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Phenol-ammonium sulfate microscopy method for diagnosis of pulmonary tuberculosis

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ABSTRACT

Background: Collection and processing of sputum samples for the detection of acid fast bacilli (AFB) is hazardous for health-workers in developing countries with limited facilities. The phenol ammonium sulfate (PhAS) method involves smear microscopy and Ziehl-Neelson (ZN) staining of precipitates/ floccules formed in sputum samples when PhAS is added. The present study has been designed to assess the performance and safety of this method.
Materials and methods: The study was conducted from January 2011 to March 2011 at the Department of Microbiology, Lala Ram Sarup Institute of Tuberculosis and Respiratory Diseases, New Delhi. A total of 1038 sputum samples were subjected to ZN staining before and after treatment with PhAS. The smear microscopy results of the PhAS treated and untreated samples were compared. In addition, 200 representative samples were inoculated after processing by petroff's method directly for culture and after treatment with PhAS.
Result: The sensitivity, specificity, positive predictive value and negative predictive value of the PhAS solution treated ZN smear microscopy method were found to be 98.8%, 88.5%, 98.0% and 92.7% respectively in comparison with direct smear microscopy. The overall correlation between the two methods was found to be 97.3%. None of the PhAS treated samples grew *Mycobacterium tuberculosis* on culture.
Conclusion: Sputum microscopy with PhAS solution is a safe, reliable and inexpensive alternative for direct microscopy. This method can be conveniently applied for usage in microscopy centers with limited bio-safety facilities.

Introduction

Detection of acid-fast bacilli (AFB) using Ziehl-Neelsen (ZN) sputum smear microscopy continues to be the mainstay in the diagnosis of pulmonary tuberculosis [1], especially in developing countries. The sensitivity of this method ranges from 34% to 84% and depends upon the collection of sufficient sputum, proper preparation of smears, good staining technique, thorough examination, and availability of a good microscope [2,3]. To facilitate the diagnosis of tuberculosis

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(TB) in India, the sputum smear microscopy centers have been established for every 1,00,000 population by the Revised National Tuberculosis Control Programme (RNTCP) so that in peripheral areas, patients do not need to travel long distances for diagnosis [2,4,5]. Direct smear preparation of clinical samples is hazardous for technicians working in centers without a bio-safety hood [3]. The technicians sometimes may not prepare sputum smears properly owing to fear of the possibility of getting the infection. Thus, it is desirable to introduce improvisations in the direct microscopy methods for effective TB diagnosis in TB control programs.

The phenol ammonium sulfate (PhAS) method of sedimentation/precipitation of sputum involves ZN staining of precipitates/floccules formed in chemically treated sputum samples. Phenol or carbolic acid is known to promptly kill the mycobacteria. It also precipitates proteins in low concentration without decreasing the AFB counts and fixes the smear firmly on the slide [3,6]. Ammonium-sulfate prevents the formation of hydrogen bonds of proteins with water and facilitates the interaction of proteins with each other to form aggregates. This causes the mucus and other proteins in the sputum to precipitate and later sediment by the "salting out" phenomenon [7]. The precipitate/sediment obtained may be used for smear preparation.

The present study was designed to compare the PhAS treated sputum microscopy with the established untreated/direct sputum microscopy method of RNTCP. In addition, the sterilizing action of the PhAS was tested in comparison with non-PhAS exposed sputum samples.

Materials and methods

Study setting

This study was carried out at the Department of Microbiology of Lala Ram Sarup Institute of Tuberculosis and Respiratory Diseases which houses the National Reference Laboratory (NRL) for tuberculosis. The solid culture system on Lowenstein-Jensen (LJ) medium is accredited for culture and susceptibility testing for first-line and second-line drugs by the Supra National Reference Laboratory (SNRL), Antwerp, Belgium. This Institute is a tertiary care specialty hospital for tuberculosis and respiratory diseases. The Department receives samples from clinically suspected and follow-up pulmonary tuberculosis patients for sputum microscopy and culture. For this study, 10–15 sputum samples per day were randomly considered from the in-patient section over three months. Patients were asked to rinse their mouths properly and collect at least 3–5 ml of deeply coughed muco-purulent sputum samples in sterile containers [8]. The study has been approved by the Institute's ethics committee.

Sample processing by direct smear microscopy

Single smear was prepared from the muco-purulent portion of sputum sample using fresh wooden-stick such that smear covers two thirds of the slide and is neither too thick nor too thin. All the smears were air dried, heat fixed and stained by ZN method as per RNTCP guidelines [4].

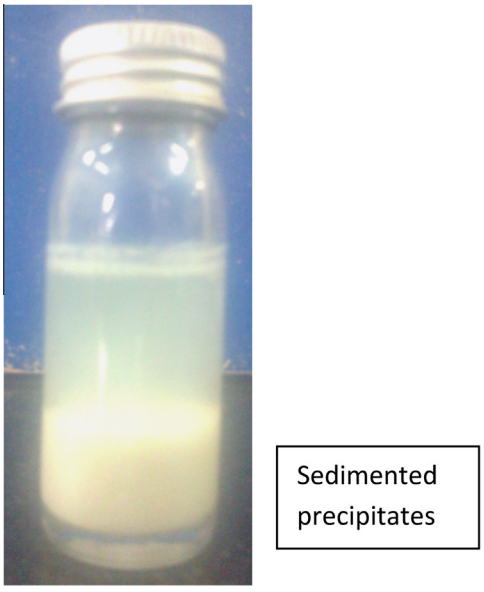


Fig. 1 – Sedimentation of precipitates formed in the PhAS treated sputum sample.

Sample processing by PhAS method

After preparing the direct smear, the remaining sample was subjected to PhAS treatment. PhAS solution was prepared as described earlier and stored for maximum of one week [3]. Around 1.5–2 times PhAS more than the volume of sputum sample was added to containers with sputum. The samples were mixed properly and kept for one and a half hours undisturbed. Addition and mixing of PhAS took around 20–25 s per sample. The sterilized inoculation loop was dipped and a loopful of the precipitates or the sediments formed in each PhAS treated sputum sample was taken. One smear was prepared from this loopful such that it covered two thirds of the slide (Fig. 1). All the smears were air dried, heat fixed and stained by ZN method as per RNTCP guidelines [4].

Smear microscopy

Both direct and PhAS smears were coded before screening by the technicians to avoid bias. Grading of direct and PhAS treated ZN smears for acid-fast bacilli (AFB) was done as per RNTCP guidelines. After examination of the coded slides by the two technicians, the results of each sample were matched by the umpire. The sets of discordant slides (PhAS and direct) were re-examined by the umpire microscopist. In all such cases, the reading of the umpire was taken as final. All the microscopists involved were asked to state their preference of the method in relation to sample handling, odor, aesthetic appearance, smear preparation, and visibility of smear in terms of AFB and background color. In addition, they were also asked about the convenience of disposal of samples.

Basic-fuchsin, methanol, phenol, concentrated sulphuric acid, ammonium sulphate and methylene blue were obtained from Merck/Qualigens, India.

Culture

Cultures were inoculated in case of the requisition for culture. For this purpose, each sputum sample was divided into two parts after conventional microscopy. One part was taken for the PhAS treatment followed by the preparation of smear from the PhAS treated sample. The other part was processed by the Modified Petroff method as per RNTCP guidelines [8]. The processed sample was inoculated onto the labeled LJ media in duplicate. The remaining processed sample was exposed to PhAS and then inoculated on a labeled separate LJ media. All media were coded, labeled and incubated at 37 °C. The cultures were examined weekly for growth, until a maximum of eight weeks [8]. Culture results of chemically treated and untreated samples were recorded independently and were compared by an umpire.

Quality control

For quality control of smear microscopy, positive control slides of *M. tuberculosis* H37Rv and negative control slide of *Escherichia coli* (direct and PhAS treated) were stained and screened with every lot. For culture, *M. tuberculosis* H37Rv was inoculated onto LJ slopes in duplicate with every new batch of LJ media to ascertain its ability to sustain growth. Each lot of LJ media was checked for sterility by incubation of un-inoculated media at 37 °C for 48 h.

Statistical analysis

The data was entered and analyzed using Microsoft Excel. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the smear microscopy results by PhAS method were calculated in comparison with direct ZN smear. Pearson Chi-Square test was performed to decipher the extent of agreement between the PhAS and direct smears. In addition, sensitivity of smear microscopy using two methods was also calculated in comparison with the available culture results.

Results

A total of 1038 sputum samples were subjected to microscopy both by direct smear method and after treatment with PhAS. Table 1 details the smear microscopy results of the two methods. A total of 881 samples were positive for AFB by direct smear and 888 samples were positive for AFB by PhAS method. The sensitivity, specificity, PPV and NPV of the PhAS treated ZN smear microscopy method were found to be 98.8%, 88.5%, 98.0% and 92.7%, respectively in comparison with direct ZN smear microscopy. Correlation of 97.2% for smear AFB positivity was found between the two methods.

Fig. 2 depicts the grade-wise distribution of total numbers of smears by the PhAS method and the direct method. A marked relative increase in number of samples with higher smear grades (2+; 52 and 3+; 33) was seen in the PhAS method along with a decrease in the number of samples in direct or pre/ZN method. Such grade-wise trend of decrease or increase between the two methods was found to be statistically significant (P value <0.01). The correlation chart for this trend is shown in Fig. 3.

A total of 205 samples received for culture requisition were processed and inoculated for solid culture on LJ medium by conventional method. Same samples after processing were also exposed to PhAS treatment and were subsequently cultured on LJ medium. Ninety-five samples (46.3%) were culture positive by conventional method, whereas all 95 were found to be negative in case of the PhAS treatment; all culture positives were M. tuberculosis. The sensitivity of the direct microscopy method in comparison with the culture was found to be 91.6% with 87 smear positives among 95 culture positive samples, whereas sensitivity of microscopy of the PhAS treated sputum samples in comparison to culture was 89.5% with 85 smear positives among 95 culture positive samples. The difference in sensitivity of the two methods with culture as gold standard was not found to be statistically significant.

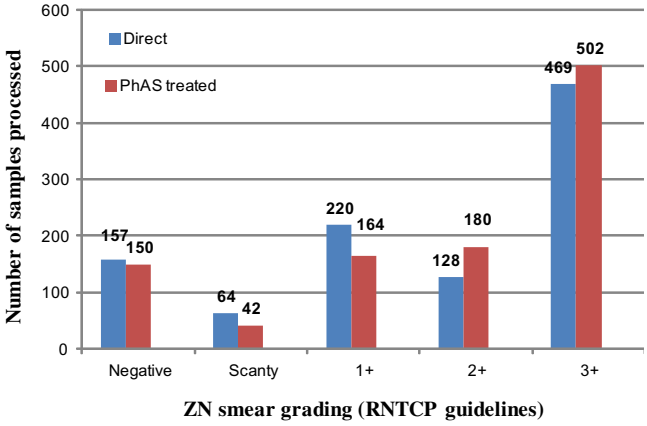
Discussion

The present study was designed to compare the performance of the PhAS treated AFB sputum microscopy with direct microscopy. A high correlation of 97.2% for smear AFB positivity was found between the PhAS treated sputum microscopy and direct microscopy. In the present study, the sensitivity

Table 1 – Comparison of Ziehl Neelsen microscopy smear results of direct & PhAS treated sputum samples.

	Microscopy result of PhAS solution treated ZN sputum smears					
	Scanty	1+	2+	3+	Negative	Total
Microscopy Result of Direct ZN Smear						
Scanty	22	23	10	2	7	64
1+	14	117	58	29	2	220
2+	1	13	80	33	1	128
3+	1	4	28	435	1	469
Negative	4	7	4	3	139	157
Total	42	164	180	502	150	1038

Scanty = 1–9 AFB in 100 fields; 1+ = 10–99 AFB in 100 fields; 2+ = 1–9 AFB per field, at least 50 fields, 3+ = >10 AFB per field, at least 20 fields; Negative = no AFB in 100 fields.



ZN Smear grading	Negative	Scanty	1+	2+	3+
Direct	157	64	220	128	469
PhAS treated	150	42	164	180	502

Pearson Chi-Square value <0.01

Fig. 2 – Comparison of PhAS treated samples with direct samples in relation to ZN microscopy grading.

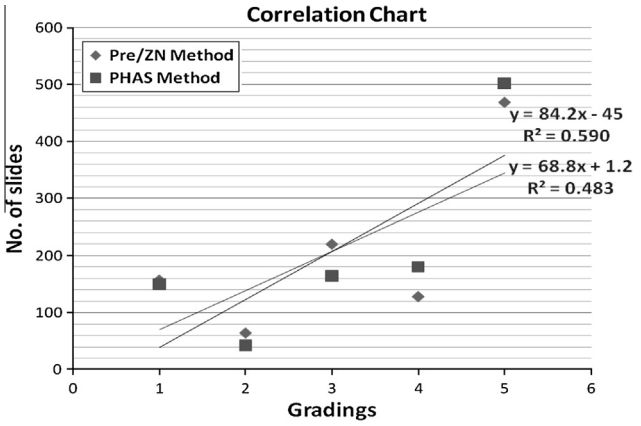


Fig. 3 – Chart depicting the correlation between the increase or decrease in the samples of PhAS method and conventional ZN method respectively as we move from lower grade to higher grade.

of the PhAS treated microscopy was found to be 98.8% in comparison with the direct ZN smear. Very few studies on AFB microscopy using the PhAS method are available in the literature review. The work on AFB microscopy using the PhAS method has been reported mainly from the Tuberculosis Research Centre (TRC), Chennai. In these studies from the TRC, Selvakumar, et al. found the sensitivity of the PhAS treated sputum smears and direct smears to be comparable with good agreement between the two methods [3,9,10]. In the first such study, the PhAS treated samples were incubated overnight to facilitate the sedimentation of the precipitates as smears were prepared from the sedimentation deposit. Though the procedure gave excellent results, the microscopy result was delayed by at least a day [3]. In the two studies conducted in 2008, PhAS solution along with basic fuschin was added to stain the sputum within the containers and checked on the same day and at a gap of seven days respectively [9,10]. In the later preliminary study, authors intended to evaluate PhAS for storing and transportation of sputum samples from peripheral areas to Microscopy Centres. They concluded that sputum samples can be stored up to seven days in the sputum container containing PhAS without any loss of sensitivity of microscopy. PhAS was added to the container containing sputum and the usual ZN staining from precipitates/ sediments formed within 1–2 h on the same day was continued, thereby giving a microscopy report on the same day.

Interestingly, in the present study, it was observed that the PhAS treatment had some concentrating effect on the sputum sample. Seven samples reported as AFB negative, 22 as scanty positive and 56 as 1+ positive in the direct microscopy were reported as 2+ or 3+ after the PhAS treatment. Thus, a statistically significant trend of increase in the number of samples in the PhAS method with higher smear grades was obtained. This concentrating ability of PhAS could be because PhAS precipitates the proteins and forms floccules which entangle the AFB [7]. Thus, a smear prepared from such portion of sputum would have more bacilli, leading to faster and easier detection of positive smears along with a decrease in false negatives. In one of the studies from TRC also, significantly higher 3+ smears were found as compared with direct ZN method [9]. However, in another study from TRC, smears from PhAS treated stored samples were found to have a lesser number of 3+ positive smears. This decrease could be because of the prolonged storage of sputum, which could have contributed to the dispersion of AFB [10].

All samples after PhAS exposure for one and a half to two hours were found to be negative on culture. The same samples when inoculated for culture without PhAS treatment were positive for 95 samples. This ascertains the sterilizing action of PhAS. Previously, it had been concluded that PhAS kills tubercle bacilli in about 30 min due to the presence of phenol, which is a good anti-mycobacterial agent [11]. In developing countries, most peripheral microscopy centers have minimal infrastructure, such as lack of bio-safety cabinets or hoods. Therefore, the direct smears are often prepared on the open work-benches using wooden sticks or wire-loops and flame [12]. The addition of PhAS to the sputum containers would ensure that highly contagious AFB positive samples are rendered sterile. This could reduce the hazard of infectious aerosol formation caused by breakage or leaking of a container during transportation of sputum samples. Smear preparation and discard of PhAS treated samples would ensure safe management of samples in microscopy centers.

Many advantages of performing the AFB microscopy on samples after PhAS treatment were found in comparison with direct AFB microscopy on untreated samples. The PhAS treated sputum was found to be aesthetically more acceptable to the technical staff as it is neutral to whitish in color and is free of odor. The smear from the PhAS treated sample was generally easier to prepare as it does not peel off easily during staining. This could be because ammonium sulphate allows smears to be firmly fixed on the slide due to precipitation of mucous. The focusing of smears and screening of slide were also easier as PhAS smears were uniformly thin with well-defined margins. In addition, the smears had a clear, whitish background along with the distinct appearance of AFB with

no change in the shape of AFB. Many of these advantages were also experienced by the TRC research groups [3,9,10].

In addition, the study concluded that the PhAS treatment had a significant concentrating effect on the sputum as compared with conventional ZN staining. Even with the PhAS treatment, the AFB report could be given to the patient on the same day as the sample, thus saving an additional visit by the patient to the hospital for report collection and treatment initiation.

One limitation of the method is that the treated sample cannot be used for culture. Also, addition of PhAS delays the smear reporting time by 2–3 h.

Conclusion

Microscopy for detection of AFB forms the basis for diagnosis and management of pulmonary tuberculosis in India and many developing countries as per the National Programme Guidelines. The PhAS method could be a reliable and rapid alternative for direct ZN smear microscopy method in peripheral microscopy centers where the infection control measures are not available. The concentrated AFB and better smear quality could ease the job of the microscopist in screening the AFB in smears. Thus, the method has a potential for implementation at a larger scale in the TB control network. The improvement in smear microscopy sensitivity would facilitate the accurate diagnosis and timely management of TB cases, especially in resource-poor countries at the lowest possible cost.

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