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## Phenol ammonium sulphate basic fuchsin staining of sputum in pot for the detection of acid-fast bacilli

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**Background & objectives:** Improper practices of making direct smears of sputum for detection of acid-fast bacilli (AFB) and of disposing sputum cups are hazardous. The present study was undertaken with the objective to stain sputum samples in their containers by 'phenol (10%) ammonium sulphate (4%) basic fuchsin (2%) solution' and to decolourize and counterstain their smears for detection of AFB- (henceforth called pot method) and to compare the smear results of pot method with the standard Ziehl-Neelsen (ZN) method.

**Methods:** A total of 575 selected sputum samples from pulmonary tuberculosis patients were stained by the standard ZN and pot methods and the proportions of AFB positive smears were compared.

**Results:** Of the 575 samples, 126 were AFB positive for both the staining methods and the difference was not statistically significant. Pot method missed 9 ZN positive smears (8 scanty and one 1+) and ZN method missed 9 pot positive smears (9 scanty) and the difference was not significant. High grade smears (3+) were seen more in pot method (42) than in ZN method (25) and the difference was significant.

**Interpretation & conclusion:** Our findings showed that pot method was comparable to standard ZN method and had many advantages. Pot method can be explored further for the detection of AFB in sputum samples obtained from pulmonary tuberculosis suspects.

**Key words** AFB - pot-staining - sputum - tuberculosis - ZN staining

In sputum acid-fast bacilli (AFB) microscopy, direct smears of sputum samples, stained by Ziehl-Neelsen (ZN) method, are examined under bright light microscope, and disposable plastic cups, used to collect sputum samples from pulmonary tuberculosis patients, are generally disinfected using phenolic / hypochlorite solutions and discarded in burial pits<sup>1,2</sup>. Preparation of direct smears from sputum samples and disposal of sputum cups have to be carried out adhering to good laboratory practices to avoid laboratory acquired tuberculosis infection<sup>1</sup>.

Adoption of poor practices while making smears and improper disposal of sputum cups can facilitate generation of aerosols and spread of infection<sup>1,2</sup>. Therefore, safe smear-making techniques and alternative ways of disinfecting sputum cups are the need of the hour to reduce further the risk of laboratory acquired tuberculosis infection.

It has been shown that AFB in sputum samples can be killed within 30 min by phenol ammonium sulphate solution<sup>3</sup> and that they can be stained in

sputum containers by 1 per cent carbol-fuchsin solution<sup>4</sup>. These observations led us to explore the possibility of simultaneously staining and disinfecting sputum in its container to prevent the generation of aerosols while making smears and disposing sputum cups. Therefore, the objectives of the present study were (i) to stain the sputum samples in their containers with phenol ammonium sulphate basic fuchsin solution and to decolourise and counter-stain the stained sputum smears for detection of AFB (henceforth called pot method), and (ii) to compare the proportion of AFB positives obtained in pot and standard ZN methods.

### Material & Methods

*Patients:* Pulmonary tuberculosis suspects with cough of more than three weeks duration reporting to a TB hospital between February 2004 and June 2005 in Chennai were studied.

*Sputum samples:* A sample of 15-20 sputum specimens a day was selected from the available sputum samples in the laboratory of the TB hospital, and it included samples for diagnosis and for follow up examination. A total of 575 sputum samples from the same number of suspects were studied. The selected samples were transported within 2 h to mycobacteriology laboratory at Tuberculosis Research Centre (TRC), Chennai, for further investigations.

*Direct smear and standard ZN staining:* At TRC, a direct smear was made from each sample and stained by the standard ZN method as described in Revised National Tuberculosis Control Programme (RNTCP) laboratory technician module<sup>1</sup>. In brief, direct smears, flooded with filtered 1 per cent carbol-fuchsin and heated until it was steaming, were left for five minutes. After rinsing the slides with a gentle stream of water, 25 per cent sulphuric acid was used to decolourise the smears for 2 to 3 min. The slides were rinsed as above and counterstained with 0.1 per cent methylene blue for 30 seconds. The slides were washed, air dried before examination under a light binocular microscope (Olympus CH30, India).

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

*Pot method:* 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 until the mucus dissolved and left at room temperature (22 to 26<sup>o</sup> C). Samples with thick mucus

material were shaken at 15 min intervals until the mucus dissolved. After an hour, a stained sputum smear was made and heat-fixed simultaneously while making smear. Then it was decolourized and counterstained using the 25 per cent sulphuric acid and 0.1 per cent methylene blue solutions by the same procedures as followed in standard ZN method. Phenol ammonium sulphate basic-fuchsin solution was prepared by dissolving 10 g of basic-fuchsin and 20 g of ammonium sulphate in 25 ml methanol and 50 ml molten phenol and the solution was made up to 500 ml with distilled water.

Both direct and stained-sputum-smears were made using bamboo sticks<sup>1</sup>.

*Smear reading and resolving discrepant results:* All the smears were coded before reading by technicians and the results matched. The smears were graded using 100 x oil immersion objective as per RNTCP guidelines<sup>1</sup>: 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. Sets (direct and stained sputum smear of a sample) of smears with discordant results were selected and read blindly by a second reader. Smears with discordant results were read blindly by a third reader. For a discordant slide the result of two concordant results was taken as final. The results of smears after resolving discrepancies were compared.

*Quality assurance:* Quality of smears and efficiency of smear reading were assessed by randomly selecting and blindly re-checking 40 AFB positive and 42 AFB negative smears by a second reader. The discrepant smears were resolved by a third reader as per External Quality Assessment guidelines<sup>5</sup>.

*Statistical analysis:* The statistical significance of the observed difference in the smear results of two methods was determined using McNemar's chi-square test and  $P < 0.05$  was considered significant.

### Results

Of the 575 samples, 126 were AFB positive in each of the methods. Eighteen samples showed discrepant results and were equally distributed between pot and ZN methods. Of the 24 scanty positives by ZN method, 8 were AFB negative by pot method. Similarly there were 22 scanty positive smears by pot method and 9 of them were missed by ZN method. The observed difference between the methods was not statistically significant. There were 42 high grade smears (3+) in

**Table.** Comparison of pot and ZN staining methods

	Pot method					Total
	Scanty	1+	2+	3+	Negative	
Scanty*	9	6	1	-	8	24
1+	3	28	7	7	1	46
2+	-	9	8	14	-	31
ZN method	3+	1	2	1	21	25
	Negative	9	-	-	-	440
	Total	22	45	17	42	449
						575

\*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

pot method compared to 25 in ZN method and the difference was significant ( $P < 0.05$ ) (Table). Quality assessment of smear reading confirmed AFB in all 40 positive smears.

### Discussion

The proportions of AFB positives in pot and standard ZN methods were similar ensuring that AFB in sputum can be stained in its containers by phenol ammonium sulphate basic-fuchsin solution. The discrepancies observed in low positives between the two methods could be due to variations in quality of sputum material taken for smears and limitations of sputum microscopy<sup>1</sup>. The assessment of smears revealed that the quality of reading was good and that colour of AFB in stained-sputum-smears was quite stable.

Phenol ammonium sulphate solution was found to kill tubercle bacilli in sputum samples in about 30 min<sup>3</sup>. Therefore, phenol ammonium sulphate basic fuchsin stained sputum smears can be made on work-benches in any clinical laboratories. Mixing of sputum with phenol ammonium sulphate solution was found to concentrate AFB in the sediments<sup>3</sup>. Similarly mixing of sputum with phenol ammonium sulphate basic fuchsin resulted in concentration of AFB as high grade (3+) smears were seen more in pot method than in ZN method (42 vs 25).

In a preliminary experiment it was found that AFB in sputum can be adequately stained by phenol ammonium sulphate basic fuchsin solution in about 60 min. The colour intensity of the bacilli and smear grades did not change much after 60 min. This can really provide an opportunity for the laboratory technicians to make smears at a convenient time. Making a smear from the floccules of phenol ammonium sulphate treated samples was reported to be less tedious and the smears

were uniformly thin<sup>3</sup>. In the present study, making stained sputum smears was found to be very easy and the smears were uniformly thin.

The concentrations of phenol and basic-fuchsin (in standard ZN method, carbol-fuchsin solution contains 5% phenol and 1% basic-fuchsin) in phenol ammonium sulphate basic fuchsin solution were doubled in order to maintain sufficient concentrations when added to sputum samples. Reduction in concentration of these two was shown to seriously affect the staining characteristics of AFB<sup>6</sup>. It is to be noted that majority of our samples measured 2-3 ml.

If the sputum can be stained in its container, this technique will have following advantages to mycobacteriologists and medical technologists working in sputum AFB microscopy centres all over the world: (i) Risk of acquired infection can be reduced as the sputum treated with phenol ammonium sulphate carbol-fuchsin solution is sterile. (ii) The technique will be more acceptable to technicians and it will improve their willingness to work with sputum AFB microscopy. (iii) Staining procedure becomes simple as it does not involve heating of carbol-fuchsin. (iv) Problems encountered during heating process, such as disfiguration of AFB in smears due to over heating and drying of stain if unattended for a long time due to busy work hours can be overcome/prevented<sup>1</sup>. (v) The sputum containers become less contagious and disposal becomes easy<sup>3</sup>. (vi) Sputum can be stained in its container during transportation from remote health posts to the microscopy centre. (vii) Risk of aerosols in breakage during transportation of sputum can be greatly reduced.

The disadvantage of pot method is that the sample cannot be used for culture of tubercle bacilli. Pot method can be used only in microscopy centres for examination of sputum smears.

In conclusion, pot method can be further explored to detect AFB in sputum samples of patients with symptoms of pulmonary tuberculosis.

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