

## Original Research Article

# Comparison of NALC-NAOH processing method with C18-carboxy propyl betaine method for the detection of mycobacterium in sputum

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**Received:** 17 April 2017

**Accepted:** 24 April 2017

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### ABSTRACT

**Background:** Tuberculosis is an infectious disease which remains to be a major public health risk worldwide even after the availability of many highly sensitive diagnostic tools. Early case detection plays an important role in control of the disease which relies solely on the detection of acid-fast bacilli in clinical samples which is low sensitive. Prior 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 (NALC, CB-18) and culture (LJ medium). 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= 48 (17.2%) were positive using NALC-NaOH method. The positivity has improved to N=69 (25%) using CB-18 and N=79 (28.3%) using culture (LJ medium).

**Conclusions:** CB-18 is sensitive than NALC-NaOH method and its sensitivity is almost comparable to the gold standard culture using LJ medium. Therefore we conclude that the identification of AFB using CB-18 concentration method can be used in resource limited health care setups which might help us to diagnose the TB at the earliest.

**Keywords:** Acid fast bacilli, C18-carboxypropylbetaine, Lowenstein-jensen, NALC-NaOH-N-acetyl-L-cysteine sodium hydroxide, Ziehl-neelsen staining

### INTRODUCTION

Tuberculosis (TB) is an important infectious with devastating morbidity and mortality. Still, TB remains to be a major public health risk worldwide even after the availability of many highly sensitive diagnostic tools and very efficacious treatment since decades.<sup>1</sup>

According to the recent data, in India TB epidemic is greater than previously estimated.<sup>2</sup>

In 2015, there was an estimate of 10.4 million new TB cases worldwide, among which six countries were accounted for 60% of new cases: India, Indonesia, China,

Nigeria, Pakistan and South Africa. TB deaths were recorded as 1.4 million and remained as one of the top 10 causes of death worldwide.<sup>2</sup>

It was estimated that about 40% of the Indian population were infected with TB bacteria, in which the vast majority have latent TB rather than TB disease.

In 2016, Global incidence of tuberculosis was recorded as 9.6 million cases annually, whereas India accounted for 2.84 million. Early case detection plays an important role in control of the disease which relies solely on the detection of acid-fast bacilli in clinical samples. Routine methods available to detect tuberculosis are Microscopy,

Culture, Biochemical test, nuclear techniques. However, Culture is considered as the gold standard. In developing countries like India, diagnosis of tuberculosis is done by sputum microscopic examination by Ziehl-Neelsen staining, since it is simple, inexpensive and provides quick results. But this technique has a low sensitivity of 22-43% because the detection of acid fast bacilli in sputum specimen requires at least 104-105 bacilli/ml for microscopy.<sup>3</sup>

Prior decontamination of sputum sample may improve the detection of mycobacterium. Conventional methods for concentration include Petroff's method which is routinely done, while N-Acetyl-L-Cysteine Sodium Hydroxide (NALC-NaOH) still remains as a standard and has sensitivity of around 60%.<sup>4,5</sup> There is a need to improve the current simple diagnostic techniques without complicating them further to increase sensitivity of case detection. In this regard, few studies reported that C18-carboxypropylbetaine method of decontamination is superior to NALC-NaOH method.<sup>6</sup> But, there is inconsistency regarding the sensitivity of CB18 method in detection of sputum AFB 7 Therefore, this study has been designed with a main objective of evaluating the sensitivity of NALC-NAOH and Cb18 compared to the gold standard culture Lowenstein-Jensen medium.

## METHODS

This prospective study was conducted in clinical microbiology laboratory of Annapoorana medical college and hospital, Salem, Tamilnadu, India from 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 CB18, NALC-NAOH methods and were also cultured on LJ medium. 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).

### 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.<sup>8</sup>

#### CB18 Procedure

Buffered CB-18 (4 mM CB-18, 50 mM Tris-HCl, 12.5 mM citrate, pH 6.0, 1.5 mM NaCl, 0.3% NALC) was added to each specimen to a final volume of 30 ml, vortexed, and then incubated at room temperature for 24 h prior to centrifugation at 1,818 g for 15 min at room temperature. All processed specimens were decanted, resuspended in the remaining supernatant backwash, and transferred to microscope slides for staining.<sup>6</sup>

#### 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 (RNTCP)

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.

#### LJ medium inoculation

Ready available LJ slants were used for the study (Hi Media). Decontaminated deposit was inoculated on the entire surface of LJ slope using a loop in a sterilized inoculation hood, by taking the necessary aseptic precautions. The date of the inoculation was noted. The slopes were incubated at 37°C for a maximum period of 8 weeks. They were inspected daily for growth or for contamination.

## 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

These AFB negative (N=279) samples were processed further using concentration methods (NALC-NaOH, CB18) and culture using LJ culture medium. Using NALC-NaOH the number of AFB positive were N= 48 (17.2%), using cb18 the number raised to N=69 (25%). However, the culture positives using LJ medium were recorded as N=79 (28.3%) (Table 2, Figure 1).

**Table 2: Comparison of concentration methods with culture.**

Total no. of AFB -ve samples	NALC-NaOH	CB-18	Culture (LJ)
279	48 (17.2%)	69 (25%)	79 (28.3%)

## DISCUSSION

Tuberculosis (TB) remains as a major public health problem in India in spite of the massive efforts by the Revised National Tuberculosis Control Programme (RNTCP) since 1997.<sup>9</sup> Ziehl-Neelsen staining is a simple, rapid, easy and low cost diagnostic technique and therefore it forms the support for the demonstration of acid fast bacilli in sputum smears especially in resource limited settings. However, it lacks sensitivity as at least 10,000 bacilli/ml of sputum are required for a positive result on direct microscopy. Though culture is considered as a gold standard, microscopy is relatively simple, inexpensive and is widely accepted diagnostic tool for the detection of mycobacterium from sputum specimens.

As sputum samples pass through the oropharynx during collection of specimen it will be contaminated not only with saliva and mucus but also with normal flora. Specimens must be homogenized to free the bacilli from the mucus, cells or tissue in which they may be fixed.<sup>10</sup> For better AFB yield, specimens need to be homogenized and decontaminated with various agents, neutralized and concentrated by centrifugation to eliminate these contaminants. There is a need for sensitive and also affordable concentration methods for the diagnosis of tuberculosis particularly in developing countries where resources are limited.

In this regard, present study has shown 17.2% (N=48) of AFB positivity among 279 direct AFB negative sputum samples using NALC-NaOH whereas using CB-18 the AFB positivity improved to 25% (N=69). This might be because of the ability of C18-carboxypropylbetaine (CB-

18) to reduce the surface tension and buoyancy there by improving the detection of bacilli.<sup>11</sup> Moreover it is a zwitter ionic detergent that can be taken up by viable mycobacteria, making them denser and thereby increasing their recovery by centrifugation.<sup>7</sup> Our results are in parallel with Charles G. Thornton 1998, K. F. Laserson 2005.<sup>12,6</sup> It has been shown CB-18 improves the recovery of mycobacteria other than tuberculosis among human respiratory specimens and also among animal products such as milk.<sup>12-15</sup>

In present study, on processing the direct AFB negative sputum samples (N=279) using LJ medium culture had displayed an improvement in the positivity of AFB to 28.3% (N=79) indicating culture as a gold standard. But, the difference in identification of AFB between CB-18 and Culture is almost comparable. Our results are in agreement with CDC, National Center for TB Prevention Division of Tuberculosis Elimination 2013.<sup>16</sup>

## CONCLUSION

CB-18 is more sensitive than NALC-NaOH method and its sensitivity is almost comparable to the gold standard culture using LJ medium. Therefore, we conclude that the identification of AFB using CB-18 concentration method can be used in resource limited health care setups which might help us to diagnose the TB at the earliest.

*Funding: No funding sources*

*Conflict of interest: None declared*

*Ethical approval: The study was approved by the Institutional Ethics Committee*

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**Cite this article as:** Amar Kumar G, Chandrasekaran S. Comparison of NALC-NaOH processing method with C18-carboxy propyl betaine method for the detection of mycobacterium in sputum. *Int J Res Med Sci* 2017;5:2315-8.