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Original Article

Microbiology Section

Evaluation of Pot's Technique and Sodium Hypochlorite Concentration Technique in Smear Negative Sputum Samples for Increased Sensitivity of Sputum Microscopy

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ABSTRACT

Introduction: In developing countries, diagnosis of pulmonary tuberculosis is primarily based on microscopy owing to its simplicity, less cost and rapidity. Various ways to improve the sensitivity with multiple samples and concentration of sputum have been used. Simple methods which can be used in microscopic centers are desirable.

Aim: This study was planned to evaluate the sensitivity of sputum smear examination by Pot's technique and Sodium hypochlorite concentration technique over conventional Revised National Tuberculosis Program (RNTCP) method.

Materials and Methods: A cross-sectional analytical study was carried out in all non-repetitive sputum smear negative samples obtained in Mycobacteriology laboratory after obtaining the Institutional Ethics Committee clearance with waiver of consent during a time period from 1st July to 31st August 2018. Two sputum samples (one morning and one spot) received from 47 outdoor and indoor patients in Mycobacteriology department of tertiary care hospital were included in study. All samples were first processed by routine Ziehl-Neelsen (ZN) staining. Sputum smear negative were subjected to Pot's technique and Sodium hypochlorite concentration technique to evaluate the efficacy of these concentration techniques. All data was maintained in MS Excel and analysed using test of proportion and test of significance.

Results: A total of 94 samples received from 47 patients were analysed. 04/94 (4.25%) samples were positive for AFB by routine RNTCP staining. With use of Sodium hypochlorite concentration method and Pots method, 05/94 (5.31%) and 06/94 (6.38%) samples were positive indicating a rise of 1.06% and 2.1% respectively over conventional. Additional two cases were detected compared to RNTCP, amounting to 100% rise in case detection which otherwise would have been missed.

Conclusion: Concentration methods are easy to perform, safe for handlers without requirement of additional human resource. Good smear quality with less debris in microscopic field aids in improving detection rate over conventional methods.

Keywords: Mycobacteriology, Phenol and ammonium sulphate, Pulmonary tuberculosis, Ziehl-Neelsen staining

INTRODUCTION

In developing countries, microbial diagnosis of Pulmonary Tuberculosis (PTB) plays a key role in routine treatment and tuberculosis control program. The rate of smear positive cases among suspected cases of tuberculosis is in the expected range of 5-20% for countries in which tuberculosis is one of the most frequent causes of chronic cough [1]. It is estimated that 60-70% of all PTB cases are diagnosed by means of sputum smear examination [2]. Bright field microscopy is inexpensive, rapid and suitable for peripheral laboratories in low-income countries and limited resources in spite of low sensitivity as compared to Fluorescent and LED microscopy [3]. Several improvements have been suggested to increase the amount of the microscopic detection like fluorescent microscopy with auramine or rhodamine staining, serial sputum specimen examination and chemical fluidization of the sputum with concentration by sedimentation or centrifugation [4].

Zeihl Neelsen (ZN) stain is commonly used throughout the world for smear examination but can detect bacilli when they are in the order of 10^5/ml of sputum. Considering the amount of sputum material that is examined under oil immersion field, chances of missing the organism are high thus reducing the sensitivity. Much of the transmission of TB can occur even before the concentration in sputum reaches a critical level when it is diagnosed [5].

A negative smear does not exclude the diagnosis of tuberculosis, as about 55% of PTB cases worldwide are said to have low bacillary load. It has also been established that sputum smear microscopy is less sensitive in HIV–TB co-infection where sputum smear tends to be negative [6]. Furthermore, pre stained smears made from positive sputum samples remain infectious and potentially hazardous [7].

The present study was undertaken to evaluate two different sputum concentration techniques viz., Pot's technique, a novel in-situ staining for Acid-Fast Bacilli (AFB) and Pre-treatment of sputum by 5% sodium hypochlorite solution (NaOCl) over conventional RNTCP method for the increase in sensitivity of sputum smear microscopy.

MATERIALS AND METHODS

This cross-sectional analytical study was carried out in the Designated Microscopic Center (DMC) in Department of Microbiology of Raipur Institute of Medical Sciences, Raipur, Chattisgarh, India, after obtaining the Institutional Ethics Committee clearance with waiver of consent during a time period of two months from 1st July to 31st August 2018. (Approval letter no. RIMS/ADMIN/DIS/06/04052018 dated 04.05.2018 from IEC RIMS with Registration no. ECR/969/Int/CG/2017)

Samples from patients of all ages and either gender received at DMC were included in the study whereas extra-pulmonary samples and repeat samples from same patients were excluded. A total of 94 sputum samples (one morning and one spot) received from 47 patients received at DMC were analysed.

Procedure for Sampling

Sputum samples collected in the universal container were first processed by conventional RNTCP method. Smears were prepared on new grease free glass slide, heat fixed and stained by ZN staining method using 25% H_2SO_4 as a decolorizer. Smears prepared were examined using bight field microscopy and graded according to RNTCP grading.

All sputum smear negative samples were again evaluated by a different observer to remove observer's bias. The samples were then divided in two portions, one subjected to Pot's technique and another to Sodium hypochlorite concentration technique.

Pot's Technique

In Pots technique, equal volume of phenol, ammonium sulphate (PhAS) and basic fuchsin solution was added to the sputum sample in falcon tube. It was rotated gently for a few seconds and left at ambient conditions (22° to 26°C) in a closed box for 24 hours. Smears were prepared (pot smears) and decolourized with 25% Sulphuric acid for 2 min and Counterstained with 0.1 percent methylene blue for 30 seconds. Remaining sample was stored in a closed box for seven days and pot smears were prepared again, stained and observed by two independent observers [8].

Sodium Hypochlorite Concentration Technique

In Sodium hypochlorite concentration technique, in screw capped disposable falcon tube 1-2 mL of sputum was taken with equal volume of 5% sodium hypochlorite and kept at room temperature for around half hour. The tubes were shaken and approximately 8 mL of distilled water was added to the test tube. Tubes were centrifuged at 3000 rpm for 15 minutes and smears prepared from sediment after carefully discarding the supernatant. The heat fixed smears were then stained by ZN method [9].

STATISTICAL ANALYSIS

All the smears prepared by either method were observed under bright field microscopy by two independent observers to remove the observer's bias and all results were tabulated. All data was maintained in Microsoft office Excel and were analysed using tests of proportion and Pearson's chi-square test for significance while comparing the results.

RESULTS

A total of 94 samples received from 47 patients in DMC. A total of 21/47 (44.68%) patients were from OPD whereas 26/47 (55.31%) were from wards. A total of 47 patients comprises of 30/47 (63.82%) males against 17/47 (36.18) females with male: female ratio of 1.76. Patient's age range was 1-75 years with majority between active age group of 20-40 years.

A total of 4/94 (4.25%) sputum samples were positive for AFB by routine RNTCP staining whereas 5/94 (5.31%) samples were reported to be positive by observers by sodium hypochlorite concentration technique. With an additional case detection apart from conventional RNTCP method, the method was found to be highly significant [Table/Fig-1].

	RNTCP Positive	RNTCP Negative	Total	Pearson's Chi-Square test	
Sodium hypochlorite Positive	04	01	05	χ^2=74.36 p <0.001 Highly Significant	SN: 100% SP: 98.88% PPV: 80% NPV: 100%
Sodium hypochlorite Negative	00	89	89		
Total	04	90	94		

[Table/Fig-1]: Comparative evaluation of sodium hypochlorite concentration method against RNTCP method.
SN: Sensitivity; SP: Specificity; PPV: Positive predictive value; NPV: Negative predictive value

Of the 94 specimens from analysed using Pot's technique, 6/94 (6.38%) were sputum positive. When evaluated against RNTCP, the results were highly significant with p-value of <0.001 with 100% sensitivity and 97% specificity [Table/Fig-2]. No observable difference was found between the smears prepared at the end of 24 hours and seven days.

	RNTCP Positive	RNTCP Negative	Total	Pearson's Chi-Square test	
Pots Positive	04	02	06	χ^2=61.27 p<0.001 Highly Significant	SN: 100% SP: 97.77% PPV: 86.66% NPV: 100%
Pots Negative	00	88	88		
Total	04	90	94		

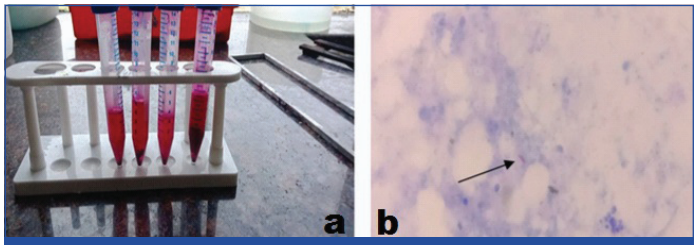
[Table/Fig-2]: Comparative evaluation of Pots method against RNTCP method.
SN: Sensitivity; SP: Specificity; PPV: Positive predictive value; NPV: Negative predictive value

An overall rise of 1.06% and 2.1% was observed over RNTCP method using sodium hypochlorite concentration method and Pot's method respectively. By RNTCP two cases (4 samples) were positive whereas two additional cases were detected by Pot's method as samples were from different patients which amounts to rise in case detection by about 100%, which would have missed by conventional method as open cases.

The sedimentation (Pots) and concentration (Sodium hypochlorite) methods have shown to have higher grades with better visibility of the *Mycobacterium* in clear fields with less debris [Table/Fig-3,4a,b].

Method ↓	Grading →	Scanty	+1	+2	+3	Total positive smear for AFB
Direct ZN		02	00	02	00	04
After Sodium Hypochlorite		01	02	00	02	05
Pot's Method		02	00	02	02	06

[Table/Fig-3]: RNTCP grading by conventional RNTCP, Sodium hypochlorite and Pots method in sputum smear positive samples.



[Table/Fig-4]: (a) Showing sedimentation in Pot's technique and (b) Showing AFB (100X objective) in smear prepared after Sodium hypochlorite concentration method.

DISCUSSION

The present study was designed to compare the performance of the Sodium hypochlorite and Pot's treated sputum microscopy with direct microscopy in smear negative cases. Bright field microscopy of Ziehl-Neelsen stained smears is rapid, inexpensive and highly specific method for detection of tubercle bacilli. The major disadvantage of this technique is a discouragingly low sensitivity. Hence an improved method which is cost effective with high bacillary yield and high sensitivity is required.

Pretreatment with sodium hypochlorite can be easily carried out with only centrifuge machine as additional requirement. Pre-treatment of sputum by 5% sodium hypochlorite solution (NaOCl) which disinfects sputum as well as ensures liquefaction of the sputum within 30 minute without destroying the acid fastness of the *Mycobacterium tuberculosis*. Sodium hypochlorite increases sensitivity of test and make samples safer for handling. Bleach itself is inexpensive and readily available almost everywhere. The half-life of NaOCl is about 12 months; it is likely to be reduced by one month if the bottle is opened and by about three months if the ambient temperature is high (around 30°C) [10].

Overall detection of an additional case with total 5/94 samples being positive over 4/94 samples being positive by RNTCP, constitutes an increase of 1.06% and highly significant with χ^2 value of 74.36 and p-value <0.001 [Table/Fig-1]. A rise of 7.11% has been observed by Kaore NM et al., in their article on increased sensitivity of sputum smear microscopy using sodium hypochloride concentrations technique with 44.11% increase in diagnosed cases with 15 additional cases of PTB detected [9]. Date K et al., also in her study found rise of 3.29% over routine method. [11]. Uddin MK et al., found 1.3% positive results by concentration method in smear negative sputum samples [12]. The small rise we have got may be because of the smaller sample size in this study.

Microscopy after sodium hypochlorite sedimentation had the sensitivity and specificity of 100% and 98.88%. Positive and negative predictive values were 80% and 100% respectively. In addition, there was a significant increase in the average number of AFB seen per field in the smears prepared with less background debris giving a better visibility after sodium hypochlorite concentration methods [Table/Fig-3]. Similar observation was seen in study by Kaore NM et al., and Gebre-Selassie S [8,13].

This study findings clearly demonstrate that results obtained from in-situ cold pot staining method using 2% basic fuchsin with PhAS were comparatively better than ZN staining results. The results of the bright field microscopy in smear prepared after 24 hours and after seven days were in concordance by both the observers. The similar results have been put forward by Selvakumar N et al., [5]. Two additional cases were detected from two separate samples which were reportedly negative using conventional RNTCP method. These cases were brought under CAT-1 treatment regimens under RNTCP which otherwise would have gone undiagnosed and untreated as open cases in the community infecting other people. The sensitivity, specificity 100% and 97.77% respectively and PPV and NPV were 86.66% and 100% [Table/Fig-2]. Das S et al., also found Pot's staining to be 100% sensitive with 99.6% specificity whereas PPV and NPV of 96.5% and 100% respectively [8]. Grading obtained in smear positive sample is much higher as compared to direct staining [Table/Fig-3] similar result have been found in one of the studies from Tuberculosis Research Centre also, significantly higher 3+ smears were found as compared with direct ZN method [6]. Marked increase in number of samples with higher grade was observed by Singhal R et al., in their study following pre treatment by PhAS method [14]. The better grades are because of sedimentation or concentration in sodium hypochlorite concentration technique thereby even scanty *Mycobacterium* could not be missed out giving a advantage in diagnosing the pauci-bacillary cases.

In resource-constrained countries where ZN staining for diagnosis is expected to remain in use for some time, the in-situ pot-staining method can have many benefits. Smear preparation is much safer; staining can be initiated in the sputum collection cup itself. Interestingly, in the present study, it was observed that the PhAS treatment had some concentrating effect on the sputum sample. This concentrating ability of PhAS could be because PhAS precipitates the proteins and forms floccules which entangle the AFB [15]. Thus, a statistically significant trend of increase in the number of samples in the PhAS method with higher smear grades was obtained.

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 [16]. It had been shown that PhAS kills tubercle bacilli in about 30 minutes due to the presence of phenol, which is a good anti-mycobacterial agent [17].

This study was having limitations in terms of sample size but in view of ease of performing the technique, without any additional human resource and better results in terms of sensitivity because of increased grading and clear fields with less debris, the potential need to be explored in large scale and/or multi-centric studies in various DMC's for its use in early diagnosis and treatment of PTB in community at large.

CONCLUSION

Sputum smear microscopy is still the backbone of tuberculosis control program in India. Increased sensitivity of microscopy is attributable by concentration technique after treatment with 5% sodium hypochlorite solution and ammonium sulphate in Pot's method by reduction in debris and leaving clear field of microscopy.

This study suggests that concentration method can significantly improve the yield of the sputum smear microscopy for the diagnosis of PTB, especially in the settings with a high prevalence of HIV, co-infection of which makes the sputum pauci-bacillary. It is also important in view of safety of the laboratory workers in developing countries as both the methods disinfect the samples and make it safer to handle.

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