JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 1980, p. 281-285
0095-1137/80/03-0281/05$02.00/0

Vol. 11, No. 3

Evaluation of Acridine Orange Stain for Detection of Microorganisms in Blood Cultures

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A pH 4.0 buffered solution of the fluorochrome acridine orange was used to stain samples of 2,704 blood cultures that failed to yield visible evidence of growth after 1 day of incubation. Results obtained by the staining method were compared with those obtained by aerobic and anaerobic subcultures simultaneously performed upon the same cultures. Of the 109 culture-positive blood specimens initially detected by the acridine orange and the subculture methods, 85 (78%) were detected by both acridine orange and subculture techniques, 14 (12.8%) were detected by subculture alone, and 10 (9.2%) were detected by acridine orange alone. The differences between the subculture and acridine orange methods were not found to be statistically significant ($P > 0.1$). The acridine orange method represents a rapid and inexpensive alternative to conventional subculture techniques for the detection of bacteria in blood cultures that fail to yield visible evidence of growth after 1 day of incubation.

The rapid detection of bacteria and fungi in blood cultures is central to the diagnosis and treatment of septicemic patients. Many have modified the traditional broth culture method to expedite and enhance the detection of bacteria and fungi. Among the modifications and improvements in blood culture technique are the use of blind subcultures (1, 13) and Gram stains (2) for the detection of organisms not yielding turbidity in broth. Automated methods also have been introduced. These include the use of radiometric (3) and impedance measurements (14) to detect microbial metabolism and growth. Mechanical methods such as membrane filtration (12) and centrifugation (4) of blood specimens have also been tested in the clinical laboratory.

Kronvall and Myhre have recently described the use of an acridine orange (AO) stain to detect microorganisms in direct smears prepared from clinical specimens (9). Because of the potentially high sensitivity of this staining method, we elected to compare the AO stain with a conventional blind subculture used for the detection of microorganisms in blood cultures that were macroscopically negative after 1 day of incubation at 35°C.

MATERIALS AND METHODS

Collection and culture of blood specimens. Different methods of collection and culture of blood specimens were employed for pediatric and adult patients. For adult patients, the venipuncture site was first cleansed with green soap and then treated with 2% tincture of iodine and 95% ethanol. Blood specimens (16.6 ml) were collected into a partially evacuated sterile collection tube (16 by 150 mm; Becton, Dickinson & Co., Rutherford, N.J.) through use of a sterile combination Vacutainer holder needle (Becton, Dickinson). Each collection tube contained 3.4 ml of 0.3% sodium polyanethol sulfonate in 0.85% saline. After collection, the specimen was mixed thoroughly with the anticoagulant and transported immediately to the laboratory. In the laboratory, the blood specimen was again mixed thoroughly, and equal portions were dispensed through a sterile, double-ended needle into either four 50-ml culture bottles or two 100-ml culture bottles (5).

When four bottles were used, two contained Columbia broth modified by the addition of L-cysteine to a final concentration of 0.05%, and the remaining two contained the modified broth supplemented with sucrose to a final concentration of 10%. One bottle of the isotonic broth and one of hypertonic broth were vented to admit air. When two 100-ml culture bottles were used, each contained the cysteine-modified medium without sucrose. One of these bottles was vented to admit air.

Specimens (4.0 ml each) from pediatric patients were collected by sterile syringe after preparing the venipuncture site by the method previously described. The stoppers of two supplemented peptone broth culture tubes (Becton, Dickinson) were disinfected with 2% tincture of iodine, and 2 ml of blood was inoculated into each tube.

Inspection and subculture of blood culture bottles. All blood cultures were incubated at 35°C for 7 days. Each bottle or tube was examined twice daily for visible evidence of growth. After 1 day and 7 days of incubation, each culture bottle was mixed thoroughly, and a 0.3- to 0.5-ml sample was aspirated into a syringe through a sterile needle. Portions (0.1 ml) of the aspirated material were applied to two chocolate

agar plates which were then incubated at 35°C in either a 5% CO_2 atmosphere or anaerobically in a GasPak (BBL Microbiology Systems, Cockeysville, Md.) jar. All subculture plates were incubated for 48 h. Culture plates incubated in CO_2 were examined daily, and anaerobic subcultures were examined only after 48 h of incubation. When visible evidence of growth was noted in any one culture bottle, all culture bottles inoculated with that blood specimen were Gram stained and then subcultured according to the Gram stain results shown in Table 1.

Preparation of AO staining solution. A stock solution of AO was prepared by dissolving 1 g of acridine orange (Fisher Scientific Co., Pittsburgh, Pa.) in 100 ml of distilled water. This solution was stored at 4°C and protected from light. Under these conditions, the stock solution was stable for at least 6 months. The staining reagent was prepared daily by adding 0.05 ml of the stock solution to 5 ml of 0.2 M Walpole acetate buffer (pH 4.0).

AO stain. Eight well slides were prepared by first placing eight evenly spaced, discrete drops of glycerol upon the surface of a glass microscope slide (75 by 25 mm) and then applying the film-bonding agent Fluoroglide (Chemplast, Inc., Wayne, N.J.) to the entire surface of the slide. The bonding agent was allowed to dry, and the glycerol was washed from the slides with distilled water, leaving eight distinct wells corresponding to areas where drops of glycerol had been applied.

Blood samples (10 μl each) from each component culture bottle or tube were applied to individual wells of the eight place slides. The slides were then warmed to 55°C until all culture samples had dried. Slides were then fixed in absolute methanol for 2 min. After fixation, the slides were stained with AO for 1 min, rinsed with tap water, and air dried. Samples of known negative and positive blood cultures were also stained with AO and examined daily. These cultures were prepared by using a blood specimen from a healthy volunteer to inoculate blood culture bottles. A known positive culture was created by inoculating a blood culture bottle containing a sample of the blood of the volunteer with *S. aureus* ATCC 25923 and incubating the culture bottle for 24 h at 35°C.

The stained smears were examined through use of an American Optical series 20 microscope fitted with a vertical fluorescence illuminator (American Optical Corp., Buffalo, N.Y.). The illuminator was fitted with the barrier-exciter combination (Fluorcluster, American Optical) specifically recommended by the manufacturer. Stained slides were examined by using 100×, 450×, and 1,000× magnifications.

When one or more wells of an eight-well slide revealed the presence of bacteria or yeast, the same slide was Gram stained without previous decolorization and examined by using light microscopy. All component culture bottles or tubes that revealed bacteria or yeast by Gram stain were subcultured by the method described earlier (Table 1).

Comparison of AO and subculture methods. All organisms recovered by cultural methods were identified by standard methods (7, 10). In the event organisms observed by AO were not grown on subculture plates after 48 h of incubation, the same culture bottle or tube was again stained by the AO method and subcultured. When organisms were detected by subculture alone, samples from the corresponding positive culture bottles were again stained with AO.

Sensitivity of the AO stain. *Escherichia coli* ATCC 25922 and *S. aureus* ATCC 25923 were grown in the cysteine-modified broth at 35°C for 24 h. Samples of these suspensions were diluted with the modified Columbia broth until their turbidity equaled that of a MacFarland suspension 0.5 turbidity standard. Four serial 10-fold dilutions of each standardized suspension were prepared in the modified Columbia broth. Quantitation of bacteria from each dilution was accomplished by transferring 0.1-ml volumes of each dilution to two individual sheep blood agar plates, and thereafter spreading the inoculum over the surface of each plate with a sterile glass rod. Inoculated culture plates were incubated at 35°C for 24 h, after which bacterial colonies were counted.

Immediately after preparation, 10-μl samples from each dilution were also placed on individual wells of an eight-well slide. These slides were processed, stained with AO, and examined by J.E.S., using the methods previously described without knowledge of the dilution prepared. The quantitative comparison of AO with quantitative plate counts was performed three times with each organism, using 10-fold dilutions.

TABLE 1. *Plating media employed for subculture of individual blood culture bottles after observation of gram-stained smears*

Gram stain observation	Subculture plates incubated at 35°C, in 5% CO_2	Subculture plates incubated anaerobically at 35°C
Gram-positive rods	Chocolate, colistin-nalidixic acid, and sheep blood agars[a]	Chocolate, sheep blood, and egg yolk agars[b]
Gram-positive cocci	Chocolate and sheep blood agars[a]	Chocolate agar
Gram-negative cocci	Chocolate and Thayer-Martin agars	Chocolate agar
Gram-negative rods	Chocolate, MacConkey, and colistin-nalidixic acid agars	Sheep blood, chocolate, and phenylethyl alcohol agars[b,c]
No organisms seen	Chocolate agar	Chocolate agar
Mixture of gram-positive and gram-negative organisms	MacConkey, colistin-nalidixic acid, and chocolate agars	Sheep blood, chocolate, and phenylethyl alcohol agars[c]
Yeastlike organisms	Chocolate and sheep blood agars[a]	Chocolate agar

[a] Sheep blood agar (at 5%) was prepared with Trypticase soy agar base.

[b] Sheep blood agar (at 5%) was prepared with brain heart infusion agar base and supplemented with 50 μg of hemin per ml and 10 μg of vitamin K per ml.

[c] Phenylethyl alcohol agar supplemented with 50 μg of hemin per ml and 10 μg of vitamin K per ml.

After the sensitivity to the AO method was determined by using 10-fold dilutions, further studies were performed by preparing 1:2 through 1:9 dilutions of the lowest 10-fold dilution which gave a positive AO smear. Plate counts of these dilutions and examination of 10-μl samples by AO staining were performed as described previously.

RESULTS

Initial examination of AO-stained blood culture samples revealed that bacteria and yeasts stained a bright red orange, leukocytes stained a pale apple green and erythrocytes failed to stain with the fluorochrome. Because of the distinct contrast between microorganisms and blood cells, we routinely examined AO-stained smears at 100×. The presence of bacteria and yeasts in smears was readily confirmed by examining "suspicious areas" of red-orange fluorescence at either 450× or 1,000× magnification. It was additionally observed that smears previously stained with AO could be Gram stained directly without requiring prior decolorization. The Gram-staining characteristics of bacteria, erythrocytes, and leukocytes in such slides were identical to those observed in smears prepared from the same blood culture bottles not previously stained with acridine orange.

Of the 2,991 blood cultures processed, 2,704 were macroscopically negative after 1 day of incubation. Each of the macroscopically negative cultures was examined simultaneously by subculture and AO methods. Of these, 109 were found to contain microorganisms by either or both methods. The microorganisms recovered from these cultures are listed in Table 2. Of the 109 positive cultures, 94 yielded only a single organism, whereas 15 yielded a mixture of two or more organisms. Gram-positive bacteria were recovered from 60 of those cultures which yielded only a single organism, gram-negative rods were recovered from 33, and a yeast was recovered from 1 culture.

A total of 10 (9.2%) cultures were detected by only the AO method, and 14 (12.8%) were detectable by only subculture and 85 (78%) by both subculture and AO methods. Evaluation of these data by the chi-square method failed to reveal a statistically significant difference between the AO and subculture methods ($P > 0.1$). Culture bottles in which organisms were detected by subculture and not by AO were again stained with AO. In each of the 14 instances, AO stained the organisms not previously detected by this staining method. All microorganisms detected initially by the AO method were also observed after Gram staining the AO smear. In a number of instances, visualization of organisms in these Gram-stained smears occurred only after a prolonged search of the smear. With the possible exception of streptococci, subculture offered no advantage over the AO method. Fourteen blood cultures yielded streptococci; nine were detected by both subculture and AO, and five were detected by subculture alone.

During the course of our evaluation we observed bacteria in eight additional blood cultures that were detected by the AO method alone. In each instance, the presence of microorganisms could not be confirmed, either by subculture or by staining additional samples from the same blood culture bottle(s). Each of these was considered to represent a false-positive result.

The sensitivity of the AO method was evaluated by staining dilutions of *E. coli* and *S. aureus* that were also quantitated by a dilution plate count method. The AO method was capable of detecting the presence of *E. coli* and *S. aureus* when 1.4×10^4 and 8.3×10^3 colony-forming units per ml, respectively, were present and when only 0.01-ml samples of the diluted suspensions were stained.

DISCUSSION

The detection of microorganisms in blood cultures relies mainly upon observation of turbidity, hemolysis, or other visible evidence of growth. Certain organisms, such as *N. meningitidis, N. gonorrhoeae, Haemophilus influenzae,* and others, fail to produce sufficient turbidity to be detected by visual methods when most medium formulations are employed. Other bacteria and yeast may grow slowly in a blood culture broth, resulting in their delayed detection when such cultures are only inspected visually. For these reasons, several authors have advocated the use of either subcultures or Gram stains of macroscopically negative blood culture bottles after a 1- or 2-day incubation period and again before the culture is assigned a negative or no growth result after 7 or 14 days of incubation (1, 2, 13). Even when radiometric methods are employed to detect microorganisms in blood culture, "blind" subcultures are needed for optimal recovery and rapid detection of microorganisms (3). A comparative study evaluating the use of subculture, Gram stain, and macroscopic methods indicated that Gram stain and subculture first detected the presence of microorganisms in 23 and 12% of positive cultures, respectively (2). As a minimum, the use of blind subculture has been advocated, since this procedure is more sensitive than the Gram stain method (1). We did not compare the AO staining method with the Gram stain, since our laboratory routinely employs only subculture methods for detecting microorganisms in macroscopically negative blood cultures. Our experience suggests that the AO method may be somewhat more sensitive

TABLE 2. *Bacteria and yeast detected in positive blood cultures and observed by AO only, subculture only, or by both methods*

Organism	No. of cultures detected			Total positive cultures
	AO positive	Subculture positive	AO and subculture positive	
Gram positive				
Bacillus cereus		1	3	4
B. megaterium			2	2
B. subtilis	1	1	5	7
Diphtheroids	1			1
Propionibacterium acnes	2	1	1	4
Staphylococcus aureus	1	1	17	19
S. epidermidis			9	9
Streptococcus bovis			1	1
S. faecalis		1		1
S. pneumoniae			6	6
S. salivarius		1	1	2
Viridans streptococci		3	1	4
Gram negative				
Acinetobacter sp.			1	1
Bacteroides fragilis			2	2
Citrobacter diversus			1	1
Enterobacter cloacae	1		1	2
Escherichia coli	1		4	5
Haemophilus influenzae			2	2
Klebsiella pneumoniae	1	1	3	5
Neisseria meningitidis			2	2
Proteus rettgeri		1		1
Pseudomonas aeruginosa	1	1	8	10
Serratia marcescens			2	2
Yeast				
Candida albicans			1	1
Mixed cultures				
E. coli, B. thetaiotaomicron			1	1
E. coli, C. diversus			1	1
K. pneumoniae, B. fragilis			1	1
K. pneumoniae, P. morganii			1	1
P. aeruginosa, B. distasonis		1		1
S. aureus, P. aeruginosa		1	2	3
S. epidermidis, enterococcus			1	1
S. morbillorum, diphtheroids	1			1
Viridans streptococci, *E. cloacae*			1	1
Viridans streptococci, *H. influenzae*			1	1
Viridans streptococci, *S. epidermidis*			1	1
Viridans streptococci, *C. albicans*			1	1
S. marcescens, enterococcus, *P. aeruginosa, P. putida*			1	1
Total	10 (9.2%)	14 (12.8%)	85 (78.0%)	109 (100%)

than the Gram stain. When we Gram stained the same smears in which organisms were detected by AO staining, we often experienced difficulty locating the microorganism(s), although each was ultimately found.

The stain was found to be optimal for examining smears from blood cultures because of the specific red-orange fluorescence of stained bacteria and yeasts, the pale green staining of leukocytes, and the lack of staining of erythrocytes. It has been observed previously that erythrocytes appear to quench the fluorescence of AO, which is believed due to the presence of hemoglobin (6). The contrast observed between organisms and blood cells was sufficient to permit routine examination of smears at 100× magnification. AO stains the nucleic acids of bacteria (8). At neutral pH, AO may be employed as a

vital stain; viable bacteria stain green, and nonviable bacteria stain red orange (11). Kronvall and Myhre evaluated the use of AO to stain methanol-fixed smears prepared from clinical specimens (9). When a neutral staining solution of AO was employed, both microorganisms and human cells stained red orange. At an acid pH, human leukocytes and epithelial cells stained a pale apple green, whereas bacteria stained red orange.

We found that, with experience, a technologist may accurately examine an eight-well slide in approximately 2 min. Since most laboratories inoculate two culture bottles with a blood culture specimen, four cultures may be examined on a single slide. The staining method is inexpensive, rapid, and quite sensitive, detecting bacteria in densities as low as 1×10^4 colony-forming units per ml. All organisms that are frequently encountered in blood cultures appear to stain well by this method. Although both the subculture and AO methods appear to be equivalent, subcultures require a 24- to 48-h incubation period, whereas same-day detection of microorganisms is possible with the AO method. Our data suggest that not all streptococci can be rapidly detected by the AO method, since all 14 strains were detected by subculture and only nine were detected by the AO method. Each of the five streptococcal strains not detected initially by AO stained with the fluorochrome when a second smear was prepared from the blood culture bottle. The ability of AO to ultimately stain these streptococci suggests that the difficulty in detecting these strains may have resulted from their low density in the sampled culture bottle. Subsequent experience in our laboratory has not revealed a consistent problem with the detection of streptococci in macroscopically negative blood cultures by the AO method.

Of some concern were the eight blood cultures in which microorganisms were only observed by the AO method. In each instance, we were unable to confirm the presence of microorganisms by repeat AO staining, Gram staining, or subculture. These organisms may have represented either procedural contaminants or nonviable bacteria in the blood culture medium introduced during the preparation of the culture bottles by the manufacturer. Subsequent to our evaluation, we have encountered two lots of commercially prepared blood culture bottles that contained nonviable bacteria detectable by the AO method. As a result of this experience, we now examine all new lots of blood culture media by the AO method before employing them for the culture of patient specimens. Lots containing nonviable bacteria in the commercially prepared broth bottles are returned to the manufacturer unused.

Based upon our results we have abandoned the 24-h blind subculture method previously employed by our laboratory and use only the AO method. An examination of our results indicated that although the AO method failed to detect 14 of the 109 positive cultures, each of these cultures yielded macroscopic evidence of growth by the next day. Our data indicate that the AO method is equivalent to conventional 24-h subculture methods employed by many laboratories, and it represents an attractive and rapid alternative to such methods.

LITERATURE CITED

1. **Bartlett, R. C., P. D. Ellner, and J. A. Washington II.** 1974. Cumitech 1, Blood cultures. Coordinating ed., J. C. Sherris. American Society for Microbiology, Washington, D.C.
2. **Blazevic, D. J., J. E. Stemper, and J. M. Matsen.** 1974. Comparison of macroscopic examination, routine Gram stains, and routine subcultures in the initial detection of positive blood cultures. Appl. Microbiol. **27**:537-539.
3. **Caslow, M., P. D. Ellner, and T. E. Kiehn.** 1974. Comparison of the BACTEC system with blind subculture for the detection of bacteremia. Appl. Microbiol. **28**: 435-438.
4. **Dorn, G. L., G. G. Burson, and J. R. Haynes.** 1976. Blood culture technique based on centrifugation: clinical evaluation. J. Clin. Microbiol. **3**:258-263.
5. **Ellner, P. D.** 1968. System for inoculation of blood in the laboratory. Appl. Microbiol. **16**:1892-1894.
6. **Gurr, E.** 1962. Staining: practical and theoretical, p. 100. The Williams & Wilkins Co., Baltimore.
7. **Holdeman, L. V., E. P. Cato, and W. E. C. Moore.** 1977. Anaerobe laboratory manual, 4th ed. Virginia Polytechnic Institute Anaerobe Laboratory, Blacksburg.
8. **Kasten, F. H.** 1967. Cytochemical studies with acridine orange and the influence of dye contaminants in the staining of nucleic acids. Internat. Rev. Cytol. **21**:141-202.
9. **Kronvall, G., and E. Myhre.** 1977. Differential staining of bacteria in clinical specimens using acridine orange buffered at a low pH. Acta Pathol. Microbiol. Scand. Sect. B **85**:249-254.
10. **Lennette, E. W., E. H. Spaulding, and J. P. Truant (ed.).** 1974. Manual of clinical microbiology. American Society for Microbiology, Washington, D.C.
11. **Strugger, S.** 1948. Fluorescence microscope examination of bacteria in soil. Can. J. Res. Sect. C **26**:188-193.
12. **Sullivan, N. M., V. L. Sutter, and S. M. Finegold.** 1975. Practical aerobic membrane filtration blood culture technique: clinical blood culture trial. J. Clin. Microbiol. **1**:37-43.
13. **Todd, J. K., and M. H. Roe.** 1975. Rapid detection of bacteremia by an early subculture technic. Am. J . Clin. Pathol. **64**:694-699.
14. **Ur, A., and D. F. J. Brown.** 1975. Impedence monitoring of bacterial activity. J. Med. Microbiol. **8**:19-28.