Comparative Evaluation of Flourescent Staining with Ziehl-Neelsen and Kinyoun Staining in the Diagnosis of Clinically Suspected Cases of Pulmonary Tuberculosis

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ABSTRACT

Introduction: Tuberculosis still remains one of the world's deadliest communicable disease. India contributes to about one fourth of the global incident case of the tuberculosis. Among the various detection methods available for detecting *Mycobacterium tuberculosis*, sputum smear microscopy is the most simple, rapid and cheapest method. Decontamination and concentration of sputum samples by N-acetyl-L-cysteine (NALC) increase the bacteriological recovery by concentrating the bacilli. Objective: To detect *Mycobacterium tuberculosis* cases before and after NALC decontamination by Ziehl-Neelsen (ZN) staining, Kinyoun staining, Fluorescent staining ((Auramine-O) and compare the case detection rates of these staining methods.

Material and Methods: Two sputum samples were collected from 100 cases of clinically suspected Pulmonary tuberculosis. Total 200 sputum samples were taken. Each sample was divided in two parts. One part of the sample was decontaminated by NALC method and subjected to ZN, Kinyoun and fluorescent (Auramine-O) staining and the other part was subjected to staining ditectly without decontamination.

Results: Out of the three staining methods, fluorescent (Auramine-O) staining has the highest case detection rates (26%) followed by ZN staining (22%) and kinyoun staining (20%). Decontamination by NALC, increases the rate of detection rate of *Mycobacterium tuberculosis* cases by 6% in ZN staining, by 10% in kinyoun staining and by 6% in fluorescent staining.

Conclusion: This study concluded that flourescent staining has higher case detection rate of pulmonary tuberculosis as compared to ZN and kinyoun staining methods. Decontamination by the NALC method increases the detection of case positivity rate of *Mycobacterium tuberculosis*.

Keywords: Ziehl Neelsen staining, Kinyoun staining, Fluorescent staining, Auramine-O, N-acetyl-L-cysteine (NALC), Mycobacterium tuberculosis.

INTRODUCTION

Tuberculosis remain a burden to mankind since 18th century. India is the second-most populous country in the world, yet it contributes to one fourth of the global incident TB cases annually. As per Annual Status Report, Revised National TB Control Programme (RNTCP) 2014, in 2012, out of the estimated total global annual incidence of 8.6 million TB cases, 2.3 million have occurred in India.[1] Tuberculosis is transmitted by inhalation of infected droplet nuclei discharged in the air when an infected and untreated sputum positive patient coughs, sneezes or even while talking. About one third of the world's population has latent TB. The chances of developing TB in those infected with TB bacteria is around 10%.[2]

In India, RNTCP was started in 1997. The program follows the WHO recommended Directly Observed Treatment Short Course (DOTS) strategy to develop ideas and data on TB treatment.[3] "The RNTCP in 2010 made a major policy decision that it would change focus and adopt the concept of Universal Access to quality diagnosis and TB treatment for all TB patients".[4]

Traditional methods for detection of *Mycobacterium tuberculosis* that can be used to improve sensitivity of detection of *M. tuberculosis* are limited by a long processing time. Newer molecular techniques like PCR, though rapid, are very expensive for wide use in developing countries like India due to limited sources. An alternative to culture is optimization of the sputum smear process and evaluation of the smear by various techniques to improve the diagnostic efficacy. In such a perspective the traditional methods of staining including the conventional Ziehl-Neelsen (ZN) stain and use of fluorescent labeled dye Auramine-O still remain the major tools of a clinical microbiologist in developing countries.

The WHO recommends the collection and examination of two sputum samples instead of three for the diagnosis of pulmonary tuberculosis.[5] WHO recommends that conventional fluorescence microscopy be replaced by LED microscopy and that LED microscopy be phased in as an alternative to the conventional ZN microscopy in all small as well as big laboratories.[6]

Most specimens received for Mycobacterial culture consist of organic debris, such as mucin, tissue, serum and other proteinaceous material contaminated with unwanted organisms. Laboratories must process these specimens to kill or reduce contaminating bacteria that can rapidly outgrow *Mycobacteria*, and to release trapped *Mycobacteria* from cells and mucus. The decontamination process and subsequent centrifugation helps to concentrate the *Mycobacteria* which can be detected more easily by staining or culture. A mucolytic agent N-acetyl-L-cysteine (NALC) combined with sodium hydroxide is the preferred method for the digestion step because it is the least toxic to the mycobacteria, and therefore provides the highest yield of positives.[7]

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Keeping in mind the above mentioned scenario, this study was conducted to detect Mycobacterium tuberculosis in clinically suspected cases of pulmonary tuberculosis by using various staining methods including Ziehl-Neelsen staining, Kinyoun and Fluorescent staining before and after decontamination by NALC method and compare the detection rates of these staining methods.

MATERIAL AND METHODS

This was a cross sectional study conducted in the Department of Microbiology, Maulana Azad Medical College in conjunction with the Chest TB Clinic of associated Lok Nayak Hospital, New Delhi for a period of 1 year.

Hundred (100) cases of clinically suspected pulmonary tuberculosis who had visited the chest clinic with sign and symptoms of cough more than 2 weeks/ fever/weight loss/ loss of appetite or positive chest X –ray were enrolled in the study after taking proper counseling and informed consent. All the relevant details were taken in a pre-designed Performa. Extra pulmonary tuberculosis cases were excluded from the study. Ethical approval was obtained from the institutional ethical board before the start of the study.

Two sputum samples (5-10ml) were collected from each case in sterile leak proof containers, one spot and the other early morning. Total 200 sputum samples were collected. Samples were transported quickly to the tuberculosis laboratory. These specimens were processed by conventional standard laboratory techniques.

The collected samples were divided in 2 parts, one part was decontaminated by NALC method and then subjected to ZN staining, Kinyoun staining and fluorescent (Auramine-O) staining. The other part was subjected directly to these three staining methods without decontamination.

Acid-fast bacilli (AFB) were seen as red, beaded and slightly curved rod against a bluish background. The smears were then graded depending on the number of bacilli observed under 100 oil immersion fields. RNTCP grading system was followed for ZN and kinyoun staining.

RNTCP grading 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, 100 fields should be examined.

In Fluorescent microscopy technique Mycobacterium tuberculosis is identified by their bright greenish yellow appearance against a dark background.

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

STATISTICAL ANALYSIS

Data was analyzed by using SPSS version 17.0. Results were expressed as total percentages/ proportions.

RESULTS

Out of the total hundred (n=100) cases, 60% were males and remaining 40% were females. The age of the patients ranged from 18 years to 90 years. Maximum number of cases (n=48; 48%) were in the age group 18-30 years. Most common complaints by the patients was cough (100%) followed by fever (88%), anorexia (64%) and weight loss (38%).

In this study 16(16%) cases were positive for AFB and 84 (84%) cases were negative for AFB by ZN stain before decontamination by NALC. Twenty two (22%) cases were positive for AFB and 78(78%) cases were negative for AFB by ZN stain after decontamination by NALC. Ten (10%) cases were positive for AFB and 90(90%) cases were negative for AFB by Kinyoun stain before decontamination by NALC method. 20(20%) cases were positive for AFB and 80 (80%) cases were negative for AFB by Kinyoun staining after decontamination by NALC method. Auramine-O Staining showed positive for acid fast bacilli in 20(20%) cases and negative in 80(80%) cases before decontamination. In this study Auramine-O Staining after decontamination showed positive for acid fast bacilli in 26(26%) cases and negative in 74 (74%) cases (Table-1).

In this study ZN stain showed positive for acid fast bacilli in 16 cases equally by both methods (before and after decontamination), however 6 cases were positive for acid fast bacilli after decontamination. There were 78 cases which were negative for acid fast bacilli by both. Kinyoun Staining showed positive for acid fast bacilli in 10 cases equally by both the methods (before and after decontamination). However 10 cases were positive for acid fast bacilli after decontamination. Auramine-O Staining showed positive for acid fast bacilli in 20 cases equally by both the methods (before and after decontamination). However 6 cases were positive for acid fast bacilli after decontamination (Table-2).

In this study, 14 cases were 1+, two case showed scanty and 84 were negative for AFB by Ziehl-Neelsen Staining before decontamination. Ten cases showed 1+ and 90 cases were negative for AFB by Kinyoun's Staining before decontamination by NALC. In this study, 2 cases showed 3+, 4 cases showed 2+, 16 cases were 1+ and 78 were negative for AFB by Ziehl-Neelsen Staining after decontamination. Two cases showed 3+, 14 cases showed 2+, 4 cases showed 1+ and 80 cases were negative for AFB by Kinyoun Staining after decontamination by NALC method (Table-3).

In this study, 20 cases were 1+ and 80 were negative for AFB by Auramine-O Staining before decontamination. In this study, 2 cases showed 3+, 10 cases showed 2+, 14 cases showed 1+ and 74 cases were negative for AFB by Auramine-O Staining after decontamination (Table-4).

Comparison of case detection by three different staining methods

The maximum no of positive cases after decontamination was detected by Auramine-O stain, 26 (26%) cases followed by ZN staining detected 22 (22%) cases and Kinyoun staining detected

Staining methods	Results	Before decontamination No. (%)	After decontamination No. (%)
Ziehl-Neelsen	Positive	16(16%)	22(22%)
	Negative	84(84%)	78(78%)
Kinyoun	Positive	10(10%)	20(20%)
	Negative	90(90%)	80(80%)
Flourescence (Auramine -0)	Positive	20(20%)	26(26%)
	Negative	80(80%)	74(74%)

Table-1: Microscopy

staining methods	Before decontamination	After decontamination	
		positive	negative
Ziehl-Neelsen	Positive	16	0
	Negative	6	78
Kinyoun	Positive	10	0
	Negative	10	80
Flourescence (Auramine-O) Staining	Positive	20	0
	Negative	06	74

Table-2: Microscopy before decontamination and after decontamination by NALC

Grading	Before decontamination				After decontamination			
	ZN staining		Kinyoun staining		ZN staining		Kinyoun staining	
	No.	Percentage (%)	No.	Percentage (%)	No.	Percentage (%)	No.	Percentage (%)
3+	0	0	0	0	2	2	2	2
2+	0	0	0	0	4	4	14	14
1+	14	14	10	10	16	16	4	4
scanty	2	2	0	0	0	0	0	0
negative	84	84	90	90	78	78	80	80

Table-3: Results of sputum smear microscopy by ZN staining and Kinyoun Staining methods (before and decontamination by NALC method)

Grading	Before decontamination		After decontamination	
	No.	Percentage(%)	No.	Percentage(%)
3+	0	0	2	2
2+	0	0	10	10
1+	20	20	14	14
Negative	80	80	74	74

Table-4: Results of sputum smear microscopy by Auramine-O Staining before and after decontamination by NALC method

the least i.e 20 (20%).

The total no. of positive cases before decontamination by Auramine-O stain, ZN stain and Kinyoun stain was 20 (20%), 16 (16%) and 10 (10%) respectively.

DISCUSSION

Ziehl-Neelsen staining is a simple, rapid, easy to perform, low cost diagnostic technique and therefore it forms the mainstay for the demonstration of acid fast bacilli in sputum smears. However it lacks sensitivity as it requires at least 10,000 bacilli/ml of sputum for a positive result on direct microscopy. Microscopy is relatively simple, inexpensive and is widely accepted as the first line of diagnosis.[8]

In the present study, on microscopy after ZN staining, 16(16%) cases were positive for AFB and 84 (84%) cases were negative for AFB by ZN staining before decontamination. 22(22%) cases were positive for AFB and 78(78%) cases were negative for AFB by ZN stain after decontamination by NALC method, clearly showing an incremental yield of 6 (6%) more cases after decontamination. Before decontamination, majority of the slide positive cases, 14 (14%) were 1+, 2 cases showed scanty bacilli. After decontamination, 16 (16%) cases were 1+, 4 cases (4%) were 2+ and 2 cases was 3+.

Decontamination helped in increasing the isolation of tubercle bacilli from sputum specimens. In our study, sputum samples were decontaminated by NALC method. It acts as a mucolytic agent, concentrates the bacilli, significantly increasing the detection rate. One study performed at Dhaka, where an extra 14 (1.5%) samples were positive on concentrated method which were negative on direct smear. The sensitivity of direct and concentrated smear microscopy was different when using positive culture as the gold standard (71% vs. 83%).The results showed that concentrated technique increased the sensitivity of microscopy up to 12%.[9]

In another study by Purusothaman K et al, it was found that out of 145 specimens that were examined, 77(53.1%) were positive by Ziehl-Neelsen method, and 67(46.21%) by cold staining method, taking culture as the gold standard.[10] The ZN staining has been shown to be highly specific in areas with a high prevalence of TB but with varying sensitivity (20– 80%).[11]

Kinyoun staining (Cold staining) method has the advantage of being simple, economical and less cumbersome and in the procedure of staining, heating, a much too precise step in ZN method for large scale application, is eliminated. ZN requires a precise heating control and experience on the part of the laboratory technician. Overheating may char the smear and under heating may not be sufficient for the bacilli to take up the stain and they can lead to false negative results.

In our study, 10 (10%) cases were positive for AFB and 90 (90%) cases were negative for AFB by Kinyoun staining before

decontamination. After decontamination, 20(20%) cases were positive for AFB and 80(80%) cases were negative for AFB by Kinyoun staining, thereby detecting 10(10%) additional cases. All the 10 positive cases before decontamination showed 1+ grading, whereas after decontamination 14 (14%) cases were 2+, four (4%) cases were 1+ and two cases (2%) showed 3+ grading respectively.

In one study, conducted at Meerut, UP, India, it was found that the 2 step cold stain method was found to be equally sensitive as the ZN method, when the primary stain was kept for a period of 20 min. Out of 1836 samples examined, AFB was detected by traditional ZN method in 368 (20.0%). Interestingly, the cold staining method was equally sensitive in detecting all the 368 samples when the primary stain was kept for a period of 20 minutes. However, when the primary stain was kept for 10 minutes as performed by Gokhale et al. (1990), they could detect only 342 (18.62%) samples positive. In each of the 26 samples which were missed by Cold staining 10 minutes method, the AFB smear showed lower concentration of bacteria (that is, scoring 1+ and scanty bacilli).[12]

The main advantage of Kinyoun staining (cold method) is that, it does not require heating of the slides, so it helps to omit the need for rectified spirit. Here the heat fixation of smears can be achieved using any source of dry heat such as the closed lid of a boiling sterilizer or even a hot plate and this makes it convenient even in remote peripheral laboratories. Also this method is very simple and no expertise is required to perform it. Other advantages of this method are, the morphology of *Mycobacteria* is well preserved and it can also be used in large volume laboratories where large no. of slides can be quickly and easily stained.

Fluorescent staining (Auramine-O stain): The main disadvantage of ZN staining is its low utility in HIV- TB co-infected patients and extrapulmonary TB. It also has low sensitivity of about 20% -80% only though highly specific. Fluorescent microscopy has better sensitivity and it takes lesser time to examine a slide as compared to ZN staining. The LED Fluorescent microscopy was more sensitive than conventional ZN microscopy and it had qualitative, operational and cost advantages over both conventional fluorescence and ZN microscopy.[11]

In our study, 20(20%) cases were positive for AFB and 80(80%) cases were negative for AFB by Auramine-O stain before decontamination. After decontamination by NALC method, the case detection increased to 26 (26%) cases and 74 (74%) cases were negative for AFB by Auramine-O stain. Before decontamination, all the positive cases, 20 (20%) were 1+. After decontamination, 14 (14%) were 1+, 10 (10%) were 2+ and 2 (2%) showed 3+ grading.

In a similar study conducted by Ben *et al* in 2008, showed that out of 221 sputum samples, 33(14.9%) samples were positive with Auramine staining and 24 (10.85%) samples were positive with Ziehl-Neelsen staining. It demonstrated superior diagnostic results by fluorescent microscopy when compared with conventional light microscopy.[13]

In another study by Laifangbam *et al* in 2009 revealed a study on 102 suspected patients, where in 44.1% patients were positive with Ziehl-Neelsen staining and 71.6% patients were positive for Auramine-O staining. Auramine-O staining was also able to detect more pauci-bacillary cases than ZN staining. It too demonstrated the superiority of Auramine-O staining over Ziehl-Neelsen staining.[14]

In another study by Saroj et al in 2010 demonstrated the superiority of Auramine-O staining over Ziehl-Neelsen staining. It stated that out of 634 sputum samples collected, 66 (10.41%) and 105 (16.56%) sputum samples were found positive for AFB by ZN staining and Auramine-O staining respectively.[15]

In our study, the case detection rate of mycobacterium tuberculosis by Flourescent microscopy, ZN, Kinyouns staining were 26%, 22% and 20% respectively. The diagnostic accuracy of fluorescent microscopy was found to be superior and much more sensitive than the conventional light microscopy. It is therefore recommended that fluorescent microscopy be phased in as an alternative to conventional ZN light microscopy in both high and low level laboratories.

CONCLUSION

In conclusion, the total number of positive cases were significantly raised after decontamination by the NALC method by all the 3 staining techniques (ZN, kinyoun and fluorescent staining). Therefore decontamination by NALC method can be a very useful and cheap substitute to increase the yield of *Mycobacterium tuberculosis* from sputum specimens in a resource limited setting. After comparing the three different staining techniques, the maximum no. of cases was detected by the fluorescent (Auramine-O) staining followed by Ziehl-Neelsen staining and then Kinyoun staining. Also the fluorescent staining method greatly saves time and a large number of slides can be screened per day.

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