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RESEARCH ARTICLE

A COMPARISON OF ZIEHL-NEELSEN STAINING AND FLUORESCENT STAINING TECHNIQUES FOR THE DIAGNOSIS OF PULMONARY TUBERCULOSIS

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ABSTRACT

Pulmonary tuberculosis is mainly a disease of the respiratory system, caused by *Mycobacterium tuberculosis*. According to WHO, one-third of world population have tuberculosis infection. The present study was undertaken to compare ZN satin with fluorescent stains. A total of 500 clinically suspected cases of pulmonary tuberculosis formed the study group. Sputum samples were screened for acid-fast bacilli by ZN stain, Rhodamine-Auramine stain and rapid fluorostain. Out of 500 patients, 9.2%, 10.2% and 10.2% were found positive for acid fast bacilli by ZN, rhodamine-auramine and rapid fluorostain respectively. Compared to ZN staining (9.2%), fluorescent staining methods were found to be more efficient (10.2%), in the detection of Mycobacterium tuberculosis from cases of pulmonary tuberculosis.

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INTRODUCTION

Pulmonary tuberculosis is mainly a disease of the respiratory system, caused by *Mycobacterium tuberculosis*. (Ba and Rieder, 1999) In the year 1993, tuberculosis was declared by World Health Organization (WHO) as a global emergency. Worldwide, one person out of three is infected with Mycobacterium tuberculosis – two billion people in total. TB currently holds the seventh place in the global ranking of causes of death. (WHO report 2006). India has about 1.8 million new cases of tuberculosis annually, accounting for a fifth of new cases in the world - a greater number than in any other country. (Steinbrook, 2007) Despite this enormous global burden, case detection rates are low, posing serious hurdles for TB control. (Pai and O'Brien, 2008) The resurgence of Mycobacterium tuberculosis in association with HIV infection has focused much attention in the rapid diagnosis of high risk cases. Infection with HIV is known to alter the presentation of pulmonary tuberculosis (Prasanthi and Kumari, 2005). In India, medical colleges have tertiary care hospitals functioning in close collaboration with Revised National Tuberculosis Control Programme (RNTCP) for TB care and control.

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These hospitals have RNTCPs designated microscopy centre (DMC) for the diagnosis of sputum smear positive pulmonary TB using ZN stain based bright field microscopy. In the year 2009/10 and 2010/11, 611683 and 689342 presumptive TB patients (PTP)were examined, out of which 92071 (15.1%) and 95272 (13.8%) were sputum smear positive respectively in 291 out of 321 medical colleges RNTCP DMC's. However, one has to admit due to high volume of cases for sputum examination at the DMC and inadequate staffing there is often delay in examination of slides and so the diagnosis. (Central TB Division 2013) Because of larger number of samples, now RNTCP has introduced fluorescent staining in the detection of tubercle bacilli in major laboratories.

The present study was undertaken to compare the efficacy of fluorescent staining methods, Rhodamine-Auramine staining (Avinash Medicals, Bangalore) and Rapid fluorescein staining (QBC diagnostics Inc, USA) with Ziehl- Neelsen staining in diagnosis of pulmonary tuberculosis.

MATERIALS AND METHODS

A total of 500 clinically suspected cases of pulmonary tuberculosis formed the study group. Study period of one year from June 2013- May 2014 .A profoma was used to collect data before the collection of sample. Early morning sputum samples were collected from each case and processed in the

department of Microbiology, AIMS, B.G. Nagara. The study protocol was approved by institutional ethical committee. Three smears were made from each sample. One heat fixed smear was stained by Ziehl Neelsen stain. (Fig. 1) Methanol fixed smear was stained by Rhodamine auramine. QBC F.A.S.T Sure Focus slides were used, heat fixed and stained by rapid fluorostain. (Fig. 2 & 3)

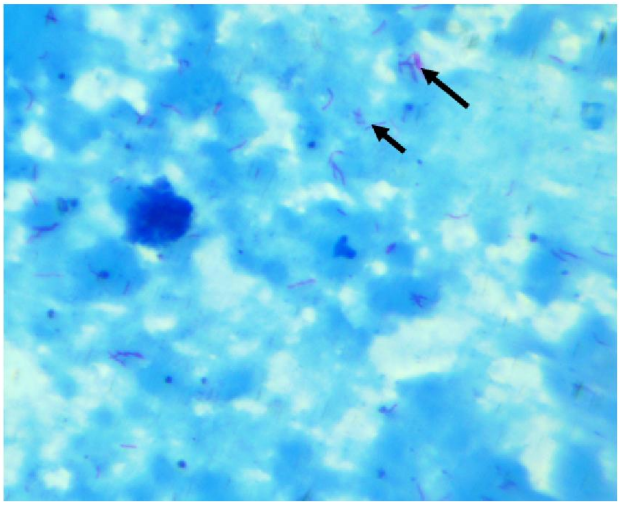

Figure 1. Zeihl Neelson staining showing Acid fast bacilli

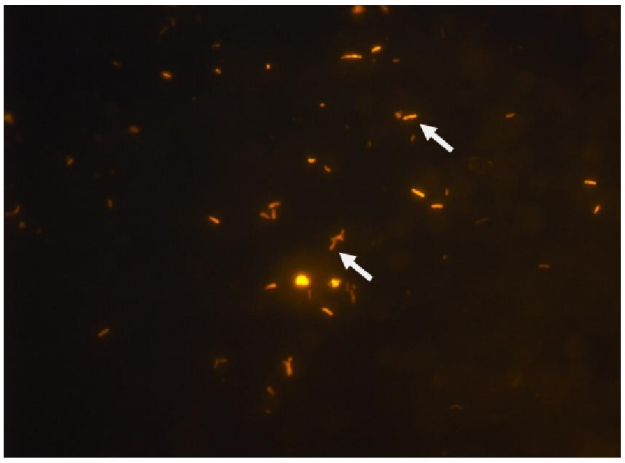

Figure 2. Yellow fluorescing bacilli on fluorescent staining

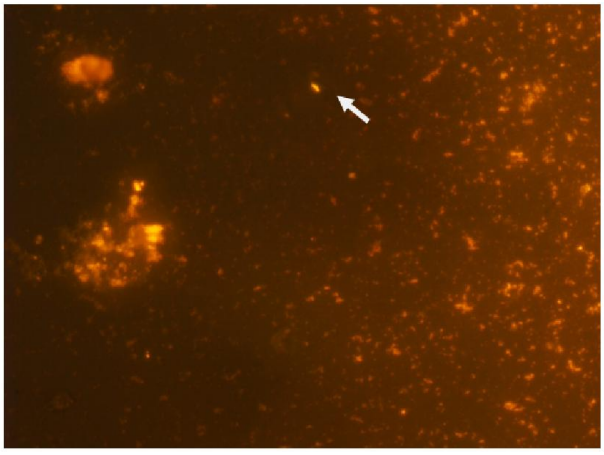

Figure 3. Single fluorescing bacillus on fluorescent staining

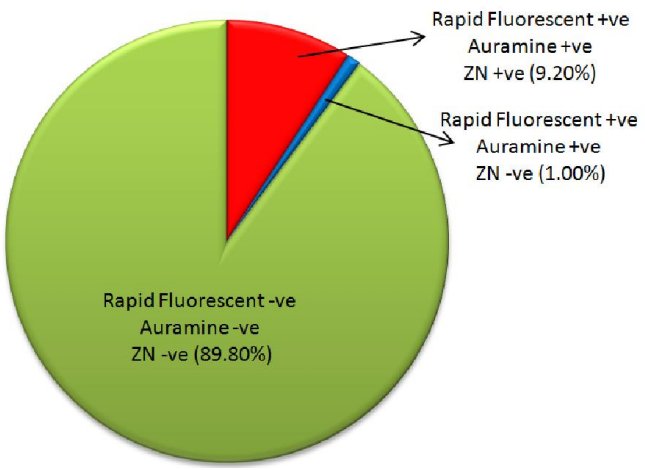

Fig. 4. Test results of various staining methods

Rhodamine-auramine staining procedure

1. Methanol fixed smear was flooded with rhodamine-auramine stain for 15 min at room temperature.
2. After washing the slides, it was decolorized with potassium hydroxide for 5 min at room temp
3. Slides were washed and flooded with potassium permanganate counterstain for 3 min at room temp.

Rapid fluorostain method

QBC F.A.S.T Sure Focus slides were used for rapid fluorostain staining.

Procedure:

1. Heat fixed smear was flooded with fluorostain –O for 1minute.
2. Washed smears were stained with fluorostain DS for 1 minute.
3. Washed and examined under fluorescent microscope.

ZN stained smears were observed under oil immersion microscope (100x) using light microscope and fluorescent stained smears were observed by fluorescent microscope (blue filter, BG-12: 540-590 nm wave length) using high power (40X). In ZN staining AFB appears pink stained bacilli. Fluorescent staining showed yellow / green fluorescing bacilli on black back ground under fluorescent microscope. (Fig 2 & 3) In case of any doubt in morphology, under fluorescent microscope, the same smear was decolorized and restained with ZN technique for confirmation. Positive and negative controls were included with each batch of staining.

RESULTS

Age of the study group ranged from 15- 85 years with mean age of 49.8 years. Males were 345 (69 %) and females 155(31%).

Table 1 shows prevalence of pulmonary tuberculosis in relation to age and sex. Figure 4 Shows results of various staining methods

Table 1. Prevalence of pulmonary tuberculosis in relation to age and sex

Age group in years	Male No – 345				Female No – 155				Total No – 500	
	+ve	%	-ve	%	+ve	%	-ve	%	+ve	%
≤ 20 No – 31	0	0	12	38.7	1	3.2	18	58.1	01	3.22
21-40 No – 160	16	10	77	48.1	5	3.1	62	38.8	21	13.1
41-60 No-163	18	11	96	58.9	0	0	49	30.1	18	11.04
61-80 No- 142	10	7	112	78.9	1	0.7	19	13.4	11	7.74
≥ 81 No – 4	0	0	4	100	0	0	0	0	0	0
500	45	9	298	59.6	07	1.4	148	29.6	51	10.2

Table 2. Results of ZN staining, Fluorescent staining and rapid fluorostain staining

	ZN stain	Rhodamine auramine stain	Rapid Fluorostain
Positive	46	51	51
Negative	454	449	449

Table 3. Sensitivity and specificity of ZN staining in relation to fluorescent staining methods

	Sensitivity	Specificity	PPV	NPV
ZN staining	88.5%	100%	100%	98.7%

Table 2 shows results of ZN staining, Rhodamine- auramine staining and rapid fluorostain staining. Out of 500 patients, 9.2%, 10.2% and 10.2% were found smear positive by ZN, Rhodamine-Auramine and Rapid fluorostain respectively.

DISCUSSION

Tuberculosis continues to be a major public health problem in India and is the single largest cause of loss in healthy life year in the productive age group. The laboratory plays a critical role in the diagnosis of tuberculosis. There are various methods for bacteriological diagnosis of tuberculosis. (Ba and Rieder, 1999; Goyal and Kumar, 2013) Smear examination is believed to be simple, cheap, quick, practicable and effective case finding method for the developing countries, as tubercle bacilli are very slow growing organisms, culture results will be available after a period of three or six weeks.

In the present study, sputum positivity was more in the age group 21 – 40 years (41.17%) correlating with other studies. (Prasanthi and Kumari, 2005; Reza *et al*., 2013) In the present study, maximum number of positivity was found in males (86.5 %) similar with other reports. (Vijaya, 2004; Saraswathi *et al*., 2013; Desai *et al*., 2009) In females, positivity was 13.5% where as Vamsedhar Annam reported 61.7% (Annam *et al,* 2009). Increased incidence of disease in males may be due to outdoor exposure. (Prasanthi and Kumari, 2005) In the present study, ZN staining showed positivity of 9.2% whereas the rhodamine - auromanie staining and rapid fluorostain staining showed positivity of 10.2%. Fluorescent methods detected 1.0% higher cases compared to ZN staining others have reported higher percentage (Goyal and Kumar, 2013; Vijaya *et al*., 2004; Hooja *et al*., 2011). Fluorescent positive smears were further confirmed by ZN staining.

Table 3 shows sensitivity and specificity of ZN staining in relation to fluorescent staining methods. The present study shows 88.5% sensitivity and 100% specificity for Zn staining correlating with Suria kumar who reported sensitivity of fluorescent is 95.4% and ZN staining 63.64%. (Kumar *et al*., 2012) Since the screening was done under high power of magnification (400x), fluorescent staining has been found to be less time consuming as compared to ZN method (1000x) in the diagnosis of tuberculosis. The tubercle bacilli stood out as bright objects against dark background in fluorescent microscopy which makes them easily identifiable, causing less eye-strain. The efficacy of fluorescent microscopy proved to be much higher than conventional light microscopy.

The disadvantage of fluorescent microscopy, that it is not available in all laboratories, requires maintenance and costlier. ZN staining is still used in most of the laboratories. For developing countries with a large number of cases and financial restraints, evaluation of rapid and inexpensive diagnostic method has greater importance. (Laifangbam *et al*., 2009). Rapid fluorostain method yielded similar sensitivity and specificity to Rhodamine-auramine staining. The advantage of Rapid fluorostain is it is rapid (2 minutes) compared to Rhodamine-auramine staining (25 minutes) but requires specific slides which are costlier. The use of fluorescent microscopy greatly improves the diagnostic value of sputum smear especially in patients with low density of bacilli that are likely missed in ZN stained smears.

Conclusion

The use of fluorescent microscopy greatly improves the diagnostic value of sputum smear to diagnose tuberculosis especially in patients with low density of bacilli that are likely

to be missed on Ziehl-Neelsen stained smears. Fluorescent microscopy is relatively more sensitive as it allows a large number of sputum specimen examination in a given time, in laboratories equipped with fluorescent microscopy.

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