

The slide should be covered with methanol and allowed to dry before staining.

2. Heat-fixing

Proper heat-fixation ensures that the bacteria and cells will stay on the slide during staining. Underfixing allows the cells to wash off, and overheating will distort morphology. At no point during this process should the slide become too hot to handle. Heat fixation does not guarantee that bacteria will be killed. A smear can be heat fixed in one of two ways:

- a. It can be passed through the flame of a Bunsen burner several times quickly, with the smear side facing away from the flame. Heat-fixing with a Bacti-incinerator involves holding the slide in front of the opening with the smear side up. Total time for these procedures is approximately 15 seconds.
- b. A slide warmer allows the slides to be dried and fixed at the same time. The slide warmer must be kept at a moderate setting, and slides must be left on the warmer for 2-3 minutes.

Regardless of the method used, the back of the slide should feel only slightly warm to the touch. Slides are always cooled before staining, to minimize precipitation during the staining process.

C. Staining procedure

1. Make a thin smear on a glass slide.
2. Air dry.
3. Cover the smear with methanol and allow slide to dry completely. (Slides may be gently heat fixed.)
4. Flood the slide with buffered crystal violet solution; allow it to remain on the slide for 60 seconds.
5. Wash the crystal violet from the slide with tap water, and then flood the slide with mordant. Leave on the slide for 1 minute.
6. Tilt slide and decolorize uniformly with 95% ethanol until colorless solvent flows from the slide. The slide will be clear, usually within 5 to 10 seconds. When multiple smears are placed on separate parts of the slide, decolorization should be done by elevating one edge of the long side of the slide to ensure that the decolorizing agent will flow off all portions of the slide at a similar rate.
7. Remove excess alcohol by rinsing the slide with water.
8. Add the counterstain, safranin, to the slide and allow to react for 60 seconds. Rinse with water.
9. Tilt slide on its side and allow to drain dry. Examine with oil immersion lens.

D. Helpful hints

Timing the decolorizer is critical. Even a gram-positive organism will decolorize. Beginners, however, who are warned of this problem, frequently overcompensate and undercolorize. This results in an unreadable slide on which even gram-negative bacteria, as well as epithelial cells, white blood cells, and background material, are stained purple instead of pink.

Both thick and thin areas often occur on smears of specimens on slides. These areas occur most often with sputum smears. Even a properly stained sputum smear may have thick areas that are unreadable. These thick areas do not present a problem, however, if there are sufficient thinner areas where proper decolorization has occurred. In reading the slide, simply avoid the areas where cells or background, or both, have stained purple rather than pink. This is true for all types of specimens: if background material, white

cells, or epithelial cells are staining purple, avoid that area when reading the smear. There is often some purple-staining precipitate, especially in thick areas of sputum smears. This is easily recognized as such, and the areas containing precipitate should be avoided when reading the smear.

Ideally, smears should air dry after staining. However, if bibulous paper is used for blotting, it should be discarded after use so that organisms blotted off one slide will not be blotted onto another. With certain specimens, for example, spinal fluid, the presence of even an occasional organism is significant; therefore, blotting a spinal fluid smear with a piece of bibulous paper previously used to blot a sputum specimen could result in an extremely misleading reading. For the same reason, care should be taken not to get fingerprints on any part of a slide where a specimen is to be placed.

Specimens that are very viscous, for example, joint fluid, must be spread out thinly on the slide. Otherwise, the smear may be so thick that it will not decolorize properly and frequently it fails to adhere to the slide and "falls off" during the staining process. Specimens that are largely water, for example, urine and spinal fluid, usually result in thin smears, which are readily decolorized, but poor adherence of the specimen to the slide during the staining process may occasionally be a problem. Methanol fixation is preferred to heat fixation. Bacteria fixed by methanol are more resistant to decolorization than bacteria fixed by heat. Placing slides for a brief period on a small electric warming plate results in better adherence to the slide as well as much shorter drying times.

The slides used for Gram staining should have frosted ends for easy marking. The slides must be clean. Precleaned slides from the slide box may be covered with an oil film. If this film is a problem, try flaming the slide. If this doesn't work, dip the slide in alcohol and wipe dry with a clean towel. Hold the slide by the edges; do not get fingerprints where the smear will be placed.

Crystal violet, on standing, may form a precipitate. If this occurs, or if yeast forms are seen on stained smears, filter the stain through Whatman filter paper. This procedure will usually eliminate the problem.

Always wipe the oil immersion lens with lens paper between slides to avoid the carry over of organisms from one slide to another.

Permanent mounts. It is helpful to keep a file of stained slides from specimen material. These slides will serve as a reference for the organisms that are isolated from the patient material. Stained slides kept can also be a valuable tool for comparing morphologies of seldom-encountered organisms and for teaching new technologists. For extended preservation, clear oil from stained slide in xylene, add a drop of Canada balsam to the smear, and place a coverslip on the slide. Excess balsam can be wiped away with a tissue moistened with xylene. Neutral mounting media consisting of plastic (polystyrene) dissolved in solvents (toluene, xylene) and containing a plasticizer (tricresyl phosphate) are marketed under such names as Flo-texx, DPX, and Permout. Flo-texx may be painted onto a stained film on a glass microscope slide without the need for a coverslip. Permout is neutral and does not become acid or discolor with age, nor does it tend to trap bubbles under the coverslip.

E. Quality control

The quality control program of every laboratory must include directions for controlling the Gram stain. The directions should be detailed in each laboratory's Standard Operating Procedure Manual. Good laboratory practice dictates that positive and negative controls for the Gram stain be run with each new batch of stain and once each week of use. If your state regulations require greater frequency than this, then you must comply. Records of this testing must be kept in the Quality Control record book.

The most often used controls are *Staphylococci* and *E. coli*. These are easy to keep in stock. The strains that are used for Kirby-Bauer sensitivity testing work well. Fresh growth can be made by transferring from the stock slants of *Staphylococci* and *E. coli* to trypticase soy broth and incubating for 18 to 24 hours. Dilute some of the broth growth in sterile physiological saline to the density of a 0.5 MacFarland standard. These may then be mixed in equal parts and used to make control smears. The broth tubes that have been standardized for Kirby-Bauer sensitivity testing are ideal for this. Some workers have had success with making multiple smears from this standardized suspension, fixing with methanol, holding these at room temperature for up to a month, and then staining a smear as needed.

Commercial quality control slides are now available from some of the laboratory supply companies.

Another important role of the Gram-stained smear is that of internal quality control, since an organism that fails to grow in culture warrants careful scrutiny. Are the procedures in the laboratory adequate for culture of the organism observed in the stained smear? Have all organisms observed in the Gram stained smear been accounted for? Identifying different types of bacteria with Gram stains also provides microbiologists with guidelines by which to select the culture media to be used and serves as a quality control comparison with the microorganisms that are ultimately recovered in culture. Confirmation that bacteria are present in a Gram stain preparation often provides useful information to the physician; if a morphologic categorization can also be provided, it may be possible to initiate specific therapy.

V. Bacterial Morphology

Before the development of the Gram stain, classification of bacteria by light-microscopic examination was possible on morphologic grounds alone. The three major forms originally described by Leeuwenhoek, the discoverer of bacteria, are still pertinent. Spherical bacteria are known as cocci, cylindrical ones are rods or bacilli, and helical ones are spirilla or spirochetes. Most bacteria encountered clinically are cocci or bacilli.

- A. The differentiation of cocci microscopically depends partly on how they are associated with one another. Cocci that adhere after one cell division appear in pairs, whereas those that multiply in one plane and adhere despite multiple divisions appear in chains. Cocci that divide at varying angles and in different planes will appear as irregular grapelike clusters of cocci.

In addition to the type of association they have with one another, microscopic differentiation also depends on the size and shape of the individual organisms.

1. Gram-negative cocci

The anaerobic gram-negative cocci is *Veillonella*. It is a small coccus appearing singly and in clusters. In clinical material it usually appears in association with other bacterial forms. Of the aerobic gram-negative cocci, two well-known human pathogens belong to the genus *Neisseria*. The *N. gonorrhoeae* and *N. meningitidis* are a gram-negative diplococcus in which the paired cells have flattened adjacent walls. The paired cocci are described as having a miniature coffee bean appearance. Figure 3 shows the typical morphology in a clinical specimen.

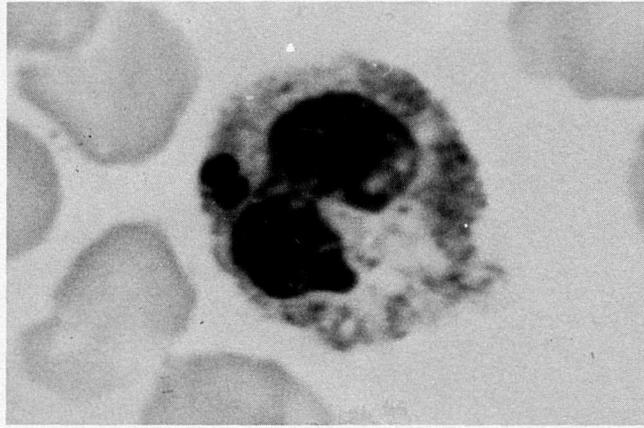


Figure 3. *Neisseria meningitidis* in a polymorphonuclear leukocyte. More typical morphology is seen in a clinical specimen.

2. Gram-positive cocci

Figures 4 through 7 show the different morphologic types of gram-positive cocci commonly encountered in clinical specimens and the most likely organism for each, without regard to the specimen source. In addition to morphology, however, the source of an organism often gives some clue about its identity.

For example, pneumococci and enterococci have a similar morphology, but pneumococci would be very unlikely in a urine specimen, whereas pneumococci are more likely in a spinal fluid. Cocci that are medium-sized and round, are never elongated, and tend to form grapelike clusters are usually staphylococci. If the source is a clinical specimen, the organism most often will be *Staphylococcus aureus*. *Staphylococcus epidermidis* is also a possibility. Micrococci tend to be somewhat larger than the cocci of staphylococci and also stain more intensely, sometimes appearing almost black.

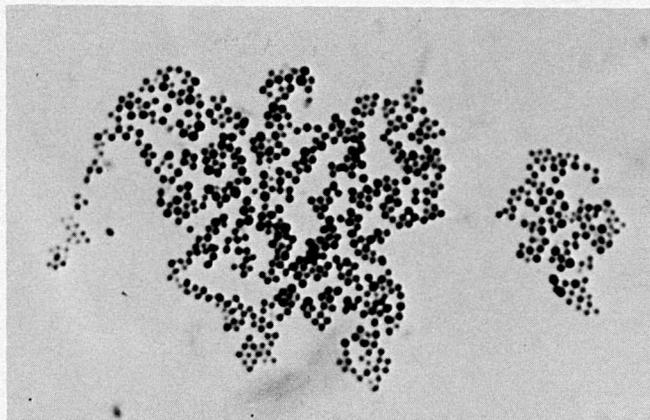


Figure 4. *Staphylococcus aureus* from a 24-hour trypticase soy broth.

- b. Below is a culture of staphylococci emulsified from a blood agar plate. Streptococci from a blood plate could look very similar. These cocci are round and occur as pairs, short chains, or clumps. The size is consistent. A Gram stain must be interpreted along with other characteristics that are available; for example, colonial morphology on plate and hemolysis.

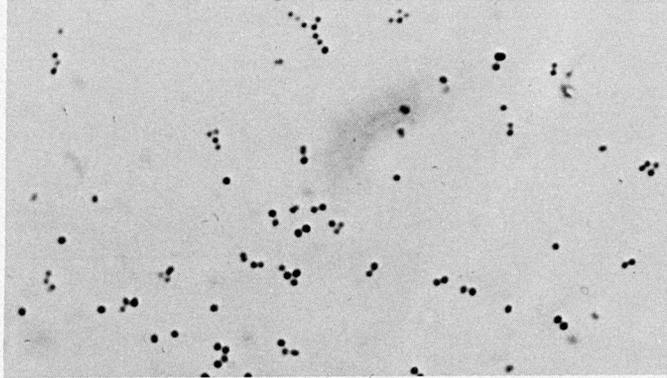


Figure 5. *Staphylococcus aureus* from a 24-hour blood plate.

- c. Round cocci that are seen in chains are usually streptococci. They divide on one plane only (longitudinally). When stained from a broth culture, these chains are long and may reach from one end of the microscopic field to another. Stains made from solid culture media have much shorter chains. Streptococci also destain very easily, giving chains of mixed gram-positive and gram-negative cocci.

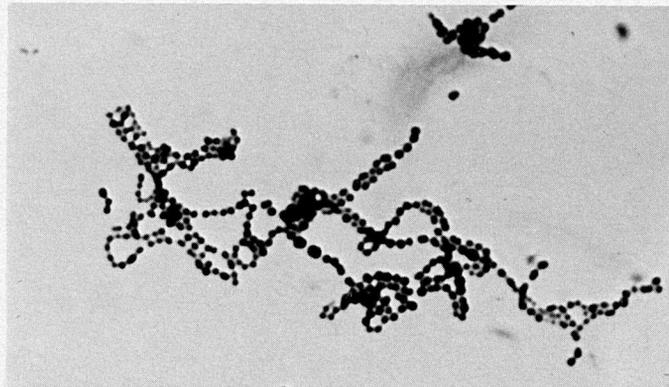


Figure 6. Group A *Streptococci* from a 24-hour trypticase soy broth.

d. Elongated cocci in diplos or chains of diplos will most likely be pneumococci, enterococci, or alpha streptococci. They, like the streptococci, divide along a longitudinal axis. It is often possible to determine which of the three is the most likely in a Gram stain of a clinical specimen by considering other factors such as specimen source, association of the organism with white blood cells or epithelial cells, and the presence of other organisms. Except for blood cultures, alpha streptococci are rarely pathogens in clinical specimens. They are commonly seen in sputum specimens because of oral contamination. Most pneumococci are elongated and tend to occur in diplos and sometimes in chains of diplos. Typically, they appear as two elongated cocci surrounded by a halo of capsular material.

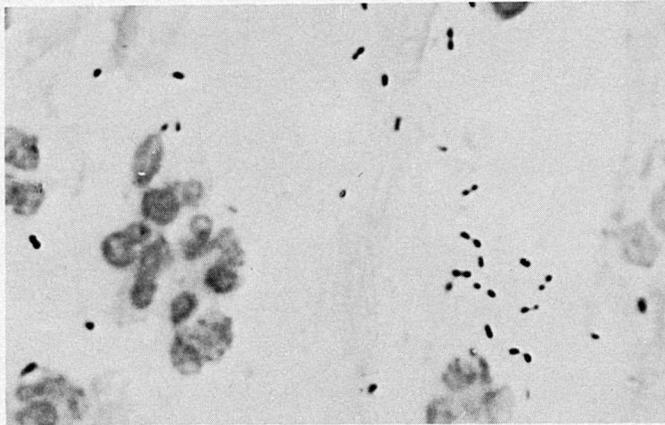


Figure 7. Cerebrospinal fluid smear with *Streptococcus pneumoniae*.

B. Differentiation of rod forms seen in clinical specimens is somewhat limited. One must pay special attention to shape, size, and arrangement. Rods will vary in size and thickness. The ends of rods may have one of several appearances. They can be rounded, tapered, or squared, and noting this will give another clue to its identity. The thickness of the bacteria may also differ from reasonably thick rods to very delicate thin rods appearing almost as a filament. Figures 8 through 29 are examples of rod forms.

1. Gram-negative rods

Many of the various gram-negative rods seen in clinical specimens have similar morphology. Large and/or fat gram-negative rods are most likely to be *Escherichia coli*, *Klebsiella*, or *Enterobacter*. *Pseudomonas* is typically quite thin. Medium-sized gram-negative rods could be any of a variety of organisms, including *E. coli*, *Klebsiella*, *Enterobacter*, *Serratia*, or *Proteus*. Tiny gram-negative rods in sputum specimens are most likely to be *Haemophilus*.

a. Anaerobic gram-negative rods usually stain more faintly than aerobic gram-negative rods and in many cases are relatively small. The stain of *Bacteroides fragilis* in figure 8 shows extreme pleomorphism. Note the differences in length of rod and the long chain of rods. The vacuoles in many cells give a "safety pin" appearance. This pleomorphism is much more evident when stained out of thioglycollate.

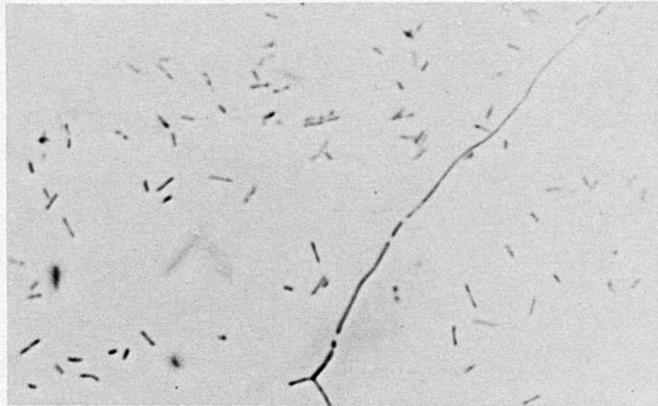


Figure 8. Gram stain *Bacteroides fragilis* from thioglycollate.

- b. Fusobacteria are long, filamentous gram-negative rods. Some have tapered ends. They stain very poorly with safranin.

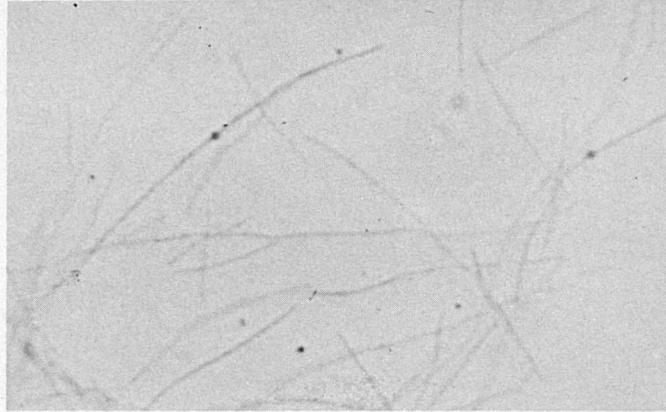


Figure 9. *Fusobacterium nucleatum* from pure culture.

2. Gram-positive rods

- a. The large-to-medium Gram-positive rods from clinical material will almost always be either *Clostridium* or *Bacillus*. These rods usually stain uniformly and have rounded or squared-off ends. The *Clostridium*, with the exception of *C. perfringens*, should produce a spore.

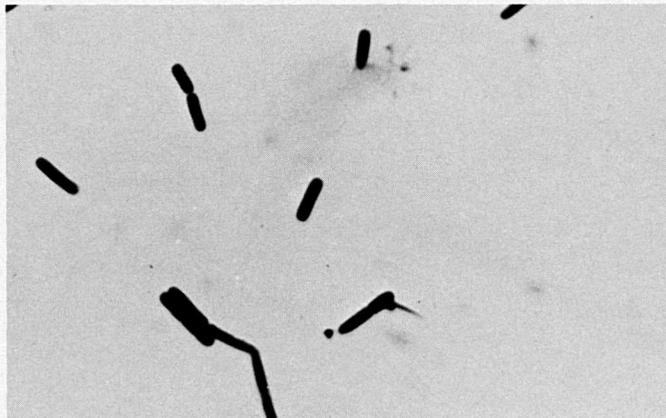


Figure 10. Large rods with rounded ends, *Bacillus subtilis*

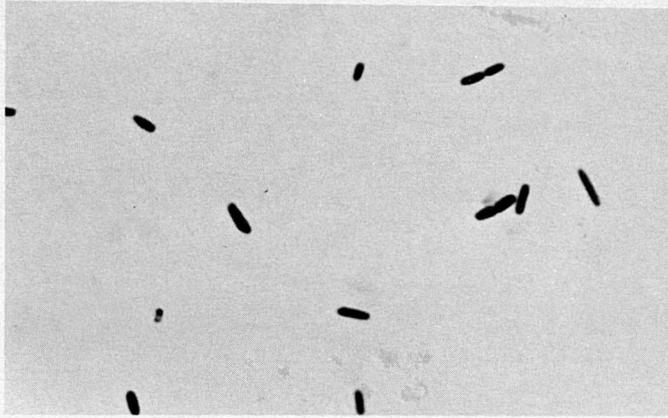


Figure 11. Medium rod with rounded ends, *Bacillus cereus*.



Figure 12. Medium rods with round terminal spores, *Clostridium tetani*.

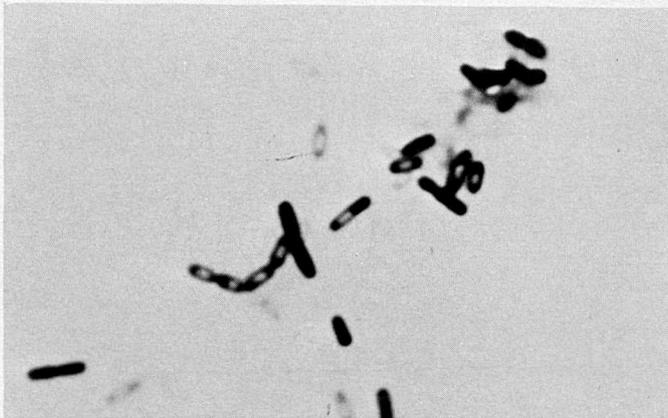


Figure 13. Large rods with oval subterminal spores, *Clostridium bifermentans*.

- b. Small-to-medium-sized, slightly pleomorphic, gram-positive rods arranged in palisades or "Chinese letters" are usually diphtheroids. The Chinese letter formation is most often exhibited by the pathogenic bacteria in this species, *Corynebacterium diphtheriae*.

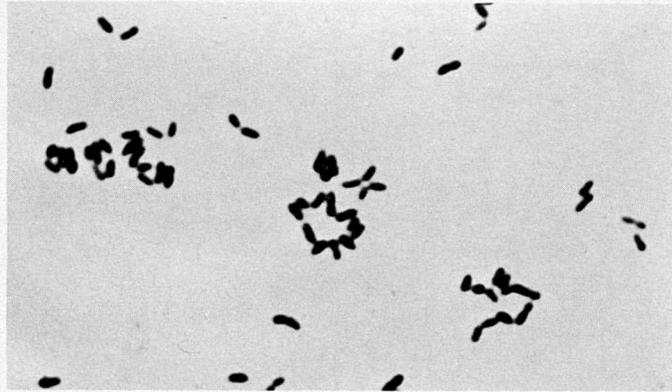


Figure 14. *Corynebacterium diphtheriae*, nontoxicogenic

- c. Small gram-negative rods may be fairly uniform in morphology with rounded ends. The *Listeria* shown below also shows some parallelism. The rods usually stain evenly.

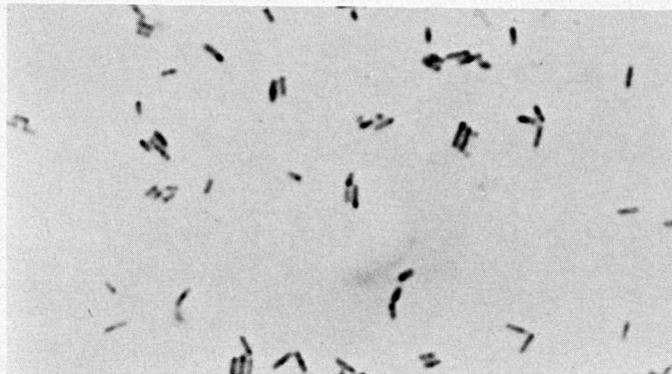


Figure 15. *Listeria monocytogenes*

- d. Very small rods can be confused with cocci, and are sometimes called "cocciobacillary." It should be noted that this is not a distinct class in itself, as an organism is either a rod or a coccus, and not both.

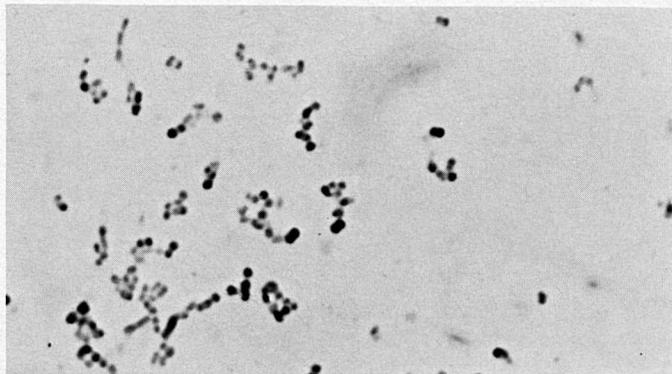


Figure 16. *Acinetobacter calcoaceticus*, a gram-negative rod, which may show as gram variable, especially from broth culture.

- e. Filamentous, branching gram-positive organisms are probably either *Nocardia* or *Actinomyces*. These two organisms were formerly thought to be fungi but are now classified with the bacteria.

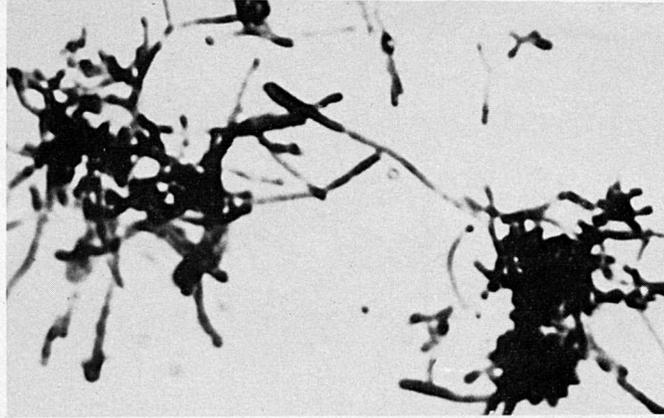


Figure 17. *Actinomyces naeslundii*

- f. The spiral-shaped bacteria include *Borrelia*, *Treponema*, and *Leptospira*. Only *Borrelia* can be seen with the Gram stain. It stains very faintly gram-negative. The others require specialized techniques for their demonstration, such as silver impregnation and dark-field microscopy.

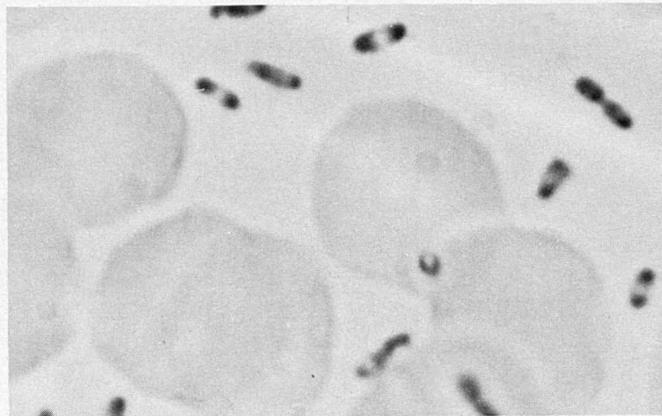


Figure 18. *Yersinia pestis* is a medium rod with bi-polar staining.

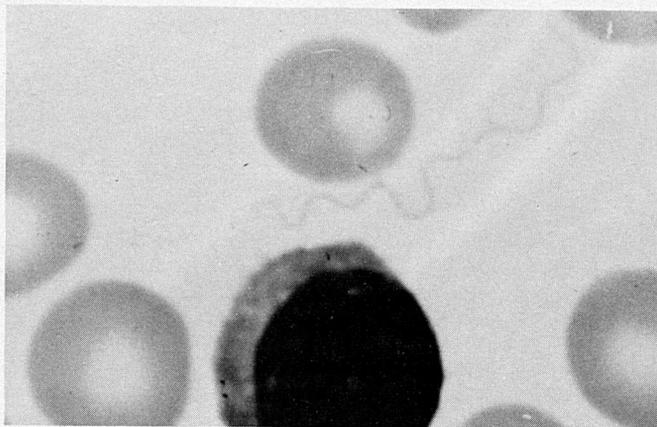


Figure 19. *Borrelia*

VI. Gram Stains of Clinical Specimens

Proper use of the Gram-stained smear requires, first, a properly collected specimen. Sputum specimens should be routinely examined microscopically before they are cultured, and another specimen should be requested, if more than 25 squamous epithelial cells are observed per low-power field (100 X). Some have advocated microscopic screening before culturing midstream, clean-voided urines and wound drainages to detect undue contamination. A specimen labeled "endometrium" containing squamous epithelial cells is contaminated with vaginal flora and should not be cultured anaerobically.

The Gram-stained smear has at least presumptive diagnostic value when used to examine:

1. Sputum from patients with pneumococcal, staphylococcal, meningococcal, and *Haemophilus* pneumonia
2. Transtracheal aspirations from patients with anaerobic pleuropulmonary infections (usually displaying mixed gram-positive and gram-negative flora) and staphylococcal, pneumococcal, meningococcal, or *Haemophilus* pneumonia
3. Cerebrospinal fluid from patients with pneumococcal, meningococcal, and *Haemophilus* meningitis
4. Pus from previously undrained abscesses due to staphylococci or anaerobes
5. Urethral exudates from males with gonorrhea
6. Other normally sterile body fluids in which the organism's morphology is distinctive or unique

Color plates 1 and 2 show some examples of Gram stains in clinical material with bacteria of typical morphology.

A telephoned and written report of the Gram-stained smear should be given to the attending physician as soon as possible. The laboratory's report should be descriptive enough to provide the clinician with information that will be of assistance in initiating antimicrobial therapy. Therapeutic decisions based on the smear can lead to a reduction in the inappropriate use of antibiotics.

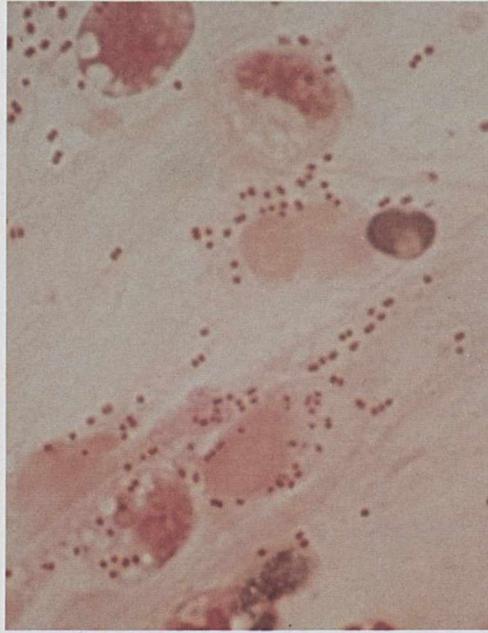
Microorganisms are seen on smear only if present in the specimen at a concentration of about 10^4 to 10^5 organisms per milliliter (2, 13). Caution should be exercised when few organisms are present, or the organism's morphology is not distinct.

Some bacteria do not stain well with the Gram stain and require special stains like Giemsa or Wright stain. When it becomes necessary to do a Gram stain on a tissue section, Churukian's modification of the Brown-Brenn (9) stain works quite well. See color plate 2, pictures K and L for examples.

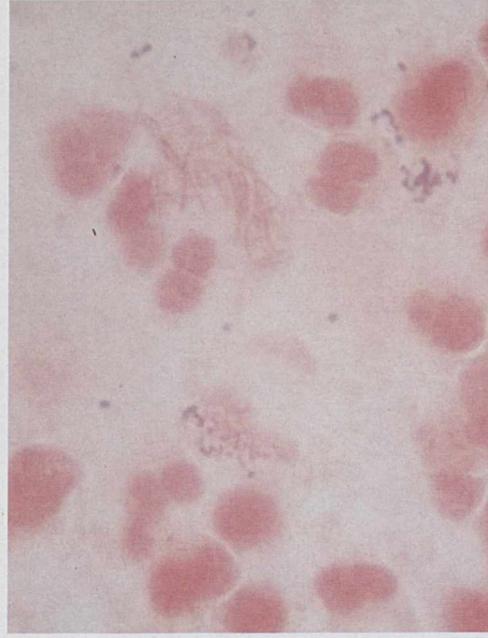
Color Plate 1
Gram Stains of Clinical Material

- A. Sputum specimen with gram-negative diplococci. Note capsular appearance. *Branhamella catarrhalis*.
- B. Vaginal smear with many polymorphonuclear neutrophils. Note the fusobacterium, cocci in chains, and possible gram-positive rods.
- C. Urethral exudate from a male showing gram-negative intercellular diplococci. *Neisseria gonorrhoeae*.
- D. Cerebrospinal fluid with a white blood cell and very tiny pleomorphic gram-negative rods. *Haemophilus influenzae*.
- E. Cerebrospinal fluid with polymorphonuclear neutrophils and gram-positive, lancet-shaped, diplococci. Note the halo of capsular material around the coccus. *Diplococcus pneumoniae*.
- F. Smear made from abscess material. Note the mixture of thin to thick rods with rounded ends, usually indicating both aerobic and anaerobic bacilli. Note the serosanguineous fluid in the background.

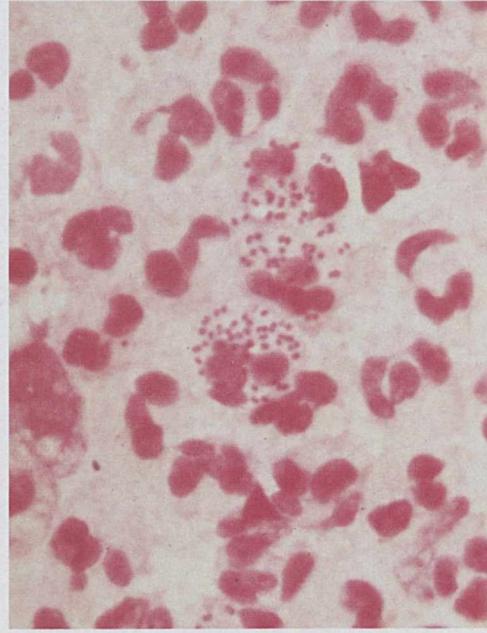
PLATE 1



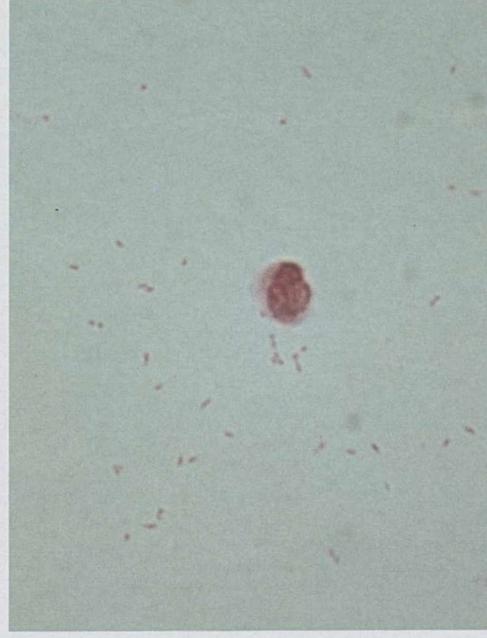
A



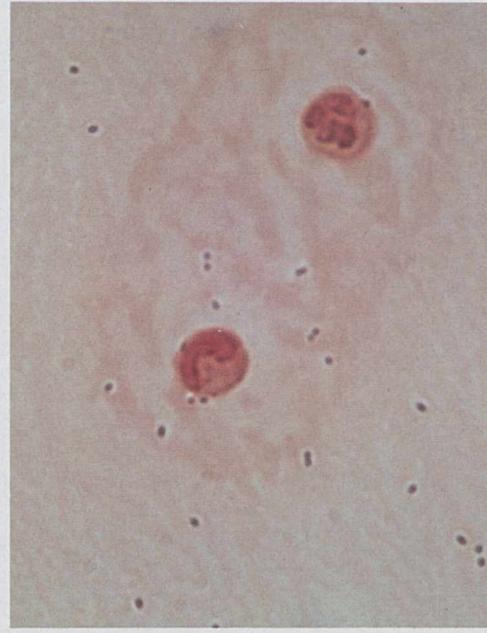
B



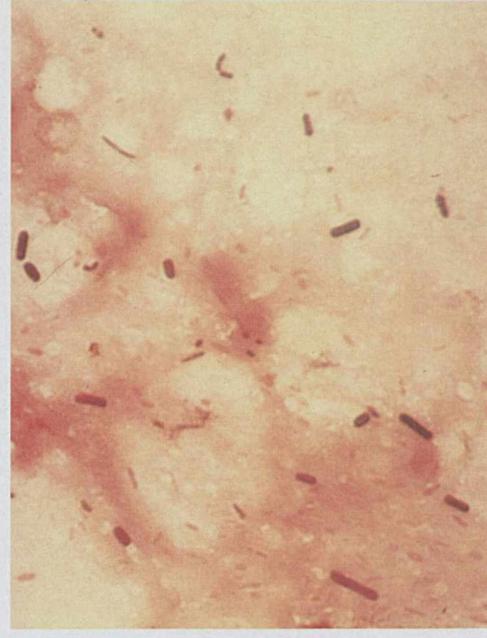
C



D



E

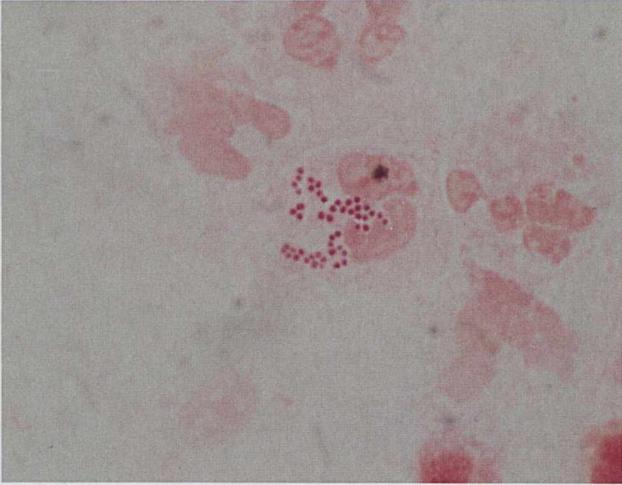


F

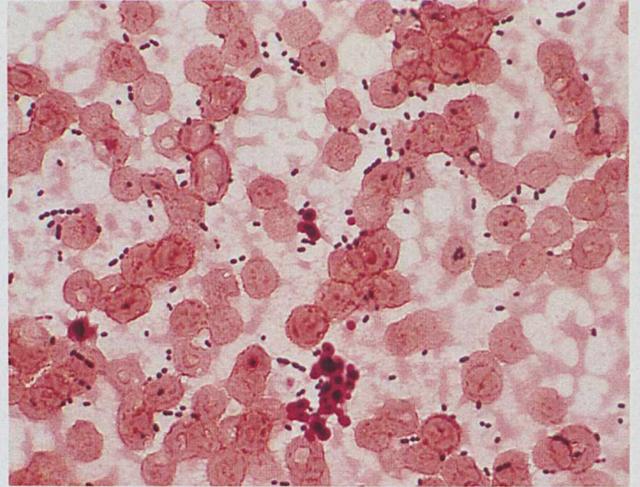
Color Plate 2
Gram stains of Clinical Material

- G. Smear made from abscess material with many polymorphonuclear neutrophils. The cocci are round and appear in clusters. *Staphylococcus aureus*.
- H. Gram stain from a 24-hour blood culture bottle taken from a patient with pneumonia. Note the lancet shaped gram-positive diplococci in pairs and short chains. *Diplococcus pneumoniae*.
- I. Gram stain from a 24-hour blood culture bottle from a patient with septicemia. Note the absence of intact red blood cells and the numerous short gram-negative rods.
- J. Gram stain of an unspun urine specimen. Note many white blood cells and the gram-negative coccobacillus.
- K. A section of lung tissue stained with Brown and Brenn. Gram-negative rods.
- L. A section of lung tissue stained with Brown and Brenn. Gram-positive cocci.

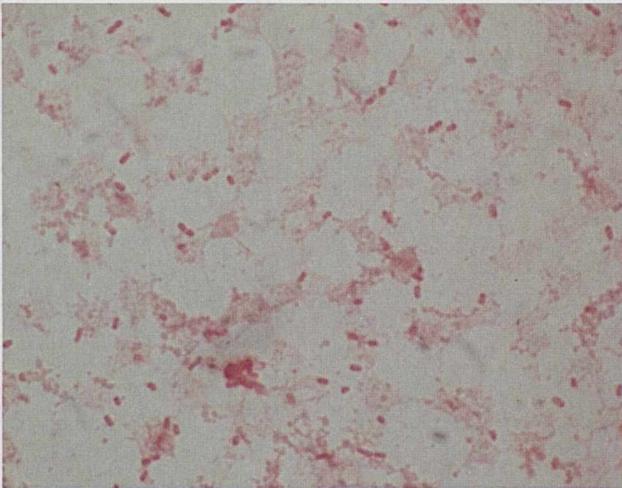
PLATE 2



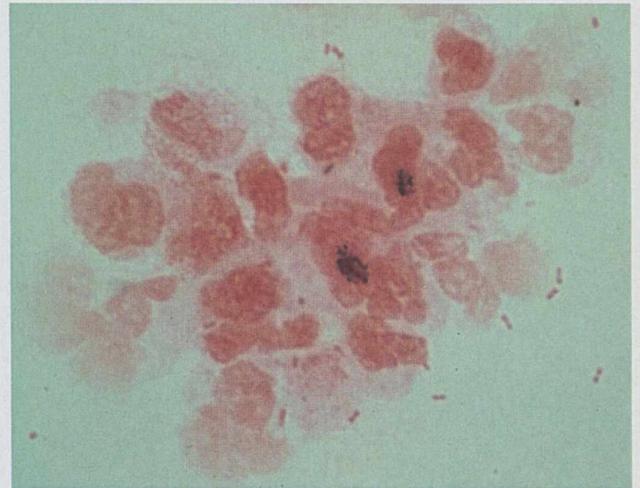
G



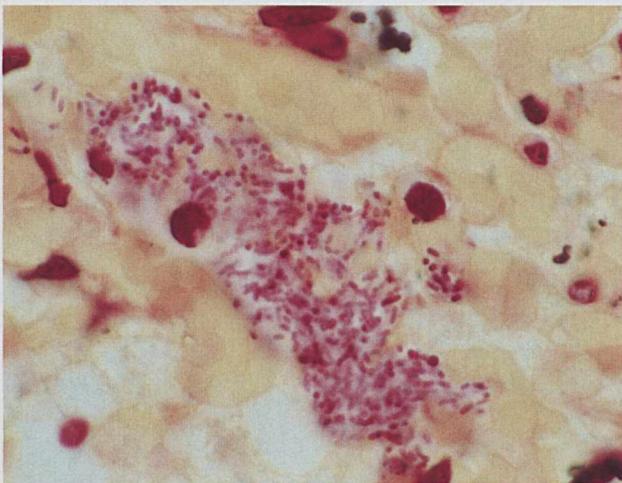
H



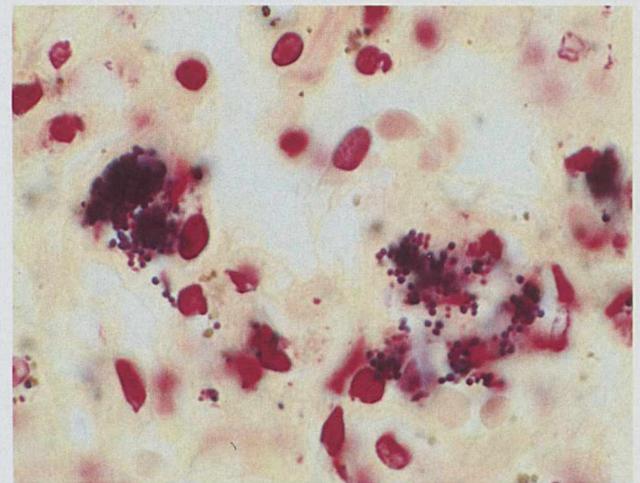
I



J



K



L

VII. Avoiding Problems with the Gram stain

Each step of the Gram stain should receive careful attention. Problems can occur at various steps of the procedure that make reading difficult or results misleading.

1. Slides

Slides are purchased from the manufacturer precleaned and ready for use. If, in preparing the smears, the smear beads up or does not adhere to the slide, further cleaning is probably necessary. The slides can be dipped in alcohol and then wiped dry with a lint-free towel. Sometimes just passing the smear side of the slide through a flame is sufficient to burn off the grease film.

2. Making the smear

Careful attention should be paid to sampling the clinical specimen. Selection of thick mucous areas or bloody specks in purulent material is most rewarding. Cultures should be diluted to a density through which print can be read. Spread the material thinly on the slide.

3. Fixing the smear

Make certain that the smear has dried adequately before fixing. If heat fixation is used, the slide should not get too hot to hold comfortably, because overheating can cause distorted or fragmented cells. If crystal violet is added to a hot slide, precipitation of the dye can occur.

If alcohol fixation is used, allow the alcohol to evaporate before staining. Never hurry the process by blotting, since some of the smear can be lost.

4. Staining

Use a staining rack in or near the sink. Make certain that it is level so that the dye will cover the slides evenly. Use slide forceps and handle the slides individually. Never try to stain more than 4 or 5 slides at one time to maintain accurate timing.

The wash steps should be done under a very gentle stream of water from the tap or poured from a flask or beaker.

The decolorizing step is especially critical. Tilt and shake the slide to remove as much water as possible before decolorizing. Remember, water dilutes the alcohol and makes it react faster.

Slides should never dry completely between steps. Air dry, never blot the stained slides.

5. General

Use the 10X objective to scan the smear for the best area to be examined under 100X.

Look at the control slide first to see that positive and negative staining are accurate.

If you see yeast forms or precipitation of dye on the slide, the crystal violet may need to be filtered or made fresh.

Always wipe the oil immersion objective with lens paper between the reading of critical slides to avoid carryover of cells.

6. Some examples of Gram stain interpretation.

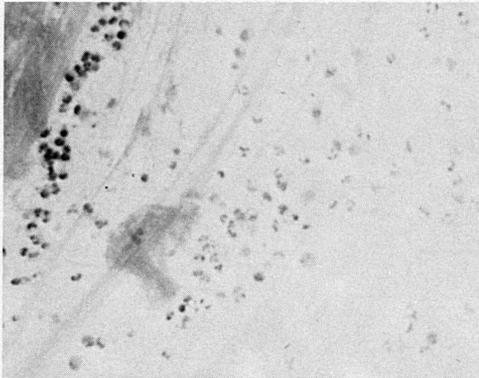


Figure 20. Sputum specimen observed on low power field (100X); 25 white blood cells and 10 squamous epithelial cells.



Figure 21. Improper sputum specimen; low power field (100X); 25 white blood cells, 10 squamous epithelial cells.

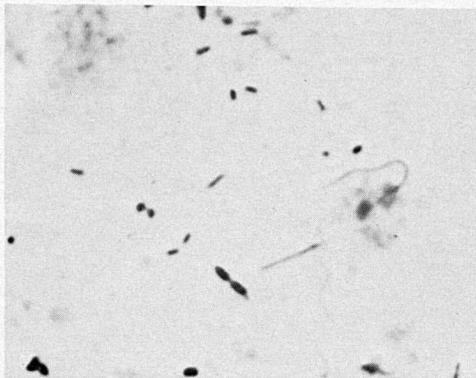


Figure 22. Improper sputum (saliva) as indicated by mixed flora.

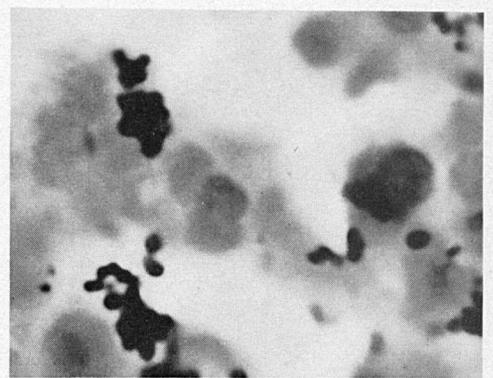


Figure 23. Smear too thick, all bacterial forms not visible.

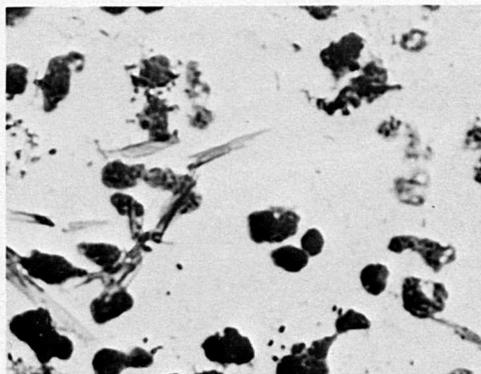


Figure 24. Crystal formation

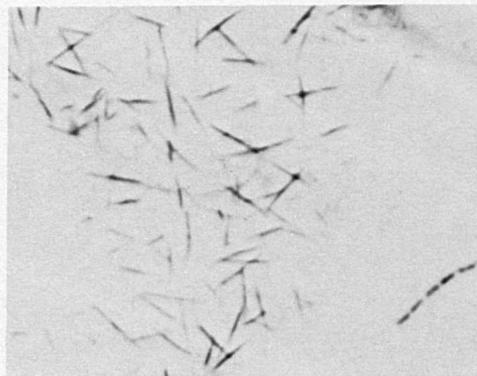


Figure 25. Crystal formation

VIII. Appendix

A. Gram Stain - CDC Modification

1. Crystal violet.

a. Crystal violet powder	13.87 gm
(99% dye content)*	
Dissolve in 95% ethanol	200.00 ml
b. Ammonium oxalate	8.00 gm
Distilled water	800.00 ml

Mix A and B. Let sit overnight or for several days until dye goes into solution. Filter through coarse filter paper.

2. Gram's iodine.

Iodine crystals	1.00 gm
Potassium iodide	2.00 gm
Distilled water	300.00 ml

3. Decolorizer.

95% Ethanol

4. Safranin.

Safranin-0	3.41 gm
95% Ethanol	100.00 ml
Distilled water	900.00 ml

*Dye content will vary. The actual dye content of each lot is given on the dye bottle label. The weight of dye to be used should be adjusted accordingly.

B. Gram Stain - Hucker Modification

1. Crystal violet.

a. Crystal violet powder	20.0 gm
(90% dye content)*	
Dissolve in 95% ethanol	200.0 ml
b. Ammonium oxalate	8.0 gm
Distilled water	800.0 ml

Mix A and B. Let sit overnight or for several days until dye goes into solution. Filter through coarse filter paper.

2. Gram's iodine.

Iodine crystals	1.0 gm
Potassium iodide	2.0 gm
Distilled water	300.0 ml

3. Decolorizer.

95% Ethanol

4. Safranin.

Safranin-O	2.5 gm
95% Ethanol	100.0 ml
Distilled water	900.0 ml

*Dye content will vary. The actual dye content of each lot is given on the dye bottle label. The weight of dye to be used should be adjusted accordingly.

C. Staining procedure (Aerobic bacteria)

1. Make thin smear on glass slide.
2. Air dry and fix GENTLY with heat or methanol.
3. Add crystal violet for 1 minute.
4. Rinse with tap water.
5. Add Gram's iodine for 1 minute.
6. Wash slide with tap water.
7. Decolorize with 95.% ethanol for about 30 seconds or until the wash is clear.
8. Counterstain with safranin for 30-60 seconds and wash off in tap water.

D. Staining Schedule (Anaerobic bacteria)

1. Make thin smear on a glass slide.
2. Air dry and fix with methanol.
3. Flood slides 1 minute with a mixture of equal parts Hucker's crystal violet solution and 1% sodium bicarbonate. Wash them off briefly in tap water (not over 5 seconds).
4. Flood slides with Gram's iodine solution for 1 minute and wash them off in tap water.
5. Flood slides with 95% alcohol and pour it off immediately. Reflood slides with 95% alcohol for 10 seconds, and wash them off in tap water.
6. Flood slides with Hucker's safranin solution for 1 minute and wash them off in tap water.

E. Brown-Brenn Gram Bacteria Stain - Churukian method on tissue sections.

Fixation: 10% neutral buffered formalin

Technique: Cut paraffin sections at 6 microns

Solutions:

1. Modified Hucker-Cohn Crystal Violet

Crystal violet (C.I. 42555), 10% alcohol	2.0 ml.
Ammonium oxylate, 1% aqueous	98.0 ml.

2. Modified Gram's Iodine

Iodine	2.0 gm.
Potassium iodide	4.0 gm.
Distilled water	400.0 ml.

Dissolve the potassium iodide in about 20 ml of the distilled water, add the iodine, and dissolve it. Add the remainder of the distilled water.

3. Absolute Alcohol-Acetone

Absolute alcohol	50.0 ml
Acetone	50.0 ml.

4. Absolute Fuchsin, Stock

Basic fuchsin (C.I. 42500 or 45510)	0.5 gm.
Distilled water	100.0 ml.

This must be prepared by placing on a magnetic stirrer and applying heat until dissolved.

5. Basic Fuchsin, Working

Basic fuchsin, stock	5.0 ml.
Distilled water	45.0 ml.

6. Picric Acid-Acetone

Picric acid	0.1 ml.
Acetone	100.0 ml.

7. Acetone-Xylene

Acetone	50.0 ml.
Xylene	50.0 ml.

F. Staining Procedure:

1. Dip sections in Xylene, absolute, 95% alcohol and distilled water.
2. Place in modified Hucker-Conn crystal violet for 2 minutes.
3. Wash briefly in distilled water.
4. Place in modified Gram's iodine for 1 minute.
5. Wash briefly in tap water.
6. Blot the slide, but not the tissue section.
7. Decolorize with alcohol-acetone until the blue color stops coming off. This usually requires 8 to 10 dips.
8. Place in working basic fuchsin. Dip several times before timing for 1 minute.
9. Rinse in distilled water.
10. Blot the slide but not the tissue section.
11. Place in acetone for 3 seconds.
12. Differentiate in picric acid-acetone for 10 seconds.
13. Dip a few quick times in acetone-xylene
14. Clear in xylene for several changes.
15. Mount with Permount.

Results:

Gram-positive	blue to blue-black
Gram-negative	red
Filament of nocardia & actinomyces	blue or blue & red
Nuclei and elastic fibers	red
Paneth cells	red
Other tissue elements	yellow

Legionella pneumophila will not stain with this method.

Reference:

Churukian, C.J.: Journal of Histotechnology Vol. 5, No. 3, 1982

REFERENCES

1. Agger WA, Maki DG. Efficacy of direct Gram stain in differentiating staphylococci from streptococci in blood cultures positive for gram-positive cocci. *J Clin Microbiol* 1978; 7:111-113.
2. Barry AL, Smith PB, Turck M. IN: Gavan TV, Coordinating ed. *Cumitech 2: Laboratory diagnosis of urinary tract infections*. Washington: American Society for Microbiology, 1975.
3. Bartholomew JW, Mittwer T. The mechanism of the gram reaction. I. The specificity of the primary dye. *Stain Technol* 1950; 25:103-110.
4. Bartholomew JW, Mittwer T. The mechanism of the gram reaction. III. Solubilities of dye-iodine precipitates and further studies of primary dye substitutes. *Stain Technol* 1951; 26:231-240.
5. Bartholomew JW, Mittwer T. The Gram stain. *Bacteriol Review* 1952; 16:1-23.
6. Brooks GF, Pribble AH, Beaty HN. Early diagnosis of bacteremia by buffy-coat examinations. *Arch Intern Med* 1973; 132:673-675.
7. Burke V. Notes on the Gram stain with description of a new method. *J Bacteriol*, 1922; 7:159-182.
8. Center for Disease Control. *Criteria and techniques for diagnosis of gonorrhea*. Atlanta: U.S. Department of Health Education and Welfare, Public Health Service, 1974.
9. Churukian CJ. *J of Histotechnology* 1982; 5:3.
10. Dalton HP, Allison MJ. Modification of laboratory results by partial treatment of bacterial meningitis. *Am J Clin Pathol* 1968; 49:410-413.
11. Edwards LD, Levin S, Balagtas R, Lowe P, Landau W, Lepper M. Ordering patterns and utilization of bacteriologic culture reports. *Arch Intern Med* 1973; 132:678-682.
12. Fadden HS. Early diagnosis of neonatal bacteremia by buffy-coat examination. *J Pediatr* 1976; 88:1032-1034.
13. Feldman WE. Concentrations of bacteria in cerebrospinal fluid of patients with bacterial meningitis. *J Pediatr* 1976; 88:549-552.
14. Gram C. *Uber die isolirte Farbung der Schizomyceten in Schnittund Trockenpreparaten*. *Fortschr Med* 1884; 2:185-189.
15. Handsfield HH, Lipman TO, Harnisch JP. Asymptomatic gonorrhea in men: Diagnosis, natural course, prevalence and significance. *N Engl J Med* 1974; 290:117-123.

16. Heineman HS, Chawla JK, Lofton WM. Misinformation from sputum cultures without microscopic examination. *J. Clin. Microbiol* 1977; 6:518-527.
17. Hodges GR, Perkins RL. Acute bacterial meningitis. An analysis of factors influencing prognosis. *Am J Med Sci* 1975; 270:427-439.
18. Hucker GJ. A new modification and application of the Gram stain. *J Bacteriol* 1921; 6:395-397.
19. Hucker GJ, Conn HJ. Further studies on the methods of Gram staining. N.Y. (Geneva) *Agr Exp Sta Tech Bull* 1927; 128:1-34.
20. Jorgensen J, Lee JC. Rapid diagnosis of gram-negative bacterial meningitis by the Limulus endotoxin assay. *J Clin Microbiol* 1978; 7:12-17.
21. Kass, EJ. Asymptomatic infections of the urinary tract. *Trans Assoc Am Physicians* 1956; 69:56-64.
22. Kopeloff N, Beerman P. Modified Gram stains. *J Infect Dis* 1922; 31:480-482.
23. Kopeloff N, Cohen P. Further studies on a modification of the Gram stain. *J Bacteriol* 1928; 15:12.
24. Kunin C. Detection, preservation, and management of urinary tract infection. 2nd ed. Philadelphia: Lea and Febiger, 1974.
25. Lasseur P. Etude de la reaction de Gram. *Ann Inst Pasteur* 1927; 41:554-575.
26. Lillie RD. The Gram stain. I. A quick method for staining gram positive organisms in tissues. *Arch Pathol* 1928; 5:828-834.
27. Mittwer T, Bartholomew JW, Kallman BJ. The mechanism of the gram reaction. II. The function of iodine in the Gram stain. *Stain Technol* 1950; 25:169-179.
28. Murray PR, Washington JA, Jr. Microscopic and bacteriologic analysis of expectorated sputum. *Mayo Clin Proc* 1975; 50:339-344.
29. Nachum R. Rapid detection of gram-negative bacterial meningitis by the Limulus lysate test. *N Eng J Med* 1973; 289:931-934.
30. Rein MF. Gonorrhoea. IN: Practice of medicine. New York: Harper and Row, 1975; Vol 3. Chap. 19.
31. Ries K, Levison ME, Kaye D. Transtracheal aspiration in pulmonary infection. *Arch Intern Med* 1974; 133:453-458.
32. Sheppe SM, Constable MG. An unsuspected source of error in the Gram stain. *J Lab Clin Med* 1923; 8:757-760.

