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Pot method for storage & detection of acid-fast bacilli from sputum samples

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***Background & objectives*: Sputum acid-fast bacilli (AFB) microscopy services are not available in all health facilities. Alternative procedures are needed to transport sputum samples to the diagnostic centres for detection of AFB. The objective of the present study was to evaluate sputum smears made by pot-method with the direct smears made immediately after sputum collection by Ziehl-Neelsen (ZN) method.**

***Methods*: Ninety three sputum samples from 49 pulmonary tuberculosis suspects were studied. Their direct smears (ZN smears) were stained by hot ZN method. The samples were then mixed with phenol ammonium sulphate basic fuchsin solution and stored at ambient conditions. The smears (pot smears), made on day 7, were then, decolourized and counter-stained for detection of AFB (pot method). The ZN and pot smears were read blind. After excluding 18 samples for various reasons, the results of pot and ZN smears of 63 samples from smear positive (2 of 3 direct smears were positive) and 12 from smear negative (3 of 3 direct smears were negative) patients were analysed. ZN method was the gold standard.**

***Results*: Pot and ZN smears were positive in 61 of 63 samples from smear-positive patients and negative in 11 of 12 smear-negative patients (kappa = 0.87). The sensitivity and specificity of pot method were 96.8 and 91.7 per cent respectively.**

***Interpretation & conclusions*: Sputum samples can be stored for up to seven days in the sputum container with phenol ammonium sulphate basic fuchsin solution. However, a comprehensive study needs to be done confirm the accuracy of the pot method for storage and transportation of sputum to microscopy centres for detection of AFB.**

Key words AFB - pot staining - sputum - storage - tuberculosis

Sputum acid-fast bacilli (AFB) microscopy services are established for every 100,000 population by the Revised National Tuberculosis Control Programme (RNTCP)[1]. Non availability of this service in many health facilities forces substantial proportion of pulmonary tuberculosis patients to travel long distances to avail diagnostic facilities[2]. These result in lose of many patients to treatment[3] and the infectious cases transmit infection in the community. In order to prevent this, Revised National Tuberculosis Control Programmme (RNTCP) has recommended transportation of sputum samples to microscopy centres[4]. However, organization of such services is difficult for several reasons and it involves transportation of highly contagious sputum samples.

Several studies in the recent past highlighted the importance of safe practices in the sputum microscopy

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centres. Giacomelli *et al*[5] demonstrated that unstained sputum smears pose a greater risk of infection to laboratory workers[5]. Only one of 38 hospitals in Malavi disposed the sputum samples after disinfection[6]. Addo *et al*[7] surveyed 114 laboratories at Ghana and found that the disposal mechanisms and safe handling of sputum material were very poor[7]. Survey of laboratory practices in an unban area in Brazil with hyperendemic pulmonary tuberculosis revealed high risk to workers and other staff with suboptimal practices while disposing infectious materials[8]. Mullu and Kassu[9] recommended improvement of safety standards in the laboratories in order to minimize the risk of infection. Chedore *et al*[10] suggested that inactivation of *Mycobacterium tuberculosis* in sputum smears before use is an important safety factor in preventing the potential transmission of tuberculosis (TB) to laboratory workers. Therefore more research studies are needed to find out safer methods for transportation of sputum samples to microscopy centres. We have earlier shown that sputum in its containers can be stained[11], in an hour, by phenol ammonium sulphate basic fuchsin solution and its smear can then be decolourised and counter-stained for detection of acid-fast bacilli (AFB)[12,13]. In the present study, the pot-method was further explored for storage of sputum samples at ambient conditions in health facilities for detection of AFB and the results were compared with direct smears made by Ziehl-Neelsen (ZN) method.

Material & Methods

Patients, sputum samples and direct-smears: A total of 93 samples from 49 pulmonary tuberculosis suspects with cough of more than three weeks, reporting to a TB Hospital, Poonamalle, Chennai, were used after their direct-smear results were recorded in the TB laboratory register. Samples were collected as per RNTCP guidelines *i.e.,* 3 samples were collected in two days. The study was carried out in first quarter 2007. The direct-smears were stained in the laboratory of the TB hospital by the standard hot Ziehl Neelsen (ZN) method as described in the RNTCP laboratory technician module[1].

ZN-smears: One additional direct smear (ZN-smear) was made from each of the above samples at Tuberculosis Research Centre (TRC), Chennai by an independent laboratory technician. These smears were then stained by the ZN method[1].

Storage of sputum, staining and pot smear: After making a direct smear, equal volume of phenol ammonium sulphate basic fuchsin solution was added to the sputum sample in its plastic container, rotated gently for a few seconds and left at ambient conditions (22 to 26^0 C) in a closed box for 7 days. The smears (pot smears), prepared then, were only decolourized with 25 per cent sulphuric acid for 2 min and counter-stained with 0.1 per cent methylene blue for 30 seconds[11].

Both direct and pot smears were made using bamboo sticks.

Phenol ammonium sulphate basic-fuchsin solution was prepared as described earlier[13]. Basic-fuchsin, methanol, phenol, concentrated sulphuric acid, ammonium sulphate, methylene blue were obtained from Hi-media/Qualigens, Mumbai, India.

Smear reading: The ZN smears and pot smears were brought to TRC. They were coded and read by technicians as per RNTCP guidelines[1]. The results were then decoded. Sets of slides (ZN smear and its corresponding pot smear) with discrepant results were read again blindly by a second reader. Slides with discordant results were again read blind by a third reader. For a discordant slide, the result of 2 concordant readings was taken as final. After resolving the discrepancies, the results were analysed.

Analysis of data: The data were entered and analysed using Microsoft Excel. After excluding, 11 samples from patients on follow up examination, 4 samples from patients who provided with less than three samples, and 3 samples with labelling/recording errors, the pot and ZN smear results of 63 sputum samples from smear-positive patients (at least two of the three samples were AFB positive in direct-smears) and 12 samples from smear-negative patients[1] (all the three samples were AFB negative in direct-smears) were analysed. The sensitivity and specificity of pot method were determined using ZN method as the gold standard. Kappa statistics was performed to know the extent of agreement between the pot and ZN smears.

Results & Discussion

Of the 75 samples analysed, 61 and 11 were, respectively AFB positive and negative in pot and ZN-smears (Table). Two samples from smear-positive patients, one with scanty positive and the other with 1+ in the direct-smears, were negative in pot smears. One sample from a smear-negative patient was AFB positive in both ZN and pot smear. These errors could be attributable to limitations of sputum AFB microscopy

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Table . Results of pot and ZN-smears (0 day), post smear (7 days)

Pot-smear	ZN-smear					
	Scanty	1+	2+	3+	Neg	Total
Scanty*	2	3		2		7
1+	2	10	3	7	1	23
2+		2	5	8		15
3+		2	6	9		17
Neg		2			11	13
Total	4	19	14	26	12	75

(Kappa = 0.87)
*Scanty = 1- 9 AFB in 100 oil immersion fields (field); 1+ = 10 - 99 AFB in 100 fields; 2+ = 1 to 9 AFB per field in at least 50 fields; 3+ = 10 or more AFB per field in at least 20 fields; Neg = no AFB in 100 fields

or true errors. The sensitivity and specificity of pot method were 96.8 and 91.7 per cent respectively. The kappa statistics revealed that the extent of agreement between the two methods was very good (k = 0.87). Twenty six ZN-smears showed '3+' grade compared to 17 in pot smears. The lower number of '3+' grade in pot smears could be attributed partly to the dispersion of AFB in the sample during prolonged storage and partly due to the dilution of sputum sample.

Storage of sputum samples has been studied earlier. Paramasivan *et al*[14] showed that sputum samples can be stored at ambient conditions for up to 28 days without loosing their smear positivity. Selvakumar *et al*[15-19] and Bobadilla-del-Valle *et al*[20] reported that storage of sputum samples in cetylpyridinium chloride solution resulted in reduced sensitivity of ZN and auramine phenol staining methods. Lumb *et al*[21] demonstrated that sputum samples can be stored in refrigerators until they were transported under ambient conditions to reference laboratory for culture of tubercle bacilli. These studies were carried out in reference laboratories using biological safety cabinets. Risk of aerosol infections is high with the liquefied sputum samples compared to fresh samples. Research is therefore needed to find out safer methods of transportation of sputum samples to microscopy centres where the direct smears are made on work benches with inadequate infection control measures. Since delay in diagnosis of pulmonary tuberculosis beyond seven days is not acceptable under RNTCP, sputum samples were stored for seven days and their smears were evaluated.

Good laboratory practices need to be followed while making direct smears of sputum on glass slides to avoid laboratory acquired tuberculosis infection[22,23]. Generally, making direct smears from sputum is unpleasant to laboratory technicians[24]. In order to make the sputum sample more acceptable to the laboratory technicians, phenol ammonium sulphate sedimentation method was explored with convincing results. Phenol ammonium sulphate solution was shown to kill tubercle bacilli in sputum samples within 30 min[24]. Therefore, the pot-method, in which phenol ammonium sulphate basic fuchsin is used as the staining agent can be practiced on work benches in microscopy centres with inadequate facilities and the risk of acquiring tuberculosis infection can be minimized.

Several advantages of pot method as have been pointed out earlier[11-13] include the following: (*i*) smear making is easy resulting in uniform spread of sputum material on glass slides, (*ii*) it is technician-friendly and will increase their willingness to work with sputum AFB microscopy, (*iii*) the background of smears is clear and the AFB is distinctly seen, (*iv*) disposal of sputum cups becomes easy, (*v*) it can greatly reduce the risk of aerosols and spread of infection among the workers and in the community, (*vi*) it is simple as it does not involve heating of carbol-fuchsin, (*vii*) it gives an opportunity for the laboratory technician to make smears at a convenient time, and (*viii*) it can be utilized to transport sputum samples to microscopy centres. The disadvantage of pot method is that the sputum samples cannot used for culture of tubercle bacilli.

In conclusion, our findings suggests that sputum samples in their containers can be stored for up to 7 days with phenol ammonium sulphate basic fuchsin solution and their smears can then be decolourised and counter-stained for detection of AFB. A large scale comprehensive study has to be carried out to assess the value of pot method for storage of sputum samples and transportation to the microscopy centres for the detection of AFB.

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