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A Comparison of Ziehl-Neelsen Staining and Fluorescent Microscopy for Diagnosis of Pulmonary Tuberculosis

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Abstract – According to WHO one third of the world population have tuberculosis. The present study was undertaken to compare the efficacy of fluroescent stain with Z-N stain in the diagnosis of pulmonary. 793 cases of suspected pulmonary tuberculosis were included in the study. All samples were screen for Acid Fast Bacilli (AFB) by Z-N & Fluorescent staining methods. Positive samples detected by fluorescent stain were 121(15.22%) when compared to Zn stain 71(8.95%).

Conclusion Compared to Z-N stain (8.95%). flurochrome staining was found to be more efficient (15.22%) in AFB detection of AFB from cases of Pulmonary Tuberculosis. Keywords: acid-fast bacilli; auramine-0; fluorescence; microscopy; tuberculosis; Ziehl-Neelsen.

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INTRODUCTION

Pulmonary tuberculosis is mainly a disease of the respiratory system, caused by *Mycobacterium tuberculosis* .Tuberculosis is a predominant infectious cause of mortality today. Tuberculosis continues to be a major health problem in our country and is the single largest cause of loss in healthy life year in the productive age group. There are various methods for bacteriological diagnosis of tuberculosis. Currently, radiometric assay allows detection of *Mycobacterium tuberculosis* growth and provides antibiotic sensitivity results more rapidly usually within 10 days. *Mycobacterium tuberculosis* strains can be detected directly in the sputum specimen within 2 or 3 hours, but in practice, this method has not become a routine laboratory technique, particularly due to lack of sufficient specificity and sensitivity. Microscopic examination and culture are still essential elements of the bacteriological diagnosis of tuberculosis in microscopic examination; the diagnosis of tuberculosis is confirmed on the basis of demonstration of tubercle bacilli in the sputum or any other pathological material . Smear examination is believed to be simple, cheap, quick and practicable and effective case finding method for developing countries. So, Microscopic examination has the advantage of the giving a result at once. The specimen most commonly examined is sputum and mucous secretion coughed up from the lungs . Microscopic examination of Ziehl-Neelson or auramine stained specimen allows detection of most strains in less than an hour. Ziehl-Neelson is the most extensively used procedure for the demonstration of *mycobacterium tuberculosis* in smear.

The requisites for the staining procedures are; basic fuchsin, phenol, absolute alcohol; sulphuric acid and methylene blue. Fluorescent staining by Auramine is other methods of staining. In this a smear is made from the specimen and stained with fluorescent stain called auramine .The auramine stain enters the wall of *Mycobacterium tuberculosis* bacterial cell and makes them glow against dark background under UV light . Therefore the present prospective study was under taken to see the efficacy of Ziehl-Neelson method versus fluorescent staining in the detection of mycobacterium in sputum sample.

MATERIALS AND METHODS:

This study is conducted at Department of microbiology, Indira gandhi institute of medical science, patna, bihar during the period of april 2013 to march2014

SAMPLE COLLECTION:

Early morning sputum samples were collected in clean, sterile, leak proof, wide mouth containers. The processing of the sample were carried out in a

biosafety cabinet. each sample was subjected to Ziehl-Neelson(ZN) staining and fluorescent Auramine-O (AO)staining

SMEAR PREPARATION;

THE Z-N STAINING METHOD: The staining solutions were prepared for Z-N method , 1%carbol-fuchsin was prepared with 1 gm of basic fuchsin dissolved in 50 ml molten phenol; 100 ml of ethanol (95%)was added to the fuchsin – phenol mixture. The solution was diluted with distilled water to make a volume of 1,000 ml then it was filtered. Decolorizing agent sulphuric acid (25%)was prepared with 250ml concentrated sulphiric acid which was slowly added to 750 ml distilled water. Methylene blue (0.1%) was prepared with 1 gram methylene blue dissolved in100 ml distilled water. Procedure of Z-N staining as perRNTCP guidelines heat fixed smears flooded with 1% carbolfuchsin and heat applied until steam rises but not boiling for 5 minutes. After cooling of slide the smear washed with tap water and decolorize step done by the 25% sulphuric acid for 4 minutes. the slides washed in tap water ,then counter stain with 0.1% methylene blue for 30 seconds, finally smear slides were washed, then air dried.

All Carbol- fuchsin Stained smears were observed under oil immersion a minimum of 10 min under compound microscope. The smears Graded as per RNTCP guidelines 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 = 1-9AFB per 100 oil immersion field; Negative = no AFB per 100oil immersion field.

FLUORESCENT AURAMINE-O STAINING METHOD: *MATERIALS REQUIRED FOR STAINING*

Auramine-Phenol solution

1% Acid alcohol

0.1% Potassium permanganate solution

The 3% stock solution of phenol was prepared with 3 g of phenol crystals dissolving in 87 ml Distilled water. Auramine phenol solution was prepared with warm 100 ml stock of three percent phenol to 40oc. To this add gradually 0.3 gm of Auramine with vigorous shaking for 10 minutes, and it was filtered and stored in a dark brown bottle. Acid alcohol was prepared with 0.5 gm sodium chloride dissolve in 25 ml Distilled water, add 0.5 ml concentrated hydrochloric acid, mix with 75ml absolute alcohol and stored in amber colored bottle**.** 1 gm of Kmno4 is added to 100 ml of distilled Water to prepare 0.1% potassium permanganate. The fluorescence staining method was carried out as follows. Mucopurulent portion sputum was taken for smear, near to the flame by using a broom stick. On a clean or fresh glass slide , at room temperature smear was allowed for air dried, and heat fixed have done by passing the slide over flame 2-3 times for about 2-3 seconds. Flooded the slides with freshly filtered auramine – phenol kept for 20 minutes without heat application .next the smears were washed with tap water, next decolorized by covering completely with acid alcohol for 3 minutes. next washed with tap water then counter stain with 0.1% KMno4 for 1minute and the slides gently rinsed with water and drain.

Auramine –o stained smears observed under LED fluorescentmicroscope in linear pattern approximately a minimum of 2min for 100 fields. The fluorescent stained smears were examined at much lower magnifications (typically 250x) than used for Z-N stained smears (1000x) each field examined under fluorescence microscopy therefore has a large area than that seen with bright field microscopy. Thus a report based on a fluorchrome stained smear examined at 250x may contain much larger number of bacilli than a similar report from the same specimen stained with carbolfuchsin and examined at 1000x . Z-N stain Microscopic examination under oil immersion objective reveals mycobacterium are red bacilli. fluorescence Microscopic examination under low power objective will reveal mycobacteria as glowing yellow white, rice like bacteria in the smear.

Table 1: Comparative grading

RNTCP ZN staining grading (using 100x oil immersion objective and 10x eye piece)	Auramine O fluorescent staining grading (using 20 or 25x objective and 10x eye piece)	Reporting /Grading
>10 AFB/field after examination of 20 fields	>100 AFB/field after examination of 20 fields	Positive, 3+
1-10 AFB/field after examination of 50 fields	11-100 AFB/field after examination of 50 fields	Positive,2+
10-99 AFB/100 field	1-10 AFB/ field after examination of 100 fields	Positive,1+
1-9 AFB/100 field	1-3 AFB/100 fields	Scanty
No AFB per 100 fields	No AFB per 100 fields	Negative

Table 2: Magnification correction factor

S.No.	FM objective magnification (power)	Magnification correction factor*
1	20x	10
2	25x	10
3	40x	5
4	45x	4
5	63x	2

To obtain the comparative grading, divide the observed count of AFB under the FM objective with this factor before grading

Figure 1 shows Acid Fast Bacilli (1+) with flourescent staining. **Figure 2** shows Acid Fast Bacilli (1+) with Zeihl Neelson's staining.

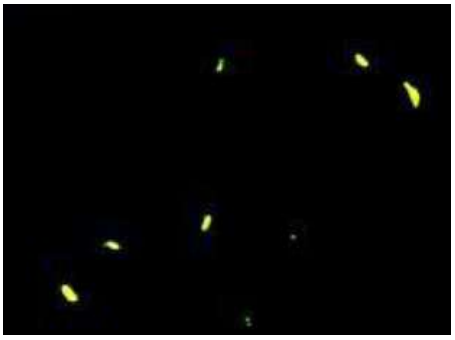

Figure 1

Figure 2

RESULTS

A comparison of the smear results obtained with Z-N method and fluorescence staining Method.

793 sputum samples 71 (8.95%) were positive for AFB with the Z-N method, and 121 (15.22%)were positive for AFB with the fluorescent staining method. 50 samples positive by the Fluorescence staining method were negative by the Z-N method. All samples are read negative by the Z-N method but positive by the fluorescence staining method were found to only have scanty AFB with the fluorescence staining method.

Table I-Result of smear examination by Ziehl-Neelson and Fluorescent staining

	ZN- STAINING	FLOURESCENT STAINING
POSITIVE	71 (8.95%)	121 (15.22%)
NEGATIVE	722 (91.05%)	672 (84.78%)
TOTAL	793	793

Table 2- Comparison of smear examination result by ZN staining and fluorescent staining

	ZN-POSITIVE	ZN-NEGATIVE	TOTAL
Flourescent positive	71	50	121
Fluorescent negative	0	672	672
Total	71	722	793

Table 1 shows, the correlation between the conventional ZN method and the modified fluorescent method. The ZN smear positivity rate and the AO smear positivity rate in this study was 8.95% (71/793) and 15.22% (121/793) respectively.

Table 2: shows 71 samples were both fluorescent and ZN positive, where 50 samples were Fluorescent positive and ZN negative. Out of which 672 samples were both fluorescent and ZN negative. Where none of the sample was ZN positive and fluorescent negative respectively.

DISCUSSION

In the present study we are compeared result of light microscopy of Z-N stain smear with that of fluorescent microscopy of auramine phenol stain smear for detection of AFB out of 793 sample of sputum,15.22% were positive by fluorescent staining &8.95% were positive by Z-N stain smear .these result show fluorescent staining is more sensitive in detection of AF Bin sputum sample as compared to ZN stain. Same result we are obtained by Githui et al8 (80% by fluorescent staining & 65% by Z-N stain). Ulukanligil et al9 also obtained 85.2% positive by fluorescent staining&67.6% by Z-N stain. Similar result obtained by S.J murray et al10 93% by fluorescent staining & 73% by Z-N stain method. K. Prashanthi et al2 69% by fluorescent staining & 50% by Z-N stain method. In this result show fluorescent staining is more sensitive in detection of AFBin sputum sample as compared to ZN stain. this may be because the organisms in fluorescent staining offer much contrast appearing as brilliant- yellow against a more or less darkened back ground.

CONCLUSION

In the present study fluorescence staining methods were compared against Z-N method, The Conclusion in the study there was a highly significant relationship

between the Z-N and Auramine o techniques in the detection of AFB. The Fluorescence staining method used here had a better sensitivity than Z-N in the detection of AFB. In this study both Z-N and Auramine-o techniques can be used in the detection of AFB. The fluorescence microscopy is better than bright field microscopy and there was a good agreement between them but Fluorescence staining method is quite economical in terms of both time and expense. However fluorescence staining technique a method of choice in this study population whenever dealing with large samples and it is more reliable thanZ-N.

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