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Comparison and Evaluation of Rapid Cold AFB Stain with Ziehl-Neelsen Staining in a Prospective Study

Authors

Sumit Kumar Rawat¹, Talha Saad²

¹Dept of Microbiology, Bundelkhand Medical college Sagar, MP India 470003

² Dept of Chest and TB Bundelkhand Medical college Sagar, MP India 470003

Corresponding Author

Dr Sumit Kumar Rawat

Assistant Professor, Dept. of Microbiology, Bundelkhand Medical College Tilli Road, Sagar MP, India

Email- rawat5000@gmail.com

Abstract

Objective: Correct and rapid diagnosis of the tuberculosis is very important to control the growing epidemic. Its laboratory diagnosis is mainly dependent on the demonstration of acid-fast bacilli in sputum by direct microscopy. Ziehl-Neelson's staining is traditionally used for detecting tubercle bacilli cold staining methods recently commercially available for use in small and remote setups; however its use and efficacy has not been adequately studied.

Methods: Total 508 consecutive sputum samples both spot and early morning were collected over a period of six months and were stained with both Z-N stain and cold AFB stain and results were compared.

Results: Out of collected 508 samples a total of 203 were AFB positive by Z-N stain and 181 were positive by cold staining method. By comparing the two we get sensitivity of 89.2%, specificity of 100%, false negative rate 10.8% and false positivity of 0% by cold staining method against Z-N stain.

Conclusion: In current scenario usage of cold staining method must be limited to less equipped setups and their results should be interpreted with caution keeping in mind that negative results need to be crosschecked at a better equipped laboratory or a RNTCP center in high suspicion cases.

Keywords: Acid fast bacilli, Z-N staining, Cold staining, Diagnosis of Tuberculosis

INTRODUCTION

Tuberculosis (TB) is second only to HIV/AIDS as the greatest killer worldwide due to a single infectious agent. In 2012, 8.6 million people fell ill with TB and 1.3 million (including 0.3 million HIV positive cases) died from TB [1]. About 20% of the world's population of tuberculosis patients resides in India [2]. Correct and rapid diagnosis of the disease is very important to control the growing epidemics [3, 4]. According to WHO expert committee, a case of tuberculosis refers to a person with 'bacteriologically confirmed disease' that is a person who expectorates tubercle bacilli in his sputum [5]. Laboratory diagnosis of pulmonary tuberculosis is mainly dependent on the demonstration of acid-fast bacilli in sputum by direct microscopy or by culture methods [6]. Smear microscopy being simpler, quicker and cheaper is widely used for detecting tubercle bacilli [7] though it lacks sufficient sensitivity, culture is more sensitive but it requires time [6]. We need urgent improvements to the implementation of existing strategies for tuberculosis control, with an emphasis on early diagnosis and effective treatments. The methods except direct microscopy are expensive, biohazardous and need special set up along with well trained staff for working, this is for that reasons these methods are not universally available [6]. Ziehl-Neelson's staining is commonly used for detecting tubercle bacilli. This technique requires heating of the smear, while staining heating is usually done either by using spirit lamps or by Bunsen burners. Since spirit lamps and Bunsen burner may not always be available at primary care centers – cold AFB staining has been considered

good for these situations. Besides this heating may sometimes be inadequate and sometimes result in charring of smear. Cold staining methods using Gabbet's methylene blue as decolouriser and counter stain [8,9] has been advocated as an alternative staining technique and is commercially available for use in small and remote setups however its use in these laboratories has not been adequately studied. Its recent commercial availability has promoted its use in small private laboratories where there is less equipment and time for Z-N staining. Current study aims to compare the efficacies of the two methods.

MATERIALS AND METHODS

508 sputum samples 315 spot and 293 early morning samples were collected from 315 patients having the symptoms of pulmonary tuberculosis like cough of more than 15 days with expectoration, chest pain, low grade fever, loss of weight, etc from January 2013 to July 2013. All these were routine patients attending the outpatient Department of Govt Bundelkhand Medical College Sagar, MP. A consent regarding use of research data on condition of anonymity was obtained.

The thickest portion of the sputum sample was spread evenly in the centre of a clean glass slide with help of disposable swab-stick. It was air dried and heat fixed. Two such smears were made from each sputum sample and labeled as A and B after writing the specimen number. "A" group of smears were stained by the standard Z-N method; "B" group of smears were stained by the cold staining method [9].

Carbol fuchsin (1%) was prepared from 10 g of basic fuchsin (Hi-Media) dissolved in 100 ml of methanol (Fischer) and 50 ml of melted phenol (Fischer) in a flask maintained at 60°C in a water bath. This solution was made up to 1,000 ml with distilled water and filtered after proper mixing. Sulfuric acid (25%) was prepared from 250 ml of concentrated sulfuric acid slowly added to 750 ml of distilled water. Methylene blue (0.1%) was prepared from 1 g of methylene blue (Hi-Media) dissolved in 1,000 ml of distilled water. Gabbett's Methylene blue was sourced commercially (Bioloab) constituted as described by Vasanthakumari et al [9]

Z-N Method-The glass slides were kept in a staining rack with the smear side facing upwards and flooded with Carbol fuchsin (1%) solution. The slides were heated from underneath using a flame until vapors started rising. After 5 minutes the slides were washed gently with running water, excess water was drained off and 25% Sulphuric acid was poured onto the slides and allowed to stand. After 2 to 3 minutes the slides were washed in running water and excess drained off. Methylene blue solution (0.1%) was poured on the slides and allowed to stand. After 1 minute the slides were rinsed in running water, air dried and examined using oil immersion objective (100x).

Cold staining Method (CS) - Slides were placed on the staining rack with the smear facing upwards. Smears were flooded with Carbol fuchsin (1%) stain and allowed to stand at room temperature for 10 minutes. After that slides were washed gently in running tap water and Gabbett's Methylene blue was poured on the smear and allowed to stand for 2 minutes. After that the slides were washed gently in

running water, air dried and examined using oil immersion objective (100 x). At least 300 fields were observed by 2 experienced observers before giving negative and the slides were graded as per RNTCP guidelines [10].

Statistical analysis: The association between results obtained by the three methods was measured using the kappa estimator [11] a proportional measure varying between -1 (negative agreement) and +1 (positive agreement). Assuming a normal distribution of kappa, confidence limits were estimated using the kappa variance [11]

RESULTS

A total of 508 sputum samples both spot and early morning were obtained from 315 patients, spot samples were obtained from all the patients, however early morning samples could be obtained from 293 patients only since some of the patients did not come back with morning samples. Some of these were given a positive report on the same day with spot sample. In our study 194 (61.6%) patients were males and 121 (38.4%) females, there was higher distribution of patients at early age and extremes of age (Shown in Figure 1). Male preponderance was noted in patients presenting with symptoms of pulmonary tuberculosis.

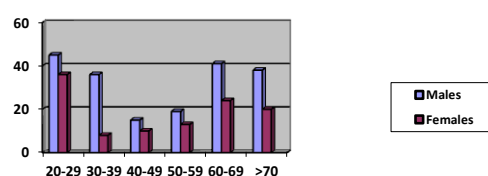


Figure 1 Age and sex distribution of Patients

Out of total sputum samples examined 203 (39.9%) were positive by Z-N method and 181 (35.6%) were

positive by cold AFB staining method. All those smears which were positive by cold AFB method were also positive by Z-N method.

Table 1-Findings of Z-N staining and AFB staining

	Z-N staining	Cold AFB stain
AFB Positive samples	203	181
AFB Negative	305	327
All Total (morning and spot)	508	508

There were 203 AFB positive samples from 126 patients out of 315 patients who were found to be AFB positive by any of the methods in at least one of the two samples sputum submitted for analysis (Table no. 1). Out of these 15 patients were completely missed by cold AFB method, most of these were only of grade 1+ positive. There were no samples or patients which were detected positive by cold AFB staining alone.

It was noted by both observers that cold AFB staining was easy to perform.

Table 2-Performance of both tests and their sensitivities and specificities

		ZN staining		Total
		disease +	disease -	
Cold AFB Stain	Test +	181	0	181
	Test-	22	305	327
	Total	203	305	508

Comparing performance of cold stain method with Z-N staining we get sensitivity of 89.2%, specificity of 100%, false negative rate 10.8% and false positivity of 0% (Table no. 2). The differences were statistically significant and comparable (Kappa =0.90)

DISCUSSION

Tuberculosis remains a major public health problem, in 2013 alone 1.4 million new cases were detected in India [1]. Every year, approximately 18 lakh people develop TB disease and about 4 lakhs die of it [10]. Control of the disease is targeted through nationwide program like RNTCP which relies heavily over detection of AFB in sputum. The approach is targeted to control the disease by early diagnosis and treatment of open pulmonary TB cases; Z-N stain occupies the key role in this program [10]. The major difficulty in staining tubercle bacilli is due to the presence of an unsaponifiable waxy substance on their surface. Tubercle bacilli are gram positive, aerobic, non-spore, acid-fast, slightly curved or straight non-motile rods [12]. They have the capacity to synthesize large amount of lipids in the form of neutral fats, free fatty acids, phosphatides and waxes [13, 14]. These waxes and the presence of complex three compartmented cell wall are mainly responsible for contributing bacilli the acid-fastness property [12, 13, 15]. Ziehl-Neelson's staining is commonly used for detecting tubercle bacilli. On staining with carbol-fuchsin followed by heating it enters into the cell [9]. This cationic dye form a complex with intracellular lipid layer prevent the trapped carbol-fuchsin from being removed and

cell-wall components prevents the entry of decolorizing agent and they appear as red or bright pink colored rods [12, 14]. But for non acid-fast bacilli the decolorizing agent removes the primary stain and they take the colour of counter stain [14]. The success of any staining technique depends on the ability of the dye to uniformly penetrate the cell wall through this waxy barrier without affecting the acid-fast character of the organism. In the conventional Ziehl-Neelsen method it is achieved by heating the slides during the staining process. This operation requires a precise heating control and experience on the part of the laboratory technician. Over heating may char the smear and under heating may not be sufficient for the bacilli to take up the stain. Both the conditions lead to false negative. While carrying out the conventional method spirit lamps are used as a source of heating. Very often this method is carried out in small laboratories and periphery places to omit the use of spirit lamp. However omitting the step may lead some of the bacilli not being penetrated by the dye in cold staining and falsely reporting these sputum samples as AFB negative. RNTCP program has conventionally relied on the Z-N staining and they have tried to make this technique uniformly available at all places. Our study intended to compare the two methods since availability of cold staining commercially has increased its use in small and less equipped laboratories. It is pertinent to mention here that in all the 18 specimens positive by ZN Method only, the number of AFB seen were less than 10 and this could be due to scanty and uneven distribution of AFB in the sputum. It cannot be overlooked that this leads to a false negative rate

of 10.8% which may lead to many diseased patients being deprived of treatment at appropriate time. On the other hand, CS Method has not shown any positive result even on a single specimen which was not positive by ZN Method. These two observations suggest that CS Method is at least as specific as ZN Method although somewhat less sensitive. Other workers also have found the results under CS Method comparable to ZN Method [9, 16,17]. Most of the small and less equipped laboratories using commercially available cold staining do not conform to RNTCP standards and most of them may be private labs, hence excessive reliance on this method may be dangerous to the whole purpose of performing the AFB stain. Thus we suggest that usage of this method must be limited to less equipped setups and their results should be interpreted with caution keeping in mind that negative results need to be crosschecked at a better equipped laboratory or a RNTCP center in high suspicion cases. Moreover good positive predictive value of the cold staining method may still make this method a method of choice at places where limited skill and limited resources are available.

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