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Alternate Gram Staining Technique Using a Fluorescent Lectin

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Fluorescence-labeled wheat germ agglutinin binds specifically to *N*-acetylglucosamine in the outer peptidoglycan layer of gram-positive bacteria. The peptidoglycan layer of gram-negative bacteria is covered by a membrane and is not labeled by the lectin. By exploiting this phenomenon, an alternative Gram staining technique has been developed.

The Gram stain is the most widely used taxonomic test of bacteria (5). The technique is relatively simple and, in experienced hands, gives reproducible results. The existing technique does, however, have its limitations (4). Most modifications of the Gram stain, such as that of Hucker (6), require at least four solutions and four staining steps. Furthermore, the stains used in the technique, particularly the primary stain (crystal violet), are concentrated and can be messy. The staining procedure is estimated to take approximately 3 min (2).

We have developed an alternative Gram staining technique which is simpler and faster, requires fewer reagents, is easier to interpret, and, in our hands, is less susceptible to errors. This technique takes advantage of the selective binding of a lectin, wheat germ agglutinin, to *N*-acetylglucosamine (1, 8). This molecule is a prominent component of the peptidoglycan layer found in all eubacteria except *Mycoplasma* spp. In gram-positive bacteria, the peptidoglycan layer is the outer portion of the cell wall. The exterior layer of gram-negative bacteria is a membrane which covers the peptidoglycan layer (9). Thus, a large molecule such as a lectin should be able to attach to the peptidoglycan layer of gram-positive bacteria but should not be able to penetrate the outer membrane and thus could not attach to the peptidoglycan of gram-negative bacteria.

A total of 92 bacterial strains were tested. Gram-positive bacteria were as follows: *Bacillus* sp. (n = 8), *Bacillus megaterium* (n = 2), *Corynebacterium* sp. (n = 1), *Lactobacillus acidophilus* (n = 1), *Lactobacillus lactis* (n = 1), *Micrococcus* sp. (n = 3), *Micrococcus luteus* (n = 2), *Mycobacterium smegmatis* (n = 1), *Sporosarcina ureae* (n = 1), *Staphylococcus aureus* (n = 9), *Staphylococcus epidermidis* (n = 7), *Staphylococcus saprophyticus* (n = 1), *Streptococcus faecalis* (n = 4), *Streptococcus mitis* (n = 2), and *Streptococcus pyogenes* (n = 4). Gram-negative bacteria were as follows: *Acinetobacter calcoaceticus* (n = 2), *Alcaligenes faecalis* (n = 2), *Cytophaga* sp. (n = 1), *Enterobacter aerogenes* (n = 1), *Enterobacter cloacae* (n = 5), *Escherichia coli* (n = 8), *Klebsiella pneumoniae* (n = 4), *Morganella morganii* (n = 1), *Proteus mirabilis* (n = 2), *Proteus vulgaris* (n = 4), *Pseudomonas* sp. (n = 1), *Pseudomonas aeruginosa* (n = 4), *Pseudomonas fluorescens* (n = 1), *Pseudomonas stutzeri* (n = 1), *Rhodospirillum rubrum* (n = 1), *Salmonella typhimurium* (n = 1), *Serratia liquefaciens* (n = 1), *Serratia marcescens* (n = 4), and *Shigella sonnei* (n = 1). All strains were initially streaked to ensure purity and then were maintained at 35°C with periodic transfer. Wheat germ agglutinin labeled with fluorescein isothiocyanate was purchased from Polysciences, Inc., Warrington, Pa. This lectin was diluted to 100 μg/ml with phosphate buffer (pH 7.2). Samples of the diluted lectin solution could be stored frozen in the dark until needed. Conventional Gram stains were prepared according to the Hucker modification (5, 6).

Smears were made on glass slides, air dried, and heat fixed. The smears were covered with freshly thawed lectin solution for 30 s and then gently rinsed with phosphate buffer. Cover slips were then placed on the wet slides. Optimal results were obtained only if the smear remained wet.

The smears were brought into focus at ×400 by using phase contrast, the visible light was turned off, the epifluorescent illumination was turned on, and the smears were observed for fluorescence. The microscope used was an Olympus BHB with a fluorite 40× phase objective. For fluorescence microscopy, a BG12 exciter filter and a Y 475 barrier filter were used. Gram-positive organisms fluoresced bright yellow-green, whereas gram-negative bacteria did not fluoresce. Attempts to use simple stains to provide contrast and thus avoid the use of phase contrast for initial observation proved unsuccessful. The stains tested interfered with the fluorescence.

To test the technique, overnight cultures were randomly assigned numbers and were stained as unknown cultures. Each numbered organism was stained at least three separate times with the fluorescent stain and was concurrently stained three times with the conventional Gram stain. The results of the two techniques were also compared by using 24-, 48-, and 72-h and 6-day cultures.

The fluorescent Gram stain technique proved to be a simple, quick (less than 1 min), reliable technique. All but one of the gram-positive organisms tested in this study fluoresced a bright yellow-green after staining (Fig. 1). The gram-negative bacteria did not fluoresce except for the *Pseudomonas* species. The difference in fluorescence between the gram-positive and the gram-negative strains was dramatic. The only exceptions were the *Pseudomonas* species, which appeared to autofluoresce and gave intermediate results. With experience, the weak reactions of these strains were easy to recognize and to categorize as gram negative.

Of 437 smears stained with the fluorescent lectin, only 13 smears stained incorrectly. In all but one of these cases, the Gram reaction was incorrect for both Gram stain techniques even though the techniques were tested independently. These cultures were presumed to be contaminated. When some of these cultures were restreaked and reisolated, pure

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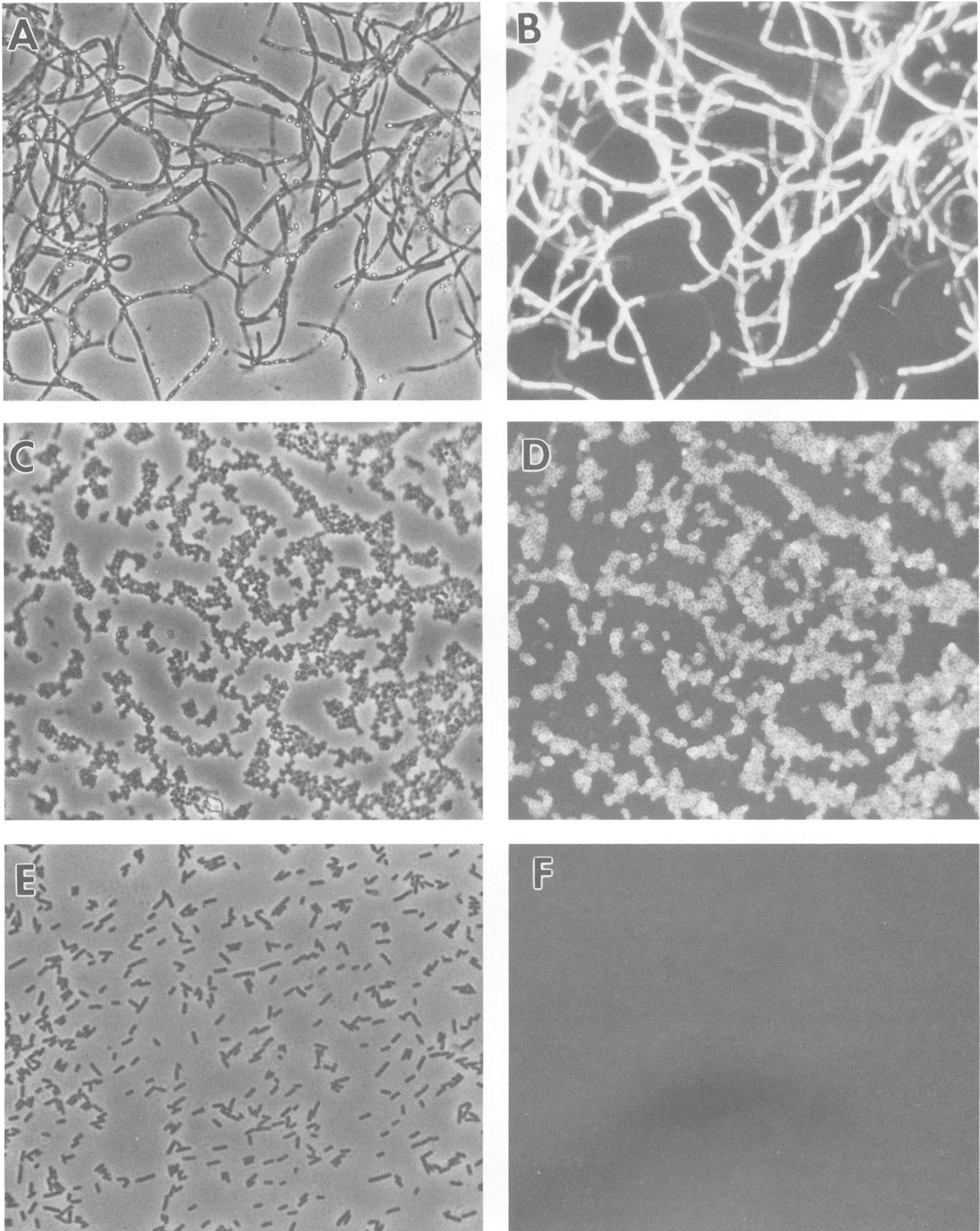

FIG. 1. (A through D) Micrographs of the gram-positive organisms *Bacillus* sp. and *Micrococcus luteus* observed by phase-contrast microscopy (A and C, respectively) and fluorescing after staining with lectin (B and D, respectively). (E and F) Micrographs of a gram-negative organism, *Escherichia coli*, by phase-contrast microscopy (E) and after lectin staining (F). Magnification, ×400.

culture consistently gave the correct result. One culture, a *Corynebacterium* sp., stained incorrectly but died in culture and could not be rechecked. We cannot conclude whether this organism resists staining by the lectin or whether the culture was simply mislabeled or contaminated. By comparison, 143 conventional Gram stains were performed, with 27 giving inconclusive results and 6 giving incorrect results.

Culture age did not effect the results of the fluorescence technique. All of the 48-h, 72-h, and 6-day cultures showed consistent fluorescence staining results. With the conventional Gram stains, however, in the 48-h cultures, 50% of the gram-positive strains appeared gram negative, and most of the 72-h (83%) and 6-day (86%) gram-positive cultures appeared negative.

This technique proved to be a simple, quick, reliable alternate to the existing Gram stain protocols. While only a limited number of strains were tested, this technique worked well across the range of the cultures. The procedure avoids exposure to messy stains, and since only one step was required, the fluorescent stain was much faster. The results were so obvious that reading the smears was quicker and less tedious than in the conventional technique and potentially could be automated. The only apparent shortcoming of this technique is the requirement for a fluorescent microscope.

Perhaps the most exciting result from this technique is the insensitivity of the technique to the age of the culture. This implies that this stain can be used directly on samples without culturing the bacteria. For example, clinical specimens with high bacterial loads can be stained and observed directly. This could be particularly helpful with fastidious, slowly growing, and/or anaerobic bacteria. Furthermore, from the results obtained, an antibiotic which is appropriate for either a gram-negative or gram-positive infection can be prescribed. A more likely application of this technique is to analyze viable but nonculturable microorganisms (11). As an example, marine bacteria are reported to be 70 to 90% gram negative (7, 10), but this result comes from cultured marine bacteria. Since most marine bacteria cannot be cultured on media (3), this technique could be used to give a better indication of the actual bacterial composition in the environment.

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