A Minimum 5.0 ml of Sputum Improves the Sensitivity of Acid-fast Smear for *Mycobacterium tuberculosis*

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Detection of acid-fast bacilli (AFB) by sputum smear supports treatment decisions with pulmonary tuberculosis (TB), but smear sensitivity for *Mycobacterium tuberculosis* is only approximately 45 to 75%. In an effort to increase sensitivity, smears were prepared using a minimum sputum volume of 5.0 ml. Sensitivity of smears during a 39-mo period (n = 1,849) using $\geqslant$ 5.0 ml of sputum was 92.0%, significantly greater (p < 0.001) than a sensitivity of 72.5% in a previous 24-mo period (n = 3,486) when all specimens were processed regardless of volume. All new cases of TB (n = 18) were smear-positive with $\geqslant$ 5.0 ml of sputum before treatment, and all were receiving antituberculosis drugs at hospital discharge. In contrast, significantly fewer new cases of TB (14 of 26, p = 0.002) were positive before treatment when smears were prepared using sputum of any volume, and significantly fewer of these new TB cases (18 of 26, p = 0.03) were receiving treatment at hospital discharge. The eight cases without treatment were smear-negative. These results indicate that acid-fast smear using $\geqslant$ 5.0 ml of sputum increases sensitivity for *M. tuberculosis* and accelerates treatment of TB.

Tuberculosis (TB) remains a major threat to global health with an estimated 8,000,000 new cases and 3,000,000 deaths annually worldwide (1). In the United States there has been an overall decrease in TB during 1992–1997 reflecting a 38% decline in cases among U.S.-born persons (2). However, the number of TB cases among foreign-born persons in the U.S. increased 6% during this period. A positive acid-fast smear of sputum supports presumptive diagnosis and treatment of pulmonary TB. Sputum acid-fast smears have a sensitivity of approximately 45 to 75% for *Mycobacterium tuberculosis* recovered by growth in culture (3–6). When acid-fast smear is negative for culture-positive sputum there are delays in the diagnosis of TB (7) often until after discharge of patients from the hospital (8). We recently implemented a minimum specimen volume required to stain and culture sputum for acid-fast bacilli (AFB). This report describes improved sensitivity of acid-fast smear for *M. tuberculosis* using a minimum sputum volume of 5.0 ml.

METHODS

Between January 1, 1992 and December 31, l993 (period 1) smears were obtained for acid-fast staining using sputum specimens regardless of volume. Between January 1, 1996 and April 1, 1999 (period 2) a minimum volume of 5.0 ml was required before sputum processing was performed. When necessary, additional sputum was requested during period 2 for specimens with a volume less than 5.0 ml. Sputum specimens < 5.0 ml were kept at 4° C for up to 48 h, and pooled for processing as a single specimen when a total volume of at least 5.0 ml

was obtained. A total of 3,486 sputum specimens were processed during period 1 and 1,849 during period 2.

Sputum was decontaminated by addition of 2.0% NaOH containing the mucolytic agent *N*-acetyl-L-cysteine (NALC) to an equal volume (5.0 to 12.0 ml) of sputum in 50.0-ml conical centrifuge tubes, followed by vortexing and incubation at room temperature for 15 to 20 min. The incubation mixture was diluted to a volume of 50.0 ml with 0.067 mol/L phosphate buffer, pH 6.8 and AFB contained in the specimen pelleted by centrifugation for 30 min at 3,100 *g*. Supernatant solution was removed by gentle decanting, 0.5 to 1.0 ml of 0.85% sterile NaCl solution containing 0.2% bovine serum albumin added, and two drops of pelleted material were placed on a glass slide for acid-fast staining. Cytocentrifugation of sputum has been reported to increase sensitivity of acid-fast smear for *M. tuberculosis* to 100% (9). Thus, during period 2 smears were prepared by cytocentrifugation. Briefly, three drops of decontaminated sputum were added to a cytofunnel with three drops of 5% sodium hypochlorite to kill mycobacteria (9). Mycobacteria were concentrated directly onto a glass slide by centrifugation for 8 min at 2,000 rpm in a cytocentrifuge (Cytopro; Wescor, Logan, UT). Smears were heat fixed at 60 to 70° C for acid-fast staining using the auramine–rhodamine fluorochrome procedure. Microscopic examination of auramine–rhodamine stains was performed at low-power ($\times$200) magnification. The entire smear was examined. The morphology of fluorescent AFB was confirmed by reexamination at $\times$400 magnification.

Results of acid-fast smear were retrospectively correlated with culture results obtained with all sputum specimens during periods 1 and 2. The recovery from sputum of *M. tuberculosis* by growth in culture was used as the reference for calculation of sensitivity, specificity, positive predictive value, and negative predictive value of acid-fast smear. Sensitivity was determined as the ratio of smear-positive specimens culture-positive for *M. tuberculosis* to smear-positive and smear-negative specimens culture-positive for *M. tuberculosis.* Specificity was determined as the ratio of smear-negative specimens culture-negative for *M. tuberculosis* to smear-negative and smear-positive specimens culture-negative for *M. tuberculosis.* Positive predictive value was calculated as (sensitivity) $\times$ (prevalence)/(sensitivity) $\times$ (prevalence) + (1 − prevalence) $\times$ (1 − specificity). Negative predictive value was calculated as (1 − prevalence) $\times$ (specificity)/(1 − prevalence) $\times$ (specificity) + (prevalence) $\times$ (1 − sensitivity). Categorical variables were assessed for statistical significance by the χ^2 test. Student's *t* test was used for statistical analysis of continuous variables. A value of p < 0.05 was considered statistically significant.

RESULTS

The sensitivity of acid-fast smear for *M. tuberculosis* during period 1 was determined with 109 culture-positive sputum specimens from 36 patients, and the sensitivity of acid-fast smear during period 2 with 112 culture-positive specimens from 29 patients. Sensitivity of acid-fast smear for *M. tuberculosis* was significantly greater when a minimum sputum volume of 5.0 ml was required (Table 1). The increased sensitivity of acid-fast smear for *M. tuberculosis* in period 2 was not accompanied by increase in sensitivity for mycobacterial species other than *M. tuberculosis* (Table 1).

Lipsky and coworkers (3) have reported that negative acid-fast smears occur with sputum specimens having lower-yield

TABLE 1

SENSITIVITY OF SPUTUM ACID-FAST SMEAR FOR MYCOBACTERIAL SPECIES

	No. Smear-positive/No. Culture-positive (%)	
Mycobacterial Species	Period 1*	Period 2*
M. tuberculosis complex	79/109 (72.5)[†]	103/112 (92.0)[†]
M. avium complex	18/122 (14.8)	9/82 (11.0)
M. kansasii	30/48 (62.5)	7/18 (38.9)
M. gordonae	0/37 (−)	3/83 (3.6)
Rapid growers[‡]	3/113 (2.7)	0/11 (−)
Others[§]	2/20 (10.0)	0/27 (−)

* Period 1 = 1992–1993, period 2 = 1996–1999. Values of sensitivity were calculated using results obtained with sputm specimens from hospital patients and outpatients.

[†] p < 0.001.

[‡] *M. fortuitum* and *M. chelonae.*

[§] Period 1: *M. xenopi*, n = 9; *M. szulgai*, n = 1; scotochromogens, n = 10. Period 2: *M. xenopi*, n = 5; *M. scrofulaceum*, n = 2; scotochromogens, n = 14; nonphotochromogens, n = 3; pigmented rapid growers, n = 3.

growth in Lowenstein-Jensen culture (≤ 100 colonies), and this was observed by us during periods 1 and 2 (Table 2). There was no significant difference between periods 1 and 2 in the sensitivity of acid-fast smear for specimens with lower-yield growth. More than one-half (55.6%) of sputum specimens in period 1 showed lower-yield growth in Lowenstein-Jensen culture as compared with one-third (33.0%) in period 2, a statistically significant difference (p = 0.002). This difference most likely reflects smaller volumes of sputum received in period 1 for smear and culture.

There were 53 smear-positive specimens positive in culture for growth of mycobacteria other than *M. tuberculosis*, and seven smear-positive specimens negative for growth in culture during period 1. Nineteen smear-positive specimens were positive in culture for species other than *M. tuberculosis* and seven smear-positive specimens were negative by culture in period 2. Based on these results, the specificity of acid-fast smear for *M. tuberculosis* in period 1 was 98.2%, and in period 2 the specificity was 98.5%.

Acid-fast smear has its greatest clinical impact on the first-time diagnosis of TB for patients who have not been treated, and who have not previously had culture-positive sputum. The medical records of patients not receiving TB drugs when admitted to the hospital and for whom the initial culture-positive sputum was collected during the hospital stay were retrospectively evaluated for signs and symptoms of TB, and for treat-

ment with TB drugs at discharge. Respiratory manifestations of pulmonary TB evaluated were productive cough, dyspnea, appearance of cavities by chest radiograph, and hemoptysis. Cough productive of sputum is associated with tissue necrosis (10). Dyspnea is a late symptom of pulmonary TB, and signifies advanced parenchymal involvement (10). The formation of lung cavities and hemoptysis also indicate advanced disease with excavation of lung tissue. Twenty-six patients in period 1 were not being treated for TB when admitted to the hospital, and the first sputum positive in culture for *M. tuberculosis* was obtained during their hospital stay (Table 3). During period 2 there were 18 newly diagnosed TB patients. No significant difference was observed with newly diagnosed TB patients between periods 1 and 2 for age, gender, the length of hospitalization, or the average number of sputum specimens per patient (Table 3). In addition, there was no significant difference between periods 1 and 2 in the frequency of clinical manifestations of pulmonary TB, or the extent of pulmonary disease as reflected in the number of signs and symptoms for individual patients (Table 4).

All but one sputum specimen obtained from newly diagnosed patients with TB during period 2 before treatment with antituberculosis drugs were positive for AFB (Table 3), and all newly diagnosed patients were smear-positive before treatment. In contrast, during period 1 less than two-thirds of sputum specimens obtained from newly diagnosed TB patients were positive by acid-fast smear before treatment, and only 14 patients (53.8%) were smear-positive. Finally, treatment at discharge with TB drugs was noted for all newly diagnosed patients in period 2, but only two-thirds in period 1, a statistically significant difference (Table 3). All eight patients not on TB drug treatment at hospital discharge during period 1 were smear-negative.

DISCUSSION

A higher frequency of smear-positive sputum has been found in advanced pulmonary TB, especially cavitary disease (11). However, the extent of pulmonary disease as reflected in signs and symptoms was equivalent for new culture-confirmed episodes of TB during our two study periods (Table 4). In addition, length of hospitalization was similar (Table 3). Thus, the greater sensitivity of acid-fast smear with respiratory specimens during period 2 (Table 1) does not correlate with more advanced pulmonary TB. We attribute this high sensitivity to use of a minimum 5.0 ml of sputum for smear.

Low sensitivity of smears causes delay in the diagnosis of TB. Mathur and coworkers (7) in their study at the Washing-

TABLE 2

SMEAR POSITIVITY AND QUANTITATIVE GROWTH OF *M. tuberculosis* IN SPUTUM CULTURE

	No. of Sputum Specimens (% Smear-positive or Smear-negative)*			
	1–100 Colonies	101–200 Colonies	201–500 Colonies	> 500 Colonies
Period 1[†]				
All sputum specimens[‡]	55	16	11	17
Smear-positive	29 (52.7)[§]	14 (87.5)	11 (100)	17 (100)
Smear-negative	26 (47.3)	2 (12.5)	—	—
Period 2[†]				
All sputum specimens[‡]	32	21	23	21
Smear-positive	23 (71.9)[§]	21 (100)	23 (100)	21 (100)
Smear-negative	9 (28.1)	—	—	—

* Number of colonies appearing in Lowenstein-Jensen culture.

[†] Period 1 = 1992–1993, period 2 = 1996–1999.

[‡] In period 1, 10 specimens were positive in Middlebrook 7H11 agar culture only; in period 2, 15 specimens were positive in ESP broth culture only.

[§] p > 0.05.

TABLE 3
DEMOGRAPHIC, LABORATORY, AND HOSPITAL FEATURES OF NEWLY DIAGNOSED PULMONARY TB

	Period 1*	Period 2*
No. of patients	26	18
Age, yr	47.9 ± 20.1	48.7 ± 17.0
Men	20 (76.9)	13 (72.2)
Mean hospitalization, d	13.4 ± 8.6	14.2 ± 14.0
Sputum specimens/patient	3.0 ± 1.3	2.6 ± 1.3
Smear-positive sputum/ culture-positive sputum[†]	25/39 (64.1)[‡]	30/31 (96.8)[‡]
Patients with smear-positive sputum[†]	14 (53.8)[§]	18 (100)[§]
Treatment at hospital discharge	18 (69.2)[∥]	18 (100)[∥]

* Period 1 = 1992–1993, period 2 = 1996–1999.
[†] Sputum specimens obtained before treatment with antituberculosis drugs.
[‡] $p = 0.003$.
[§] $p = 0.002$.
[∥] $p = 0.03$.

TABLE 4
MANIFESTATIONS OF NEWLY DIAGNOSED PULMONARY TB

	No. (%) of Patients with Indicated Manifestation	
Feature	Period 1*	Period 2*
Productive cough	17 (65.4)	14 (77.8)
Dyspnea	16 (61.5)	9 (50.0)
Cavitary disease	6 (23.1)	4 (22.2)
Hemoptysis	3 (11.5)	3 (16.7)
Number of pulmonary manifestations		
0	3 (11.5)	2 (11.1)
1	8 (30.8)	6 (33.3)
2	11 (42.3)	7 (38.9)
3	4 (15.4)	3 (16.7)

* Period 1 (1992–1993), n = 26 patients. Period 2 (1996–1999), n = 18 patients.

ton Hospital Center and Veterans Administration Medical Center observed a median time to diagnosis with smear-positive TB patients of 3 d compared with 38 d with smear-negative patients. At the Akron City Hospital, Counsell and co-workers (8) observed the diagnosis of TB prior to hospital discharge with 19 of 21 patients having positive smears, in contrast to only 2 of 10 patients with negative smears. The clinical utility of high sensitivity obtained with acid-fast smear using ≥ 5.0 ml of sputum for prompt diagnosis and treatment of TB is clearly shown in our study (Table 3).

The sensitivity, specificity, positive predictive value, and negative predictive value of acid-fast smear for *M. tuberculosis* observed in this and other studies are reported in Table 5. The prevalence of respiratory specimens positive in culture for *M. tuberculosis* shown in Table 5 is representative of hospital-based mycobacteriology laboratories in the United States. There was no indication of a minimum sputum volume for acid-fast smear in the cited reports, except the study of Woods and co-workers (5) in which a minimum sputum volume of 2.0 ml was required with 1.0 to 2.0 ml used for cytocentrifugation and the remainder conventional processing and centrifugation. The sensitive fluorochrome staining procedure was used in all studies. Sensitivity and negative predictive value obtained with a minimum sputum volume of 5.0 ml during our period 2 were unusually high, and indeed the highest reported in Table 5. The high sensitivity achieved with smears prepared from 5.0 ml or more of sputum thus provided the strongest indication of culture-negative sputum. Not unexpectedly, specificity of acid-fast smear for *M. tuberculosis* decreased with an increase in sensitivity (Table 5). False-positive smears result from sputum culture positive for mycobacteria other than tuberculosis, or sputum that is culture-negative. Even though use of 5.0 ml or more of sputum raised sensitivity without significantly changing specificity in our laboratory (Table 1), the false-positive smears that did occur limited the positive predictive power of acid-fast smear to the lower range of values (Table 5). Our results suggest that use of 5.0 ml or more of sputum for acid-fast smear has strong negative predictive power for the absence of *M. tuberculosis*, but constrained positive predictive power for presence of *M. tuberculosis* rather than mycobacteria other than tuberculosis or nonviable mycobacteria. Nucleic acid amplification for specific detection of *M. tuberculosis* with smear-positive sputum ≥ 5.0 ml in volume would be particularly effective as an adjunctive test (12), given the high sensitivity and negative predictive value obtained using larger specimen volumes. Use of nucleic acid amplification with smear-positive spu-

tum would prevent treatment by TB drugs and respiratory isolation for infection by mycobacteria other than tuberculosis.

The detection of AFB in sputum represents a dynamic end-result of the numbers of organisms shed into respiratory secretions and the volume of sputum collected for smear and culture. Use of at least 5.0 ml of sputum appears to provide a sufficient volume for the near-uniform (92%) detection (Table 1) of AFB intermittently shed into respiratory secretions in pulmonary TB. Cytocentrifugation used in period 2 facilitated microscopic evaluation of smears by depositing AFB in a well-defined 6-mm circle compared with variable 15 to 25-mm areas on slides prepared by conventional centrifugation. However, as seen in Table 2 cytocentrifugation did not significantly increase smear sensitivity for paucibacillary sputum specimens with 100 or fewer colonies of *M. tuberculosis* in Lowenstein-Jensen culture. Our results agree with those reported by Woods and coworkers (5) (Table 5) that cytocentrifugation does not increase smear sensitivity for *M. tuberculosis.*

A 5- to 10-ml volume of sputum is recommended for the diagnosis of pulmonary TB (13), but most clinical laboratories and hospitals are not compliant with this guideline (14). In 1994 ongoing monitoring was implemented in our laboratory for the volume of respiratory specimens, and by 1996 (the first year of period 2) a protocol was uniformly applied by which only sputum specimens 5.0 ml or greater in volume were accepted for smear and culture. During period 2 of our study, the

TABLE 5
SENSITIVITY, SPECIFICITY, AND PREDICTIVE POWER OF ACID-FAST SMEAR WITH RESPIRATORY SPECIMENS FOR *M. tuberculosis**

Study (Reference)	Sensitivity	Specificity	Prevalence	PPV	NPV
Warren, *et al.* Period 2	92.0	98.5	6.1	79.9	99.5
Woods, *et al.* (5)[†]	74.4	97.5	4.6	58.9	98.8
Warren, *et al.* Period 1	72.5	98.2	3.1	56.3	99.1
Lipsky *et al.* (3)	65.0	99.6	3.9	86.8	96.3
Gordon and Slutkin (6)[‡]	55.3	99.9	7.2	97.7	96.6
Greenbaum, *et al.* (4)	48.1	99.8	2.8	87.4	98.5
Gordon and Slutkin (6)[§]	45.7	99.9	3.2	93.8	98.2

Definition of abbreviations: NPV =negative predictive value; PPV = positive predictive value.
* Values (%) were calculated using culture and acid-fast smear results reported in the cited references, or observed in our two study periods.
[†] Values based on acid-fast smears prepared by conventional processing and centrifugation. Identical values were obtained for sensitivity and NPV with smears prepared by cytocentrifugation with the same specimens. The specificity by cytocentrifugation was 97.4% and PPV 58.0%.
[‡] 1979–1982.
[§] 1975–1978.

average volume of smear-positive sputum was 7.3 ml with a range of 5.0 to 20.0 ml. The 100% sensitivity of acid-fast smear for clinical TB with $\geq$ 5.0 ml of sputum is an important practical result, and provides direction for prospective studies to further assess its utility in conjunction with other technologies (especially nucleic acid amplification) and in a variety of patient settings (hospital and community) for the diagnosis of TB.

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