Evaluation of a Rapid Fluorescent Staining Method for Detection of Mycobacteria in Clinical Specimens[▽]

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Rapid detection of mycobacterial disease is essential. Using multiple specimen types and concentrations of mycobacteria, we compared two commercial auramine O stains. The more rapid stain permitted consistent acid-fast bacillus quantitation and exhibited less debris staining, and the staining procedure required less time (~2 min) to perform. These results suggest that the rapid stain may be more cost-effective and efficient for use in clinical laboratories.

Due to the steady increase in both tuberculous and nontuberculous mycobacterial disease, the rapid detection of mycobacteria in clinical specimens is essential for the early diagnosis and treatment of patients (1, 3, 6). Currently, many laboratories in which a fluorescence microscope is available use fluorescence microscopy for rapid screening of clinical specimens for the presence of mycobacteria. Fluorescence microscopy has been shown in numerous studies to be at least 10% more sensitive than traditional light microscopy (2, 5). Thus, fluorescent stains are of paramount importance, not only in confirming the presence of mycobacteria in a given specimen, but also in providing an estimated quantification of organisms. In high-volume laboratories such as ours at the Johns Hopkins Medical Institutions, rapid laboratory turnaround times can prove to be crucial in the diagnosis of presumptive mycobacterial disease. In such settings, test characteristics such as staining time and background fluorescence contribute to overall laboratory efficiency in reporting results.

In this study, we evaluated the effectiveness of a rapid fluorescent staining method employing a rapid modified auramine O fluorescent stain (hereinafter referred to as rapid-AO; Scientific Device Laboratory, Inc., Des Plaines, IL) compared to that of the staining method employing TB auramine O stain (hereinafter referred to as standard-AO; Remel, Lenexa, KS) currently in use in our laboratory. The rapid-AO procedure requires six steps, two stains, and 2 min to complete (rapid-AO fluorescent stain set package insert; Scientific Device Laboratory, Inc., Des Plaines, IL). The standard-AO procedure requires eight steps, three stains, and 22 min to complete (standard-AO stain kit package insert; Remel, Lenexa, KS). Testing included excess specimens from the following digested and decontaminated sources: tissue, sputum, bronchoalveolar lavage (BAL) fluid, peritoneal fluid, and cerebrospinal fluid. Specimens were pooled according to the source to provide sufficient volume for division into separate aliquots. Each pooled specimen type was subsequently inoculated with a dilution series of each *Mycobacterium* species to be tested.

Viable-cell counts confirmed the actual number of bacilli per milliliter in a given sample. The concentrations (in CFU per milliliter) used for all staining experiments were selected to reflect a range of bacilli (10^3 to 10^6) commonly encountered in smear-positive clinical specimens. All samples were evaluated blindly, and duplicate slide sets for each species, strain, and dilution tested were prepared according to the manufacturers' instructions (standard-AO stain kit package insert [Remel, Lenexa, KS] and rapid-AO fluorescent stain set package insert [Scientific Device Laboratory, Inc., Des Plaines, IL]). The *Mycobacterium* species tested included a control strain of *Mycobacterium gordonae* and two strains of each of the following: *M.*

FIG. 1. Representative staining of AFB and background debris with the rapid-AO stain (top panel) versus the standard-AO stain (bottom panel).

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TABLE 1. Comparisons of results from rapid-AO versus standard-AO staining of mycobacterial species in spiked sputum samples

Species	Stain	AFB quantitation score (avg)	Concordance (%)	Debris brightness score (avg)	Concordance (%)
<i>M. tuberculosis</i>	Standard-AO	4+	100	3.2	80
	Rapid-AO	4+	100	1.2	80
<i>M. avium</i>	Standard-AO	3.2+	80	2.1+	80
	Rapid-AO	3+	100	1+	100
<i>M. fortuitum</i>	Standard-AO	4+	100	1.4	80
	Rapid-AO	4+	100	1.2	80

abscessus, *M. chelonae*, *M. avium*, *M. kansasii*, *M. fortuitum*, *M. neoaurum*, *M. mucogenicum*, *M. lentiflavum*, *M. scrofulaceum*, and *M. tuberculosis*. A total of 110 duplicate slide sets for the control organism were prepared. For each of the other strain and dilution combinations tested, 30 duplicate slide sets were made. Slide sets were divided into groups A (standard-AO stained) and B (rapid-AO stained). Slides were read and graded independently by five qualified technologists, and the results were compared. Microscopy was performed using an Optiphot fluorescence microscope and a universal fluorescence illuminator (Nikon, Melville, NY) with a catalog no. 102DH fluorescent bulb. All slides were scanned using a magnification of $\times 10$, with a total of 15 fields observed before a slide was termed negative. Results included the quantification of the acid-fast bacilli (AFB) observed using a modification of the current WHO-International Union against Tuberculosis and Lung Disease guidelines, as follows: rare (1 to 9 AFB per 100 fields), 1+ (10 to 99 AFB per 100 fields), 2+ (1 to 10 AFB per individual field), 3+ (10 to 100 AFB per individual field), and 4+ (more than 100 AFB per individual field) (6). The levels of brightness of both the bacilli and background debris were also determined using a similar scale developed in our laboratory for the purposes of this study. Brightness was graded as dull (1+), bright (2+), very bright (3+), or brilliant (4+). The two stains were also evaluated using real-time clinical specimens ($n = 30$). These specimens included 13 sputum samples, 7 BAL samples, 5 tissue samples, 3 pleural fluid samples, and 2 cerebrospinal fluid samples. All slides were prepared in duplicate. All specimens were processed and/or concentrated using standard laboratory procedures (4). Slides were read and graded as detailed above.

The two staining methods demonstrated equivalent quantifications of acid-fast organisms regardless of the specimen type or the *Mycobacterium* species present. The lower limit of detection for both stains was $\sim 10^4$ CFU/ml. Figure 1 demonstrates representative differences between the two stains both in the brightness of the AFB present and in the background debris fluorescence. As shown, bacilli stained brighter (Fig. 1, top panel) in approximately 40% of the rapid-AO slides than in the standard-AO counterparts (Fig. 1, bottom panel). Of importance, only 4% of the rapid-AO slides showed marked background debris fluorescence, compared to 30% of the standard-AO slides. This result is made evident by little to no visible rapid-AO-stained debris (Fig. 1, top panel) versus patches of standard-AO-stained debris (Fig. 1, bottom panel). The overall concordance of the results recorded by five technologists for all isolates considered together varied between

the two stains (rapid-AO, 96%; standard-AO, 84%). However, *Mycobacterium* species-specific differences were noted with both stains. Table 1 lists the average AFB quantitation and debris brightness scores obtained with the rapid-AO stain versus the standard-AO stain for three of the *Mycobacterium* species most commonly encountered in our laboratory, *M. tuberculosis*, *M. avium*, and *M. fortuitum*. Averages reflect results recorded by five trained technologists examining spiked sputum samples. As shown, rapid-AO concordance in AFB quantitation by five technologists was 100% for these representative *Mycobacterium* species. The standard-AO stain consistently yielded less than 100% concordance (average, 80%) for these and all other species tested, with the exceptions of *M. tuberculosis* (100%) and *M. fortuitum* (100%). In contrast, the rapid-AO stain gave 100% concordance for all species but two, *M. chelonae* (80%) and *M. scrofulaceum* (80%) (data not shown).

All real-time clinical specimens were negative for AFB by both stains. Of these specimens, only one, a BAL sample, was culture positive following 4 weeks of incubation. Given this finding, it is likely that the burden of organisms in the original specimen was below the limit of detection ($< 10^4$ CFU/ml) for both stains. However, background debris fluorescence in the clinical specimens treated with the rapid-AO stain (average brightness, 1+) was markedly reduced compared to that in the specimens treated with the standard-AO stain (average brightness, 2+).

This study is the first documented laboratory evaluation of the rapid-AO stain versus the standard-AO stain. The rapid-AO stain outperformed the standard-AO stain according to a number of measurable parameters. Bacilli appeared brighter with the rapid-AO stain, while background debris fluorescence was markedly reduced. The decrease in background fluorescence made the detection of acid-fast organisms easier, even in samples with heavy background debris. The rapid-AO staining was completed within 2 min, which not only reduced the technologist time in preparation but also improved the turnaround time in reporting results. Thus, the rapid-AO stain has the capacity to improve overall laboratory efficiency.

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