

Original Research Article

Effectiveness of Concentration Technique in Smear Microscopy for *M. Tuberculosis* Diagnosis

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ABSTRACT

Background: Revised National Tuberculosis Control Program (RNTCP) started as pilots in October 1993 and in 1997 it was launched as a national programme. By March 2006, entire country has been covered under the programme. But still the figures are high. In RNTCP, microscopic examination of sputum for Acid Fast Bacilli (AFB) plays an important role in the initial diagnosis of tuberculosis. Bacillary concentration after decontamination and liquefaction by 4% sodium hydroxide (Petroff's method) is useful in providing increased sensitivity and safety for handling of specimen. **Materials and Methods:** In this cross-sectional, prospective study, carried out at MLB Medical College and Hospital, Jhansi, patients were included according to RNTCP criteria. One set of smears was made according to the RNTCP guidelines while another set was prepared by concentration after decontamination with 4% sodium hydroxide (Petroff's method). Both set of smears were stained according to RNTCP method and were screened and graded according to the RNTCP guidelines. A total of 1152 sputum samples from 384 patients were included in the study with 254 males (66.14%) and 130 females (33.85%). **Results:** A total of 578 samples (50.17%) from 198 patients were positive by routine method whereas by concentration method 653 samples (56.68%) from 223 patients were found positive diagnosing 25 additional patients. This rise of 6.51% in sputum positivity over routine is highly significant ($P=0.002$, $\chi^2=9.81$) with 12.62% increase in diagnosed cases. **Conclusion:** There is a statistically significant rise in smear positive cases after concentration with 4% sodium hydroxide solution (Petroff's method). Considering its increased sensitivity of sputum microscopy, low cost, decontaminating and liquefaction properties, this method is safe and can be of vital importance, at least for smear negative cases.

INTRODUCTION

Tuberculosis (TB) continues to be one of the most fatal infections of the present time despite a well-planned therapeutic regimen recommended by the WHO. TB is a common and often deadly infectious disease caused by mycobacteria in human, mainly by *M. tuberculosis*.

TB is a major airborne, infectious bacterial disease. Mycobacterium tuberculosis infects almost the third part of the world population killing around 2 million people worldwide each year. [1-2]

As per the WHO Global TB Report 2013, there were an estimated 8.6 million incident cases of TB globally in 2012 with 1.3 million deaths.

Out of this estimated global annual incidence of TB cases, 2 million were estimated to have occurred in India, thus contributing to a fifth of the global burden of TB. It is estimated that about 40% of Indian population is infected with TB bacillus.

Microscopy is perhaps the easiest and most rapid procedure that can be performed in the laboratory to detect the presence of acid-fast bacilli. It is,

however, much less sensitive than culture for detecting mycobacteria.

David [3] has estimated there must be 5,000 to 10,000 bacilli/milliliter of sputum to permit their routine detection in stained smears. [4]

Smear microscopy is still the most used amongst all methods employed worldwide due to its simplicity, low cost, speed *and* minimal requirements of equipment's and technical skills. The major objectives of the tuberculosis control program are early detection and treatment of the infectious case of pulmonary tuberculosis.[5]

In Revised National Tuberculosis Control Program (RNTCP), microscopic examination of sputum for AFB plays an important role in the initial diagnosis of tuberculosis.

Considering the amount of sputum material that is examined in 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. A negative smear does not exclude the diagnosis of tuberculosis, as about 55% of pulmonary tuberculosis cases worldwide harbors low bacillary load.[6]

It has also been established that sputum smear microscopy is less sensitive in HIV-TB co infection where sputum smear tends to be negative.[7]

Increasing the number of samples to be tested increases the smear positivity rate marginally when compared to three samples being tested in RNTCP.

Pre-treatment of sputum by 4% sodium hydroxide solution (NaOH) ensures liquefaction of the sputum as well as disinfects it within 30 min without destroying the acid fastness of the Mycobacterium tuberculosis.[8]

Apart from that it is simple, the reagents are inexpensive *and* easy to obtain, sterilized NaOH solution can be kept for several weeks. Concentration of these pre-treated samples by centrifugation has been tried as a possible mean to increase the sensitivity of sputum direct microscopy

by providing clean fields with less debris and making it safe for handling by laboratory workers. With this background, we planned our study to look for increase in sensitivity of direct microscopy by concentration after pre-treatment with sodium hydroxide over routine RNTCP method.

MATERIALS AND METHODS

This cross-sectional study was carried out in the Department of Microbiology from February 2007 to January 2010. Patients of all ages and either sex coming to RNTCP center were included according to RNTCP criteria. Individual aged 13 years or above were included in the study.

Attempt was made to collect three sputum samples (two spot and one morning sample) in a clean, wide mouth labelled container. Two sets of sputum smears were prepared from each sample.

For the first set, the mucopurulent portion of the sputum was taken on a new, clean and grease free glass slide and the smears were heat fixed and stained by ZN staining technique using 25% H₂SO₄ as a decolorizer as per RNTCP guidelines.[9]

In view of potential aerosol formation during manipulation of sputum samples for second set of smears, Bio-Safety Cabinet was used. 1-2 ml of sputum was taken in screw capped disposable test tubes. Equal volume of 4% sodium hydroxide was added to the test tube and kept at 37°C for 15-20 minutes.

The test tube was shaken intermittently after which it was centrifuged at 3000 g for 20 min. The supernatants were carefully discarded and add a small drop of indicator and neutralize with 8% HCl. Centrifuge again for 3 minutes. Discard the supernatant fluid. Smears were prepared from the sediments.[1] The smears were heat fixed and stained similarly.

Slides prepared by both the methods were observed by bright field microscopy and graded according to the RNTCP guidelines.[9]

All the data was managed in Microsoft Excel and statistical analysis was done using Epi Info. Pearson's chi-square test was used for comparative evaluation between two groups.

RESULTS

A total of 384 patients were included in the study with 254 males (66.14%) and 130 females (33.85%), a ratio of 1.95 to 1. Totally, 1152 sputum samples collected from the patients were studied. From all the 384 patients three samples were collected, two spot and one morning sample. Of the total 1152 samples, 578 (50.17%) samples were positive by routine method employed by RNTCP, whereas by concentration method 653 (56.68%) samples were found positive, thus an increase of 75 samples from RNTCP method. [Graph 1]

When compared with the RNTCP method the increase of 6.51% over routine is highly significant with $P < 0.002 (\chi^2 = 9.8)$ [Table 1].

A total of 198 patients were diagnosed by RNTCP method. Additional 25 patients were diagnosed using concentration method which otherwise would not have been diagnosed by RNTCP method [Table 2].

Nine smear negative patients by RNTCP method were smear positive in two smears by concentration technique with a grading of 2+ and were put on DOTS.

Out of these 25, 4 patients which were negative by routine method, and one smear positive by concentration method showing grading of 1+ were subjected to X-ray examination as per guidelines of RNTCP and were reported as radiologically positive for tuberculosis.

Twelve patients were one smear positive by routine RNTCP method but were two smears positive by concentration technique with a grading of 2+ in 7 out of 12 patients and 3+ in rest, thus obviating the need for X-ray examination.

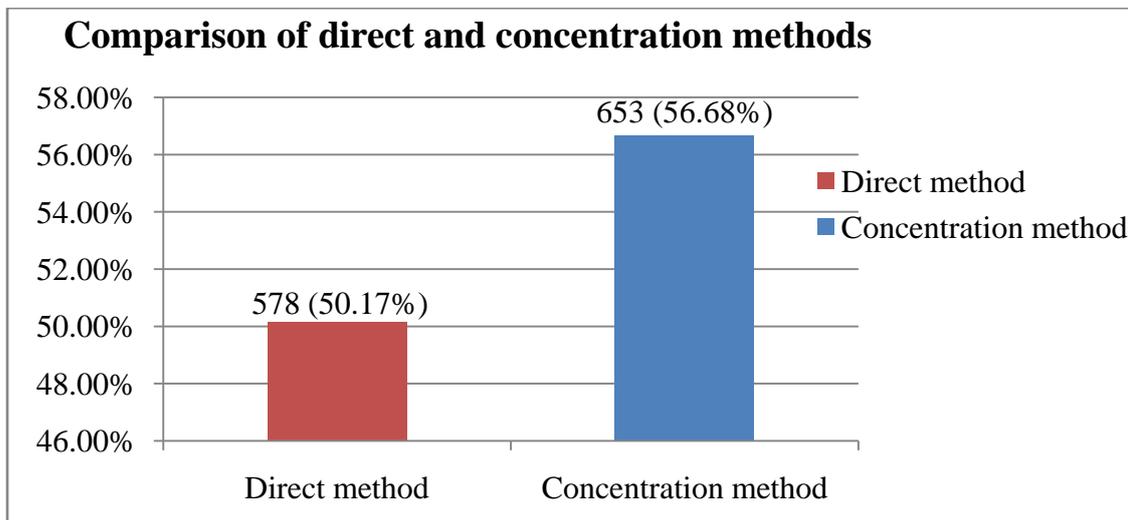
These 25 additional cases diagnosed constituted to 12.62% increase in case detection when compared to RNTCP method. Thus concentration method is highly sensitive.

Table 1: Comparison of Petroff’s concentration method (4%NaOH) over RNTCP method

	Sample Positive	Sample Negative	Total	Chi-square value = 9.81 $P = 0.002$
Concentration method	653	499	1152	
RNTCP method	578	574	1152	
Total	1231	1073	2304	

Table 2: Comparison of additional 25 patients diagnosed by Petroff’s method against the routine RNTCP method

Additional patients diagnosed over RNTCP (n=25)	Status by RNTCP method	Status by concentration method	Grading by concentration method	Remark
4	Smear negative	1 smear positive	1+	X-ray positive (treatment started)
9	Smear negative	2 smear positive	2+	Treatment started
7	1 smear positive	2 smear positive	2+	Treatment started
5	1 smear positive	2 smear positive	3+	Treatment started



Graph 1: Result of Microscopy after various methods

DISCUSSION

Undoubtedly, in developing countries direct microscopy of sputum is currently the backbone for diagnosing pulmonary tuberculosis. It is a rapid, inexpensive and highly specific method for the detection of AFB in sputum. Major disadvantage is the discouragingly low sensitivity when used in overburdened control programs like the RNTCP.

The study aimed at increasing the sensitivity of paucibacillary samples by concentration after pre-treatment with sodium hydroxide which also makes sputum samples safe to be handled by laboratory workers in RNTCP setup.

Generally, making direct smear from sputum is considered hazardous by laboratory technicians working in developing countries with limited facilities.

Sodium hydroxide is added to the original wide mouth container itself which will liquefy the sputum and will also disinfect the sample within 15-20 min, [8] so that it will be safe to be handled laboratory workers even at places where BSC is not available.

Use of sodium hydroxide for pre-treatment before concentration is not labor intensive and could be carried out with same technical team with additional requirement of centrifuge machine only.

In our study, we observed an increase of 6.51% sputum sensitivity after concentration method. Hooja S [9] et al, has reported 6.67% increased

sensitivity after concentration method, which is similar to our study.

In our study, we observed sputum smear positivity by concentration method is 56.68% and by routine method is 50.17%. Kiran Tripathi [10] et al, has also reported sputum smear positivity by concentration method is 56.44% and by routine method is 54.67%.

In our study, there were 25 additional cases diagnosed over 198 cases diagnosed by routine RNTCP method which is marked increase of 12.62% over the routine. Four patients having one smear positive required X- ray examination to confirm the diagnosis while in 21 patients X-ray was not needed as they had two smears positive by concentration method while by RNTCP method the smears were either negative or only one was positive. All these 25 patients got the benefit of early detection and prompt treatment which otherwise would have gone undetected as open cases spreading infection in the community.

An increase of 12.62% in the new case detection in our study definitely shows that improved sensitivity, which can compensates for a 24 h delay in the reporting because one undetected sputum positive case missed by direct microscopy can infect 10-15 other persons/year and thus transmit infection in the community resulting in patient suffering and death.[11-12]

If not all, only those samples which are negative by routine RNTCP method can be retested by sodium

hydroxide concentration method. This can be done on the same day as procedure requires only half hour of pre-treatment with time required for centrifugation and staining.

False results in AFB sputum smear microscopy in diagnosis of TB under RNTCP affect treatment. False negative results lead to a patient being denied TB treatment *and* subsequent risk of spreading the TB disease in the society. False negative results also lead to incomplete treatment *and* being wrongly declared as cured.

To meet RNTCP objectives, a newer strategy has been developed as a comprehensive National Strategic Plan (NSP) 2012-2017,[13] this coincides with RNTCP Phase-III. The new objective is 'Universal Access' to quality diagnosis *and* treatment for all TB patients in the community.[14]

Six targets to be achieved under Universal Access by 2015, out of which one is early detection *and* treatment of at least 90% of estimated TB cases in the community, including HIV-associated TB.[12-14], In this plan, Petroff's concentration method can play a key role for early detection with increased sensitivity of smear microscopy and so rapid treatment.

CONCLUSION

To conclude, sodium hydroxide is cheap and easily available; it also liquefies the purulent sputum samples and increases detection rate by concentration method, by increasing the number of bacilli per fields as well as give clean fields with less debris, so it will become less strenuous for lab technicians. The decontaminating property of sodium hydroxide has an advantage of limiting laboratory infections. This sputum concentration method after treatment with sodium hydroxide can be of vital importance, at least for samples which are coming negative with routine methods. Majority of the designated microscopic centers under RNTCP do not have centrifuge or Bio-safety cabinets but due to increased sensitivity of sputum microscopy by this method, the centers can be provided with the facility of centrifuge, till the new and better tool which could replace sputum microscopy in the near future. This shall be recommended in the RNTCP in order to reduce the rate of transmission of TB in the community.

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