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Comparison of Acridine Orange and Gram Stains for Detection of Microorganisms in Cerebrospinal Fluid and Other Clinical Specimens

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Acridine orange, a fluorochrome stain, is potentially superior to the Gram stain in the direct microscopic examination of clinical specimens because it gives striking differential staining between bacteria and background cells and debris. Its value in clinical laboratories was evaluated by testing 209 cerebrospinal fluids and 288 other body fluids, tissues, and exudates by both techniques. Smears were made in duplicate, fixed with methanol, stained, and examined without knowledge of the result of the companion smear or culture. Overall, acridine orange was slightly more sensitive than the Gram stain (acridine orange, 59.9%; Gram stain, 55.8%) and equally specific in detecting microorganisms. One smear was falsely positive by the Gram stain; none was falsely positive by the acridine orange stain. We conclude that acridine orange staining is a sensitive method for screening clinical specimens and reviewing selected specimens that are purulent, but negative by the Gram stain. Bloody fluids, thick exudates, and other normally difficult-to-read specimens were easily and quickly examined. We recommend, however, that positive smears be reexamined with the Gram stain to confirm the result and determine the Gram reaction of the microorganisms.

Direct microscopic examination of cerebrospinal fluid (CSF) and other clinical specimens with the Gram stain is often extremely helpful to clinicians because microorganisms are detected rapidly and the morphology and Gram reaction help guide the choice of antimicrobial therapy. Unfortunately, the Gram stain is less sensitive than culture because about 10^5 colony-forming units per ml are required for bacteria to be detected microscopically (3, 10). Many samples contain fewer than 10^5 colony-forming units per ml. For example, only 75 to 85% of CSF specimens that subsequently grow bacteria are positive by the Gram stain (7, 17). Another difficulty with the Gram stain is that only gram-positive organisms stain differentially. Gram-negative bacteria are frequently difficult to see, especially if they are few and there is much cellular debris in the specimen from blood or inflammatory cells.

Recently, Kronvall and Myhre described enhanced differential staining of bacteria in clinical specimens with a fluorochrome dye, acridine orange (AO), buffered at a low pH (11). AO binds to the nucleic acids of bacteria and stains them orange. The authors concluded that this fluorescent technique is superior to the Gram stain or the methylene blue stain, but they did not present their data in a manner that permitted a comparison of the sensitivity and specificity of the stains (11). The purpose of this study was to compare the sensitivity, specificity, and predictive value of AO and Gram staining of CSF and other clinical specimens to determine whether AO offers any advantages over the Gram stain in clinical microbiology laboratories.

MATERIALS AND METHODS

Selection of specimens. We examined microscopically 209 specimens of CSF and 288 specimens of other body fluids, tissues, and exudates submitted to the clinical microbiology laboratories of the University of Colorado Hospital and the Denver Children's Hospital. Specimens were processed according to standard laboratory procedures, except that duplicate direct smears of each specimen were made. Both slides were air dried and fixed with absolute methanol for 1 to 2 min. One slide was stained immediately with the Gram stain and examined at a ×1,000 magnification with an oil immersion objective, and the result was reported by the technologist on duty. The unstained slide and the Gram-stained slide were both saved. Later, we selected smears of CSF specimens from all patients with culture-proved bacterial meningitis (n = 73) and an additional set of smears of sterile CSF specimens (n = 146). Negative CSF specimens were chosen to

include as many as possible that contained erythrocytes or many leukocytes or both. Specimens growing *Staphylococcus epidermidis* or *Propionibacterium acnes* were presumed to be contaminated and were included in the set of negative specimens unless patients had other evidence of meningitis. We also selected a similar set of smears with an equal number of culture-positive and culture-negative body fluids, tissues, and exudates matched according to the source of the specimen (n = 144 positive and 144 negative cultures). Urine specimens and blood cultures were excluded from the study.

AO stain. The AO stain was prepared by adding 20 mg of AO powder (J. T. Baker Chemical Co., Phillipsburg, N.J.) to 190 ml of sodium acetate buffer (stock solution of 100 ml of 1 M $CH_3COONa \cdot 3H_2O$ and 90 ml of 1 M HCl); 1 M HCl was added as necessary to yield a final pH of 3.5 and a final AO concentration of about 100 mg/liter. The staining solution was stored in a brown bottle at room temperature. Slides were flooded with the AO stain for 2 min, washed with tap water, dried, and examined. Each batch of the AO and Gram stains was quality controlled with smears of *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923.

Microscopic examination. The AO-stained slides were coded to ensure that the technologist examining them had no knowledge of the results of the Gram stain or culture. Each smear was examined for no more than 5 min. We used a Leitz Dialux microscope equipped with a quartz halogen lamp for incident light fluorescence and the following combination of filters: BG 23, KP 500, and TK 510/K515 (Lietz/Opto-Metric Div. of E. Lietz Inc., Rockleigh, N.J.). Smears were surveyed at ×100 and 400 magnifications and were read definitively at a ×540 magnification with an oil immersion objective. The presence, quantity, and morphology of microorganisms and the presence of leukocytes were noted. A smear was considered positive if ≥2 bacteria per smear were seen. The results of the AO-stained smears were compared with the results of the Gram-stained smears and cultures reported by the diagnostic laboratories.

Quantitative studies. We compared microscopically the concentrations of bacteria necessary for detection by the Gram and AO stains. *S. aureus* ATCC 25923, *E. coli* ATCC 25922, and a stock strain of *Haemophilus influenzae* were inoculated into bottles of supplemented peptone broth without blood (BD Vacutainer Systems, Rutherford, N.J.), and after overnight incubation, smears of 0.01 ml were made from serial 10-fold dilutions of the broth. Colony counts were done by the Miles-Misra technique (15).

Statistical methods. The sensitivity, specificity, and predictive value of each stain were calculated by standard formulas (9), and differences between stain results were measured statistically by the McNemar test (14).

RESULTS

The staining quality of the AO stain was excellent. Bacteria and yeast cells usually stained a brilliant orange, and the background was black, light green, or yellow. Occasionally, bacteria were seen that did not pick up the stain and appeared as faint, grey silhouettes; however, other brightly staining organisms were always present in large numbers on the same smears. The brightness of organisms faded with time, but not so quickly as to create difficulty. Leukocyte nuclei and granules stained yellow, orange, or red. Until we became familiar with the stain, orange-staining granules from disintegrating leukocytes sometimes were confused with cocci. Negative smears generally had little or no confusing orange artifacts; stain or stain precipitate adhering to the slides was not a problem.

Many positive AO-stained smears could be read at a low magnification (×100), and most could be read definitively at a ×400 magnification. The 540× oil immersion objective was helpful in confirming the morphology of microorganisms seen at a lower magnification and in scrutinizing suspicious areas before calling a smear negative. The enhanced contrast between the organisms and the background permitted even excessively thick smears and smears from bloody specimens to be examined at a low magnification.

The ability to survey AO-stained smears at a low magnification facilitated rapid and thorough microscopic examination. Although the microscopist was permitted up to 5 min to examine each slide, positive smears seldom required more than 1 min to locate organisms. Because the time required in the diagnostic laboratories to examine the matched Gram-stained smears was not controlled or recorded, we could not objectively compare the amount of microscope time needed to examine AO-stained smears with that needed to examine Gram-stained smears. Our impression, based upon 2 years of experience with AO staining, is that often much less time is required with the AO stain than with the Gram stain.

Table 1 shows the results of the two stains and the types of clinical specimens used. A total of 497 specimens were examined by the two staining techniques.

H. influenzae type b was the single most common CSF isolate, accounting for 43 (59%) of the 73 positive cultures (Table 2). Five of seven specimens growing *Klebsiella pneumoniae* and three of four growing *Proteus mirabilis* were from two patients who were refractory to antimicrobial treatment.

Direct microscopy of culture-positive CSF specimens revealed bacteria by both the Gram and the AO stains in 56 (77%) of 73 cultures, including 42 (98%) of the 43 specimens growing *H. influenzae*. Neither stain detected *Listeria monocytogenes* in four specimens. Four speci-

TABLE 1. *Types of clinical specimens and results of direct microscopy of Gram- and AO-stained smears*

Types of specimens	Total no. of specimens	No. of positive cultures	No. (%) of smears positive by following stain[a]:		
			Gram and AO	Gram only	AO only
CSF	209	73	56 (76.7)	0	4 (5.5)
Other body fluids, tissues and exudates	288	144	60 (41.7)	5 (3.5)	10 (6.9)
Peritoneal fluid	100	50	11	2	3
Bile	50	26	21	0	2
Pleural fluid	50	25	8	0	2
Synovial fluid[b]	40	20	10	1	0
Culdocentesis fluid	12	6	5	1	1
Miscellaneous[c]	34	17	5	1	2

[a] Positive cultures by: both the Gram and AO stains, 53.4% of total; the Gram stain only, 2.3%; the AO stain only, 6.4% ($P = 0.06$).

[b] One falsely positive Gram-stained smear is not included in this tabulation.

[c] Included were amniotic fluid, semen, vitreous fluid, tracheal aspirates, sinus drainage, biopsies, and purulent material from wounds and abscesses.

TABLE 2. *Identification of bacteria isolated from 73 culture-positive CSF specimens*[a]

Bacteria	No. (%) of positive cultures
Haemophilus influenzae type b	43 (58.9)
Klebsiella pneumoniae	7 (9.6)
Streptococcus pneumoniae[b]	6 (8.2)
Listeria monocytogenes	4 (5.5)
Proteus mirabilis	4 (5.5)
Escherichia coli	3 (4.1)
Neisseria meningitidis	2 (2.7)
Group A streptococcus	1 (1.4)
Group B streptococcus	1 (1.4)
Enterobacter aerogenes	1 (1.4)
Staphylococcus epidermidis	1 (1.4)

[a] A total of forty-three additional cerebrospinal fluid cultures that grew contaminants, predominantly *S. epidermidis* and *P. acnes*, were considered negative.

[b] Includes one specimen positive for *S. pneumoniae* by counterimmunoelectrophoresis, but sterile by culture.

mens (6%) were positive only by the AO stain. There were no false-positive smears by either method.

Culture-positive body fluids, tissues, and exudates were positive by both staining techniques in 60 (42%) of 144 comparisons (Table 1). Five specimens were positive by the Gram strain only, and 10 were positive by the AO stain only. There was one false-positive smear by the Gram stain; it was judged to be falsely positive because the accompanying culture was negative.

The sensitivity, specificity, and predictive values of the two stains are compared in Table 3. Overall, the AO stain was more sensitive than the Gram stain, and the difference approached statistical significance ($P = 0.06$).

In blood culture bottles inoculated with stock strains of bacteria, organisms were detected by the Gram stain at a concentration of about 1×10^5 colony-forming units per ml (10^3 bacteria per smear); organisms were consistently detected by the AO stain at lower concentrations of about 2×10^4 colony-forming units per ml (10^2 bacteria per smear).

DISCUSSION

The acridines are a large family of related compounds used as dyes, antimicrobial agents, and reagents (1). Some, such as AO, are fluorochromes; i.e., they absorb ultraviolet light and emit visible light. AO [3,6-bis(dimethylamino)acridine] is readily available commercially.

AO stains the nucleic acids of bacteria and other cells. Under certain conditions, ribonucleic acid stains orange, and deoxyribonucleic acid stains green. This feature has been applied by cytologists to differentiate the deoxyribonucleic acid and ribonucleic acid components of cells and by virologists to distinguish deoxyribonucleic acid from ribonucleic acid viral inclusions (2, 16). The stain is unfamiliar to most clinical microbiologists, although it has been used to detect malaria and other blood parasites in humans and animals (6, 8, 18) and to identify *Trichomonas vaginalis* in vaginal secretions (5, 12).

Recently, Kronvall and Myhre reported that AO buffered at a low pH produced differential staining of bacteria and background material in clinical specimens (11). They experimented with the AO stain at concentrations of between 2 and 100 mg/liter and over a pH range of 3.5 to 9. Over the entire pH range, bacteria stained bright orange. At a pH of >6, human cells and debris also stained orange, but at a pH of 3.5 to 5.0, the background was yellow to pale green, which

TABLE 3. *Comparison of Gram and AO stains in the direct microscopic examination of clinical specimens*[a]

Test	Stain results (%)					
	CSF		Other specimens		Total specimens	
	Gram	AO	Gram	AO	Gram	AO
Sensitivity	76.7	82.2	45.1	48.6	55.8	59.9
Specificity	100	100	98.8	100	99.6	100
Predictive value (+)	100	100	98.5	100	99.2	100
Predictive value (−)	88.9	91.3	64.4	66	74.4	76.3

[a] A total of 209 CSFs and 288 other body fluids, tissues, and exudates were examined.

enabled the bacteria to be seen distinctly. In a small series of clinical specimens, these authors compared the AO stain (pH 4.0 and concentration of 25 mg/liter) with matched Gram and methylene blue stains and found that the AO stain was sometimes more sensitive than the standard stains.

Specific applications of the AO stain in clinical laboratories have been studied by others. Forsum and Hallén compared AO and methylene blue stains of urethral exudates with culture results in 83 patients with suspected gonorrhea (4). The two methods gave similar results. McCarthy and Senne compared the AO stain with blind subcultures of blood cultures (13). They concluded that the AO method was a rapid and inexpensive method for the detection of positive blood cultures.

Our results support the original work of Kronvall and Myhre (11). In our controlled comparative trial, the AO stain was more sensitive than the Gram stain and was equally specific. Smears were easy to interpret because organisms stained intensely and distinctly, the background stained differentially, and seldom were any confusing orange-staining artifacts present on the slides. Moreover, the colors, orange, yellow, and green on a black field, were aesthetically pleasing. Although the difference between the final results for the two stains was not great, our experience has convinced us that, in selected situations, the AO stain has advantages over the Gram stain.

The AO staining technique also has disadvantages, the chief one being that the technique requires a fluorescent microscope. Many clinical laboratories possess a fluorescent microscope, but for those that do not, the initial expense in purchasing such a microscope may not be warranted. Also, misinterpretation of smears can be a problem. Granules from disintegrating leukocytes may be mistaken as cocci by the unwary, and dead bacteria or contaminants may be stained and lead to erroneous interpretations. We have occasionally noted rare rods in AO-stained smears of CSF that grew *P. acnes* that were contaminants, based on clinical evidence. Thus, at times, the AO stain may be overly sensitive. Finally, positive smears will still have to be Gram stained to determine the Gram reaction of the organism(s) seen. If necessary, the Gram stain can be done directly on the AO-stained smear (13).

Our results of dilutions of simulated positive blood cultures agree with the findings of McCarthy and Senne (13). In general, the AO stain was capable of detecting bacteria in concentrations of about 10^4 colony-forming units per ml, 10-fold fewer than with the Gram stain. These dilution studies confirm the findings with clinical specimens that the AO stain is somewhat more sensitive than the Gram stain.

We conclude that the AO stain is slightly more sensitive than the Gram stain in detecting microorganisms in clinical specimens and that it is a valuable addition to clinical microbiology laboratories. The staining method is simple and permits rapid, thorough, and accurate microscopic examination. The AO stain complements the Gram stain. It is particularly helpful in rapidly screening large numbers of specimens and in examining bloody or thick smears that are normally difficult to interpret by the Gram stain. In our laboratory, we are now using the AO stain routinely to screen blood cultures, to examine smears of blood or buffy coat when indicated, and to confirm the results of Gram-stained smears that reveal leukocytes, but no microorganisms.

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