

Original Research Article

Bleach method in comparison with NALC-NaOH specimen processing method for the detection of mycobacterium in sputum specimen

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ABSTRACT

Background: Tuberculosis is an infectious disease still remains to be a foremost public health risk worldwide. Even though there is an availability of many highly sensitive diagnostic tools, early case detection plays a significant role in control of the disease which relies specially on the detection of acid-fast bacilli in clinical samples which is low sensitive. Earlier decontamination of sputum sample may improve the detection of mycobacterium.

Methods: A prospective study was conducted with a total of (N=464) clinically suspected TB patient sputum samples which were collected and processed directly for AFB. From which AFB +ve samples were excluded. The direct AFB -ve (N=279) samples were processed using concentration methods (NALC, Bleach). After concentration, smears were examined under oil immersion for acid fast bacilli by ZN staining.

Results: Out of 464 sputum samples, 185 direct ZN stain +ve were excluded. Among the ZN-ve (N=279) samples, n=44 (15.7%) were positive using bleach method. The positivity has improved to n=48 (17.2%) using NALC-NaOH.

Conclusions: Bleach method of sputum decontamination is comparable to standard NALC-NaOH method. Therefore, we conclude that the identification of AFB using bleach concentration method can be used in resource limited health care setups especially in laboratories where mycobacterial culture is not performed which might help us in early diagnosis of tuberculosis.

Keywords: Acid fast bacilli, Bleach, NALC-NaOH-N-acetyl-L-cysteine sodium hydroxide, Ziehl-neelsen staining

INTRODUCTION

Tuberculosis (TB) remains a worldwide public health problem in spite of the fact that the contributing organism was discovered more than 100 years ago. It was estimated that two billion people being infected with tubercle bacilli worldwide even after the availability of many highly sensitive diagnostic tools and very efficacious treatment since decades.¹ According to the recent data, in India TB epidemic is greater than previously estimated.² There was an estimate of 10.4 million new TB cases worldwide in 2015, among which Asian, Nigerian and South African countries were accounted for 60% of new cases. TB mortality was recorded as 1.4 million and remained as

one of the top 10 causes of death worldwide.² About 40% of the Indian population was infected with TB bacteria, in which the vast majority has latent TB rather than TB disease. The Global incidence of tuberculosis was recorded as 9.6 million cases annually, whereas India accounted for 2.84 million in 2016.

Early case detection plays a significant role in containment of the disease which relies merely on the detection of acid-fast bacilli (AFB) in clinical samples. Regular methods available to detect tuberculosis are microscopy, culture, biochemical test and nuclear techniques. Culture is however considered as the gold standard.

In developing countries like India, identification of tuberculosis is done by sputum microscopic examination by Ziehl-Neelsen (ZN) staining, since it is easy, low-cost and provides speedy results. However, the sensitivity of this technique is low (22-43%). For the microscopic detection of sputum acid fast bacilli, at least 104-105 bacilli/ml is essential.³

Prior sputum decontamination may improve the revealing of mycobacterium. Among which, N-Acetyl-L-Cysteine Sodium Hydroxide (NALC-NaOH) method is considered as standard.^{2,4}

In this regard, few studies reported that bleach method of concentration is cheaper and safer method for the detection of AFB.⁵ There are very less comparison studies between the NALC-NaOH and bleach methods of AFB concentration. So, a study has been designed to compare standard NALC-NaOH method with cheaper bleach method in resource limited settings.

METHODS

This prospective study was conducted in clinical microbiology laboratory of Annapoorana medical college and hospital, Salem, Tamilnadu for 3 years from August 2013 to October 2016 after obtaining the institutional ethical committee clearance and patient's informed written consent. A total of 464 sputum samples were obtained from both in-patients and out-patients of various departments. These samples were subjected to direct ZN stain. The AFB negative samples were processed for concentration using NALC-NAOH, Bleach methods. After concentration, smears were examined under oil immersion for acid fast bacilli by conventional Ziehl-Neelsen staining method.

Inclusion criteria

- Clinically suspected tuberculosis patients with cough more than 2 weeks.

Exclusion criteria

- Known smear positive pulmonary tuberculosis patients
- Patients under anti-tubercle drug treatment
- HIV positive patient
- Inadequate sample volume (< 10ml)
- Salivary specimen which is not representative of lower respiratory tract.

Decontamination methods

NALC-NaOH Procedure

NALC-NaOH procedure 2ml of a mixture composed by 1.0 ml 1.0% N-acetyl-L-cysteine in 2.9% citric acid and 1.0 ml 4.0% NaOH were added to 2 ml volumes of each respiratory specimen and vortexed in a tube for 15–20

seconds and incubated at 37 °C for 20 minutes. Phosphate buffer pH 6.8 was then added and the tubes centrifuged at 3000 g for 15 minutes. The supernatant was then carefully discarded, and the sediment resuspended in 1-2 ml of phosphate buffer pH 6.8. This last suspension was used to prepare smears for microscopic examination.⁶

Bleach method

An equal amount of bleach (5% NaOCl) was added to the sputum sample in a screw cap tube and was shaken for 30 seconds. Then, the tube was left for 10-15 minutes at room temperature and was hand shaken for 30 seconds, at five minutes interval. An equal amount of distilled water was added and the tube was centrifuged at 3000 rpm for 15 minutes. After 15 minutes, the supernatant was discarded and the pellet was suspended in a few drops of the remaining fluid. Smear was prepared from the suspended sediment.⁷

ZN sputum smear preparation

AFB sputum smears preparation done in bio safety cabinet. One drop of each suspended pellet was used to prepare slides for AFB microscopy using the standard Ziehl- Neelsen stain.

Each slide was reported according to the Revised National Tuberculosis Program.

Smears were reported as follows

Each slide was examined and graded according to the RNTCP guidelines.

- Grade 0 - No Acid-Fast Bacilli (AFB) observed in a total of 100 oil immersion fields
- Scanty (Sc) - 1-9 AFB in 100 microscopic fields (actual number of bacilli)
- 1+ - 10-99 AFB in 100 fields
- 2+ - 1-10 AFB per field in at least 50 fields
- 3+ - >10 AFB per field in at least 20 fields.

RESULTS

Out of 464 microbiological sputum samples, 185 were found to be positive for acid fast bacilli by direct ZN staining method and remaining 279 samples were negative by direct staining method (Table 1).

Table 1: Direct ZN staining of sputum samples.

Total no. of sputum samples	AFB +ve	AFB -ve
464	185	279
% of positivity	41%	59%

These AFB negative (N=279) samples were processed further using NALC-NaOH, bleach concentration methods.

Table 2: Comparison of NALC-NaOH and bleach concentration methods.

Total no. of AFB –ve samples	NALC-NaOH	Bleach
N=279	N=48	N=44
% of positivity	17.2%	15.7%

Using bleach, the number of AFB positive were N= 44 (15.7%) whereas using NALC-NaOH the positivity has increased slightly (N=48 (17.2%) (Table 2).

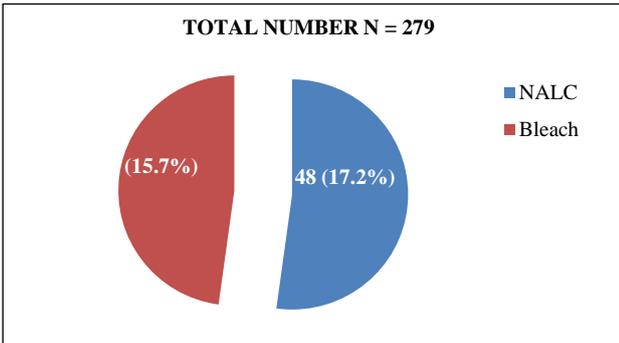


Figure 1: Comparison of NALC-NaOH concentration method with bleach method.

Table 3: Comparison between NALC-NaOH and Bleach methods using Fisher exact test.

Concentration methods	Positive	Negative
NALC-NaOH	n=48	n=231
Bleach	n=44	n=235

The Fisher exact test statistic value is 0.65. The result is not significant at $p < 0.05$; * A p value of < 0.05 is considered as highly significant

DISCUSSION

In India tuberculosis (TB) remains to be a main public health problem in spite of the immense efforts by the Revised National Tuberculosis Control Programme (RNTCP) since 1997.⁸ Ziehl-Neelsen staining, being a simple, rapid, easy and low-cost diagnostic technique forms the provision for the demonstration of acid fast bacilli in sputum smears especially in resource inadequate setups. Equally Ziehl-Neelsen staining lacks in sensitivity as at least 10,000 bacilli / ml of sputum is required to obtain an optimistic outcome on direct microscopy. The sputum samples pass through the oropharynx at the time of specimen collection. It will be contaminated with saliva, mucus and normal flora. Sampling essentially is homogenized to free the bacilli from the mucus, cells or tissue in which they may be fixed.⁹ For increasing detection of AFB, samples have to be homogenized and decontaminated with various agents, neutralized and concentrated. In this respect, comparison of the standard NALC-NaOH with bleach method might benefit the diagnosis of tuberculosis in resource limited

settings since bleach is cheaper and also does not require technical expertise to perform the method compared to NALC-NaOH.

NALC-NaOH processing method increases the detection rate of Mycobacterium tuberculosis not only by ZN staining, also by fluorescent staining methods.¹⁰⁻¹² However, few studies failed to find a difference in sensitivity between direct and concentrated sputum smear microscopy especially when the AFB smear is negative and scanty.^{13,14}

Application of 2-5% NaOCl digests sputum products and inactivate mycobacteria without altering their morphology, so that even when killed they can still be stained and observed under microscope. This provides greater safety for laboratory use.¹⁵ Further centrifugation concentrates the AFB in the mixture and increases the rate of positivity.

This study has shown 15.7% (N=44) of AFB positivity among 279 direct AFB negative sputum samples using Bleach whereas using NALC-NaOH the AFB positivity improved to 17.2% (N=48) respectively indicating there is low marginal difference between both the methods and almost comparable. In addition, our Fisher exact test analysis has not shown any significant difference between both the methods ($\chi^2=0.65$, P value= > 0.05).

The increased sensitivity by bleach method is probably due to the greater concentration of AFB and to the fact that NaOCl removes debris and leaves the microscopic field free for easy examination.¹⁶

In these regards, study results are in parallel with it has been also shown in other studies that bleach method improves the recovery of mycobacteria in extra pulmonary tuberculous lymphadenopathy.^{13,17-20}

CONCLUSION

Bleach method of sputum decontamination is comparable to standard NALC-NaOH method. Therefore we conclude that the identification of AFB using Bleach concentration method can be used in resource limited health care setups especially in laboratories where mycobacterial culture is not performed which might help us in early diagnosis of tuberculosis.

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