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Comparative Evaluation of Different Staining Techniques for Diagnosis of Pulmonary Tuberculosis

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ABSTRACT

Introduction: We have various diagnosis methods for tuberculosis, however, sputum smear microscopy is the simplest, rapid and cheapest method.

Aims and Objectives: The purpose of this study was to detect Mycobacterium tuberculosis (MTB) in clinically suspected cases of pulmonary tuberculosis by using staining methods.

Methods: A prospective examination of samples were performed in the department of microbiology in Government Medical College, Amritsar. During this study, a total of 100 sputum samples from patients clinically suspected of pulmonary tuberculosis were collected in sterile leak-proof containers as per RNTCP guidelines. The collected samples were divided into the following three parts: initial part was processed before decontamination; Second part processed post-NALC-NaOH method and third post Bleach method and then subjected to ZN, Kinyoun and Fluorescent staining.

Results: Among the following methods, the detection rate of acid-fast bacilli (AFB) by prior decontamination was more with Fluorescent (27%), followed by Ziehl Neelson(ZN) (25%) and then by Kinyoun staining (20%). Furthermore, post decontamination, the detection rate of AFB by post-NALC-NaOH method increased as the fluorescent method (37%), ZN (30%) and Kinyoun method (23%). Whereas the detection rate of AFB by post bleach method was less effective i.e. fluorescent staining (32%), ZN (26%), and Kinyoun (20%).

Conclusion: The rate of detection and efficiency of acid-fast bacilli was found to be more with Fluorescent staining. This research also highlighted the increase in the detection rate of AFB after decontamination with NALC-NaOH method as compared to the bleach method thus it aided in increasing detection of bacilli.

Key Words: Ziehl Neelsen staining, Kinyoun staining, Fluorescent staining, Tuberculosis, Mycobacterium tuberculosis, N-acetyl-L-cysteine (NALC), Sodium Hydroxide, Bleach method

INTRODUCTION

One of the crucial health problems which are suffered by the globe is Tuberculosis (TB). It would be shocking to know that, India ranks first among high TB burden countries contributing 26% of estimated global incident TB cases in 2012, with a population of around 1.24 billion. In India, more than 40% of the population is infected with *Mycobacterium tuberculosis*.¹ In 2017, approximately 10 million individuals had suffered chronically with TB and among them, 2.79 million individuals were active cases.² It is also one of the most common opportunistic infection in patients living with HIV/AIDS.³ According to the prestigious World Health Organi-

zation, tubercular infections are currently spreading at the rate of one person per second per million people in India by inhalation of infected droplet nuclei released in the air. Most of the time Tuberculosis affects the lungs of the ailing person lungs but it can also be extrapulmonary.⁴

Conventional methods like Conventional Culture Methods are treated as a golden standard but they are very time consuming and requires long processing time. On the other side of the coin, newer molecular techniques like Polymerase Chain Reaction are more expeditious but they are very expensive for an ever-changing economy like India, which is still suffering from the pain of colonialization and there is a

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lack of health infrastructure both in terms of technology and we are also socially backward due to our regressive thinking. For example, India has many underreported cases due to poverty and illiteracy. In economics, there is a concept of scarce resources and alternative means and it's of no use in developing countries like India due to limited sources.⁵

The most widely used method for detection and diagnosis of TB is the direct microscopic examination of AFB. RNTCP emphasize on early detection of cases based on sputum smear microscopy which is very essential to interrupt the transmission of disease. Identification of tuberculosis is done by sputum microscopic examination by Ziehl-Neelsen (ZN) staining because it is easier, cost-efficient and provides rapid results.⁶ Whereas Fluorescent microscopy using Auramine-O fluorescent staining method which has been introduced in some laboratories detects 10% more TB cases than light microscopy using ZN method and requires only 25% of the time to read a ZN-stained smear.⁷ Another potential alternative to ZN Staining is Kinyoun's Cold Acid Fast staining which differs from former as in that case heating is not required, phenol concentration in carbol fuchsin is increased and duration of carbol fuchsin staining is more.⁸ The only demerit of microscopy is low sensitivity(50-80%) relative to culture. The sensitivity of microscopy is influenced by various factors like a method of sample collection, staining technique and technical expertise.⁹ Sensitivity of Sputum microscopy can be improved by prior sputum decontamination. N-Acetyl-L-Cysteine Sodium Hydroxide (NALC-NaOH) method is considered as a standard among the others, as it is the least toxic to the mycobacteria, and therefore provides the highest yield of positives. The other important method of decontamination is bleach method using 5% Sodium Hypochlorite which is a more economical and safer method for the detection of AFB.¹⁰ The purpose of the study was to detect *Mycobacterium tuberculosis* (MTB) in clinically suspected cases of pulmonary tuberculosis by using various staining methods including Zeihl-Neelson (ZN), Kinyoun and Fluorescent staining method prior NALC-NaOH and bleach processing and post-NALC-NaOH and bleach processing.

MATERIALS AND METHODS

The present cross-sectional study was performed in the Microbiology Department in collaboration with Chest & TB hospital and other wards of Guru Nanak Dev Hospital, Amritsar. A total of 100 Sputum samples from Patients with clinical suspicion of pulmonary tuberculosis including symptoms of cough with or without expectoration for >2 weeks, weight loss, fatigue, hemoptysis, and loss of appetite and attending outpatient and indoor service at this prestigious institute, were included in the study. Patients already taking anti-tubercular drugs and cases of extrapulmonary tuberculosis were not part of this study should need to be noted. We

are very much obliged to The Institute ethical committee for their Ethical approval.

Sample Collection and Transport

Two Sputum samples (5-10 ml) one spot and other early morning sample were collected from these cases in sterile leak-proof containers as per the Revised National Tuberculosis Control Program (RNTCP) guidelines.¹¹ These were taken quickly to the tuberculosis laboratory, where these specimens were processed by conventional standard laboratory techniques.

Sample Processing

These collected samples were divided into the three main parts: the initial part was processed before decontamination, then the second part was processed post-NALC-NaOH method and third post Bleach method and then subjected to Zeihl Neelson, Kinyoun and Fluorescent staining.¹²

Decontamination Methods

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 15–20seconds 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.¹³

Bleach method

An equal amount of bleach (5% NaOCl/Sodium Hypochlorite) was put in 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 poured 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.¹⁴

Direct Microscopy

Smears were made by spreading the specimen over clean grease-free slides spread over 2cm x 3 cm areas with a sterile loop and was heat fixed for various staining methods. Acid-fast bacilli (AFB) were seen as red, beaded rods when assessed under oil immersion (X 100) lens. The smears were graded contingent to the number of bacilli observed in the stained smear under oil immersion lens of the light microscope as per RNTCP guidelines for ZN and Kinyoun stain-

ing. We have inspected at least 300 fields before reporting the smear as negative.

RNTCP grading was followed for ZN and kinyoun staining

Grade 3+: More than 10AFB per oil immersion field, 20 fields should be examined.

Grade 2+: 1-10 AFB per oil immersion field, 50 fields should be examined.

Grade 1+→10-99 AFB per 100 oil immersion field, 100 fields should be examined

Scanty→1-9 AFB per 100 oil immersion field, 100 fields should be examined

No AFB → No AFB seen, 300 fields should be examined.⁵

Fluorescent Microscopy

The Tubercle bacilli appear bright brilliant green against dark background using 40 X lens using Fluorescent microscope.

RNTCP grading for Fluorescent microscopy using Auramine-O stain

Reporting scale → AFB seen (400 magnification; one length=40 fields=200 HPF in bright field microscopy

Negative : No AFB seen in at least 40 fields

Actual Number : 1-19 AFB per 40 fields

1+ : 20-199 AFB per 40 fields

2+ : 5-50 AFB per field at least 20 fields

3+ : More than 50 AFB per fields in at least 8 fields.⁵

RESULTS

Among all of the samples (n=100) cases, 56% were males and the remaining 44% were females. The age group of patients which was studied for this analysis ranges from 8 years to 80 years. The maximum number of cases (n=40; 40%) were in the age group 41-60 years. At the end of this analysis, cough (100%) was the most common complaint which was succeeded by fever (78%), generalized weakness (58%) and followed by weight loss (30%).

In the present study, 25 (25%) cases were positive for AFB and 75 (75%) cases were negative for AFB by ZN staining before decontamination. 30(30%) cases were positive for AFB and 70(70%) cases were negative for AFB by ZN stain after decontamination by NALC-NaOH method, with an increase of 5(5%) more cases after decontamination. On the other side, 26(26%) cases came out to be positive and 74(74%) were negative after decontamination by Bleach method having just increase in 1(1%) case (Table 1).

Staining by Kinyoun Method showed that 20(20%) cases

were positive before decontamination and post NALC method positivity increased to 23(23%) and by Bleach Method positivity remained the same as before decontamination (Table 1). Also, Fluorescent Microscopy showed a remarkable increase in positive cases to 27(27%) before decontamination as compared to staining performed by the other two methods. After decontamination by NALC-NaOH positivity came to be 37(37%) and by Bleach, it came out to be 32(32%) (Table 1)

The maximum number of positive cases after decontamination by NALC-NaOH was detected by Auramine-O stain, 37(37%) cases followed by ZN staining detected 30 (30%) cases and Kinyoun staining detected the least i.e. 23 (23%). Whereas by Bleach method it came out to be Auramine-O stain, 32(32%) cases followed by ZN staining detected 26(26%) cases and Kinyoun staining detected the least i.e. 20(20%) (Table 1, 2)

DISCUSSION

The most efficacious and constructive method used in the diagnosis of TB is the direct microscopic examination of appropriately stained sputum specimens for Acid-fast bacilli. This method is easy and cost-efficient. It is also useful to study the response to treatment and to establish a cure or failure at the end of treatment.¹⁵ Although microscopy has a low sensitivity rate; it is still the most commonly used AFB detection method especially in resource-limited developing countries like India.¹⁶

Out of the all three staining methods, the detection rate of acid-fast bacilli (AFB) by prior decontamination was more with Fluorescent (27%), followed by Ziehl Neelson (ZN) (25%) and then by Kinyoun staining (20%). Studies conducted by Lawrence et al. and Purusothaman K et al. were also in concordance with the present study.^{5,17} Another study by Saroj et al. demonstrated the superiority of Auramine-O staining over Ziehl-Neelsen staining.¹⁸ Though Fluorescent microscopy is more sensitive than Zeihl- Neelson staining, but the real disadvantage of fluorochrome method is that fluorescence fades with time along with the cost of microscopy. Then Slides must be read within 24 hours. The fluorescent method can give false-positive results as compared to the ZN method, which can be futile.

ZN staining is simple, rapid, and easy to perform and cost-effective diagnostic technique. This is one of the main reasons that this method is preferred by most of the countries in the globe, especially in developing countries because of its simplicity and cost-efficiency. The only disadvantage of ZN staining is its low utility in HIV- TB co-infected patients and extrapulmonary TB.¹⁹ The sputum is contaminated with saliva, mucus and normal flora. Therefore, Sample to be processed needs to be homogenized to free the bacilli from

the mucus, cells or tissue in which they may be fixed. For increasing detection of AFB, samples have to be homogenized and decontaminated with various agents, neutralized and concentrated. NALC acts as a mucolytic agent, concentrates the bacilli, significantly increasing the detection rate. In this respect, comparison of the standard NALC-NaOH with bleach method might benefit the diagnosis of tuberculosis in resource-limited settings.¹¹

In the present study, Post decontamination, the detection rate of AFB by post-NALC-NaOH method increased as Fluorescent method (37%), ZN (30%) and Kinyoun method (23%). Whereas the detection rate of AFB by post bleach method was less effective i.e. fluorescent staining (32%), ZN (26%), and Kinyoun (20%). Both Decontamination methods were comparable and we found the NALC-NaOH method to be more effective than Bleach method, as reported previously.^{10,20}

CONCLUSION

In this study, we have concluded that, the Detection rate of *Mycobacterium tuberculosis* was more with Fluorescent microscopy as compared to the others and which is followed by ZN and Kinyoun staining. Therefore, under RNTCP, LED Fluorescent microscopy has been phased in as a substitute for traditional ZN microscopy. Fluorescent staining method is more effective and saves time and at the same time, a large number of slides can be screened in a single day. Also, the detection rate of Acid-fast bacilli was remarkably raised after decontamination by the NALC method by all the three staining techniques (ZN, kinyoun and fluorescent staining) as compared to Bleach method. Hence, decontamination by NALC method can be a very useful alternative to increase the yield of *Mycobacterium tuberculosis* from sputum specimens in a resource-limited setting as we are facing in a developing country like India which is still lagging behind the western world in terms of health infrastructure.

Table 1: Results of Sputum smear microscopy by three staining

Staining Methods	No. AFB Detected prior Decontamination (n=100)	No. AFB Detected Post-Decontamination with NALC-NaOH Method (n=100)	No. AFB Detected post-Decontamination with Bleach Method (n=100)
Zeihl-Neelson Staining	25 (25%)	30 (30%)	26 (26%)
Kinyoun Staining	20 (20%)	23 (23%)	20 (20%)
Fluorescent Staining	27 (27%)	37 (%)	32 (32%)

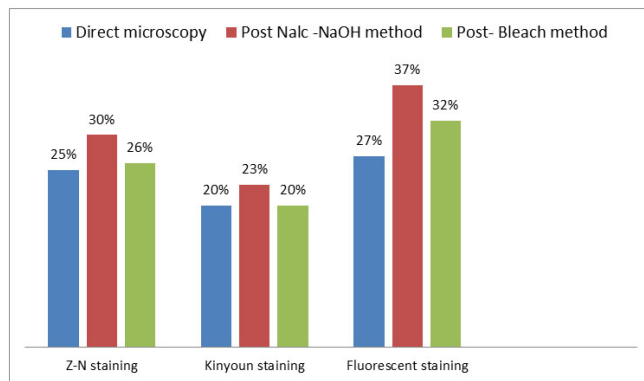


Figure 1: Effect of direct microscopy and decontamination methods on staining observation.

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