Rapid Method for the Differentiation of Gram-Positive and Gram-Negative Bacteria on Membrane Filters

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Microfiltration has become a popular procedure for the concentration and enumeration of bacteria. We developed a rapid and sensitive method for the differentiation of gram-positive and gram-negative bacteria, utilizing a polycarbonate membrane filter, crystal violet, iodine, 95% ethanol, and 6% carbol fuchsin, that can be completed in 60 to 90 s. Gram reactions of 49 species belonging to 30 genera of bacteria were correctly determined by the filter-Gram stain. The sensitivities of the filter-Gram stain and conventional slide-Gram stain were compared by testing dilutions of *Escherichia coli*, *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae* suspensions in the presence and absence of whole human blood. The filter-Gram stain was approximately 100-fold more sensitive than the slide-Gram stain. The filter-Gram stain detected 2 to 100 bacteria, whereas the slide-Gram stain failed to detect less than 1,000 bacteria. The sensitivities of the methods were not significantly altered by the addition of whole human blood to the dilutions of bacteria tested. The filter-Gram stain could be a useful tool for the examination of body fluids with very low numbers of bacteria.

More than a hundred years after its introduction, the Gram-stained slide (8) continues to be a very valuable tool for the identification and diagnosis of bacterial infections (5, 20, 21; J. A. Washington, Clin. Microbiol. Newsl. 8:135-138, 1986). Despite its long history, the exact mechanism of the Gram reaction for differentiating gram-positive from gram-negative bacteria is not clearly understood (2, 6). There have been several modifications of the original procedure (8) to produce a more rapid and sensitive method (1, 3, 7, 10, 11, 13). Even with these modifications, there has not been a sufficient increase in sensitivity to allow the detection of bacteria on glass slides when present in low concentrations (e.g., 10^3 bacteria per ml).

Alternative procedures have been developed to increase sensitivity, including the acridine orange (14, 16) and ethidium bromide (15) stains. These techniques, however, lack the differential value of the Gram stain. A technique that has gained acceptance for various bacteriological procedures is microfiltration. It has been used for sterilization (25), concentration and identification (23), and enumeration and recovery (17-19) of bacteria from various sources. The concentration of samples by means of filtration has been used to improve the detection of bacteria (9, 19, 22).

We combined the diagnostic value of the Gram stain with the concentration of bacteria by means of filtration to develop a sensitive method for the differentiation of gram-positive and gram-negative bacteria. We compared this filter-Gram stain with the slide-Gram stain by using 49 species of aerobic, anaerobic, and facultatively anaerobic bacteria. The sensitivities of the two methods were compared by testing various dilutions of bacteria in the presence and absence of whole blood.

MATERIALS AND METHODS

Microorganisms. A total of 49 clinical and reference isolates belonging to 30 genera of aerobic, anaerobic, and facultatively anaerobic bacteria (*Achromobacter* sp., *Acinetobacter calcoaceticus* subsp. *anitratus*, *Aeromonas hydrophila*, *Bacillus cereus*, *Bacteroides fragilis*, *Bifidobacterium bifidum*, *Branhamella catarrhalis*, *Clostridium perfringens*, *Corynebacterium diphtheriae*, *Enterobacter agglomerans*, *Escherichia coli*, *Eubacterium lentum*, *Flavobacterium* sp., *Fusobacterium nucleatum*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Lactobacillus* sp., *Listeria monocytogenes*, *Morganella morganii*, *Neisseria meningitidis*, *Peptococcus saccharolyticus*, *Peptostreptococcus magnus*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Salmonella enteritidis* serovar typhimurium, *Serratia marcescens*, *Shigella sonnei*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, and *Yersinia enterocolitica*) were used to compare the Gram reaction of the filter-Gram stain and the slide-Gram stain. *E. coli*, *N. meningitidis*, *S. pneumoniae*, and *H. influenzae* were also used to compare the sensitivities of the filter-Gram stain and the slide-Gram stain. All organisms were identified by conventional biochemical reactions performed at the Wisconsin State Laboratory of Hygiene, Madison.

Staining reagents. The staining reagents used in the filter-Gram stain were crystal violet and iodine solution (GIBCO Laboratories; Hucker modification of the Gram stain [10]), 95% ethanol (decolorizer), and 6% carbol fuchsin (GIBCO; Kinyoun modification of the acid-fast stain [12]). The same reagents were used for the slide-Gram stain, except that acetone-alcohol (GIBCO) and safranin (GIBCO) were used for decolorization and counter staining, respectively. All reagents used for the filter-Gram stain technique were sterilized by filtration (Nuclepore; pore size, 0.2 μ m).

Filter-Gram stain procedure. Tenfold dilutions ranging from 10^1 to 10^8 bacteria per ml were made from 0.5 McFarland suspensions of fresh cultures (4 h) of *E. coli*, *N.*

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meningitidis, *S. pneumoniae*, and *H. influenzae*. The dilutions of bacteria were filtered through polycarbonate membrane filters (Nuclepore; pore size, 0.4 μm) supported by a metallic screen mounted on a hydrosol manifold (Micro Filtration Systems, Dublin, Calif.). The filters were marked with a pen containing alcohol-resistant black ink to locate the area inoculated with the bacterial suspensions. An inoculum of 100 μl of each bacterial dilution was concentrated in a circular area (0.5-cm diameter) by means of a vacuum pump (Motor Division, St. Louis, Mo.). Microorganisms were fixed to the filter with 200 μl of 95% ethanol (vacuum on) and stained with 20 μl of crystal violet for 15 s (vacuum off). The vacuum was turned on to eliminate excess crystal violet, and 20 μl of iodine solution was added for 15 s (vacuum off). Iodine was flushed from the filter with 500 μl of distilled water, and decolorization was performed with 100 μl of 95% ethanol. The filter was then flushed twice with 500 μl of distilled water. After the vacuum was closed, 20 μl of carbol fuchsin was added for 20 s. The carbol fuchsin was removed by flushing twice with 500 μl of distilled water. The filter-Gram stain was completed in 60 to 90 s. The filter was then dried on a hot plate (50 to 60°C) for 1 to 3 s, mounted on a glass slide, and clarified with 1 drop of immersion oil (Cargille; index of refraction, 1.584 ± 0.0002). A glass cover slip (no. 1; 22 by 22 mm; Cida, Leakwood, Kans.) was placed over the membrane with care to avoid air bubbles, and the filter was examined by light microscopy with the oil immersion lens ($\times 1,000$ total magnification) and the same immersion oil. Fifty oil immersion fields were examined for 10 to 15 min. Uninoculated stained areas of the filters were used as controls. A filter was considered positive if 10 or more morphologically defined bacteria were found in 50 fields.

Slide-Gram stain. Glass slides, precleaned overnight in 95% alcohol, were used in the slide-Gram stain. A 100- μl drop of the above-described 10-fold dilutions of bacterial suspensions ranging from 10^1 to 10^8 bacteria per ml was placed on the slide and allowed to air dry. The area of inoculation was approximately 1 cm^2 . Bovine serum was added to the suspensions of bacteria at a final concentration of 1% to increase the adhesion of the bacterial cells to the glass surface. Slides were heat fixed twice and stained by the method recommended by Hucker and Conn (11). Gram-stained slides were allowed to air dry and were examined with a light microscope ($\times 1,000$ total magnification). The slides were examined for 10 to 15 min. A slide with 10 or more morphologically defined bacteria in 50 fields was considered positive.

Sensitivity study. The relative sensitivities of the filter-Gram stain and slide-Gram stain were determined (Fig. 1). Bacterial suspensions (approximately 10^6 organisms per ml) in the logarithmic phase were prepared by inoculation into Trypticase soy broth (BBL Microbiology Systems) after 4 h of incubation at 35°C. The bacteria included *E. coli*, *H. influenzae*, *S. pneumoniae*, and *N. meningitidis*. A 100- μl inoculum from each of the 10-fold serial dilutions (0.85% sterile saline solution) of the original suspensions was stained by the filter-Gram stain and the slide-Gram stain. For each dilution, viable counts were determined by one of two methods: (i) culture of filters inoculated with 100 μl of the bacterial dilutions on chocolate agar plates (Difco Laboratories) and (ii) placement of 100 μl of each dilution directly on chocolate agar plates. Plates were then incubated for 18 to 24 h at 35°C under aerobic conditions, and the number of bacteria per 100 μl was determined.

A similar study was performed with bacterial suspensions

FIG. 1. Schematic representation of the protocol used for the comparison of the filter-Gram stain and the slide-Gram stain.

and various concentrations of whole human blood. Whole human blood was added to dilutions of *E. coli*, *H. influenzae*, *S. pneumoniae*, and *N. meningitidis* suspensions to obtain one to two leukocytes per oil immersion field. In addition, whole human blood was added to dilutions of *H. influenzae* suspensions to obtain zero to one, one to two, and two to four leukocytes per oil immersion field. The microscopic evaluation was performed by examining 50 oil immersion fields per filter or slide. A slide or filter was considered positive when 10 or more morphologically well defined microorganisms were observed in the 50 fields.

Statistical analysis. All results were tested by analysis of variance. The Fisher least-significant-difference test (24) was used to examine pairs of means when a significant *F* ratio indicated reliable mean differences. The alpha value was set at the 0.05 level before the experiments were performed.

RESULTS

The filter-Gram stain procedure was tested with 49 species of gram-positive and gram-negative bacteria. The filter-Gram stain yielded appropriate Gram reactions and did not alter the morphologic characteristics of the bacteria (Fig. 2).

A comparative study was performed to determine the relative sensitivities of the filter-Gram stain and the slide-Gram stain (Fig. 3). The filter-Gram stain detected significantly fewer microorganisms ($P < 0.025$). The lowest bacterial counts detected were 2, 46, 8, and 38 by the filter-Gram stain and 2,400, 4,600, 7,600, and 38,000 by the slide-Gram stain for *E. coli*, *N. meningitidis*, *S. pneumoniae*, and *H. influenzae*, respectively. The addition of whole human blood to the bacterial suspensions did not significantly alter the number of bacteria detected by either method ($P > 0.05$) (Fig. 3). The range of detection observed in the presence of blood cells was 3 to 100 bacteria for the filter-Gram stain and 350 to 32,000 bacteria for the slide-Gram stain. The bacterial counts determined by microscopy did not differ significantly from the number of viable bacteria detected by plating dilutions of bacterial suspensions on chocolate medium or inoculation of polycarbonate filters on chocolate medium.

FIG. 2. Representative bacteria stained by the filter-Gram stain technique. (A) *H. influenzae* and *E. coli*; (B) *S. pneumoniae* and *N. meningitidis*; (C) *S. pyogenes* and *E. coli*; (D) *B. cereus* and *P. aeruginosa*. Color photographs are available on request.

When various concentrations of whole human blood were added to dilutions of *H. influenzae* suspensions (Fig. 4), similar results were obtained. The filter-Gram stain detected lower numbers of *H. influenzae* (38 to 100 bacteria) in the presence of various concentrations of whole human blood than did the slide-Gram stain (approximately 10^4 bacteria).

FIG. 3. Minimum number of bacteria detected by the filter-Gram stain and the slide-Gram stain in the absence (□) and presence (▨) of whole human blood.

FIG. 4. Minimum number of *H. influenzae* organisms detected by the filter-Gram stain and the slide-Gram stain in the absence (□) or presence of various concentrations of whole human blood. Leukocytes were used as an indicator of the total erythrocyte concentration. Number of leukocytes per oil immersion field: zero to one (▨), one to two (▨), and two to four (▨).

DISCUSSION

Polycarbonate membrane filters are well suited for the filter-Gram stain procedure. These membranes are resistant to staining by the Gram reagents and present bacteria on a microscopically flat surface suitable for observation at $\times 1,000$ magnification. Furthermore, when polycarbonate filters are saturated with an immersion oil having a refractive index equivalent to that of the membrane, the filters become transparent, allowing light transmission from under the slide and unaltered viewing of stained microorganisms. Although we used 0.4- μm -pore-size filters, smaller pore sizes can be used.

Preliminary studies were performed to standardize the filter-Gram stain technique. Mixed cultures of gram-positive and gram-negative bacteria were initially filtered and stained by the conventional Gram stain (11). Because this technique did not yield morphologically well defined bacteria on the filters, modifications were required. These modifications included fixation of the sample with 95% ethanol, rapid decolorization, a different counter stain (6% carbol fuchsin), and decreased exposure times to each stain. Other concentrations of carbol fuchsin were tested, but optimal staining reactions were obtained only with a 6% solution. The whole process was completed in 60 to 90 s. With these modifications, the filter-Gram stain technique could concomitantly stain and differentiate bacteria.

Bacteria were retained on the filters by fixation with 95% ethanol. This treatment allowed a better attachment of the bacterial inoculum to the surface, reducing bacterial loss and clumping during the filtration-staining procedure. Although the same bacterial inoculum, with and without blood cells, was placed on the glass slide, the slide retained fewer bacteria. Examination of more microscopic fields did not increase the sensitivity of the slide-Gram stain. The high loss of bacteria during the staining step of the slide-Gram stain procedure has been a concern in clinical microbiology laboratories. The filter-Gram stain proved an alternative approach that solves this well-known problem.

The filter-Gram stain did not alter the staining reaction or the microscopic morphology of the 49 bacterial species tested. As with the slide-Gram stain (4), some variable reaction with the Gram stain was detected with aged cultures. In a properly stained filter preparation, the bacteria were pinkish red or dark blue against a faint blue background. Occasionally, crystals of crystal violet and iodine were seen on the filters. When this crystallization occurred, newly filtered reagents were used. With appropriate technique, the image of the bacteria with the filter-Gram stain was equivalent to or better than that observed with the Gram-stained slide. The presence of blood cells did not complicate the microscopic examination. In fact, the blood cells were a valuable reference point for focusing, and bacteria were more readily observed on the filter surface. Proper adjustment of the light source made the microscopic examination more effective. The condenser was adjusted to eliminate the background of the filter. The presence of cells or debris made this adjustment for the location of the plane of focus easier.

In the filter-Gram stain procedure, the stained filter must be carefully dried and transferred to a clean glass slide for clarification with the proper immersion oil. The filter should not be disturbed or touched at the inoculum site. A semipermanent mount can also be obtained after clarification of the filter on the glass slide; therefore, microscopic examination is not required immediately after slide preparation.

In summary, the filter-Gram stain is a rapid method that differentiates gram-positive and gram-negative bacteria with a sensitivity higher than that of the slide-Gram stain. The filter-Gram stain was approximately 100-fold more sensitive than the slide-Gram stain, detecting as few as 2 to 100 bacteria. These studies suggest that the filter-Gram stain may be an important procedure for the rapid detection and differentiation of bacteria. Sage et al. (23) have previously developed a filtration-staining device for the early detection and recovery of bacteria from blood cultures. The application of microfiltration-staining techniques could be useful for the examination of clinical samples, including cerebrospinal fluid, which may contain very few bacteria. Additional studies are needed to adequately assess the usefulness of the filter-Gram stain with clinical specimens.

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