



## A COMPARATIVE STUDY OF ZIEHL NEELSEN, GABBET'S AND FLUORESCENT STAINING METHODS TO DETECT ACID FAST BACILLI IN SPUTUM SAMPLES.

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### ABSTRACT

Tuberculosis remains world's leading cause of death from a single infectious agent. Pulmonary tuberculosis (PTB) is the most common presentation of tuberculosis. Here we compare Ziehl-Neelsen (ZN), Gabbet's and Fluorescent staining (FS) methods to determine efficiency and cost effectiveness of diagnosing pulmonary tuberculosis from sputum samples. Three smears from 148 sputum samples of PTB cases were prepared and stained by the above methods. Of the samples examined, 71.6% and 53.4% and 85.8% cases were detected by Ziehl-Neelsen, Gabbet's and Fluorescence staining respectively. Fluorescent staining was found to be more reliable and cost effective method particularly when dealing with large sample loads than Ziehl-Neelsen and Gabbet's staining technique.

**KEYWORDS** : Pulmonary tuberculosis, Ziehl-Neelsen, Fluorescent staining, Gabbet's staining.

### Introduction:

Tuberculosis, the captain of death, has been and is still lingering as a major threat to human health.<sup>1</sup> World Health Organization (WHO) in 1993 declared tuberculosis as a global emergency and its prevalence in India is of paramount significance as it is said to be a leading cause of death.<sup>2</sup>

Robert Koch identified and described the tubercle bacilli as a causative agent of tuberculosis on March 24<sup>th</sup> 1882 for which he received the Nobel Prize for his discovery. These acid fast bacilli (AFB) can cause pulmonary tuberculosis as well as extra pulmonary tuberculosis like, tuberculous lymphadenitis, pleural effusion, bone and joint tuberculosis, meningitis, enteritis, peritonitis, genitourinary tuberculosis, pericarditis and miliary tuberculosis.<sup>3</sup>

Sputum microscopy is useful to assess the success of treatment and to affirm whether patient is cured or not. The principle of staining is that *Mycobacteria* retain the primary stain even after exposure to a strong decolorizing agent, hence called acid fast.<sup>1</sup>

Sputum examination by microscopy is the cornerstone to diagnosis and treatment of pulmonary tuberculosis. This study envisages the need to analyze the pros and cons of currently available staining techniques for *Mycobacterium tuberculosis* from sputum samples and suggest a cost effective method for routine diagnosis both in urban and rural health care facilities.<sup>4</sup>

Microscopic examination of sputum for detection of acid fast bacilli is of utmost importance. The WHO or Revised National Tuberculosis Control Program (RNTCP) states an individual with at least one sputum smear positive for AFB or culture positive for tubercle bacilli is labeled to be suffering from pulmonary tuberculosis. In developing countries like India with deficit of resources and high TB burden, culture facility is not adequately available. Hence most of the PTB cases are diagnosed based on Sputum Smear Microscopy (SSM).<sup>4</sup>

### Materials and methods:

148 well coughed up early morning sputum samples were collected from adults with lower respiratory tract infection with a clinical suspicion of pulmonary tuberculosis. Three smear from each sample were prepared, stained by the three methods, first using ZN staining, second and third by Gabbet's and Fluorescent stain respectively and then screened by direct light microscopy in case of ZN and Gabbet's stain and Fluorescent microscopy in case of fluorescent stain for the acid fast bacilli. Grading of the sputum

smears was done as per the current RNTCP guidelines which is different for ZN / Gabbet's and FS.

In ZN staining heat fixed smears flooded with 1% strong carbol fuchsin and heat applied until steam rises but not boiling for 5 minutes. After cooling of slide the smear washed with tap water and decolorized by 25% sulphuric acid for 4 minutes. The slides were washed in tap water then counter stain with 0.1% methylene blue for 30 seconds, finally smear slides were washed, then air dried and observe under oil immersion objective of light microscope for AFB.

For Gabbet's method heat fixed smears were flooded with strong basic fuchsin phenol solution and allowed to stand for 10 minutes without heat application. The smear was then washed with tap water, then decolorized and counter stained with Gabbet's methylene blue for 2 minutes. Gabbet's methylene blue contains both decolorizer and counter stain. The slides were then washed and air dried and observed under oil immersion objective of a light microscope for AFB.

In fluorescent staining, the slides were flooded with freshly filtered Auramine-phenol and kept for 20 minutes without heat application. Next the smears were washed with tap water and then decolorized by covering completely with acid alcohol for 3 minutes. The slide were then washed with tap water and counter stained with 0.1% KMNO<sub>4</sub> for 1 minute. The slides were then gently rinsed with water, drained and dried and observed under the LED (Light emitting diode) fluorescent microscope under low power (10X).

### Results:

Of the 148 samples examined, 71.6% and 53.4% and 85.8% TB cases were detected by ZN, Gabbet's and FS staining methods respectively. 21 sputum smears which were negative by ZN method were positive by FS method (**Table 1**) and 48 sputum smear which were negative by Gabbet's method were positive by FS. When we compared Gabbet's and ZN methods (**Table 2**) we observed 27 sputum smear which were negative by Gabbet's method were positive by ZN.

In relation to reliability of these methods, we found that the sensitivity of the staining methods showed, FS and ZN was 83.5%, FS and Gabbet's (**Table 3**) was 62.2%, and the ZN and Gabbet's was 74.5%. Specificity of each comparison was 100%. The overall accuracy of the different comparisons mentioned above was 85.8%, 67.6% and 81.8% respectively.

**Table 1:** Cross comparison of the ZN and Fluorescent staining method

		FS					Total
		1+	2+	3+	Neg	SC	
ZN	1+	0 0% 0%	34 97.1% 97.1%	0 .0% .0%	0 .0% .0%	1 2.9% 4.5%	35 100.0% 23.6%
	2+	0 0% 0%	0 .0% 0%	45 100.0% 100.0%	0 .0% 0%	0 0% 0%	45 100.0% 30.4%
	Neg	0 0% 0%	0 .0% 0%	0 .0% 0%	21 50.0% 100.0%	21 50.0% 95.5%	42 100.0% 28.4%
	SC	25 96.2% 100.0%	1 3.8% 2.9%	0 .0% 0%	0 .0% 0%	0 0% 0%	26 100.0% 17.6%
Total		25 16.9% 100.0%	35 23.6% 100.0%	45 30.4% 100.0%	21 14.2% 100.0%	22 14.9% 100.0%	148 100.0% 100.0%

**Table 2:** Cross comparison of the Gabbet's and ZN staining method.

		ZN				Total
		1+	2+	Neg	SC	
GABBETS	1+	0 0% 0%	45 97.8% 100.0%	0 .0% 0%	1 2.2% 3.8%	46 100.0% 31.1%
	Neg	2 2.9% 5.7%	0 .0% 0%	42 60.9% 100.0%	25 36.2% 96.2%	69 100.0% 46.6%
	SC	33 100.0% 94.3%	0 .0% 0%	0 .0% 0%	0 0% 0%	33 100.0% 22.3%
	Total	35 23.6% 100.0%	45 30.4% 100.0%	42 28.4% 100.0%	26 17.6% 100.0%	148 100.0% 100.0%

**Table 3:** Cross comparison of the Gabbet's and Fluorescent staining method

		FS					Total
		1+	2+	3+	Neg	SC	
GABBETS	1+	0 0% 0%	1 2.2% 2.9%	45 97.8% 100.0%	0 .0% 0%	0 0% 0%	46 100.0% 31.1%
	Neg	25 36.2% 100.0%	1 1.4% 2.9%	0 .0% 0%	21 30.4% 100.0%	22 31.9% 100.0%	69 100.0% 46.6%
	SC	0 0% 0%	33 100.0% 94.3%	0 .0% 0%	0 .0% 0%	0 0% 0%	33 100.0% 22.3%
	Total	25 16.9% 100.0%	35 23.6% 100.0%	45 30.4% 100.0%	21 14.2% 100.0%	22 14.9% 100.0%	148 100.0% 100.0%

**Discussion:**

*M. tuberculosis* is not a commensal, and the demonstration of the typical acid fast bacilli cultured from a specimen is presumptive evidence of infection.<sup>5</sup> There are different staining procedures for the diagnosis of tuberculosis such as hot methods and cold methods. Thus sputum smear microscopic examination is the corner stone of diagnosis and treatment and has been successfully implemented by various governmental organizations.<sup>4</sup> However the need to analyze the pros and cons of currently available staining techniques for *Mycobacterium tuberculosis* from sputum samples and suggest a cost effective method for routine diagnosis both in urban and rural health care facilities invariably was of utmost concern.

Early detection is crucial and can prevent further complication. The ZN stain was superior to Gabbet's cold staining method in our study although there was a good agreement between them similar to the findings by Balakrishna et al.<sup>2</sup> The morphology of tubercle bacilli in Gabbet's staining appeared more or less like *Mycobacteria* but were fainter than those seen with the ZN stain, which may be reason for the false negative results compared to ZN method. In the conventional ZN method there is a better penetration of stain through the complex cell surface structure due to the heating therefore the organism appears brighter against a blue background. The use of alcohol and a heating process in ZN stain, was cumbersome and hazardous compared to the easy two step process in Gabbet's cold staining method which was also true in Balakrishna et al study.<sup>2</sup> A drawback of FS is that, all staff who examine sputum samples should become familiar with FS in order to prevent false positive and or negative results.<sup>3</sup> A highly significant relationship was seen between the ZN and FS techniques in the detection of AFB than the Gabbet's method. The fluorescent staining method had a better sensitivity than ZN and Gabbet's methods in the detection of AFB from sputum samples.

The fluorescence microscopy technique with the use of LED is better than bright field microscopy and although there was a good agreement between both the fluorescent staining method was found quite economical in terms of both time and expense. Fluorescent staining technique was found to be a cost effective method in our study population especially when dealing with large samples and smears with low density of AFB and more reliable than ZN and the Gabbet's staining method. Fluorochrome microscopy permits rapid and easy visualization of AFB and doubtful smears can always be cross checked with ZN stain. Another fluorescent stain Acridine orange is also available which has given comparable results with Auramine as seen in various studies and can be used as an alternate stain in the diagnosis of pulmonary tuberculosis.<sup>6</sup>

In recent years, several radiometric and molecular techniques have been developed for the diagnosis of TB having reduced the turnover time for AFB considerably. However the scope of these techniques have their drawbacks due to high cost and need for expensive specialized equipments.<sup>7</sup>

**Conclusion:**

A highly significant relationship was seen between the ZN and FS techniques in the detection of AFB than the Gabbet's method. The fluorescent staining method however had an edge in better sensitivity even in low densities than ZN and Gabbet's methods in the detection of AFB from sputum sample. The use of LED fluorescent microscopy make FS quite economical in terms of both time and expense especially when dealing with large number of samples.

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