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Research Article

# A Comparative Assessment of Petroff's and N-Acetyl-L-Cysteine- Sodium Hydroxide Method in the Diagnosis of Pulmonary Tuberculosis

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## ABSTRACT

Tuberculosis (TB) stays one of the deadliest communicable disease and responsible for almost two million deaths every year worldwide. The objective of the present study is to compare Petroff's and N-acetyl-L cysteine- sodium hydroxide methods used for the diagnosis of pulmonary tuberculosis. This present study was conducted in the department of ST John's Medical college and Hospital, Bangalore, from October 2011 to September 2012. Total 100 sputum specimen was collected from patients under the Revised National Tuberculosis Control Program (RNTCP) Guidelines. These samples were decontaminated with Petroff's and NALC- NaOH Method and same were processed for L J culturing and incubated at 37°C. As per result analysis, out of total 100 sputum sample, 64 % smears were positive by petroff"s methods and 69 % smears were positive by NALC - NAOH methods. The positivity rate was increased by NALC – NAOH method. All samples were cultured on LJ medium for bacterial growth. A maximum number of cultures were positive by NALC – NAOH method (53 %) and Petroff"smethod (51 %). This study concludes that NALC-NaOH method is effective and provides valid and rapid results. This method can be used for routine diagnosis and for better sensitivity of Mycobacterium growth. There is further multicentric research is required in respect of targeting larger population for better effective outcomes.

**Keywords:** Tuberculosis, Petroff's, NALC- NaOH Method, Sputum, L J Culture

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## INTRODUCTION

Mycobacterium tuberculosis complex can cause tuberculosis, which is one of the oldest diseases of mankind, and is a major cause of death worldwide. It affects the lungs, and other organs. In 98 % of cases disease spread when patients are sneezing and coughing [1]. Tuberculosis remains to be a major health concern worldwide. Pulmonary tuberculosis is a contagious disease and to control the spread of TB, the disease must be diagnosed early and treated effectively [2].Tuberculosis (TB) remains one of the major global health threats leading to morbidity and mortality [3,4]. All over the world One in three people is affected by tuberculosis, which is instead of 2–3 billion persons are infected with Mycobacterium Tuberculosis (M. Tuberculosis) which are expected to develop active TB disease during their life, Among them 5–15% developed vital Tuberculosis throughout their life[5].

Majority of TB sufferers live in low income and middle income countries, especially in regions such as Sub-Saharan Africa and South East Asia [4]. In 2014, an estimated 9.6 million people fell ill due to TB, around 1.5 million people died from the disease including 1.1 million HIV-negative persons and 400,000 HIV patients [5]. Over the past decade, significant progress has been made towards TB control with most of the TB targets set as part of the Millennium Development Goals (MDGs) having been achieved [5]. TB mortality for instance, has declined by 47% since 1990, with nearly all of that happening in the era of the MDGs. In all, effective diagnosis and treatment of TB has been estimated to have saved over 40 million lives between 2000 and 2014 [5].

India, contributing to a fifth of the global burden of TB. It is estimated that about 40% of the Indian population are infected with TB bacillus [6]. For a definitive diagnosis of pulmonary tuberculosis identification of mycobacteria in sputum by microscopy and culture is essential [7]. Currently,

for diagnosis of TB, Ziehl – Neelsen staining, smear microscopy is still the most used amongst all methods employed worldwide due to its simplicity, low cost, speed and minimal requirement of equipment and technical skills [8]. Digestion and decontamination of contaminated unsterile specimens, like sputum, are a basic step in the separation of mycobacteria. Decontamination procedure promotes the resistance of mycobacteria to acids, bases or other antibacterial agents [9]. The use of sodium hydroxide (NaOH) sodium citrate along with the mucolytic agent N-acetyl-L-cysteine (NALC, the NALC-NaOH method) is one of the most widely used methods, as it is rapidly effective in reducing the number of contaminating organisms [10]. In developing countries, culture on Lowenstein-Jensen solid medium is the gold standard for microbiological diagnosis of TB [8].In this study, Petroff's method is compared withN-acetyl-Lcysteine-sodium hydroxide methods.

## MATERIALS AND METHODS

This present study was conducted in the department of ST John's Medical college and Hospital, Bangalore, from October 2011 to September 2012. Total 100 sputum specimen was collected from patients under the Revised National Tuberculosis Control Program (RNTCP) Guidelines. Suspected case of pulmonary tuberculosis. Before the collection of specimen instruction given to the patients to obtain a good sputum specimen. Explain properly to patients the importance of collecting a good sputum sample, as many people do not understand the difference between saliva and sputum, demonstrate the patients action how to open and close the sputum container and how to bring up sputum. Before collection of the sputum, the patients are instructed to rinse his/her mouth well with water. Stand facing the wall away from the wind, away from the public place. Keep his/her both hands on the hip, take deep breaths, cough forcefully, collect sputum in his mouth and spit carefully into the cup and close the cup tightly. This is called as spot sample. Given two more labeled cups to the patients. Once he/she has to collect next day early in the morning after rinsing the mouth well. The third sample is collected on the spot with the same instruction when the patients come to deliver the second sample [11].

**Inclusion Criteria:** All the patients visited the hospital cough with sputum and blood at times, chest pains, weakness, weight loss, fever and night sweats were included.

**Exclusion Criteria:** Some patients was not able to give a sputum sample after giving all the instructions that the sample was excluded from this study.

### Microscopic Examination

All sputum samples were collected in sterile container's and processed immediately in the laboratory. Each smear was prepared on a new clean glass slide, dried and heat fixed and finally stained with Ziehl-Neelsen (Z-N) method and graded [12]. Grading was made from scanty to + to +++ as per Revised National Tuberculosis Control Program (RNTCP) guidelines [13]. Each sample was then subjected to decontamination by two different methods, Petroff's and N-acetyl-L cysteine-sodium hydroxide method and then ZN staining the smear was prepared again after decontamination of the concentrated sample [14].

### Specimen Digestion, Decontamination

Specimen were vortex lightly or hand mix, than specimen was divided in two parts, one half of the specimen was by processed by NALC- NaOH method and another half specimen were processed by Petroff's method. In addition, for this study, we inoculated all the samples directly on Lowenstein-Jensen (LJ) medium without doing decontamination method.

**Petroff's Method:** The sputum sample was pouring into germfree test tube and the same quantity of germfree 4% NaOH was added. The tube was incubated at 37˚c for 30 minutes with vigorous shaking every 5 minutes. The mixture was centrifuged at 3,000 RPM for 30 minutes and the supernatant discarded. The sediment was neutralized by N/10HCL using a drop of phenol red as an indicator. Than inoculated in LJ culture media and incubate 37˚C L.J [15].

### NALC-NAOH Method

The most widely used digestion-decontamination procedure is the N-acetyl- L-cysteine (NALC) -NAOH method. The critical reagent used in this procedure includes NALC the mucolytic agent; sodium hydroxide the decontaminating reagent and sodium citrate, which stabilizes the acetylcysteine. This method may be also used to process sputum, gastric lavage specimen, tissue, urine and other body fluids [16,17].

### Procedure of Sputum Decontamination

a. 50 ml sterile 4% NaoH.

b. 50 ml sterile 2.9 % sodium citrate.

c. 0.5g NALCpowder.

This solution must be used within 24 hours of the preparation because the mucolytic activity of NALC is inactivated on exposure to air. Label centrifuge tubes with specimen numbers and place on a rack in the BSC (Biosafety cabinet). Open always one sample at a time and then transfer up to 10 ml of specimen into 50 ml germfree not reusable pointed tube. For extremely small or thick specimens, put in a small quantity of phosphate buffer to the sample container and mix to loosen the sample to make the easy sputum sample transfer.

Add the same amount of new NALC-NaOH-sodium citrate working solution to all tube, opening only one tube at a time. Adding up of the same quantity of NALC-NaOH-sodium citrate working solution is significant and a smaller amount will lead to under-decontamination and will cause contamination of culture. More than equal quantity will lead to elevated concentration of NaOH and will reduce the number of sufficient bacilli.

Recap the tube tightly and agitate on a vortex mixer for no more than 30 seconds. Avoid excessive agitation, as it may inactivate NALC and cause the specimen to gel. Let the tube place for 15 min at room temperature to disinfect the sample. Make secure the sample is completely liquefied. If still marked put in small quantity of NALC powder (30 – 35 mg) directly to the sample tube. Mix well by inverting the tube a number of times. Processing time can be extended for up to 20 – 25 min but no longer because prolonged incubation in the presence of sodium hydroxide greatly affects the recovery and time-to-detection of mycobacteria.

Add sterile phosphate buffer, pH 6.8 up to the 45 ml mark. This will decrease the continuous action of the NaOH and lesser the thickness of the mixture. Recap the tubes strongly and mix well by inverting more than a few times. Using aerosol-free sealed centrifuge cups centrifuge the specimen tubes at 3,000*g* for 20 minutes. Open the aerosol-free sealed centrifuge cups inside the biological safety cabinet only. Remove the centrifuge tubes. Opening

one tube at a time with awareness with one uninterrupted movement decant the supernatant into the splash-proof throw away container containing roughly 5cm depth of appropriate decontaminator making sure that the residue is not lost during decanting. Recap the tube.

Opening one tube at a time draw up an appropriate volume of phosphate buffer, pH 6.8 into a sterile disposable Pasteur pipette (usually 1-2ml, depending on the number of tests being performed) put in the buffer along the wall of the tube holding the end of the pipette close to the sediment to prevent aerosolization. Mix carefully with the pipette and throw away the pipette into sharp container and continue for inoculation of solid media [18].

All slant was observed for rate of growth daily for the first week and then at weekly intervals for 8 weeks. Absence of growth at the end of 8th weeks was recorded as negative culture

## RESULTS

Out of 100 sputum sample, 61% smear was positive by direct staining technique, after that again we have decontaminated and concentrated the sample by both method, Petroff''s method and NALC - NAOH method, and made the smear to rule out any modify in smear microscopy, which showed 64 % smear positive by petroff''s methods and 69 % smear positive by NALC - NAOH methods. So smear positivity was increase in NALC – NAOH method.All sample at the same time (direct and after decontamination by different methods) we have a culture on LJ medium to rule out the bacterial growth. Maximum number of culture positivity achieves by NALC – NAOH method (53 %), Petroff''s method (51 %) and direct culture it was (41 %). Some culture media, we observed contaminated growth, again we have studied about the rate of contamination it was maximum (29%), by direct method (17 %) by Petroff''s method and (13 %) by NALC – NAOH method.

**Table 1: Allocation of direct smear examination and culture results (n=100)**

| Result | Microscopy | | | Culture | | |
|---|---|---|---|---|---|---|
| | **Direct (%)** | **Petroff's (%)** | **NALC- NaOH (%)** | **Direct(%)** | **Pettoff's (%)** | **NALC- NaOH (%)** |
| **Positive** | 61 | 64 | 69 | 41 | 51 | 53 |
| **Negative** | 39 | 36 | 31 | 30 | 32 | 34 |
| **Contamination** | --------- | ---------- | --------- | 29 | 17 | 13 |

**Table 2: Comparative assessment - Growth on LJ medium**

| Method | Total Samples | 1st week (%) | 2nd week (%) | 3rd week (%) | 4th week (%) | 5th week (%) | 6th week (%) | 7th week (%) | 8th week (%) |
|---|---|---|---|---|---|---|---|---|---|
| **Direct** | 100 | 0 | 2 | 22 | 34 | 36 | 36 | 41 | 41 |
| **Petroff's method** | 100 | 0 | 3 | 24 | 39 | 39 | 48 | 51 | 51 |
| **NALC- NaOH methods** | 100 | 0 | 6 | 27 | 42 | 45 | 53 | 53 | 53 |

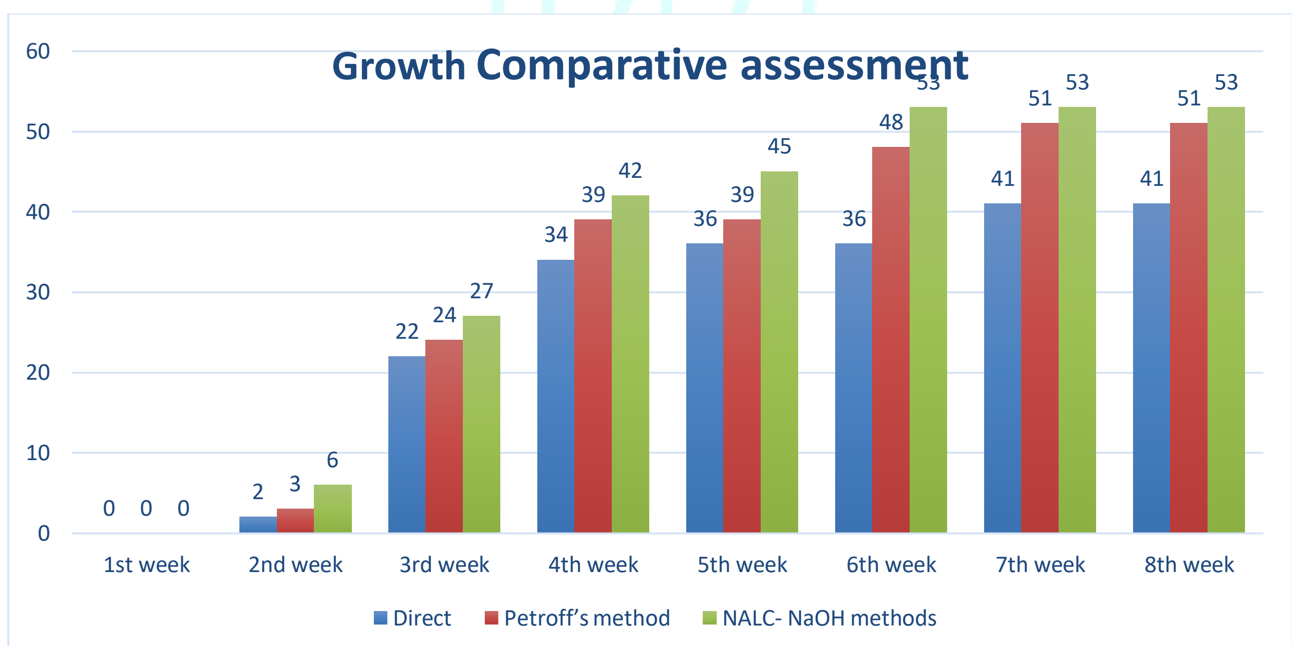


**Figure 1: Growth comparative assessment of three different technique.**

**Table 3: Comparison of two decontamination methods for rate of contamination, negative culture and culture failure**

| Methods | Number of contaminated Slopes | Negative culture (No growth up to 8 weeks) | Total culture (Negative/failure) |
|---|---|---|---|
| **Direct** | 29 | 30 | 59% |
| **Petroffs** | 17 | 32 | 49% |
| **NALC- NaOH** | 13 | 34 | 47% |

## DISCUSSION

For diagnosing Tuberculosis, smear microscopy and sputum culture are significant methods. Sputum culture is the gold standard and more susceptible method compared with smear microscopy as it detects as few as 10–100 bacilli/ml. It also helps in drug susceptibility testing. But contamination of culture specimens limits the diagnostic yield of sputum culture [19]. Sputum decontamination methods most commonly used for the separation of mycobacteria and to kill the oral normal commensal present in the sample. These decontamination methods also kill the mycobacteria and other microorganisms which are present in the specimen. Varies according to the method used and the mycobacterial type present in the sample [20]. A number of studies have reported that the use of decontamination methods with more concentration of NaOH (3-4%) will decrease bacterial contamination rates, but it is harmful for mycobacteria may occasionally give a negative culture report [21].

In this study, we used the NALC-NaOH sputum digestion method of Kent and Kubica et al [22] as it is widely used and recommended by standard laboratory manuals [16] and because the final 2% NaOH concentration employed results in the destruction of fewer mycobacteria than 4% NaOH used by Petroff's method. In the Present study, we have been using Petroff's and NALC-NAOH method of sputum digestion. Petroff's method sputum is digested with 4 % NaOH and the centrifuged deposit is neutralized by adding 8% HCI and phenol red indicator and if neutralization is not vigilantly carrying out the medium may be acidic which is also harmful to the mycobacteria. In NALC-NAOH method digestion is arrested by dilution with sterile phosphate buffer, pH 6.8. This will also decrease the continuous action of the NaOH and lesser the thickness of the mixture. Centrifuged and deposit is used for culture which is simpler, safer and seems to give a greater number of positive smears and culture for mycobacteria with minimum overgrowth by contaminants.

In this study,100 sputum samples were taken for the diagnosis of TB, AFB microscopy alone should not be used as it does not always give accurate results and in doubtful cases LJ culture should be used for confirmation. The number of culture positives by NALC-NAOH method was53%, by Petroff's method were 51 % and 41%by Direct method (Table 1). Culture positives from Modified Petroff's the decontaminated by the NALC-NaOH method provided isolation of M. Tuberculosis in a higher percentage (53 %) than obtained by Kang et al. And Chakravorty et al. (39.7%) study [23]. Both result from Pathak, Deshmukh and Menon (1973) who reported superiority of swab method Nassau (Nassau, 1954) 92.3% over NALC NaOH 79.4% method [24,25].

As per our examination in this study, we noticed that in NALC-NaOH growth were higher (53 %) during V and VI work as compared to Petroff's method (48%) (Table: 2) the probable reason are, 4% NaOH is used for Petroff's method as compared NALC-NaOH method which uses 2% NaOH while concentrating the sputum may kill or seriously injure few Mycobacteria. Hence recovery by NALC-NaOH was faster and better than Petroff's method. In our study, we found smear positivity higher than the culture positivity. The cause may be that microscopy sometimes gives false positive results and in our circumstance, it cannot differentiate between dead and live bacteria. In such cases, the patients might be treated with anti-tubercular drugs and in the microscopy of these samples, the AFB may be dead. For these reasons, the dead isolates did not grow in the L-J culture media.

This also reveals that AFB microscopy does not always give perfect results for the diagnosis of TB. The contamination rate by NALC-NaOH method was 13 % in our study, which is lower than that reported by other workers. Engback et al. (1961) reported 30.4%, while Pathak et al. (1973) reported 27% as contamination rate with NALC-NaOH method [25]. In our study the NALC-NaOH method for AFB smear and culture improves the sensitivity when compared with the Direct and Petroff's method.

## CONCLUSION

The results of present study conclude that NALC-NaOH method is effective and provide the valid and rapid results. This method can be used for routine diagnosis and better sensitivity of mycobacterium growth. It is more suitable for routine diagnostic and research purpose.

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