

Detection of Acid-Fast Bacilli in Concentrated Primary Specimen Smears Stained with Rhodamine-Auramine at Room Temperature and at 37°C

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Received 28 February 1994/Returned for modification 10 May 1994/Accepted 28 June 1994

Many laboratory workers prefer the rhodamine-auramine method of staining acid-fast bacilli (AFB) in primary specimen smears rather than carbol fuchsin stains because the stain is more readily interpreted and yields greater sensitivity. The increasing incidence of AFB infections serves as an impetus to optimize the rhodamine-auramine stain. A total of 782 primary smears were evaluated blindly by the rhodamine-auramine method at both room temperature and 37°C. Thirty-five smears (4.5%) were positive for AFB, 30 were positive by both methods, and 5 were positive at 37°C only. Room temperature staining detected only 85.7% of the positive primary smears. Of the 30 smears positive by both methods, 13 (43.3%) had equal numbers of AFB on both smears, 13 (43.3%) had more AFB on the smear stained at 37°C, and 4 (13.3%) had greater numbers of AFB on the smear stained at room temperature. No smears were positive only when stained at room temperature. The increasing diagnostic emphasis placed on the primary smear underscores the importance of optimizing AFB smear methods, and rhodamine-auramine staining at 37°C enhances the detection of AFB compared with conventional staining at room temperature.

Between 1953 and 1984, the number of reported cases of tuberculosis in the United States declined. Since then, the upward trend in the incidence of tuberculosis has continued steadily. The increase in the number of human immunodeficiency virus-infected individuals with tuberculosis and outbreaks of multidrug-resistant tuberculosis underscore the need for rapid identification of infectious patients (2, 4, 5, 7). Although the definitive diagnosis of tuberculosis is dependent on the isolation and identification of the causative agent, *Mycobacterium tuberculosis*, acid-fast microscopy remains the most rapid initial step in diagnosis. Two types of acid-fast stains are commonly used, one employing basic fuchsin (Ziehl-Neelsen and Kinyoun) and the other using a fluorochrome (rhodamine-auramine). The rhodamine-auramine method of staining acid-fast bacillus (AFB) smears is preferred because fluorescing bacilli are more readily detected than fuchsin-stained bacilli. Rhodamine-auramine-stained smears are scanned under lower magnification than fuchsin-stained smears, thus permitting a larger area of the smear to be examined in a shorter period of time (8). Recently, Tenover et al. recommended the exclusive use of a fluorescent acid-fast staining method to decrease smear turnaround time (10). Conventional rhodamine-auramine staining is performed at room temperature; however, we have observed that the *M. avium* complex may not stain well (unpublished data). Truant et al. reported that rhodamine-auramine staining could be performed at 37°C with no compromise in smear quality (11). However, there have been no reports of the use of staining at 37°C in routine clinical laboratories. Therefore, we evaluated the ability of rhodamine-auramine staining at 37°C, compared with that of conventional staining at room temperature, to detect AFB in primary specimen smears prepared from concentrated specimens.

MATERIALS AND METHODS

Specimens. A total of 782 clinical specimens received for mycobacterial culture in the Division of Microbiology at Hartford Hospital were included in the study. Stool specimens were excluded from the study because they are routinely stained by the Kinyoun method. Blood, cerebrospinal fluid, and urine specimens were excluded because they do not routinely receive a primary direct smear. Of the specimens received, 551 (70.5%) were respiratory secretions, including 421 sputum samples and 130 bronchoscopy specimens; 65 (8.3%) were tissues, including lymph node, lung, bone marrow, liver, brain, and bone samples; 142 (18.2%) were fluids, including pleural, peritoneal, pericardial, joint, and bile samples; 19 (2.4%) were abscess exudate samples; and 5 others included three gastric aspirate samples, one maxillary sinus drainage sample, and one sinus tract drainage sample.

Specimen processing and smear preparation. Specimens from nonsterile body sites were decontaminated and digested, as necessary, with 2% NaOH (Ricca Chemical Co., Arlington, Tex.) and *N*-acetyl-L-cysteine (Mucomyst; Bristol Laboratories, Evansville, Ind.) for 15 min. The reaction was stopped by addition of phosphate buffer, followed by centrifugation at $2,500 \times g$ for 15 min. Specimens from normally sterile body sites were centrifuged at $2,500 \times g$ for 15 min without decontamination or digestion. Tissues were ground and processed directly without a concentration step.

All specimens were inoculated into one Bactec 12B bottle (Becton Dickinson, Sparks, Md.) and one Middlebrook 7H11 slant (Becton Dickinson, Cockeysville, Md.). One drop of each specimen was added to both wells of two two-well Teflon-coated slides. Conditions were controlled so that the order of slide preparation was equalized between the two staining protocols. Slides were placed on a heating tray to dry at 54°C for at least 2 h. Before staining, slides were fixed in methanol for 1 min and allowed to dry.

Staining. The identical staining reagents used at each temperature included Bacto TB auramine O-rhodamine T (Difco,

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TABLE 1. Rhodamine-auramine staining procedure

Step no.	Procedure for rhodamine-auramine staining at:	
	Room temp	37°C
1	Use rhodamine-auramine stain directly from bottle	Pour rhodamine-auramine into staining container, prewarm in 37°C incubator for 20 min
2	Place slides on staining tray	Place slides in slide holder
3	Flood slides with rhodamine-auramine, stain for 15 min at room temp	Place slide holder into prewarmed rhodamine-auramine in incubator, stain for 15 min
4	Wash slides with running water	Wash slides with running water
5	Flood slides with decolorizer, decolorize for 5 min at room temp	Place slide holder in decolorizer, decolorize for 5 min at room temp
6	Wash slides with running water	Wash slides with running water
7	Flood slides with potassium permanganate, counterstain for 3 min at room temp	Place slide holder in potassium permanganate, counterstain for 3 min at room temp

Detroit, Mich.), Bacto TB decolorizer (0.5% HCl in 70% ethanol; Difco), and Bacto TB potassium permanganate (Difco). One slide was stained at room temperature by conventional staining methods, and one slide was stained at 37°C as described in Table 1. Positive and negative control smears were stained in the same fashion, except that controls stained at 37°C were stained separately, to avoid cross-contamination, in a coplin jar containing the same lot number of rhodamine-auramine.

Interpretation. Smears were interpreted by technologists without knowledge of the other staining method results. Slides were screened for the presence of acid-fast organisms with a fluorescence microscope (BH 2-RFCA; Olympus, Lake Success, N.Y.) by using the 20× objective. The presence of characteristic fluorescing rods was confirmed with the 40× objective. Positive smears were graded in accordance with the following criteria: rare, 1 to 9 organisms per 10 20× fields; few, 1 to 9 organisms per field; moderate, 10 to 90 organisms per field; numerous, >90 organisms per field.

When questionable or rare AFB were observed, the presence of AFB was confirmed by Ziehl-Neelsen staining.

RESULTS

A total of 782 duplicate primary smears were stained and blindly evaluated. Thirty-five (4.5%) of the smears were positive for AFB. All 35 (100%) of these smears were positive when stained with rhodamine-auramine at 37°C. In contrast, 30 smears (85.7%) were positive when stained with the conventional rhodamine-auramine room temperature protocol. Thus, 14.3% of the smears were positive only with staining at 37°C. No smears were positive only when stained at room temperature.

Relative quantities of organisms per smear were compared for the 30 smears positive by both methods. Thirteen (43.3%) of 30 smears demonstrated greater quantities of AFB when stained with rhodamine-auramine at 37°C. Thirteen (43.3%) of 30 smears had equal amounts of AFB on both smears, and only 4 (13.3%) of 30 smears had greater quantities of AFB when stained with rhodamine-auramine at room temperature (Table 2).

Of the 782 specimens cultured for mycobacteria, 72 (9.2%) were positive. *M. tuberculosis* was isolated from 21 (29.2%) of the specimens; the *M. avium* complex was isolated from 29 (40.3%) of the specimens; *M. gordonae* was isolated from 20

(27.8%) of the specimens; and 1 specimen each grew *M. chelonae* and *M. simiae*. One specimen that was smear positive by both staining methods was culture negative. In addition, two specimens that were smear positive when stained at 37°C but negative by conventional staining were culture negative. Cul-

TABLE 2. Positive AFB smear and culture results

Culture result	Smear result obtained with rhodamine-auramine at:	
	Room temp	37°C
<i>M. tuberculosis</i>	Rare	Few
<i>M. tuberculosis</i>	Rare	Moderate
<i>M. tuberculosis</i>	Few	Few
<i>M. tuberculosis</i>	Moderate	Few
<i>M. tuberculosis</i>	Moderate	Moderate
<i>M. tuberculosis</i>	Moderate	Moderate
<i>M. tuberculosis</i>	Moderate	Numerous
<i>M. tuberculosis</i>	Moderate	Numerous
<i>M. tuberculosis</i>	Numerous	Moderate
<i>M. tuberculosis</i>	Numerous	Numerous
<i>M. avium</i> complex	Negative	Rare
<i>M. avium</i> complex	Negative	Rare
<i>M. avium</i> complex	Negative	Rare
<i>M. avium</i> complex	Rare	Rare
<i>M. avium</i> complex	Rare	Rare
<i>M. avium</i> complex	Rare	Numerous
<i>M. avium</i> complex	Rare	Numerous
<i>M. avium</i> complex	Few	Numerous
<i>M. avium</i> complex	Few	Numerous
<i>M. avium</i> complex	Moderate	Numerous
<i>M. avium</i> complex	Numerous	Numerous
<i>M. avium</i> complex	Numerous	Numerous
<i>M. avium</i> complex	Numerous	Numerous
<i>M. avium</i> complex	Numerous	Numerous
<i>M. gordonae</i>	Rare	Few
<i>M. chelonae</i>	Few	Rare
<i>M. simiae</i>	Few	Rare
Culture negative ^a	Negative	Rare
Culture negative ^a	Negative	Rare
Culture negative ^a	Few	Moderate

^a Presence of AFB confirmed by Ziehl-Neelsen staining.

ture results of smear-positive specimens are summarized in Table 2. The *M. avium* complex was isolated from three specimens that stained positive with the 37°C staining method but were negative by conventional staining. Of the 26 cultures in which equal or greater numbers of AFB were detected in the 37°C-stained smear, 10 yielded the *M. avium* complex, 14 yielded *M. tuberculosis*; 1 yielded *M. gordonae*, and 1 was culture negative. *M. simiae* and *M. chelonae* and two strains of *M. tuberculosis* were isolated from cultures in which greater numbers of AFB were seen in the conventionally stained rhodamine-auramine smear.

DISCUSSION

The laboratory plays a critical role in the diagnosis of tuberculosis. With the resurgence of tuberculosis, particularly multidrug-resistant tuberculosis, in the United States, greater emphasis is now placed on the use of rapid laboratory methods for the detection and identification of AFB (3, 6). Detection of AFB in primary specimen smears is often the first evidence of infection (1), and in institutions where the prevalence of *M. tuberculosis* is high, rapid identification and isolation of potentially infectious patients are essential.

The use of a fluorochrome acid-fast stain, such as rhodamine-auramine, is recommended because of its increased sensitivity and ease of interpretation compared with carbol fuchsin stains. The accepted practice is to stain smears with rhodamine-auramine at room temperature. With the exception of the report by Truant et al. (11), we are unaware of any reports on the results of staining at a higher temperature. In the present study, staining with rhodamine-auramine at 37°C increased smear positivity by 14.3% over the conventional room temperature method. Not only did 43.3% of the smears positive by both staining methods show greater quantities of AFB when stained at 37°C, but 4 (30.8%) of those 13 smears showed at least 2 orders of magnitude more organisms than did the smears stained at room temperature. Thus, staining at 37°C increased overall smear sensitivity and may enable the visualization of greater numbers of AFB in a smear.

The majority of cultures with positive smears grew the *M. avium* complex. This undoubtedly reflects the higher incidence of the *M. avium* complex at our institution. In institutions in which the isolation of *M. tuberculosis* is predominant, the increase in smear sensitivity could contribute to quicker isolation and treatment of infected patients. In this study, there were three instances in which a positive smear was not confirmed by culture. These specimens, a lung tissue sample and two sputum samples, were from patients with a recent history of treated tuberculosis or *M. avium* complex infection. This previous treatment is a likely cause of the negative culture.

Staining at 37°C is no more difficult than staining at room temperature and requires only that the rhodamine-auramine stain be prewarmed prior to use. In addition, technologists may prefer the ease of using a staining container for the staining

and washing steps. We did not observe any cross-contamination as a result of staining slides in a staining container. However, Kent and Kubica (9) have indicated that transfer of material in bulk staining tanks may result in false-positive smears. Acid-fast smears may be stained on a staining rack at 37°C after the rhodamine-auramine stain has been prewarmed. Staining of primary specimen smears with rhodamine-auramine at 37°C appears to be more sensitive than staining at room temperature. Therefore, we recommend the use of rhodamine-auramine at 37°C for routine staining of smears for AFB.

ACKNOWLEDGMENTS

The technical assistance of Pamela Abbott and Carol Bisailon is gratefully acknowledged.

REFERENCES

1. American Thoracic Society. 1990. Diagnostic standards and classification of tuberculosis. *Am. Rev. Respir. Dis.* **142**:725-735.
2. Centers for Disease Control. 1990. Guidelines for preventing the transmission of tuberculosis in health-care settings, with special focus on HIV related issues. *Morbidity and Mortality Weekly Rep.* **39**:1-29.
3. Centers for Disease Control. 1992. Meeting the challenge of multidrug resistant tuberculosis: summary of a conference. *Morbidity and Mortality Weekly Rep.* **41**:51-57.
4. Dooley, S. W., M. E. Villarino, M. Lawrence, L. Salinas, S. Amil, J. V. Rullan, W. R. Jarvis, A. B. Bloch, and G. M. Cauthe. 1992. Nosocomial transmission of tuberculosis in a hospital unit for HIV-infected patients. *JAMA* **267**:2632-2635.
5. Edlin, B. R., J. I. Tokars, M. H. Greico, J. T. Crawford, J. Williams, E. M. Sordillo, K. R. Ong, J. O. Kilburn, S. W. Dooley, K. G. Castro, W. R. Jarvis, and S. D. Holmberg. 1992. An outbreak of multidrug-resistant tuberculosis among hospitalized patients with the acquired immunodeficiency syndrome. *N. Engl. J. Med.* **326**:1514-1521.
6. Ellner, J. J., A. R. Hinman, S. W. Dooley, M. A. Fischl, K. A. Sepkowitz, M. J. Goldberger, T. M. Shinnick, M. D. Iseman, and W. R. Jacobs, Jr. 1993. Tuberculosis symposium: emerging problems and promise. *J. Infect. Dis.* **168**:537-551.
7. Fischl, M. A., R. B. Uttamchandani, G. L. Daikos, R. B. Poblete, J. N. Moreno, R. R. Reyes, A. M. Boota, L. M. Thompson, T. J. Cleary, and S. Lai. 1992. An outbreak of tuberculosis caused by multiple drug-resistant tubercle bacilli among patients with HIV infection. *Ann. Intern. Med.* **117**:177-183.
8. Huebner, R. E., R. C. Good, and J. I. Tokars. 1993. Current practices in mycobacteriology: results of a survey of state public health laboratories. *J. Clin. Microbiol.* **31**:771-775.
9. Kent, P. T., and G. P. Kubica. 1985. Public health mycobacteriology: a guide for the level III laboratory, p. 58-59. Centers for Disease Control, Atlanta.
10. Tenover, F. C., J. T. Crawford, R. E. Huebner, L. J. Geiter, C. R. Horsburgh, Jr., and R. C. Good. 1993. The resurgence of tuberculosis: is your laboratory ready? *J. Clin. Microbiol.* **31**:767-770.
11. Truant, J. P., W. A. Brett, and W. Thomas, Jr. 1962. Fluorescence microscopy of tubercle bacilli stained with auramine and rhodamine. *Henry Ford Hosp. Med. Bull.* **10**:287-296.