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NOTES

Evaluation of the Phenol Ammonium Sulfate Sedimentation Smear Microscopy Method for Diagnosis of Pulmonary Tuberculosis

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We compared the sensitivity and specificity of the phenol ammonium sulfate (PhAS) sediment smear microscopy method for detection of acid-fast bacilli with those of direct smear microscopy, using culture results for *Mycobacterium tuberculosis* as the "gold standard." The sensitivities of the PhAS and direct smear methods were 85% (465 of 547) and 83% (454 of 547), respectively, and the specificity of each method was 97%. The PhAS method was better accepted by the laboratory technicians and safer but necessitates an overnight sedimentation, which delays reporting of results until 1 day after sputum collection.

Each year, there are an estimated 2 million tuberculosis deaths and 8 million new cases of tuberculosis worldwide (3, 13). In developing countries, diagnosis of pulmonary tuberculosis depends primarily on the identification of acid-fast bacilli (AFB) using Ziehl-Neelsen sputum smear microscopy, a technique more than 100 years old. The sensitivity of this method varies (7) and depends upon collection of sufficient sputum, proper preparation of smears, good staining technique, careful examination of smears, and availability of a good microscope. Several methods have been tried to improve smear microscopy for AFB (1, 4–6, 9, 11, 12), but these have limitations under field conditions. Developing new smear microscopy methods which would be feasible under field conditions remains a priority for improving the diagnosis of pulmonary tuberculosis. In developing countries, laboratory technicians sometimes tend to sidestep the sputum examination owing to apprehensions about the infectiousness of sputum samples and due to the cumbersome method of preparing direct smears from the mucus portion of the sample. We evaluated the sensitivity, specificity, and acceptability of a new smear microscopy method using phenol ammonium sulfate (PhAS) for sedimentation of sputum.

The study was conducted at a large tertiary-care hospital in Chennai, Tamil Nadu, South India. Each patient attending the outpatient department of the hospital was screened for chest symptoms. One spot sputum specimen was collected in a McCartney bottle from each patient who reported having a cough for 3 weeks or longer. Patients who were on antituberculosis treatment at the time were excluded from the study. All samples were collected during the months of August and September 2001.

Processing of sputum samples. Sputum specimens were transported and processed within 4 h of collection. Each sample was divided into two portions of 3 to 5 ml each by pouring the sample from one McCartney bottle to another, such that both portions were approximately equal in volume and appeared similar in quality. The two portions were randomly allocated; one to the modified Petroff method for culture of *Mycobacterium tuberculosis* (10) and the other to the PhAS sediment smear method. For the Petroff method, sputum was homogenized for 15 min in a shaker by using an equal volume of 4% sodium hydroxide. After centrifugation at 3,000 rpm for 15 min in a Megafuge 1.0 (Heraeus), the deposit was neutralized with about 20 ml of sterile distilled water. Samples were again centrifuged, and the deposit was inoculated into Lowenstein-Jensen medium and incubated for 8 weeks at 37°C. The isolated cultures were confirmed for *M. tuberculosis* by a niacin test, a 68°C catalase test, and growth on *para*-nitrobenzoic acid.

The PhAS reagent was prepared by dissolving 50 g of phenol crystals (Qualigens, Chennai, India) and 40 g of ammonium sulfate (E. Merck, Mumbai, India) in 950 ml of distilled water. The reagent was prepared and kept in the laboratory until use (3 to 5 days). The portion allocated to the PhAS sediment smear method was used to prepare a direct smear and a PhAS sediment smear. First, a direct smear was prepared by using a wire loop; the smear was left to dry for 10 to 15 min and was then heat fixed and stained by the Ziehl-Neelsen method (8). To the remaining sputum sample an equal volume of PhAS reagent was added. The sample was mixed well and left to stand overnight at room temperature. Next morning, a PhAS sediment smear was prepared after the clear supernatant was discarded and a drop of the sediment was placed on a glass slide by using a wire loop. Sputum samples were not centri-

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TABLE 1. Comparison of PhAS sediment smear and direct smear results with culture results

Smear method and result[a]	No. of samples with the following culture result[b]:						
	3+	2+	1+	Colonies	Any positive	Negative	Total
PhAS[c]							
3+	173	45	5	4	227	2	229
2+	67	39	5	1	112	4	116
1+	37	37	18	4	96	18	114
Scanty	6	8	10	6	30	34	64
Any positive	283	129	38	15	465	58	523
Negative	9	12	40	21	82	1,736	1,818
Total	292	141	78	36	547	1,794	2,341
Direct[d]							
3+	138	37	2	3	180	1	181
2+	85	33	9	1	128	4	132
1+	57	47	15	4	123	22	145
Scanty	6	7	8	2	23	30	53
Any positive	286	124	34	10	454	57	511
Negative	6	17	44	26	93	1,737	1,830
Total	292	141	78	36	547	1,794	2,341

[a] Smear results: 3+, more than 10 AFB per oil immersion field in at least 20 fields; 2+, 2 to 10 AFB per oil immersion field in at least 50 fields; 1+, 1 to 99 AFB in 100 oil immersion fields; scanty, 1 to 9 AFB in 100 oil immersion fields.
[b] Culture results: 3+, confluent growth; 2+, innumerable colonies; 1+, 20 or more but fewer than 100 colonies; colonies, 1 to 19 colonies.
[c] For the PhAS smear method versus culture, sensitivity was 85%, specificity was 97%, and the kappa value was 0.83.
[d] For the direct smear method versus culture, sensitivity was 83%, specificity was 97%, and the kappa value was 0.82.

fuged to obtain the sediment. The direct and PhAS sediment smears were prepared by experienced technicians.

Reading of smears. All smears were read within 1 to 2 days after sputum collection. For each patient, the direct and PhAS sediment smears were read by the same technician; however, the smears were coded such that the technician reading them was unable to identify which PhAS smear and direct smear came from the same patient. Because the PhAS and direct smears are distinct in appearance, it was not possible to blind the reader to the type of smear. To minimize potential bias in reporting, a duplicate PhAS sediment smear was prepared from every fifth sputum sample. The sensitivity and specificity of the PhAS sediment smear method were determined by using the culture results as the "gold standard."

Sterilizing activity of PhAS. To ascertain the sterilizing activity of PhAS, 20 smear-positive sputum samples were aliquoted into two portions. One portion was processed for culture on Lowenstein-Jensen medium; the other portion was treated with PhAS reagent, and the sediment was then cultured for *M. tuberculosis*.

Acceptability of the methods. To assess the acceptability of each method, a structured questionnaire was given to seven laboratory technicians who had used both methods. Information collected from respondents included the safety of the method, the time taken for making and reading each type of smear, advantages and disadvantages of each method, and overall preference.

Data were entered and processed by using Microsoft Excel and were analyzed by using SPSS (version 4.0). The chi-square test with the Yates correction and a 5% level of significance was used to determine the statistical significance of the difference observed between the direct smear and PhAS sediment smear methods. The kappa value was calculated in order to determine the agreement between the results of each smear method and the culture results.

The results of the duplicate PhAS smears were used to measure the magnitude of bias in reporting. The discordance between the original and the duplicate PhAS smear was 2.7%, indicating insignificant bias in reporting the results of PhAS sediment smears.

Table 1 presents the results of comparison of the PhAS sediment smear method and the direct smear method with the bacteriologic culture method. *M. tuberculosis* was isolated from 547 of 2,400 samples. Fifty-nine samples were found to be contaminated and were excluded from further analysis. Of the culture-positive samples, 465 (85%) and 454 (83%) were positive for AFB by the PhAS sediment smear method and direct smear method, respectively. The observed difference in sensitivities was not statistically significant. The specificity of each method was 97%.

Of the 535 specimens positive by PhAS sediment smear, 233 (44%), 117 (22%), 119 (22%), and 66 (12%) had grades of 3+, 2+, 1+, and scanty, respectively. In comparison, of the 523 specimens positive by direct smear, 183 (35%), 136 (26%), 148 (28%), and 56 (11%) had grades of 3+, 2+, 1+, and scanty, respectively (data not shown).

Treatment with the PhAS reagent sterilized all the 20 smear-positive sputum samples that were used to test the sterilizing activity of the reagent. While the aliquots from the above 20 samples which were not treated with PhAS grew *M. tuberculosis* in culture, all the aliquots that were treated with PhAS were culture negative.

All seven of the laboratory technicians interviewed preferred the PhAS method over the direct smear method. Reasons stated for preferring the PhAS method included ease of specimen handling, ease of making smears, ease of reading and grading smears, and ease of disposal of specimens.

Although the sensitivity of the PhAS sediment smear method was not significantly higher than that of the direct smear method, the PhAS method has several advantages. Be-

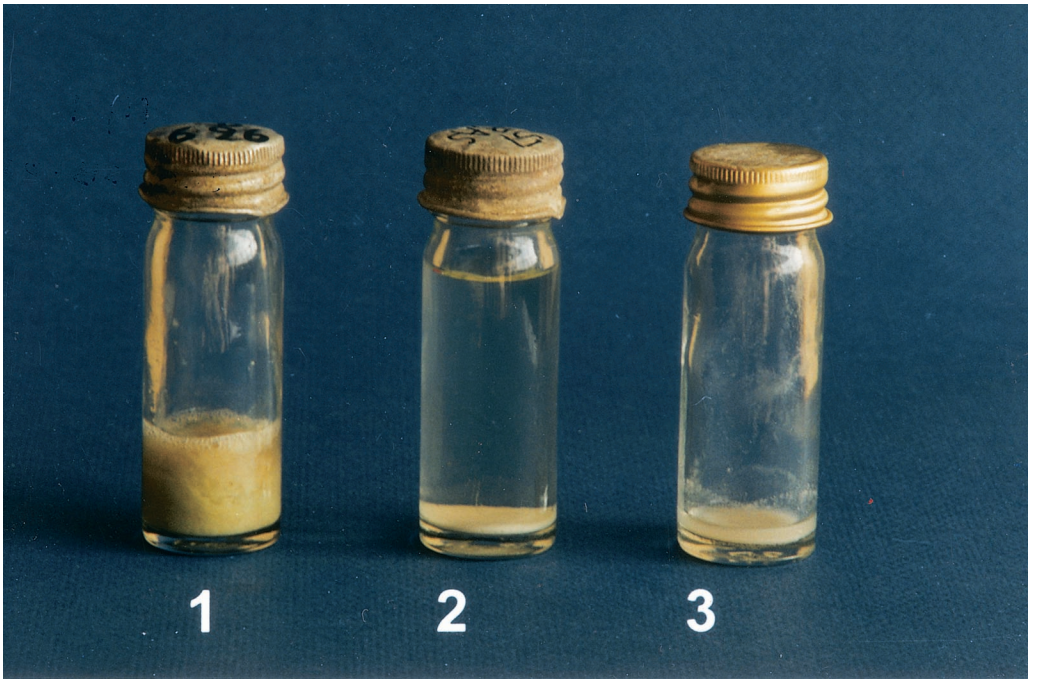

FIG. 1. Bottle 1, sputum sample; bottle 2, PhAS-treated sputum sample with visible sediment; bottle 3, sputum sediment after decantation.

cause PhAS-treated samples lose resemblance to sputum samples (Fig. 1), they are less aesthetically offensive to laboratory technicians. Additionally, the PhAS sediment smear method is safer, since the sputum is rendered sterile and PhAS-treated samples can be easily disposed of along with other hospital wastes. The time taken to read PhAS sediment smears, especially the high-grade smears, can be reduced. It has been reported previously that the concentrated smears were washed off from the slides during processing (2). In the present study, all the smears were found to be intact; it is likely that ammonium sulfate precipitated the mucus component of the sputum, allowing firm fixation of the smears on the slides. PhAS sedi-

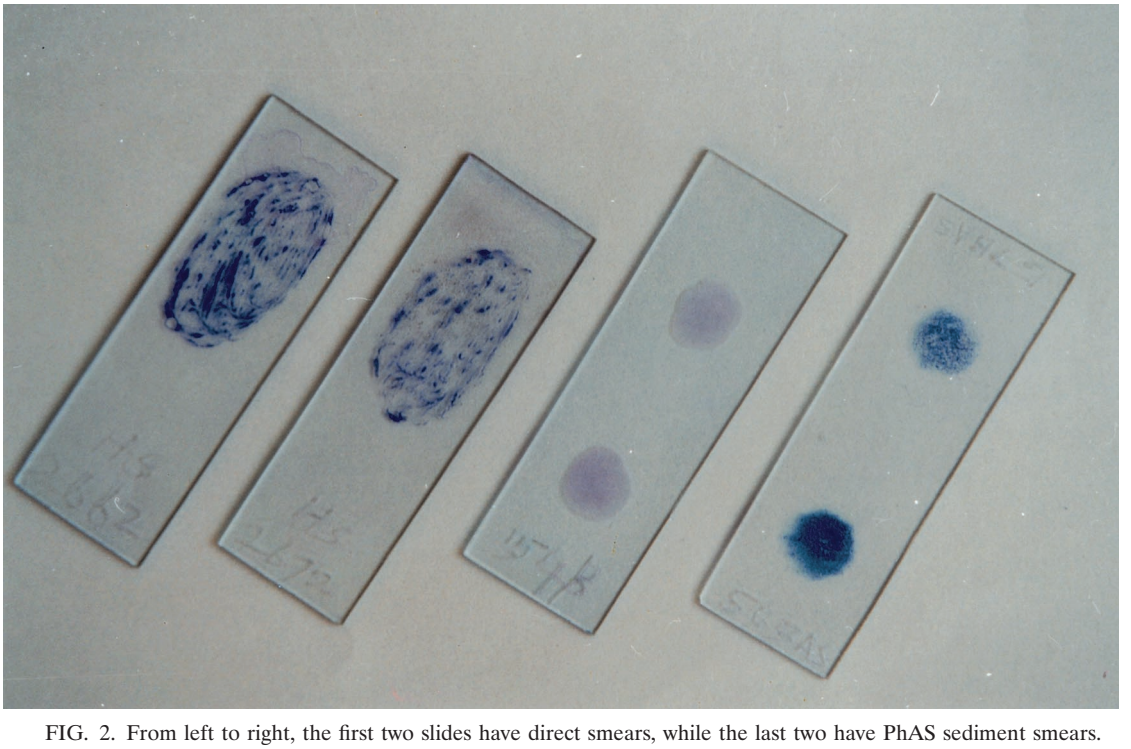

FIG. 2. From left to right, the first two slides have direct smears, while the last two have PhAS sediment smears.

ment smears were reported to be easy to read, with well-defined margins (Fig. 2) and with distinct AFB against a clear background. In large laboratories where several samples are processed, the PhAS method can substantially increase the efficiency of sputum smear microscopy. Other advantages of the method are that phenol is inexpensive, is stable at room temperature, and can be prepared at reference laboratories and supplied to peripheral health centers.

The main disadvantage of the PhAS method is that it necessitates an overnight sedimentation, which delays reporting of results until 1 day after sputum collection and therefore requires an additional visit by the patient to the clinic. If the PhAS treatment time could be reduced and the benefits of the method maintained, the PhAS method would find more application under field conditions.

In conclusion, the PhAS sediment smear method is as sensitive and specific as the direct smear method in the diagnosis of smear-positive pulmonary tuberculosis and would be safe and suitable for use in peripheral microscopy centers.

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REFERENCES

1. **Angeby, K. A. K., C. Alvarado-Galvez, L. Pineda-Garcia, and S. E. Hoffner.** 2000. Improved sputum microscopy for a more sensitive diagnosis of pulmonary tuberculosis. Int. J. Tuberc. Lung Dis. **4:**684–687.
2. **Collin, C. H., J. M. Grange, and M. D. Wates.** 1985. Organisation and practice in tuberculosis bacteriology, p. 36–43. Butterworths and Co. Publishers, Ltd., Cambridge, United Kingdom.
3. **Dye, C., S. Scheele, P. Dolin, V. Pathania, and M. C. Raviglione.** 1999. Consensus statement. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. WHO Global Surveillance and Monitoring Project. JAMA **282:**677–686.
4. **Farnia, P., F. Mohammadi, Z. Zarifi, D. J. Tabatabee, J. Ganavi, K. Ghazisaeedi, P. K. Farnia, M. Gheydi, M. Bahadori, M. R. Masjedi, and A. A. Velayati.** 2002. Improving sensitivity of direct microscopy for detection of acid-fast bacilli in sputum: use of chitin in mucus digestion. J. Clin. Microbiol. **40:**508–511.
5. **Gebre, N., U. Karlsson, G. Jonsson, R. Macaden, A. Wolde, A. Assefa, and H. Miorner.** 1995. Improved microscopical diagnosis of pulmonary tuberculosis in developing countries. Trans. R. Soc. Trop. Med. Hyg. **89:**191–193.
6. **Miorner, H., G. Ganlov, Z. Yohannes, and Y. Adane.** 1996. Improved sensitivity of direct microscopy for acid-fast bacilli: sedimentation as an alternative to centrifugation for concentration of tubercle bacilli. J. Clin. Microbiol. **34:**3206–3207.
7. **Peterson, E. M., A. Nakasone, J. M. Platon-De Leon, Y. Jang, L. M. de La Maza, and E. Desmond.** 1999. Comparison of direct and concentrated acid-fast smears to identify specimens culture positive for *Mycobacterium* spp. J. Clin. Microbiol. **37:**3564–3568.
8. **Revised National Tuberculosis Control Programme.** 1998. Manual for laboratory technicians. [Online.] Central TB Division, Directorate General of Health Services, Ministry of Health and Family Welfare, New Delhi, India. http://www.tbcindia.org/LABMANUAL.pdf.
9. **Selvakumar, N., M. Gomathi, F. Rahman, and P. R. Narayanan.** Evaluation of two-reagent cold staining method for acid-fast bacilli. Int. J. Tuberc. Lung Dis., in press.
10. **Tuberculosis Chemotherapy Center, Madras.** 1960. A concurrent comparison of isoniazid plus PAS with three regimens of isoniazid alone in the domiciliary treatment of pulmonary tuberculosis in South India. Bull. W. H. O. **23:**535–585.
11. **Van Deun, A., A. K. Mang, E. Cooreman, M. A. Hossain, N. Chambuganj, V. Rema, H. Marandi, A. Kawria, and F. Portaels.** 2000. Bleach sedimentation method for increased sensitivity of sputum smear microscopy: does it work? Int. J. Tuberc. Lung Dis. **4:**371–376.
12. **Vasanthakumari, R.** 1988. Concentrated sputum smear microscopy, a simple approach to better case detection in pulmonary tuberculosis. Indian J. Tuberc. **35:**80–84.
13. **World Health Organisation.** 2000. Global tuberculosis control: W. H. O. Report 2000. W. H. O./CDS/TB/2000. World Health Organisation, Geneva, Switzerland.