

# Comparative Study of Z N Staining vs. Flurochrome Staining and Impact of Sample Processing on Diagnosis of Tuberculosis from Various Clinical Samples

Yogita Mistry<sup>1</sup>, Sangita Rajdev<sup>2</sup>, Summaiya Mullan<sup>2</sup>

<sup>1</sup>TB-IRL Laboratory, Government Medical College, Surat, India

<sup>2</sup>Department of Microbiology, Government Medical College, Surat, India

Email: dryogitamistry@gmail.com

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## Abstract

**Background:** Tuberculosis is a highly infectious disease and India has the highest burden with it. Diagnosis of tuberculosis in many countries is still dependent on microscopy. Although its sensitivity is low in comparison to culture and molecular methods, its sensitivity can still be improved by using fluorescence staining method and processing of samples by homogenization and concentration method. **Material and methods:** Samples were collected from all newly registered suspected cases of tuberculosis in tertiary care hospital from outward and indoor department during a period of one year. Smears were prepared for Ziehl Neelsen stain and fluorescence stain both before and after homogenization and concentration procedure by 4% NaOH-2.9% sodium citrate method and results of them were interpreted according to RNTCP criteria for grading of sputum samples. All the samples were cultured in liquid culture MGIT system (Mycobacterial Growth Indicator Tube) and results of microscopy were compared with liquid culture taken as gold standard. Data were analyzed by using SPSS software version 16. **Result:** 350 samples were collected during study period. Out of 350 samples, 48 samples were positive for *M. tuberculosis* by MGIT system. In comparison with MGIT system, sensitivity of Z N stain for detection of acid fast bacilli was 77% before decontamination procedure, which was increased up to 85.42% after decontamination and concentration process. Sensitivity of fluorescence stain was 85.42% before processing, which was increased up to 91.67% after processing of samples. **Conclusion:** Sensitivity of smear microscopy can be enhanced by use of fluorescence microscopy and concentration method.

## Keywords

Ziehl-Neelsen Staining, Fluorescence Staining,

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## 1. Introduction

Tuberculosis (TB) is a leading cause of morbidity and death worldwide, with approximately two billion people infected and approximately two million annual deaths attributable to it. In 2010, there were an estimated 8.8 million incident cases of TB globally, equivalent to 128 cases per 100,000 population, and an estimated 12.0 million prevalent cases of TB. This is equivalent to 178 cases per 100,000 population. Thus, approximately 1.4 million people died of TB in 2010 [1]. India accounts for nearly one third of global burden of tuberculosis, where diagnosis of tuberculosis relies mostly on smear microscopy method due to its simplicity, reliability, cost effectiveness and high specificity but its sensitivity is low in comparison with culture. It needs experienced person to screen the smear, it takes more time to screen negative smear, and it often misses the paucibacillary TB cases especially when the patient is co-infected with HIV [2].

Another microscopical technique which is available now is fluorescence staining, it is simpler and more rapid as it screens the smear in 40 $\times$ . This advantage would be more beneficial in overburden laboratories in low resource settings where culture facilities are not available. Even the cost of this microscopy method can be reduced by use of LED microscope instead of fluorescence microscope which contains the bulb with life up to 50,000 hours [3].

Also processing of samples for decontamination and concentration method can also increase the sensitivity of Z N stain and fluorescence stain as compared to direct smear preparation from slide.

The aim of this study was to compare both Z N stain and fluorescence stain and also to compare the results of smears both before and after decontamination procedure.

## 2. Material and Methods

Study was planned after getting ethical approval for the same for one year period from newly registered suspected cases of tuberculosis from outdoor and indoor department of tertiary care hospital. From all the samples smears were prepared for Z N and fluorescence stain using Carbol fuchsin-phenol and Auramine phenol stain respectively. Then all the samples were processed for homogenization and concentration method by using 4% NAOH-2.9% sodium citrate method. This process was done in biosafety cabinet with all personal protective measures. Equal volume of 4% NAOH and 2.9% sodium citrate were mixed to make final concentration of NAOH 2%. Samples which were collected in wide mouthed sterile container were transferred in to falcon collection tube of 50 ml capacity, in to which equal amount as that of sample is added of mixture of NAOH and sodium citrate were added and tubes were left at for 20 minutes at room temperature. After which phosphate buffer solution (pH 6.8, 0.067 M) was added to neutralize the pH and then samples were centrifuged. Supernatant was discarded and in

from deposit smear were prepared for ZN and fluoro-chrome staining. Results of the smears were graded by RNTCP criteria for both the staining technique. Also all the samples were cultured in liquid culture MGIT system (Mycobacterial Growth Indicator Tube system). For which 800 µl PANTA-OADC supplement is added in MGIT tube containing 7 ml of Middlebrook 7H9 broth, then 500 µl of processed sample was inoculated within 30 minutes with all aseptic precaution to reduce contamination and tubes were incubated in 37°C incubator and reading are taken daily in first 3 weeks and twice a week in next 3 weeks by using semi-automated MGIT reader (micro MGIT) which was standardized using Micro-MGIT caliber. Growth of *M. tuberculosis* was appear as granular turbidity while uniform turbidity suggest contamination which were again decontaminated and cultured in MGIT system. Positive MGIT tubes results were also confirmed by smear microscopy. Data were analyzed using SPSS software version 16.

### 3. Result

Out of 350 samples, 216 (61.71%) were sputum samples, other samples were 115 (32.85%) pleural fluid, 3(0.85%) lymph node biopsy, 5 (1.42%) ascetic fluid, 6 (1.71%) CSF, 4 (1.14%) pus and 1 (0.28%) pericardial fluid. 256 (73.14%) samples were from outpatient department. Direct smear microscopy result of all samples by Z N stain had shown 37 (77.0 %) samples positive, out of which 4 were pleural fluid and 33 were sputum sample. While 41 (85.41%) samples were positive by fluorescence staining method out of which 6 were pleural fluid and 35 were sputum sample. When both the methods were compared it had shown a statistically significance difference in rate of positivity by fluorescence staining when tested by fisher extract/mid P extract with p value <0.05.

Smear results after homogenization and concentration method by Z N stain had shown 41 (85.41%) samples to be smear-positive out of which 6 were pleural fluid while 35 were sputum sample. while fluorescence stain had shown 44 (12.57%) positivity out of which 7 were pleural fluid while 37 were sputum sample. When result of both the process was compared it had shown statistically significance difference when tested by fisher extract test with p value 0.05. Smear which were missed by Z N stain were mostly paucibacillary (smear grading with scanty or +1) with less bacterial load.

Out of 350 samples, 48 samples positive by MGIT. In comparison with liquid culture sensitivity of ZN stain was 77% which was increased by 85.92% after decontamination processing. Sensitivity of fluorescence stain was 85.92% before processing which is equal to sensitivity of ZN stain after processing. Sensitivity of fluorescence stain after processing is 91.67%. Specificity of both staining technique is equal (98.55%).

### 4. Discussion

In the present study we compared results of light microscopy by Z N stain smear with that by fluorescent microscopy of auramine phenol stain smear for detection of AFB. These results shows fluorescence staining technique is more sensitive in detection of AFB in sputum as well as extra pulmonary samples compared to ZN stain. Smear result obtained by Githui *et al.* [4] had shown 80% by florescent microscopy & 65% by Z N

staining; Ulukanligil *et al.* [5] also demonstrated 85.2% positivity by fluorescence microscopy and 67.6% by Z N method. Similar result obtained by S J Murry *et al.* [6] had shown 93% positivity by FI microscopy and 73% by Z N staining. Jain *et al.* [7] had shown 41% by FI and 32% by Z N stain.

All these results show that fluorochrome staining method is more sensitive as compared to Z N staining method for demonstration of AFB. This may be due to appearance of brilliant yellow colored bacilli against more dark back ground which is easily observable.

Sputum smear microscopy is a simple, rapid and inexpensive technique which is highly specific in areas with a very high prevalence of tuberculosis. It also identifies the most infectious patients and is widely applicable in various populations with different socioeconomic levels. Hence, it has been an integral part of the global strategy for TB control [8].

In comparison with Z N stain fluorescence microscopy which was introduced in 1930, requires the fluorochrome dye, halogen or high pressure mercury lamp to excite the dye. It is more sensitive in screening the low grade smear positive sputum samples and also extra pulmonary samples and samples from HIV patients as compared to Z N staining. As it screens the smear in low power objectives, time required to screen the smear is less as compared to Z N stain which requires at least 10 - 15 minutes screening one negative smear, which is important in high burden countries like India with high work load. Cost constraints are major issues with fluorescent microscopy. This may be circumvented by the use of light emitting diodes (LEDs) which cost less than 10 per cent of a mercury vapor lamp. With a life >50,000 h, it can run on batteries and thus has been used in peripheral areas with definite operational advantages. Although sensitivity of fluorescence microscopy is 10 percent higher than Z N stain, specificity is same as Z N stain and chances of false positivity are more in fluorochrome stain while chances of false negativity are more with Z N stain. Overall microscopy method results are affected by type of sample, quality and quantity of sample, thickness of smear, experience of observer and sensitivity is low in comparison to culture method and molecular method [8].

As Z N stain will be positive with bacterial load of  $10^5$ /ml and fluorochrome  $10^4$ /ml of sample. So aim of this was to increase the of both the microscopy result especially in low grade positive smear and also in extra pulmonary sample with less bacterial load. Use of concentration method had yielded higher positivity of microscopy as compared to direct microscopy. This is due to concentration of sample, increase in number of bacilli per field as well as clean field without debris in which mycobacteria may be trapped sometimes. Any decontamination and concentration method can be used based on requirement. If we have to concentrate the sample only for smear microscopy then we can use the method that can kill the bacilli, so it can limit the laboratory infection. If we have to do culture from processed sample then we have to use Petroff's method or any method that will kill only contaminant bacilli and normal flora and that does not harm or damage the mycobacteria or its growth in culture medium either in liquid or on solid culture medium. Although its implication will depend on availability of biosafety

cabinet, centrifuge, requires standardization of process. But still we can hope its implication for better management of patients [9] [10].

## 5. Conclusion

Sensitivity of smear microscopy for diagnosis of tuberculosis can be enhanced by use of fluorescence microscopy over light microscopy using ZN stain and concentration method which can help in diagnosis of tuberculosis. As it is cost effective, it can be applied for use in countries like India. LED microscope has made the use of fluorescence microscopy more convenient.

## Disclosure of Interest Statement

The present study was funded from research funds of the authors. No pharmaceutical grants were used for this study.

## Ethical Clearance

Approved by HREC committee of Government Medical College, Surat.

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