Document heading doi: 10.21276/apjhs.2019.6.4.8 **Original Research Article**

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

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ABSTRACT

Background: Bacteriological examination of sputum is the cornerstone in diagnosis of pulmonary tuberculosis in developing world, which is usually done using a Ziehl-Nelseen (ZN) method. However, due to limited laboratory facilities that can satisfy the procedure, applicability of this procedure appears to be adversely affected in field conditions and at peripheral health institutions. Hence, it has become necessary to look for a procedure which can be used as alternative in such conditions. **Material and Methods:** This was a cross sectional study conducted in the Department of Microbiology, Index Medical College Hospital & Research Centre, Indore, Madhya Pradesh in conjunction with the Chest TB Clinic of Index Hospital, New Delhi for a period of 1year [from February 2018 to January 2019]. 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 and Kinyoun staining. 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, 67% were males and remaining 33% were females. The age of the patients ranged from 18 years to 73 years. Maximum number of cases (n=37; 37%) were in the age group 18-30 years followed by 29 cases (n=29; 29%) were in the age group 31-40 years. Most common complaints by the patients was cough (100%) followed by fever (83%), anorexia (69%), weight loss (44%), expectoration (37%) and hemoptysis (17%). Of the 100 direct smears examined 55 (55%) were positive by the cold staining methods in contrast to 62 (62%) by the conventional ZN method (Table 2). Statistically using the unpaired students 'T' test method there was no significant difference between the results of the two methods ($p > 0.5$). **Conclusion:** This study concluded that kinyoun staining has almost similar case detection rate of pulmonary tuberculosis as compared to ZN staining method. Decontamination by the NALC method increases the detection of case positivity rate of *Mycobacterium tuberculosis.*

Keywords: Pulmonary Tuberculosis, Suspected Case, Acid fast bacilli, Ziehl-Neelsen Staining, Kinyoun Staining

INTRODUCTION

India accounts for about a quarter of the global TB burden. Worldwide India is the country with the highest burden of both TB and MDR TB. [1]

There are an estimated 79,000 multi-drug resistant TB patients among the notified cases of pulmonary TB each year. India is also the country with the second highest number (after South Africa) of estimated HIV associated TB cases.

In 2016 an estimated 28 lakh cases occurred and 4.5 lakh people died due to TB.[2] India also has more than a million "missing" cases every year that are not notified and most remain either undiagnosed or

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unaccountably and inadequately diagnosed and treated in the private sector. There are some more TB statistics for India. [2]

Staining Characteristics of *Mycobacteria*

Acid-fast stains or other procedures are necessary for demonstrating the bacilli, which are not visible in hematoxylin and eosin-stained preparations. Acid-fast stains include the Ziehl-Neelsen stain, the Kinyoun stain, and the Fite stain. Due to their waxy cell wall components, the bacilli of MTB are acid fast; that is, they retain the red dye, carbol fuchsin, after rinsing with acid solvents. Often, the bacilli have a beaded appearance. Detection of one or more mycobacteria in an area of granulomatous inflammation is highly specific and indicative of infection. [3,4] Unfortunately, however, over 100 mycobacteria per milliliter of tissue are usually necessary before the organisms can be visualized by light microscopy, so a negative stain does not exclude a diagnosis of TB. Also, mycobacteria cannot be speciated based on morphologic features; other techniques (culture, molecular assays) must be used to determine the mycobacterial species. *Mycobacteria* inconsistently stain with a Gram stain. In some cases, mycobacteria can be stained by Gomori methenamine silver stain or periodic acid–Schiff stain. Fluorochrome stains are more sensitive, though not as specific, but may be useful for screening of the tissue specimens. These include auramine-O and the rhodamine stains. Organisms visualized using Gomori methenamine silver or periodic acid–Schiff or fluorochrome stains should be confirmed by a Ziehl-Neelsen or Kinyoun stain.[3]

Acid Fast Strain.[3]

Acid fast stains are used to differentiate acid fast organisms such mycobacteria. Acid fast bacteria have a high content of mycolic acids in their cell walls. Acid fast bacteria will be red, while nonacid fast bacteria will stain blue/green with the counter stain with the Kinyoun stain. The steps include:

Step 1. Apply carbol fuchsin to a fixed slide for 1 minute followed by rinsing.

Step 2. The decolorizing agent, 3% hydrogen chloride (HCl), is applied for 2 minutes and remove the primary stain and rinse.

Step 3. Apply the counterstain, methylene blue, for 2 minutes then rinse.

Step 4. Allow to dry and observe slide with a light microscope.

Ziehl Nielson can also be used to stain mycobacteria but uses heat while the Kinyoun method does not. The Kinyoun method can be modified as a weak acid fast stain, which uses 5% sulfuric acid instead of hydrochloric acid. The weak acid fast stain in addition to staining mycobacteria will stain organisms that are not able to maintain the carbol fuchsin after decolorizing with HCl, such as *Nocardia* spp., *Rhodococcus* spp., *Tsukamurella* spp., and *Gordonia* spp. The weak acid fast stain also helps differentiate among the organisms that appear as Gram-positive branching filamentous rods such as **Nocardia** spp. and *Streptomyces* spp. Nocardia will stain positive with a weak acid fast stain and **Streptomyces** spp. will not. [5,6]In the cold staining technique, Kinyoun used higher concentrations of basic fuchsin and phenol. [7]Tan Thia Hok devised a method by combining the staining techniques of Kinyoun and Gabbet, and found it quicker than ZN method. [8]Vasantha Kumari et a reported a variation of ZN method that permitted cold staining of tubercle bacilli by avoiding the use of heat and by increasing the staining time. We report here a study of these 2 methods using sputum from 100 cases of pulmonary tuberculosis. 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. [9]Graded as per RNTCP guidelines like 3+ = more than 10 AFB/ oil immersion field; 2+ = 1-10 AFB per oil immersion field; 1+ = 10-99 AFB 100 oil immersion field; Scanty = 1-9 AFB per 100 oil immersion field; Negative = no AFB per 100 oil immersion field. [9,10]

MATERIAL AND METHODS

This was a cross sectional study conducted in the Department of Microbiology, Index Medical College Hospital & Research Centre, Indore, Madhya Pradesh in conjunction with the Chest TB Clinic of Index Hospital, New Delhi for a period of 1 year [from February 2018 to January 2019]. 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 counselling 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 [IMCHRC/IEC/2017/44, Dated 20.02.2018]. 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 and Kinyoun staining. Two direct smears were prepared from the thickest portion of each sputum sample One slide was stained by the ZN method while the other was stained by the cold staining method. Data was analyzed by using SPSS version 17.0. Results were expressed as total percentages/ proportions.

RESULTS

Table 1: Demographic and clinical characteristics of suspected pulmonary TB cases [n=100]

Age (in years)	Male [n=67] (%)	Female [n=33] (%)	Total (%)
18-30	25%	12%	37%
31-40	20%	9%	29%
41-50	11%	6%	17%
51-60	5%	4%	9%
61-70	3%	2%	5%
71-80	3%	-	3%
Sex	67%	33%	100%
Clinical Symptoms			
Cough	100%	100%	100%
Fever	54 (80.59%)	29 (87.87%)	83%
Anorexia	45 (67.16%)	24 (72.73%)	69%
Weight loss	25 (37.31%)	19 (57.57%)	44%
Expectoration	22 (32.84%)	15 (45.45%)	37%
Hemoptysis	11 (16.42%)	6 (18.18%)	17%
Chest pain	6 (8.95%)	3(9.09%)	9%

Out of the total hundred (n=100) cases, 67% were males and remaining 33% were females. The age of the patients ranged from 18 years to 73 years. Maximum number of cases (n=37; 37%) were in the age group 18-30 years followed by 29 cases (n=29; 29%) were in the age group 31-40 years. Most common complaints by the patients was cough (100%) followed by fever (83%), anorexia (69%), weight loss (44%), expectoration (37%) and hemoptysis (17%) [Table 1].

Table 2: Comparison of the results of direct smears examination of sputum by ZN method and cold staining (Kinyoun stain) method

ZN method	**Cold staining (Kinyoun stain) method**		**Total**
	Positive	**Negative**	
Positive	48 (48%)	14 (14%)	62 (62%)
Negative	7 (7%)	31 (31%)	38 (38%)
Total	55 (55%)	45 (45%)	100

Of the 100 direct smears examined 55 (55%) were positive by the cold staining methods in contrast to 62 (62%) by the conventional ZN method (Table 2). Statistically using the unpaired students 'T' test method there was no significant difference between the results of the two methods ($p > 0.5$) [Table 2].

Table 3: Results of sputum smear microscopy by ZN staining and Kinyoun staining methods

Grade	ZN staining		Kinyoun staining	
	No.	Percentage	No.	Percentage
3+	14	14%	10	10%
2+	20	20%	16	16%
1+	25	25%	27	27%
Scanty	3	3%	2	2%
Negative	38	38%	45	45%

The number of bacilli seen was more by the ZN method since 14 out of 62 smears showed +++ bacilli as compared to only 10 such smears by cold staining method [Table 3]. After decontamination by NALC method, both ZN and cold staining method showed equal positivity and negativity [Table 3].

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. [9,11]. Applicability of the Ziehl-Nelseen technique appears to be adversely affected, especially at peripheral health institutions, due to limited laboratory facilities that can satisfy the procedure.[12] Hence, the present study aimed to evaluate CS method which can be used as alternative in such conditions. In the present study, the interesting finding is that the CS Method has not shown any positive result even on a single specimen which was not positive by the ZN method. Possible explanations for the slight difference in yield of results by the ZN and cold stain could be defined as follows: in the present study, generally, in microscopic examination of smear slides prepared by the cold staining method, it was observed that AFB organisms appeared more delicate, fainter (which is closer to their natural morphology) and less brighter against background than those seen with the Z-N stain, which may be the reason for the false negative results compared with by the Z-N method.[12] Whereas, in Z-N method since there is a better penetration of stain through the complex cell surface structure due to heating effect, organisms appeared brighter against background though there is slight change in morphology. Although it is difficult to conclude, since none of the specimens could be cultured for want of facilities, the ZN could give a few false positive results. Similar observation was seen by other scholars too. [12-14] Throughout the country in all Primary health centers sputum smear microscopic examination successfully implemented by the RNTCP. Due to the presence of unsaponable wax substances in the cell wall of the tubercle bacilli the Z-N method shows major difficulty in staining it requires heat application to the microscopic slide for the uniform penetration of dye in to the cell wall through its waxy barrier.[15] However for this operation possess problem like fairly precise control of the temperature to the slide ,and regular supply of the alcohol or liquid propane gas (LPG) is require for the heating and fixing steps with the Z-N staining method. A desire to develop an alternate staining procedure has resulted in several modifications of the Z-N staining method to overcome this drawbacks. [16,17]In this study, Of the 100 direct smears examined 55 (55%) were positive by the cold staining methods in contrast to 62 (62%) by the conventional ZN method [Table 2]. Statistically using the unpaired students 'T' test method there was no significant difference between the results of the two methods ($p > 0.5$). Neelu Sree P et al [18] study revealed total number of sputum smear-positive samples detected by microscopic examination by ZN staining method was found out to be 76 (8.4%). This result by microscopic examination by ZN staining method could be correlated to the study by Makesh kumar *et al.*, which reports that 12.13% were smear positive for AFB in Kanchipuram district, South India[19]. Lt Col KK Lahiri et al study (1994) revealed that out of the 105 direct smears examined 59 (56.2%) were positive by the cold staining methods in contrast to 65 (61.8%) by the conventional ZN method.[21] Statistically using the unpaired students 'T' test method there was no significant difference between the results of the two method ($p > 0.5$). The number of bacilli seen was more by the ZN method since 13 out of 65 smears showed +++ bacilli as compared to only 7 such smears by cold staining method. After concentration by the Petroffs method, both ZN and cold staining method showed equal positivity of 68.6% and negativity of 31.4%. [20] Decontamination helped in increasing the isolation of tubercle bacilli from sputum specimens. In Lawrence et al study (2016), sputum samples were decontaminated by NALC method. [9] 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. [21] In the present study the number of bacilli seen was more by the ZN method since 14 out of 62 smears showed +++ bacilli as compared to only 10 such smears by cold staining method (Table 3). After decontamination by NALC method, both ZN and cold staining method showed equal positivity and negativity. In Lawrence et al 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. [9]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. [14]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. [9,10]

CONCLUSION

The difference in results obtained by both methods was not very significant and compared well with results of other workers. The cold staining method has the distinct advantage of being a simple procedure which can be carried out easily without the need of trained personnel and it does not require spirit for heating purposes. Although the cold staining method is a longer procedure as compared to the ZN method, its simplicity, low cost and efficiency make it a useful alternative technique in laboratories for early identification of acid fast bacilli. 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.

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