USE OF THE GRAM STAIN FOR DIAGNOSIS OF INFECTIOUS DISEASE

Part Two: Infectious Agents

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One can provide useful suggestive information as to the identification of bacterial forms in clinical specimens, as suggested by these kissing cocci.
Gram positive cocci in clusters provide a presumptive identification of staphylococci. One cannot distinguish coagulase-positive from coagulase-negative staphylococci; the anaerobic staphylococci (peptococci) have a similar appearance. If well-defined clumps of cocci are present in a clinical specimen and are extracellular, the identification of these organisms as staphylococci is quite accurate.
If the clumps of bacteria are intracellular, however, then one must use caution in assigning a staphylococcal designation to them. Once the bacteria have been phagocytized they will of necessity clump within the cytoplasmic phagosomes of the cell. This slide represents a streptococcal infection in which most of the bacteria have been engulfed by phagocytes. The classic description of the genus *Streptococcus* is gram-positive cocci in chains.
SPECIMEN WITH LONG CHAINS OF COCCI
If one has a clinical specimen with long chains of cocci, the identification of these organisms as streptococci is quite reliable. An arbitrary designation of five cocci as a chain is usually used. By using this definition one can avoid a mistake caused by mistaking two pairs of diplococci for a chain. On occasion certain gram-positive bacilli, particularly lactobacillus (which forms chains of rods), can resemble streptococci sufficiently to cause problems, but this is an unusual situation.
In this Gram-stained smear of a positive blood culture bottle one can see gram-positive cocci in pairs, chains (arrows), and clusters (arrowhead). It is often possible to differentiate streptococci (chains) from staphylococci (clusters). When they are seen together, however, it is best to remain non-committal, reporting only gram-positive cocci. Both genera may be present, but it is particularly important not to direct attention away from the possible presence of staphylococci by reporting streptococci even if they are the predominant form, as the staphylococci will not respond to penicillin therapy. Group B beta hemolytic streptococci were recovered in culture in this case.
Evaluation of the morphology of cocci on smears made from growth on agar media may be misleading. This smear of a Group B streptococcus taken from a blood agar plate contains gram-positive cocci in pairs and chains, but tetrads and clumps are also present. If the morphology is uncertain, the gram stain reaction and morphology from a four-hour broth culture should be evaluated.
DISTINCTIVE AND CLASSIC MORPHOLOGY OF PNEUMOCOCCUS
(defined as lancet-shaped diplococci)
Sometimes a clearing around bacteria may suggest the presence of a capsule (arrows), but it is easy to be fooled when evaluating a Gram smear. Pseudocapsules may appear when focusing up and down. The following slide demonstrates a pneumococcal capsule.
The “Quellung” test is a precipitin reaction between a polysaccharide capsule (here type 3 pneumococcus) and a specific antiserum. The immunologic reaction renders the capsule easily visible. Unfortunately, the antisera are very expensive and the procedure works much less well on isolated bacteria than on bacteria in direct smears.
The pneumococcus may form short chains, but usually does not form very elongated tangled chains of cocci. The lancet shape refers to the pointed ends of the pair of cocci (arrows). In the appropriate clinical situation this morphology is very suggestive of pneumococcus, but the differentiation from other streptococci requires rather more experience than does the characterization of staphylococcal clumps and streptococcal chains. Enterococci also occur in pairs and may appear lancet-shaped. Furthermore, the group of pleomorphic gram-positive rods that are sometimes lumped together as diphtheroids may on occasion resemble lancet-shaped pairs of cocci.
Winn’s Law

• All gram-positive organisms aren’t blue
• All gram-negative organisms aren’t red
• All cocci aren’t round
• All bacilli aren’t long
• Bonus: Some bacteria stain poorly
• Super Bonus: Some bacteria don’t stain at all
Some authorities describe a “chain” as more than four cocci in a row, by which definition two pairs of pneumococci that haven’t separated do not qualify as a “chain.” Of course, there are always exceptions! In this field the number of cocci in some chains “squeaks” over the arbitrary limit of the definition. Notice that one of the cocci is more elongated (“a bigger lance”) than others (arrow).
In this field most of the bacteria are coccal, but some of the bacteria are elongated (arrows). They can be judged here by the “company they keep” but the potential problem in interpretation is obvious.
Detection of Pneumococcus in Sputum Specimens
### ACCURACY OF GRAM’S STAIN IN IDENTIFYING PNEUMOCOCCI IN SPUTUM

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Sensitivity (%) n=29</th>
<th>Specificity (%) n=13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any Gram Positive Diplococci, Any Shape</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Any Gram Positive Lancet Shaped Diplococci</td>
<td>83</td>
<td>38</td>
</tr>
<tr>
<td>Preponderant Flora of Gram Positive Doplococci, Any Shape</td>
<td>85</td>
<td>46</td>
</tr>
<tr>
<td>More Than 10 Gram Positive Lancet Shaped Diplococci/Oil Immersion Field</td>
<td>62</td>
<td>85</td>
</tr>
<tr>
<td>Preponderant Flora of Gram Positive Lancet Shaped Diplococci</td>
<td>48</td>
<td>100</td>
</tr>
</tbody>
</table>

Illustration of the Criteria Set Forth in the Previous Table
SUMMARY OF ATTEMPT TO ASSESS ACCURACY OF GRAM’S STAIN

The previous slides show a summary of an attempt to assess the accuracy of Gram’s stain for identifying pneumococci in sputum. They demonstrate the importance of careful analysis of bacterial morphology. The investigators used various criteria to predict the presence of pneumococci in the culture of the sputum specimen. (Note: Definitive diagnosis of pneumococcal pneumonia is difficult and this paper did not even make an attempt to provide that most important assessment).

Note that if a loose criterion for identifying pneumococci was employed (any gram positive diplococci of any shape) all cases in which pneumococci were present were detected, but the specificity was zero. Every sputum specimen contains streptococci which fit such a loose definition.

Progressing through more rigorous definitions, one finally arrives at the most restrictive – the presence of preponderant gram-positive lancet-shaped diplococci. Sensitivity of the Gram stain for identifying pneumococci is then ~ 50%, but the specificity is very high. With such criteria, therefore, one can depend on the subsequent recovery of pneumococci from the specimen. The more interesting question, which was not addressed in this investigation, would be the relative predictive values of Gram’s stain and culture for the diagnosis of pneumococcal pneumonia. It might well be true that the culture positive specimens which were negative by the most restrictive Gram stain criteria represent oral colonization rather than true infection in the lower respiratory tract. Pneumococci are clearly inhibited by the oral flora on some cultures and some of the apparently false positive Gram smears may have reflected this deficiency on the part of sputum culture.
LISTERIA MONOCYTOGENES
Listeria monocytogenes is a major pathogen in the diphtheroid group. Some of these short rods appear rather like diplococci and confusion has resulted in evaluation of a purulent cerebrospinal fluid. Other members of the diphtheroid group include various aerobic and anaerobic bacteria, such as Corynebacterium or Propionibacterium, which are usually indigenous skin flora but may occasionally cause infections. The most important pathogen, fortunately infrequently encountered, is Corynebacterium diphtheriae.
In addition, the filamentous gram positive bacilli, of which *Actinomyces* sp and *Nocardia* sp. are the most important members, may fragment and resemble gram-positive coccobacilli.
Large plump gram-positive bacilli suggest the identification of either *Clostridium* sp or *Bacillus* sp., although some lactobacilli may be rather broad. The clinical situation often helps to provide a presumptive diagnosis. The clostrio-dial species that most characteristically has this morphology is the most important pathogen, *Clostridium perfringens* (shown here).
Notice that these fat gram-positive rods are becoming decolorized. The clostridia in general decolorize easily and may actually appear gram-negative. Notice also that, although *Clostridium* is a genus characterized by spore formation, no spores are evident in this slide. *Clostridium perfringens* is a species in which spores are very difficult to demonstrate in the laboratory; spores essentially never occur in human infections produced by this organism.
Finally, among the gram-positive bacilli it is worth remembering that *Mycobacterium tuberculosis* is a member of the *Actinomycetales*. Although this bacillus does not usually stain with Gram’s stain, it may be demonstrated if large numbers are present, as in an overwhelming pulmonary infection. This slide demonstrates a Gram-stained smear of tuberculosis. Thin, variably stained gram-positive rods are present. Mycobacteria may also appear as negatively stained ghosts against the inflammatory background.
NEISSERIA SP.: CHARACTERISTIC MORPHOLOGY OF GRAM-NEGATIVE ORGANISMS
In this smear of urethral exudate, there are large numbers of intracellular gram-negative diplococci and a smaller number of extracellular organisms. The diplococcal morphology characteristically has a kidney bean shape with the flat ends of the cocci back to back. Several of the cocci in the smear have such a morphology, although it is difficult to demonstrate with a picture. One cannot determine the species of *Neisseria* from the morphology. For the diagnosis of gonorrhea, it is important to demonstrate large numbers of intracellular organisms.
Gram-Negative Coccobacilli

Some short negative bacilli (particularly *Acinetobacter* sp. and *Moraxella* sp., which are indigenous flora of a number of body sites) can mimic *Neisseria* rather closely. The difficulty is deciding whether one has two cocci back to back or a short rod, the stained ends of which are separated by a clear center. In addition overdecolorized staphylococci may have a kidney bean morphology and mimic *Neisseria*.

The predictive value of the Gram stained smear of a male urethral exudate is greater than a smear from the female tract, because of the lower frequency of indigenous flora in the male urethra.
SPUTUM SMEAR FROM A PATIENT WITH A MENINGOCOCCAL PNEUMONIA
Large numbers of intracellular gram-negative diplococci are present. Recently, increasing numbers of nosocomial infections with an organism, *Moraxella catarrhalis*, have been recognized, particularly in the respiratory tract. This organism used to be a member of the genus *Neisseria* and the morphology of the bacteria is identical.
In the proper clinical setting, the presence of small pleomorphic gram-negative coccobacilli is presumptive diagnosis of *Haemophilus* species. In a case of meningitis, bacteremic infection, or otitis media the most likely organism would be type B *Haemophilus influenzae*, but one cannot make such a distinction from the Gram smear. Notice in this smear that many of the organisms are very short and resemble cocci; others are more elongated and have a central vacuole or perhaps are in the process of dividing, On occasion *Haemophilus* can get rather long and filamentous, even in a clinical specimen.
Historical Note

*Haemophilus influenzae*, type B (contains type B capsular polysaccharide) was historically the most important pathogen in this genus and essentially the type to cause bacteremia and meningitis. Attempts to produce a vaccine with capsular material were unsuccessful until a protein conjugate was added as a hapten. Subsequently, immunization was so successful that disease caused by type B *H. influenzae* has essentially disappeared.
A SMEAR OF PREVOTELLA/PORPHYROMONAS
Other bacteria may assume a morphology similar to *Haemophilus*. Here a smear of *Prevotella/Porphyromonas* is demonstrated. The coccobacillary morphology is obviously rather similar to that of *Haemophilus. Prevotella* and *Porphyromonas* spp include species formerly known as pigmented *Bacteroides*. They are members of the oral flora and participate in many mixed anaerobic infections. Other *Bacteroides* species, including *B. fragilis* group have a similar morphology. The clinical situation in which the infection takes place, therefore, makes a major difference in interpretation.
This slide demonstrates the typical appearance of enteric gram-negative bacilli. They are plump gram-negative rods that usually stain rather well. They may, however, be short as seen on the left hand side of this group of bacteria, and may even be quite coccal in shape. One cannot distinguish among the enteric bacteria; \textit{E. coli} is identical to \textit{Salmonella, Proteus}, etc., in morphology.
Demonstrated here is the classic morphology of *Pseudomonas aeruginosa* in clinical specimens. These gram-negative bacilli are thin and elongated. They may occur in pairs or short chains. Although one can make a suggestive distinction between pseudomonads and enteric bacilli, this differentiation is rather more difficult than some of the others we have discussed and is usually not attempted in official reports.
Some other gram-negative bacilli, particularly some of the anaerobic bacteria may have rather distinctive morphologies. This slide demonstrates the morphology of a *Fusobacterium* sp. The bacteria are enlarged and filamentous. Very large swollen forms are evident in the center of the smear. A second smaller gram-negative rod, probably a *Bacteroides* sp. is also present. These elongated and enlarged fusobacteria (most commonly *Fusobacterium necrophorum*) may appear so bizarre that one is tempted to dismiss the possibility that they are bacteria.
This slide demonstrates another morphology of *Fusobacterium* (*Fusobacterium nucleatum*). These organisms have a very distinctive thin, pointed, needle-shaped morphology. In addition, in this smear from a fusospirochetal infection, one can see very faintly staining wavy oral spirochetes in the center of the slide. Occasionally, one may demonstrate these organisms in other mixed anaerobic infections of the upper and lower respiratory tracts, but they are very hard to see in clinical specimens.
This slide demonstrates another gram-negative bacterium with a characteristic morphology. In this smear from a positive blood culture bottle one can see innumerable small thin gram-negative bacilli, many of which appear curved, s-shaped, or c-shaped. Sometimes the morphology has been referred to as gull-winged. This is an isolate of *Campylobacter* sp., but the morphology is also typical of *Vibrio* sp. The characteristic rapid, darting motility of these organisms is reasonable presumptive identification in the appropriate clinical setting.
This smear is from a case of *Legionella pneumophila* pneumonia. Notice that these thin irregular bacilli are rather similar to some of the other organisms we have seen previously. Filamentous forms occur occasionally in clinical specimens and frequently when grown on suboptimal media. Detection of pale staining organisms such as *Legionella* and many anaerobes is facilitated by addition of 0.05% basic fuchsin to the safranin counterstain in Gram’s stain.
Some fungi may also be stained by Gram’s stain. The Gram stain is a rather good way of identifying *Candida* sp. In this smear from a lesion of oral thrush one can see the blastoconidia (yeast cells), which are budding and also the elongated pseudohyphae that this genus produces. Other genera of yeast, which are much less rarely encountered in clinical specimens, may not stain as well by Gram’s stain.
On rare occasions, _Cryptococcus neoformans_ may also be detected. _Cryptococcus_ often stains rather irregularly if it is seen in the Gram-stained smear. Dimorphic yeast, such as _Histoplasma_ and _Blastomyces_, and most moulds do not usually stain as well as _Candida_ and _Cryptococcus_.

This large, thick-walled yeast with a broad-based bud was found in a skin lesion. It is a rare example of *Blastomyces dermatitidis* demonstrated by Gram’s stain. Note that it stains gram-negative in this preparation. Photograph courtesy of Mike Rein, M.D.
Aspergillus fumigatus in Sputum
In this sputum specimen from a patient with invasive aspergillosis the hyphae of *Aspergillus fumigatus* are demonstrated with unusual clarity. The branching, segmented hyphae stain gram-negative and are enmeshed in a proteinaceous exudate. *Aspergillus* spp. may be isolated from sputum in the absence of infection from contamination with environmental spores. Demonstrating hyphae, however, indicates either colonization or infection of tissue or secretions. The final differentiation can be made only by biopsy of tissue.
A hyphal fragment of *Aspergillus fumigatus* has been phagocytized by a macrophage.
Pneumocystis carinii in Gram-stained Respiratory Specimens
In this cytospin preparation of a bronchoalveolar lavage two masses of “foamy exudate” (arrows) are demonstrated. The dots within the exudate are the nuclei of trophozoites.
In this cytospin preparation of a bronchoalveolar lavage from another patient a cyst of *Pneumocystis* is clearly visible. The thin cyst wall is delimited from the surrounding exudate and the multiple internal trophozoites are clearly shown.
Interpretation of the Gram-stained smear may be simple, if the issues are as overt as in this smear from a woman who entered the hospital with bacterial meningitis. So many pneumococci were present in this CSF that the slide was visibly blue. Host defenses in this situation were clearly overwhelmed. Such patients usually do not survive their infection.
SMEAR FROM AN ABDOMINAL WOUND INFECTION
This smear contains large plump gram-positive bacilli that are *Clostridium perfringens*. In addition, there are smaller gram-negative coccobacilli that represent *Bacteroides* sp. Some elongated gram-negative rods that could be enteric bacilli or might be decolorized *Clostridium* are present. In addition, there are a few gram-positive cocci in pairs that appear rather lancet-shaped. In this instance, these organisms were *Enterococcus* sp. It is important, when approaching the Gram-stained smear not to be so distracted by gram-positive elements that gram-negative organisms in the background are overlooked.
In this smear of sputum there are large numbers of lancet-shaped gram-positive diplococci that represent pneumococcus and tangles of filamentous gram-positive organisms. In addition, however, in the background there are an even larger number of pleomorphic gram-negative bacilli that represent *Haemophilus influenzae*. 
This slide is also from a respiratory specimen. It is even more subtle. The pneumococci are present; once again, in the background the predominant organism is *Haemophilus influenzae*, but these gram-negative bacteria fade much more easily into the proteinaceous background.
Winn’s Law Revisited

• All gram-positive organisms aren’t blue
• All gram-negative organisms aren’t red
• All cocci aren’t round
• All bacilli aren’t long
• Bonus: Some bacteria stain poorly
• Super Bonus: Some bacteria don’t stain at all
It is important to recognize that many bacteria will have aberrant or distorted morphologies in clinical specimens. When bacteria are senescent because large numbers are present in the specimen or because of antimicrobial therapy, they decolorize easily. This slide is from a case of pneumococcal pneumonia which was being treated. The organisms are mostly intracellular. Many are gram-negative or weakly gram-positive in their staining reactions.
The tendency for *Clostridium* to decolorize is demonstrated in this smear from a culture in which at least half of the organisms appear gram negative and many of the others are stained irregularly. In fact, one species of *Clostridium* (*clostridiiformis*) is always gram-negative and was once included in the genus *Bacteroides*. 
Under certain conditions some cocci, particular gram-positive cocci, may elongate. This slide is from a patient with pneumococcal pneumonia. Note the elongated forms of several of the pneumococci. On occasion, they may even become rather filamentous. A presumptive identification of the bacteria in this field as pneumococci would be impossible. The identification was made from observation of the slide as a whole.
Additional fields in which elongated pneumococci are found among more conventionally shaped diplococci
1. *Gardnerella vaginalis* is a gram-variable coccobacillus that is one of the polymicrobial causes of bacterial vaginosis. At one time it was classified as *Haemophilus vaginalis*, later as *Corynebacterium vaginale*. In a previous edition of Bergey’s Manual of Determinative Bacteriology, it was placed in the genus *Haemophilus* – with a note saying that the authors didn’t know where the organism belonged, but figured that most people would look for it under *Haemophilus*! Later it was transferred to a new genus, *Gardnerella*.

2. *Acinetobacter* is a gram-negative coccobacillus that tends to retain the crystal violet stain, producing the appearance of a gram-positive organism. When the coccobacillary shape is predominantly coccical it can even be confused with a gram-positive coccus, such as pneumococcus.
This smear is from a culture of *Gardnerella vaginalis*. The organisms may be characterized by the ultimate hedge – gram-variable coccobacilli. They are quite pleomorphic and may stain with either gram reaction or with both, as seen in this slide.
Acinetobacter Bacteremia

These Gram stains from a positive blood culture bottle contain clumps of bacteria that stain gram-positive. Most of the organisms appear to be short bacilli, but some have the appearance of diplococci. If one is not careful, such a smear can be misinterpreted as containing pneumococci. The culture yielded a pure culture of *Acinetobacter lwaffi*. 
For some time it has been recognized that bacilli, particularly enteric gram-negative bacilli may assume bizarre morphologies if they are exposed to sub-inhibitory levels of antimicrobial agents that effect the formation of cell walls.
In this sputum Gram smear elongated and swollen bacterial forms are present (arrows). *Klebsiella pneumoniae* was cultured from the specimen.
Artefacts
Finally, it is important to consider some artifacts that may appear in clinical specimens and that have on occasion confused experienced viewers. This slide demonstrates a clump of epithelial cells from a sputum specimen. There are a few bacteria scattered around the edge of the clump. On top of the cells are elongated refractile crystals, which may have a bluish appearance depending on the plane of focus. In such a specimen, when other bacteria are present, these crystals do not usually cause a problem. When the specimen is from a sterile body fluid and no bacteria are present, however, such crystals may cause difficulty.
A different focal plane of the field shown in the previous slide. Note that the appearance of the crystals can vary in color. Focusing up and down helps to delineate the lack of bacterial characteristics of the staining.
Another example of crystals assuming a bacillary form. At some focal planes the crystals may have a blue coloration, increasing the likelihood of misinterpretation as bacteria.
Another artifact that may cause problems is the presence of stain debris. Such stain debris tends to be worse in thicker areas of the smear. The stain at the edge of the heavy clump of material on the right would probably not be misinterpreted. On the left, however, the blue staining elongated debris in a thinner and more decolorized area of the smear might cause a problem. If cells are present, one aid in assessment of the degree of decolorization is the color of the cell nuclei, which should be gram-negative.
Sometimes the Gram stain debris is round and may resemble gram-positive cocci. If the debris is present all over the slide, the stain must be refiltered.
Gram-negative material may cause problems also. In body fluids, particularly CSF, proteinaceous globs may raise the suggestion of gram-negative organisms, particularly *Neisseria*. In respiratory specimens one may occasionally encounter thin, wavy, poorly staining structures, such as seen on the right-hand half of the slide. There are several present in the picture with the arrow pointing to one of the more obvious structures. These are cilia, as is evident on the left hand side of the smear where they are still attached to the ciliated epithelial cell. Away from their cell of origin, however, they may mislead individuals into diagnosis a gram-negative infection.
Finally, one must consider contamination of the material into which the specimen was put or the slide on which the Gram smear was made. Occasionally fingerprints on the slide may leave skin flora amidst the specimen.

Tubes and reagents for collecting and transporting clinical specimens are usually sterilized, but not all manufacturers are concerned about the presence of dead bacteria and fungi. Pseudo outbreaks of yeast or bacterial infection may result from detection of organisms that have been killed by autoclaving of the material, but have not disintegrated morphologically.
In summary, Gram’s stain provides an important, simple rapid, and expensive diagnostic tool. Spending the time to become proficient in interpretation of clinical smears provides a rewarding sense of direct involvement in laboratory diagnosis of important infections. Recognition of the artefacts is equally as important as the appreciation of the morphology of infectious agents.
THATS ALL FOLKS!