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Phenolic Acridine Orange Fluorescent Stain for Mycobacteria

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A new fluorescence acid-fast staining method with acridine orange as the specific stain is presented. Only two reagents are required: the acridine orange-specific stain and a destaining-counterstaining reagent. Compared with auramine fluorescence acid-fast staining, there was less nonspecific staining of non-acid-fast debris which fluoresced a pale green contrasting color to provide a background in which to search for the red-to-orange fluorescing acid-fast bacilli. The results of the study indicate that the acridine orange method is superior to the auramine method in detecting acid-fast bacilli in specimen smears.

Auramine O has been used in fluorescence acid-fast microscopy since it was first introduced by Hageman in 1938 and reported by Richards et al. (8). Although this method is a time-saving improvement over basic fuchsin acid-fast microscopy, there have been some difficulties with its use. Fluorescing debris can be mistaken for acid-fast bacilli (AFB), and potassium permanganate quenching of the background fluorescence frequently leaves little visible background material in which to search for AFB (10).

Katila and Mantyjarvi evaluated a fluorescence acid-fast staining method that used acridine orange as the specific dye (6). The procedure requires a prestaining acidification step. The specific staining reagent does not contain phenol but includes a barbital buffer and was indicated to have only a 2-week shelf life.

The new acridine orange fluorescence acid-fast staining method presented in this report is similar to the traditional acid-fast staining methods that use phenol to accelerate dye penetration through mycobacterial cell walls (2). The acridine orange reagent was prepared by dissolving 5 g of colorless phenol crystals (Fisher Scientific, Pittsburgh, Pa.) in a solution containing 50 ml of deionized water, 25 ml of glycerol (Sigma Chemical Co., St. Louis, Mo.), and 25 ml of 95% ethanol (Midwest Grain Products, Pekin, Ill.). Then 0.1 g of acridine orange (Eastman Organic Chemicals, Rochester, N.Y.) was added, and the mixture was stirred briefly. The solution was set overnight to allow the acridine orange to completely dissolve and then was stirred. The acid-alcohol destaining solution was prepared by mixing 74 ml of 95% ethanol, 26 ml of deionized water, 0.5 ml of concentrated hydrochloric acid (Fisher Scientific), and 0.2 g of methylene blue (Fisher Scientific). The auramine O staining reagents were prepared as previously described (7, 9); the additional products used, auramine O and potassium permanganate, were from Fisher Scientific. Products used to prepare the stock solutions were classified as either reagent grade, for biological use, or certified.

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These reagents were stored in brown bottles at room temperature. After 6 months, no precipitates or other signs of deterioration were seen, and the fluorescence of AFB stained with these reagents was good.

Glycerol has been used as a component of fluorescence acid-fast stain solutions in the past (5). Glycerol was incorporated into the acridine orange acid-fast stain to make the solution more viscous, which tended to keep the stain solution on the smear during staining. Methylene blue in the acid-alcohol destaining reagent reduced background fluorescence in the stained smears. A combination destaining-counterstaining reagent that used methylene blue was used in previous acid-fast staining methods (4).

For staining, heat-fixed specimen smears on clean glass slides were covered with the acridine orange staining reagent for 15 min and then rinsed with deionized water and drained. The smears were covered with destaining reagent for 2 min, rinsed with water, drained, and dried. In initial trials, AFB staining with acridine orange was inhibited when paper strips were placed over the smears during staining; therefore, paper strips should not be used. All procedures were done at 22 to 25°C.

Viable tubercle bacilli in the smears were not likely to survive staining because the acid-fast staining solution used in this study contained 5% phenol (1).

Duplicate sputum smears were prepared at four laboratories, primarily from NaOH-NALC specimen concentrates (7), and sent to the Centers for Disease Control and Prevention for staining and observation (R.W.S.). One of the smears was stained by the acridine orange method, and the other smear was stained by the auramine O fluorescence acid-fast stain (3, 9). AFB-positive control smears were used with each day's staining. The same staining reagent solutions were used throughout the study.

The smears were observed with an Olympus BH2 microscope with a 100-W mercury vapor lamp, a light filter system for use with auramine O, and incident illumination (Olympus Optical Co. Ltd., Tokyo, Japan). The scanning magnification was ×200, and morphology was confirmed at ×600 without immersion oil. Thirty ×200 fields were observed before a smear was reported negative for AFB. The observation of one or two AFB was reported as negative (9).

FIG. 1. Orange fluorescing AFB in a pale green fluorescing background. Original magnification, ×600.

Acridine orange-stained AFB fluoresced a bright red to orange. The methylene blue in the acid-alcohol destaining solution stained the non-acid-fast debris pale blue, as seen by transmitted light microscopy. During fluorescence microscopy, the methylene blue partially blocked fluorescence of the background debris, but there was a pale yellow-green fluorescing background to search for the bright red fluorescing AFB. The AFB were easily seen in the contrasting background (Fig. 1). Red fluorescing debris was seen rarely but was easily differentiated from AFB at ×600 magnification. The fluorescence intensity of the acridine orange-stained AFB was stable over several days when the stained smears were kept in the dark.

Mycobacterium species stained with acridine orange in this study were *Mycobacterium tuberculosis* complex, *M. avium* complex, and *M. leprae.*

The results of the two staining methods were compared (Table 1). The results on the smears from laboratories A, B, and D show that the two methods are comparable. The results of acridine orange microscopy from laboratory C include a report of 19 false-negatives compared with the auramine method. Also, culture results were negative for 15 of those specimens. Of the four specimens reported positive by culture, no more than four AFB were seen on smears stained with auramine O. Only one or two AFB were seen on each of the smears stained with acridine orange that were reported as negative for AFB for these four AFB-positive cultures. An AFB-contaminated reagent used to decontaminate and concentrate the specimens in laboratory C is believed to have caused the higher than expected reports of one to four AFB per smear by both methods. Although other possibilities may be suggested, there was insufficient information available to speculate on any other explanation for the cause of these unexpected results.

TABLE 1. Results of the acridine orange acid-fast stain versus the auramine O acid-fast stain on duplicate smears from 392 specimens

Laboratory	No. of specimens					Sensitivity	Specificity
	Positive	Negative	False-positive	False-negative	Total		
A	12	87	1	0	100	1.00	0.99
B	11	80	1	0	92	1.00	0.99
C	22	57	2	19	100	0.54	0.97
D	9	90	1	0	100	1.00	0.99

In this preliminary evaluation of the proposed acridine orange acid-fast staining method, there was less nonspecific fluorescence, a contrasting background fluorescence, and results comparable to the auramine method in this group of laboratories. Therefore, we have demonstrated that the acridine orange fluorescence acid-fast staining method is superior to the auramine method. We recommend its evaluation and use in other laboratories.

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