JOURNAL OF CLINICAL MICROBIOLOGY, June 1981, p. 1109-1113
0095-1137/81/061109-05$02.00/0

Vol. 13, No. 6

Acid-Fast Microscopy on Polycarbonate Membrane Filter Sputum Sediments

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Received 18 December 1980/Accepted 11 March 1981

Polycarbonate membrane filters were used to concentrate 916 sputum specimens for detecting acid-fast bacilli by microscopic examination. These results were compared with those of smears prepared from centrifugates and direct smears of the same specimens. Culture isolation, the control procedure, demonstrated the presence of acid-fast bacilli in 76 specimens. The number of positive specimens detected by microscopy was 82 on polycarbonate membrane filter concentrates, with an 80.2% sensitivity; 53 on centrifugate smears, with a 62.2% sensitivity; and 44 on direct smears, with a 55.8% sensitivity. Acid-fast microscopy results demonstrated that the sensitivity of the polycarbonate membrane filter sputum concentration method was superior to that of the recommended centrifuge concentration method and that the former method may be considered a rapid alternative when culture for acid-fast bacilli is impractical.

Culture isolation and identification of mycobacterial species from sputum specimens are more sensitive and definitive than acid-fast microscopy on sputum smears, but they are more complex and require several weeks for a final report. The rapidity and simplicity of acid-fast microscopy on sputum smears continue to be a valuable first bacteriological test for diagnosing pulmonary tuberculosis.

As a specimen is progressively concentrated for smear preparation, the sensitivity of acid-fast microscopy should approach that of culture isolation. In preliminary trials, a ca. 200-fold increase was seen in the number of acid-fast bacilli (AFB) in polycarbonate membrane filter (PMF) sediments compared with direct smears of the same sputum specimens. These promising results prompted this investigation, in which the acid-fast microscopy observations of direct smears, centrifugate smears, and PMF sediments are compared with AFB isolation by culturing sputum specimens.

(This paper was presented in part at the 24th World Conference of the International Union Against Tuberculosis, 5 to 9 September 1978, Brussels, Belgium).

MATERIALS AND METHODS

Direct smears were prepared by spreading a 3-mm loopful of sputum over a 2-cm^2 area of a glass microscope slide (25 by 75 mm). The sodium hydroxide-*N*-acetyl-L-cysteine method (20) was used to decontaminate 8 to 10 ml of each sputum for culture isolation on Lowenstein-Jensen medium at 35 to 37°C. The decontaminated specimens were centrifuged at approximately 2,000 × *g* before the centrifugate was resuspended in 1 ml of 0.2% bovine albumin fraction V and inoculated onto media. A smear of the resuspended centrifugate was prepared in the same manner as the direct smear.

Polycarbonate filter material was chosen because it is not affected by ethanol or NaClO, is strong and flexible, and has a very smooth surface. To prepare the PMF sediments (PMFs were obtained from Nucleopore Corp., Pleasanton, Calif.), we mixed 2 ml of each sputum sample with a 5 to 6% squeous solution of NaClO in 50-ml plastic, screw-capped centrifuge tubes. Clear to light-colored and liquid to moderately mucoid specimens received 4 ml of NaClO; dark-colored and very mucoid specimens received 8 ml of NaClO. The most mucoid specimens were first mixed with an equal volume of NaClO in the collection container. Then, 4 ml of the mixture was transferred to the processing tube, and 6 ml of NaClO was added, bringing the volume to the 8 ml of NaClO used for processing. The processing tube was capped and inverted until the sputum and NaClO were mixed, and then the tube was placed in a rack. After a 10-min reaction, 95% ethanol, which was previously filtered through a 0.4-μm pore size PMF, was added to the mixture in a volume equal to twice the amount of NaClO used. The ethanol thinned the liquefied sputum specimen to facilitate flow through the filter. The liquefied specimen was then pulled by vacuum through a 25-mm, 1.0-μm pore size PMF in a glass filter holder, with the shiny side of the filter up. After initial filtration, 1 ml of filtered 95% ethanol and 1 ml of filtered deionized water were successively passed through the sediment on the PMF. The filter funnel was removed

and placed in a pan of fresh tap water. Two drops of agar adhesive, prepared by boiling 0.1 g of agar in 100 ml of filtered deionized water, were placed on a pre-labeled glass slide. PMF was removed from the holder with a pair of forceps and laid on the liquid adhesive, with the sediment or shiny side down for drying. The lumen and bottom of the filter funnel were cleaned with a test tube brush in tap water, and the excess water was wiped off with a clean cloth before the funnel was placed over a new filter. Three filter holders were used continuously to process an average of 10 specimens per h.

Since NaClO kills tubercle bacilli at the concentrations and time of exposure used in this investigation (18, 20, 21), the sediment cannot be used for culture isolation. One advantage, however, is that after the specimen has been disinfected, it would be noninfectious for the individuals processing it.

All smears and PMF sediments were stained by the fluorescence acid-fast method by using auramine-phenol as the specific stain and acridine orange as the counterstain (18). The air-dried filter was left on the slide during initial staining and was removed from the sediment before the first rinse. The stained material was observed with a Carl Zeiss RA 38 microscope equipped with a 6-V, 15-W incandescent lamp operated at 8 V; a BG-12, 4-mm exciter filter, a GG-50 barrier filter; a 1.3 N.A. bright-field dry condenser; 25× and 63× dry objective lenses; and 10× ocular lenses. Thirty 250× fields were observed before a smear negative for AFB was reported. Morphology was confirmed, and counts of more than 9 AFB per 30 250× fields were observed at 630×.

The results of acid-fast microscopy on the smears from the three methods of preparation were compared with the results of culture isolation, the control, in 2 × 2 contingency tables (1, 3, 15, 16, 19). A 2 × 2 contingency table and methods selected to evaluate test results in this report are shown in Table 1. Since these evaluations are used for other kinds of tests, they are defined for use in this report. Sensitivity is the proportion of true-positive results that are test-positive. Specificity is the proportion of true-negative results that are test-negative. Predictive value is the proportion of test-positives that are truly positive. Absolute false-positives are the proportion of true-negatives identified by the test as positive. Absolute false-negatives are the proportion of true-positives identified by the test as negative.

TABLE 1. *2 × 2 contingency table and selected evaluations*[a]

Control	Test		
	+	−	Total
+	A	B	T1
−	C	D	T2
Total	T3	T4	

[a] Sensitivity = A/T1; predictive value = A/T3; specificity = D/T2; absolute false-positive = C/T2; absolute false-negative = B/T1.

RESULTS

In this study, 916 sputum specimens were examined. Those positive for AFB by microscopy were 44 by direct smear, 53 by centrifugate smear, and 82 by PMF sediment. Seventy-six specimens were positive for mycobacteria by culture isolation. In Table 2, results of acid-fast microscopy are compared with culture isolation results. Other investigators have shown that patients on antituberculosis chemotherapy may produce specimens positive for AFB by microscopy but negative by culture isolation (3, 7, 13). The occurrence of 25 PMF-positive, culture-negative (false-positive) specimens was considered unusual, and previous specimen reports for the patients were sought. Of these 25 patients, 20 had previous specimens that were culture-positive for mycobacteria. The status of the remaining five patients had not been submitted to the Georgia Department of Human Resources. The 20 AFB microscopy false-positive specimens from the patients known to be under therapy were then assumed to be truly positive, since these patients had produced previous specimens that were positive for AFB by culture isolation (12, 14, 15). The corrected evaluations reflect this assumption.

Of the 76 culture-positive specimens, 50 were *Mycobacterium tuberculosis* 22 were *Mycobacterium avium* complex, and 1 each was *Mycobacterium kansasii*, *Mycobacterium fortuitum*, and *Mycobacterium terrae*. One culture was an unidentified mycobacterium. Nine *Mycobacterium tuberculosis* cultures were negative by acid-fast microscopy on PMF sediments. Seven of these had less than five colonies per isolation culture tube. Similarly, three of eight PMF false-negative *M. avium* complex isolates had less than five colonies on primary isolation.

Thirty-five and forty-six percent more AFB-positive specimens were detected in the PMF sediments than in the centrifugate smears and direct smears, respectively. The evaluations of the three preparation methods for acid-fast microscopy are shown in Table 3. The initial and corrected evaluations demonstrate how the false-positive smear results on specimens from patients on chemotherapy affect the evaluations. The fourth row of evaluations demonstrates the detection of only *M. tuberculosis* in sputum specimens when the PMF concentration method is used.

Food and blood in the specimens either made some sediments too thick for effective microscopic observation or prevented the sediment from adequately adhering to the slide.

TABLE 2. *Detection of acid-fast bacilli in specimens prepared for microscopy by three different procedures compared with detection by culture*[a]

Culture	Direct				Centrifuge				PMF			
	A		B		A		B		A		B	
	+	−	+	−	+	−	+	−	+	−	+	−
+	42	34	43	34	45	31	51	31	57	19	77	19
−	2	838	1	838	8	832	2	832	25	815	5	815

[a] A, Initial results; B, results corrected for patients under treatment with previous culture isolations positive for mycobacteria.

TABLE 3. *Results of acid-fast microscopy on direct, centrifugate, and PMF smears compared with culture results*[a]

Smear	Sensitivity	Corrected[b]	Specificity	Corrected	Predictive value	Corrected	Absolute false-positives	Corrected	Absolute false-negatives	Corrected
Direct	55.3	55.8	99.8	99.9	95.5	97.7	0.2	0.1	44.7	44.2
Centrifugate	59.2	62.2	99.1	99.8	84.9	96.2	1.0	0.2	40.8	37.8
PMF	75.0	80.2	97.0	99.4	69.5	93.9	3.0	0.6	25.0	19.8
M. tuberculosis-PMF[c]	82.0		99.4		89.1		0.6		18.0	

[a] Numbers represent percentages.

[b] Corrections are based on the assumption that 20 patients under therapy would have produced positive cultures if the acid-fast bacilli seen by microscopy had been viable.

[c] These evaluations demonstrate the ability of the PMF method to detect acid-fast bacilli in specimens containing only *M. tuberculosis*.

DISCUSSION

Sputum specimens from patients with active cases of tuberculosis frequently contain so few AFB that they are difficult to detect. This has prompted an ongoing effort to develop new methods for concentrating specimens to facilitate the detection of AFB as a diagnostic aid or to determine the effect of chemotherapy. Many early methods of concentrating specimens were reviewed by Willis and Cummings (21). These included flotation on liquid hydrocarbons and centrifuging liquefied sputum specimens with or without a chemical flock. Several investigators have used membrane filters to concentrate pathological specimens for culture of mycobacteria (8, 11) and to concentrate *Mycobacterium leprae* from tissue for acid-fast microscopy (17).

Carvalho (4) determined that a concentration of 10,000 to 100,000 AFB per ml of sputum was necessary to consistently find smears positive for AFB. Cruickshank concluded that the equipoint for reporting smears positive or negative by microscopy was a concentration of 6,900 AFB per ml of specimen (5). Smithwick determined that approximately 6,000 AFB per ml of specimen was required to find at least three bacilli 50% of the time when searching 300 microscopic fields at a magnification of × 1,000 in a 2-cm^2 smear prepared with 0.01 ml of specimen (18). At this concentration, the entire smear contains about 60 AFB. The same results are expected from similar AFB concentrations in resuspended centrifugates. Theoretically, there would have to be 600 AFB per ml of sputum before the specimen is concentrated 10-fold to prepare a similar smear containing 60 AFB. On isolation, this concentrate should produce 600 mycobacterial colonies from a 0.1-ml inoculum.

For the PMF procedure, the concentration of AFB in the specimen would have to be 30 per ml to produce a 2-cm^2 area of sediment containing 60 AFB. This is approximately 1/20 of the theoretical concentration required to prepare equivalent smears from centrifugates. This determination is supported by the photographs of microscopic fields of the three methods of preparation used on the same specimen as shown in Fig. 1.

A proportional increase in the number of AFB detected before and after centrifuging a specimen should not be expected, although Rickman and Moyer recently achieved a significant increase in the specificity of acid-fast microscopy on centrifugates by increasing the relative centrifugal force from 1,260 × *g* to 3,800 × *g* (15).

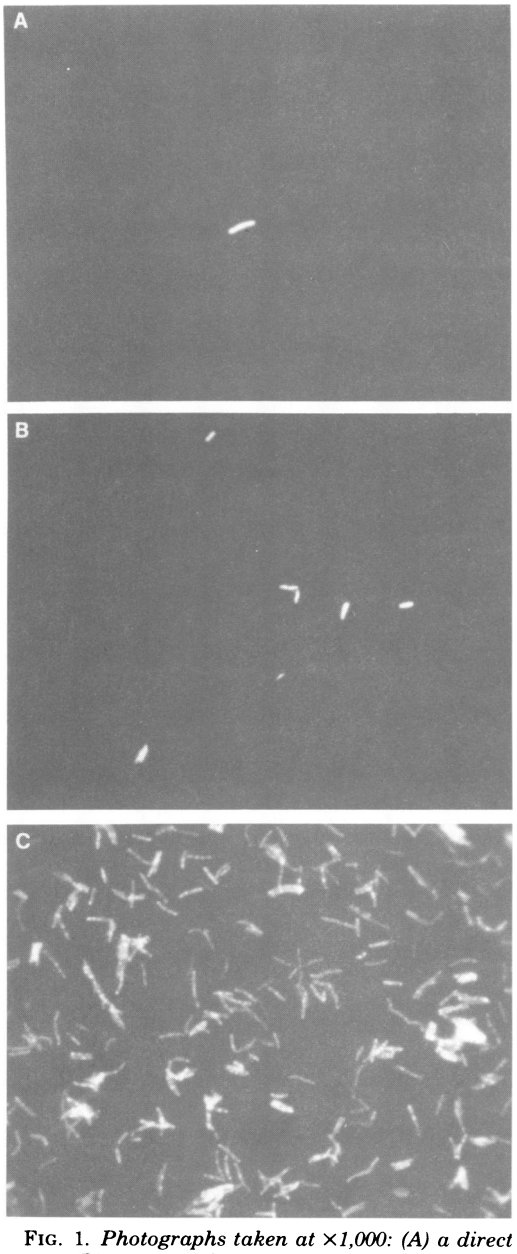

FIG. 1. *Photographs taken at ×1,000: (A) a direct smear, (B) a smear of resuspended centrifugate, and (C) a polycarbonate membrane filter sediment, all prepared from the same sputum specimen. Fluorescence acid-fast staining was done with auramine-phenol as the specific stain, and the background fluorescence was quenched with potassium permanganate.*

The relative centrifugal force now recommended is 1,800 × *g* to 2,400 × *g* (20). Klein et al. (9) reported that L. Silverstolpe had determined that the range of specific gravity of tubercle bacilli was 1.07 to 0.79. If this is true, not all of the tubercle bacilli in a liquefied sputum will be sedimented at any relative centrifugal force. When a liquefied sputum was centrifuged at 2,000 × *g* for 15 min, the PMF sediment of the supernatant liquid had almost as many AFB by microscopy as the centrifugate (R. W. Smithwick, unpublished data). Klein et al. had similar results (9). Darzin's review of the subject concluded that even high-speed centrifugation would not recover all of the tubercle bacilli in a specimen (6). In addition, Yegian and Budd reported that almost 50% of the tubercle bacilli in culture-seeded sputum were killed during a 5-min exposure to 2% sodium hydroxide (22). These factors tend to make evaluations of AFB detection methods questionable when isolation from centrifuged specimens is used as a standard, although it is the most sensitive method available.

In this study, culture isolation from centrifugates was used as the control procedure. More AFB true-positives were found by microscopy in the specimens concentrated by centrifugation or PMF, both of which gave higher sensitivity and lower absolute false-negatives than the direct smear results. Increased concentration also increased the false-positives and is reflected in the decrease in the specificities and predictive values.

In 1975, the reliability of acid-fast microscopy as a diagnostic procedure was challenged (2, 10). False-positive reports were the target of the criticism. For over 25 years, chemotherapy has been recognized as a major contributing factor in causing the AFB seen in smears to be noncultivable (7), and recently it has been an important factor in evaluating results of acid-fast microscopy (3, 12, 14, 15). Rickman and Moyer adjusted their results to account for false-positives caused by chemotherapy, and this adjustment increased the sensitivity values for acid-fast microscopy in their study (15). In our study also, false-positive microscopy reports were believed to be caused by chemotherapy. All evaluations were improved when the results were corrected for the patients under treatment who were assumed to have caused 20 false-positive microscopy reports on PMF sediments, 6 on centrifugates, and 1 on direct smear. This observation supports the proposition that specimens must be identified as being from either "new-untreated" or "under-therapy" patients when acid-fast microscopy reports are evaluated.

Although it did not agree 100% with culture isolation, the PMF method was rapid and more sensitive than the method of examining direct or centrifugate smears.

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