Evaluation of Sputum Smears Concentrated by Cytocentrifugation for Detection of Acid-Fast Bacilli

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The continuously increasing number of tuberculosis (TB) cases in recent years and the emergence of strains with multidrug resistance present a public health problem that requires rapid intervention. Patients with active pulmonary infection expectorate a large number of mycobacteria in the form of droplet nuclei which remain suspended in air for hours.

Early identification and isolation of TB patients is of utmost importance in minimizing the risk of further epidemic spread. The definitive diagnosis of TB is made by the clinical laboratory upon growth on culture of Mycobacterium tuberculosis from patient specimens. However, these usually slowly growing organisms have a generation time of up to 22 h. Thus, it could take 4 to 8 weeks for a positive culture (1, 8). Smears from respiratory specimens are crucially important for a presumptive diagnosis of TB, as well as for the purpose of monitoring the patient's response to chemotherapy (8).

When a concentration technique is used, approximately 10^5 mycobacteria per ml of specimen are needed to be microscopically detected (8). False-positive (acid-fast bacillus) (AFB) smears (i.e., smear positive, culture negative) due to artifacts or false-negative AFB culture due to nonviable mycobacteria occur in 5 to 10% of specimens (1, 8).

The expected overall sensitivity for the traditional acid-fast smear has been reported to be from 22 to 43% (1). While a positive smear will serve to alert the physician to a probable AFB infection, a false-negative smear may lead to a false sense of security. Since the positive culture will not appear for at least 4 to 6 weeks, failure to take appropriate measures during this interval may have a seriously adverse effect upon the control of a TB epidemic.

The specificity, simplicity, and rapidity of the acid-fast smear have made this procedure a popular method for early detection of active pulmonary TB. Although the sensitivity of this technique could be influenced by a variety of factors (e.g., quality of specimen, g factor of centrifugation, staining artifacts, etc.), most authors consider the acid-fast smear to be a valuable method for identifying contagious patients (5, 7, 8). Knowing that the cytocentrifugation technique is successfully used in bacteriology and cytology for concentrating specimens (2, 3), we decided to evaluate the applicability of this method to the preparation of concentrated sputum smears for the detection of AFB.

MATERIALS AND METHODS

Specimens. Two hundred samples were examined in this study. From the clinical specimens submitted to the Mycobacteriology Laboratory over a 3-month period from Pulmonary Clinic patients, 120 respiratory secretion samples from patients with a "rule out TB" diagnosis were selected. In addition, 80 simulated sputum samples were prepared with crude mucin (type II, M2378; Sigma, Inc.) and seeded with mycobacterial suspensions in various concentrations. This resulted in known positive AFB specimens at a concentration range from 10^3 to 10^5 CFU/ml. The simulated positive specimens were prepared from broth culture of five mycobacterial species: M. tuberculosis, M. kansasii, M. avium, M. gordonae, and M. fortuitum. To estimate the concentration of bacilli in the simulated specimens, colony counts on 7H10 agar plates were performed (twice) for the M. gordonae strain. This mycobacterial species was chosen in view of its specific characteristics (e.g., opportunistic pathogen,
scotochromogen, smooth colony, etc.). The concentration of the M. gordonae broth culture was compared with the McFarland turbidity standards, and then dilution with sterile distilled water was performed to achieve a turbidity similar to that of the 0.5 McFarland tube (i.e., \(10^6\) CFU/ml). Further 10-fold dilutions were carried out to achieve concentrations of \(10^2\), \(10^4\), \(10^5\), and \(10^6\) CFU/ml. Colony count plates were read from the last four concentrations (\(10^3\) to \(10^4\)), and the numbers of CFU were found to be within the expected ranges.

A mixed culture of Corynebacterium sp. and a yeast (Rhodotorula sp.) was also serially diluted to serve as an AFB-negative control. All specimens were processed according to the standard laboratory procedure for acid-fast smear and culture (1, 8). The 3% sodium hydroxide digestion-decontamination concentration method routinely used in our laboratory was employed (1). An aliquot (1 to 2 ml) of each specimen was transferred from the original sample to a 10-ml plastic tube to be processed in parallel by the cytocentrifugation technique.

**Sample preparation for Cyto-Tek smear.** Within the biological safety cabinet, equal amounts of 5% sodium hypochlorite (household bleach) were added to the aliquoted specimens in the 10-ml plastic tubes. The tube caps were tightly closed, and then the specimens were shaken for 1 min on a vortex mixer. The mixture was allowed to stand for 5 min to react with the liquefying-decontaminating reagent, and then 2 ml of the mixture was dispensed into two assembled Cyto-Tek chambers (1-ml disposable units) for preparation of duplicate smears. All specimens were centrifuged in the Cyto-Tek (Ames Division, Miles Labs., Inc., Elkhart, Ind.) at 2,500 rpm for 5 min as specified by the manufacturer. After centrifugation, the slides were carefully removed from the Cyto-Tek chambers, air dried for 1 to 2 min, and then heat fixed for 1 min by passing them through a flame until the slide was hot enough to be felt when touched to the back of the hand. One smear was stained by the Kinyoun method, and the duplicate slide was saved for additional staining if needed.

**Sterility control.** In order to determine the efficiency of the 5% sodium hypochlorite to kill M. tuberculosis, 1 drop of the bleached sample was inoculated into two agar slants (7H10 and L-J) prior to cytocentrifugation.

**Culture evaluation.** All cultures planted on an agar slant of Middlebrook 7H10 and two Lowenstein-Jensen media were examined twice a week for growth of mycobacteria. If growth was detected, a Kinyoun smear was made from the colony to confirm the presence of AFB. Complete identification of the isolate was then performed by Gen-Probe and conventional methods (1).

**Smear evaluation.** All Kinyoun-stained smears, permanently mounted, were microscopically examined for 5 to 10 min. First, the smears were scanned with the 20× objective to select the best fields and then they were viewed with a 100× oil immersion objective (7, 8). The smears were independently read (blinded) by two investigators who quantitively recorded the cells, bacteria, and other features of the stain. The routinely prepared AFB smears from the parallel concentrated specimens were reviewed, and the results were compared with those obtained by the cytocentrifugation method (7, 8).

**RESULTS**

A retrospective review of our laboratory reports for acid-fast studies showed that the correlation between positive culture and positive smears ranged from 18% to 38% with 33% average.

Further analysis of available data indicated the apparent causes for this poor correlation to be concentration technique limitations (e.g., revolutions per minute versus g force) or the quality of specimen (e.g., quantity not sufficient, saliva).

The correlation between positive AFB smears and positive AFB cultures increased to 100% when the Cyto-Tek method was employed.

The results for the parallel testing evaluated in this study for Cyto-Tek versus the concentration technique are shown in Table 1.

A total of 120 lower-respiratory-tract specimens collected from selected patients between August and October 1992 were examined by the traditional AFB smear, the new Cyto-Tek smear technique, and culture. Out of 120 specimens, there were 43 negative cultures with negative smears by both Cyto-Tek and concentration techniques. Ten cultures were overgrown with bacteria and fungi within 10 days after incubation. These cultures were discarded, although two of them had positive AFB smears by the Cyto-Tek technique.

Sixty-seven cultures were AFB positive. All 67 cultures were also positive by the Cyto-Tek smear, whereas only 34 were positive by the traditional smear technique. Cultures made from the Cyto-Tek bleached samples were all negative for growth of any microorganisms after 8 weeks of incubation.

In order to further assess the sensitivity of the Cyto-Tek smear, 80 simulated positive AFB specimens were prepared with five strains of mycobacteria in various concentrations from \(10^5\) to \(10^6\) CFU/ml. These simulated samples were processed by both methods in the same fashion as the patients’ specimens. The results are summarized in Table 1.

All 80 simulated specimens had positive AFB cultures and yielded positive Cyto-Tek smears. Only 72 of the 80 smears prepared by the traditional technique were positive. All cultures of simulated specimens at \(10^5\) and \(10^6\)-CFU/ml concentrations grew numerous AFB within 3 weeks. A
FIG. 1. Microscopic (oil immersion) appearance of sputum AFB smear by conventional (A) and Cyto-Tek (B) methods.

A moderate number of colonies were seen on culture of the $10^3$-CFU/ml concentration after 6 weeks, and very few colonies grew from the $10^2$-CFU/ml concentration after 8 weeks of incubation.

The sterility control bleached sample cultures were all negative for growth of *M. tuberculosis* as well as growth of any other microorganism. The number of bacilli seen on Cyto-Tek positive smears was consistently higher than on
the parallel smears made by the traditional technique. For example, on the same sputum specimen, the Cyto-Tek smear was quantified as 4+ while the traditional smear was 1+ for the presence of AFB (Fig. 1). Eight of the simulated specimens at the 10^2-CFU/ml concentration that had negative smears by the traditional technique showed a few AFB (8 to 12 per entire smear) on the parallel Cyto-Tek smears.

DISCUSSION

Subsequent to abnormal radiologic findings, purified protein derivative conversion, and clinical symptoms, a positive acid-fast smear from a patient specimen provides a presumptive diagnosis of active TB. Historically, the control of TB was accomplished by isolating patients with active disease from the healthy population by putting them in sanatoria. In modern days, there are a variety of new socioeconomic factors to be considered.

However, the early detection of contagious patients followed by respiratory isolation and treatment remains the key for effective TB control. Although new methods have been developed recently for the rapid detection of mycobacteria from clinical specimens, further research is required to make them cost-effective laboratory tools (4). Until then, the current laboratory methods should be improved upon. The Cyto-Tek sputum smear has many advantages: (i) rapid results, given within 30 min after receiving the specimen; (ii) sensitivity, readily detecting AFB in samples even when small numbers of bacilli are present; (iii) safety, because the bleached specimen culture has no viable organisms; and (iv) cost-effectiveness and simplicity, enabling laboratories of all sizes to perform accurate AFB smears around the clock.

The only limitation of this method is the inability to produce viable mycobacterial cultures from the bleached cytocentrifuged specimens. However, when epidemiologic factors are considered, patients with positive smears could be immediately isolated. Duplicate or subsequent specimens could then be used for culture and direct sensitivity studies (6).

In a centralized laboratory system for TB control, the identification of mycobacterial species and anti-TB drug susceptibility studies will be limited to large medical centers or public health laboratories. The Cyto-Tek smear technique will be a cost-effective and rapid procedure for detecting patients with AFB-positive sputum at any hospital or outpatient clinic for referral to the specialized laboratories.

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REFERENCES