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Evaluation of the Wayson Variation of a Methylene Blue Staining Procedure for the Detection of Microorganisms in Cerebrospinal Fluid

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Meningitis of bacterial origin is a severe infection that must receive immediate attention and prompt treatment. We evaluated a basic fuchsin-methylene blue, ethyl alcohol-phenol staining procedure (Wayson stain) and compared it with the Gram stain procedure for evaluation of cerebrospinal fluid. All smears were prepared within 30 min of receiving the specimen and examined without knowledge of the results of the companion smear or culture. Of 546 specimens entered into the study, 84 were culture positive. Based on the culture results, the sensitivity and specificity of Wayson stain were 90 and 98%, respectively, compared with 73% sensitivity and 99% specificity by Gram stain. We observed that Wayson staining is a particularly sensitive method for screening clinical specimens that contain proteinaceous background material. The Wayson staining procedure is a simple and sensitive technique for early detection of meningitis.

Meningitis of bacterial origin is a severe infection that must receive immediate attention and prompt treatment. Thorough initial examination of cerebrospinal fluid (CSF) in the laboratory can yield information which can provide invaluable perspective for proper therapy of patients with this condition. Direct microscopic examination of CSF with the Gram stain has proven to be very helpful in the rapid provision of relevant information. Unfortunately, the Gram stain is less sensitive than culture, since approximately 10^5 CFU/ml are required for bacteria to be detected microscopically (3). Many specimens contain fewer than 10^5 CFU/ml, and most studies have reported that only 70 to 85% of culture-positive CSF specimens are positive by Gram stain (7, 9). The lack of contrast staining with gram-negative organisms is another problem with the Gram procedure, particularly if the specimen contains inflammatory cells and proteinaceous material with only a few organisms.

In 1926, Meyer and Batchelder used a stain developed by N. E. Wayson to improve contrast staining of microorganisms in exudates of infected rats (9). The purpose of the present study was to compare the sensitivity and specificity of Gram staining and Wayson staining (basic fuchsin-methylene blue, ethyl alcohol-phenol) of CSF to determine whether Wayson stain offers any advantage over Gram stain in clinical laboratories.

MATERIALS AND METHODS

Specimens. We examined 546 specimens of CSF submitted to the clinical laboratories of Primary Children's Medical Center and the University of Utah Medical Center. Specimens were processed by standard laboratory procedures, including duplicate direct smears of each specimen (8). Because of the small amount of CSF available with many patients, no concentration of the specimens was attempted before processing to maintain uniformity. The first two-thirds of the specimens represent consecutive acquisitions. However, only cloudy specimens were accepted for the remainder of the study to increase the yield of positive specimens. Specimens growing viridans streptococci, diphtheroids, or *Propionibacterium acnes* were considered to be contaminated and were treated as negative specimens unless patients had clinical, biochemical, and cytological evidence of meningitis.

Gram stain. Air-dried, heat-fixed smears were stained sequentially with crystal violet solution (Difco Laboratories, Detroit, Mich.) and iodine solution (Difco), decolorized by acetone-alcohol (Difco) and counterstained with safranin solution (Difco) at the time of initial processing of the specimens (8).

Wayson stain. Solution A consisted of 0.20 g of basic fuchsin (90% dye content; Eastman Kodak, Rochester, N.Y.), 0.75 g of methylene blue (90% dye content; J. T. Baker Chemical Co., Phillipsburg, N.J.), and 20 ml of 95% ethyl alcohol (Mallinckrodt, Paris, Ky.). Solution B consisted of 200 ml of 5% phenol (J. T. Baker Chemical Co.). Solution A was poured slowly into solution B, after which the staining solution was filtered and stored in an opaque bottle at room temperature. Heat-fixed, air-dried smears were stained for 10 s, washed with water, and blotted dry. When new Gram reagents and Wayson stain solution were made, quality control consisted of smears of *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923.

Bacteriological cultures. All CSF specimens were inoculated onto Columbia base agar (BBL Microbiology Systems, Cockeysville, Md.) or tryptic soy base agar (Micro-Media, Phoenix, Ariz.) with 5% sheep blood, onto chocolate agar (BBL or Micro-Media), and into supplemented tryptic soy broth with a 0.15% agar gradient. All plates and tubes were incubated at 35°C; in addition, chocolate agar was maintained under an atmosphere of 5 to 8% CO_2. Daily examination of the media for bacterial growth was performed for at least 3 days. Turbid broth was Gram stained and, if positive, subcultured onto chocolate agar and prereduced base agar with 5% sheep blood. The prereduced base agar with 5% sheep blood was incubated in an anaerobic jar (BBL) with a GasPak (BBL) generator at 35°C. Bacterial isolates recovered from cultures were identified by conventional methods (8).

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TABLE 1. Bacteria isolated from 84 culture-positive CSF specimens

Bacterium	No. (%) of positive cultures
Haemophius influenzae type b	63 (75)
Klebsiella pneumoniae	5 (6)
Streptococcus pneumoniae	4 (5)
Escherichia coli	2 (2)
Group B *streptococcus*	2 (2)
Neisseria meningitidis	2 (2)
Proteus mirabilis	2 (2)
Enterobacter aerogenes	2 (2)
Staphylococcus epidermidis	2 (2)

Microscopic examination. Coded (the technologist had no knowledge of results of culture or alternatively stained smear) Wayson-stained and Gram-stained smears were examined for approximately 5 min each. Smears were surveyed at ×100 and ×400 magnification and were read definitively at ×1,000 magnification with an oil immersion objective. The quantity and morphology of microorganisms and the presence of leukocytes were noted. A smear was considered positive if any bacteria were seen. A smear was considered false-positive if organisms considered to be contaminants grew in culture or if the culture was sterile.

Statistical methods. Standard methods were used to calculate the sensitivity and specificity of each stain (5).

RESULTS

Of the 546 CSF specimens evaluated, 84 were from patients with bacterial meningitis as indicated by culture (Table 1). *Haemophilus influenzae* type b was the most common CSF isolate, accounting for 63 (75%) of the 84 positive cultures.

Culture-positive CSF specimens were positive by both staining techniques in 61 (73%) of 84 comparisons. Fifteen (18%) were positive with Wayson stain alone, and none were positive with only Gram stain. Wayson stain was more sensitive (90%) than Gram stain (73%), and the specificity of Wayson stain was 98% compared with 99% for Gram stain. Of the 462 negative specimens, 7 smears were false-positive by Wayson stain, and 3 smears were false-positive by Gram stain. Ten percent (8/84) of the smears stained with the Wayson reagent were false-negative, and 27% (23/84) of the Gram-stained smears were false-negative.

The staining quality of the Wayson stain was excellent. Bacteria stained dark blue, whereas background material stained light blue. Organisms were readily observed and easily distinguished from polymorphonuclear leukocytes.

DISCUSSION

Considerable interest exists in rapid diagnostic methods for detection of microorganisms in various body fluids, especially CSF. In many laboratories, rapid diagnostic methods are used as adjuncts to more conventional means of diagnosis. Although culturing the organism from the CSF is one method for definitive diagnosis of bacterial meningitis, culture is relatively slow (often 2 to 3 days before final results are available).

Rapid diagnostic methods include techniques for the detection of antigens (countercurrent immunoelectrophoresis, staphylococcal coagglutination, latex agglutination) or other products in the CSF (gas-liquid chromatography, *Limulus* amoebocyte lysate assay). However, the more conventional direct microscopic techniques are often faster, easier, and more cost effective than antigen detection.

Several varieties of methylene blue stains have been reported for smears from culture media as a counterstain for acid-fast organisms, for detecting metachromatic granules of *Corynebacterium diphtheriae*, and for direct counting of bacteria in milk (2). Kronvall and Myhre compared acridine orange-, methylene blue-, and Gram-stained smears of buccal scrapings, CSF specimens, and urethral secretions and found equal sensitivities and specificities with methylene blue and Gram stains (6). In addition, urethral and cervical smears stained with methylene blue and acridine orange were found to be equally sensitive (4). A recent comparison of acridine orange, methylene blue, and Gram stains for blood cultures demonstrated 38% sensitivity and 99% specificity with both methylene blue and Gram stains (10). The Wayson stain variation has been used successfully to detect plague bacilli in rat secretions (9) and amoebae (1) in CSF.

This comparison of conventional Gram staining and Wayson staining demonstrated that the Wayson stain was more sensitive than Gram stain and was also as specific. Interestingly, Lauer et al. (7), reported a Gram stain sensitivity of 76.7%, which is very comparable to the results of this study, whereas in their study, acridine orange staining demonstrated a sensitivity of 82.2% and specificity of 100% for bacterial detection in CSF.

An added advantage of Wayson staining of infected CSF is that it is easy to interpret. Microorganisms stain dark blue, whereas proteinaceous material stains light blue and polymorphonuclear leukocytes stain light blue and purple. Our impression is that much less time is required to detect bacteria with Wayson staining than with Gram staining because of this contrast, although we did not monitor the time required for detection in this comparative study.

Wayson staining has minor disadvantages. When the stain was stored, even in an opaque bottle, the tinctorial qualities of stained bacteria gradually changed over a 6-month period, although the microorganisms still stained a shade of blue which contrasted with amorphous background material and leukocytes. In addition, the use of Gram stain to stain material previously stained with Wayson stain is unsatisfactory because gram-negative microorganisms do not stain with the appropriate contrast. It is, therefore, necessary to perform an additional stain on positive smears to determine classic Gram stain morphology.

Since only a small amount of CSF was available from many patients, specimens were not concentrated by centrifugation to maintain uniformity. Although some authors recommend centrifugation of CSF at 1,500 × *g* for 15 min (11, 12), others suggest that 10,000 × *g* is optimal for maximal recovery of organisms, especially *Haemophilus influenzae*, when more than 1 ml of specimen is available (13). Since many clinical laboratories may not have access to a centrifuge with the capability of generating 10,000 × *g*, the use of Wayson stain with uncentrifuged CSF specimens may be a worthwhile alternative.

In summary, Wayson stain is a rapid, cost-effective, simple, and sensitive technique to detect organisms in CSF specimens. It is particularly helpful in evaluating CSF specimens containing large amounts of background material.

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