To Compare the Efficacy of Fluorescent Microscopy Versus ZN Stain Microscopy in Detecting the Pulmonary Tuberculosis

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ABSTRACT

Introduction: By preparing sputum with sodium hypochlorite and applying fluorescent microscopy, the sensitivity of smear microscopy for the diagnosis of tuberculosis could be enhanced. The objective of this study was to establish the agreement between direct fluorescent microscopy and the concentration technique of Ziehl-Neelsen by their ability to detect fast acid bacilli in poor resource settings. Aim of the present study was to compare the efficacy of Fluorescent microscopy versus ZN Stain microscopy in detecting the Pulmonary Tuberculosis under DOTS Programme.

Material and methods: It is Comparative study comprised of suspected cases of Pulmonary Tuberculosis coming to Inpatient and Outpatient department of Pulmonary Medicine. Study was conducted for a period of six months in Clinically suspected cases of Pulmonary Tuberculosis and cases with radiological evidence, pulmonary TB suspect is any person with cough for 2 weeks, or more.

Results: The majority of patients i.e. 52 (20.71%) were found to be in the age group of 41- 50 years, followed by 47 (18.72%) patients among 21-30yrs. Out of 251 patients studied, 179 (71.31%) were males while 72 (28.68%) were females. ZN smear positivity rate and the AO smear positivity rate in the study was 8.76% (22/251) and 14.35% (36/251) respectively(P <0.0001) in detection of AFB from cases of Pulmonary tuberculosis. Out of 251 patients, the ZN Smear Positive and Fluorescent Positive are 22 and 36 respectively. The ZN Smear Negative and Fluorescent negative are 229 and 215 respectively. Fluorescent stain with sensitivity of 100%, specificity of 94.4%, NPV of 100%, is more efficient over ZN stain in detecting Tubercle bacilli in sputum.

Conclusion: Since screening is performed under low magnification power (40X), fluorescence in the diagnosis of tuberculosis has been found to take less time compared to the ZN approach (100X). It is easy to recognise the fluorescent bacilli and cause less eye strain.

Keywords: Zeihl Neelson Stained Smears, Fluorescent Staining, Pulmonary Tuberculosis

INTRODUCTION

Tuberculosis is mainly a disease of the respiratory system, caused by Mycobacterium tuberculosis. Tuberculosis is a predominant infectious cause of mortality ranked as 12th leading cause of death in world presently.[1] According to World Health Organization (WHO), tubercular infections are currently spreading at the rate of one person per second per million people. With three millions dying from it.

Tuberculosis continues to be a major health problem in our country and is single largest cause of loss in healthy life year in the productive age group. There are various methods for bacteriological diagnosis of tuberculosis.[1] Currently, radiometric assay allows detection of mycobacterium tuberculosis growth and provides antibiotic sensitivity results more rapidly usually within 10 days. However, use of the technique is limited because culture medium contains radioactive carbon. Genetic probes are on the other hand quite easy to use and allow identification of culture media in only a few hours by polymerase chain reactions. Mycobacterium stains 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. Serological tests are currently not reliable enough for the diagnosis of Tuberculosis. 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 sputum or any other pathological material.[2] Smear examination is believed to be simple, cheap, quick and practicable and effective case finding method for developing countries. As tuberculosis bacilli are very slow growing organisms, culture results are available after a period of three to six weeks.

Direct Microscopic examination has the advantage of giving a result at once. The specimen most commonly examined is sputum and mucus 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 most extensively used procedure for the demonstration of mycrobacterium tuberculosis in smear.[2] The requisites for staining procedure are; basic fuchsin, phenol, absolute alcohol, Sulphuric acid and methylene blue. Microscopic examination under oil immersion objective reveals mycobacterium are red bacilli.

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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.[3] So, microscopic Examination under low power objective will reveal mycobacteria as glowing yellow white, rice like bacteria in the smear.

Therefore the present prospective study was undertaken to see the efficacy of Ziehl-Neelson method versus fluorescent staining in the detection of mycobacterium in sputum sample.

MATERIAL AND METHODS

The Comparative study comprises of the suspected cases of Pulmonary Tuberculosis coming to Inpatient and Outpatient department of Pulmonary Medicine, MNR Medical college, Sangareddy, under DOTS programme. The study was conducted for a period of six months from 24th August, 2012 to 24th February, 2013.

Inclusion criteria: Clinically suspected cases of Pulmonary Tuberculosis and cases with radiological evidence, pulmonary TB suspect is any person with cough for 2 weeks, or more

Exclusion criteria: Patients on antitubercular therapy.

At all outpatient clinics, hospitals and health facilities, both in the public and private sectors, all patients need to be systematically screened for cough by medical officers and health staff manning the health facilities. Additionally, in medical colleges and hospitals, in-patients also need to be screened for identification of TB suspects. Persons with cough for 2 weeks, or more, with or without other symptoms suggestive of TB, should be promptly identified as pulmonary TB suspects and steps taken to subject them to sputum smear microscopy for acid-fast bacilli, for diagnosis of TB.

Sputum collected from patient as instructed to take a deep breath, hold it momentarily and then cough deeply and vigorously. Patient was also instructed to cover their mouth carefully and spit in to the cup. Saliva, nasal secretion and patient who had used oral antiseptics were rejected.

Induced sputum taken by aerosol (3% hypertonic saline) induction procedure was done.

2 sputum samples were collected within a day or two consecutive days from each patient- spot specimen on 1st day, one early morning by patient at home. Samples were collected in clean, sterile, leak-proof, wide mouth containers. The processings of the sample were carried out in a biosafety cabinet. Each sample was subjected to Ziehl-Neelsen (ZN) staining, Fluorescent Auramine-O (AO) staining.

Decontamination

Modified Petroff's Technique: was used for decontamination of sputum sample.4-ml of sputum was taken in a screw capped bottle and an equal volume of 4% NaOH was added to it. Mixture was homogenized by shaking on vortex machine for 15 minutes and then incubated at 37°C for 20 minutes. The mixture was centrifuged at 3000 rpm, for 15 minutes. The supernatant was poured off in a disinfectant solution and the deposit was resuspended in 15 ml of sterile distilled water. The solution was centrifuged once again at 3000 rpm, for 15 minutes. Supernatant fluid was poured off and the deposit was used for further processing.[4]

ZN staining [5]

Numbered slides were placed on rack and flooded with Ziehl-Neelsen carbol fuchsin. Slide heated slowly from underneath until steaming, steaming maintained for 3-5min by using low and intermittent heat. Slides were rinsed under stream of running water. Slides were flooded with decolorizing agent (25% sulphuric acid) till smear becomes pale then rinsed thoroughly with water and counterstaining was done with 1% Loeffler"s methylene blue for 1 minute. Rinsed with water, smears were allowed to air dry and observed under immersion objective of light microscope, tubercle bacilli were observed as red coloured rods, slightly curved in pairs or in groups against blue background.

Fluorescent staining (Auramine O) [6]

Slides were flooded with Auramine O and allowed to stand for 15 minutes and Rinsed with distilled water. Decolourisation was done with 0.5% acid alcohol for 2 minutes then rinsed with distilled water and drained and slides were flooded with KMN04 and allowed to counterstain for 2 minutes. Rinsed with distilled water and allowed to air dry and observed under fluorescent microscope as early as possible. The film is examined with a 40x objective and a 10x eyepiece. Tubercle bacilli emitting a bright yellow fluorescence against a dark background were seen.

The smear reporting is done according to IUATLD/ WHO Scale[7]

IUATLD/ WHO Scale (1000 X field = HPF) Result	Bright field (1000 X magnification : 1 length = 2cms = 100 HPF)	Fluorescence (400 X magnification: 1 length = 40 fields = 200 HPF)
Negative	Zero AFB/1 Length	Zero AFB / 1 Length
Scanty	1-9 AFB /1 Length or 100 HPF	1-19 AFB /1 Length
1+	10-99 AFB /1 Length or 100 HPF	20-199 AFB /1 Length
2+	1-10 AFB /1 HPF on average	5-50 AFB /1 field on average
3+	>10 AFB /1 HPF on average	>50 AFB /1 field on average

RESULTS

The majority of patients i.e. 52 (20.71%) were found to be in the age group of 41- 50 years, followed by 47 (18.72%) patients among 21-30yrs. Out of 251 patients studied, 179 (71.31%) were males while 72 (28.68%) were females (table-1).

The correlation between conventional ZN method and the modified Fluorescent method. The ZN smear positivity rate and the AO smear positivity rate in the study was 8.76% (22/251) and 14.35% (36/251) respectively.

Out of 251 patients, the ZN Smear Positive and Fluorescent

Age in Years	Number of Patients	%
Upto 10	2	0.79
10-20	23	9.16
21-30	47	18.72
31-40	35	13.94
41-50	52	20.71
51-60	34	13.54
61-70	39	15.53
71-80	15	5.97
81-90	4	1.59
Total	251	100.0
Gender		
Male	179	71.31
Female	72	28.68
Total	251	100.0

Table-1: Demographic distribution of patients studied

	ZN Staining	Fluorescent staining
Negative	229 (91.24%)	215 (85.65%)
Positive	22 (8.76%)	36 (14.35%)

Table-2: Result of Smear examination by ZN and Fluorescent staining.

	ZN Positive	ZN Negative	Total
Fluorescent Positive	22	14	36
Fluorescent negative	0	237	237
Total	22	251	273

Table-3: Comparision of smear examination result by ZN staining and Fluorescent staining.

Grading	Positive by	
	ZN Staining	Fluorescent staining
Scanty	0	2
1+	9	16
2+	0	2
3+	13	16
Total	22	36

Table-4: Correlation of ZN Staining and Fluorescent staining grade wise

Measures	%
Sensitivity	100.0
Specificity	94.4
PPV	61.1
NPV	100.0
Accuracy	94.8
Chi-square value (x^2)	149.39 (Yate's corrected)
P Value	<0.0001
x2 = 149.39 (Yate's corrected) d.f (degree of freedom) =1, P Value is <0.0001 shows highly significant.	

Table-5: Evaluation of sensitivity, specificity, PPV, NPV, Accuracy, P Value of Auramine stain method (Fluorescent method) against ZN Stain method.

Study	ZN	AO
Jain A et al[10]	32.7%	41.6%
Githui et al[11]	65%	80%
Ulukanligil et al[12]	67.6%	85.7%
Prasanthi k & Kumari AR[13]	50%	69%
Present study	8.76%	14.35%

Table-6: Comparision of our findings with other studies

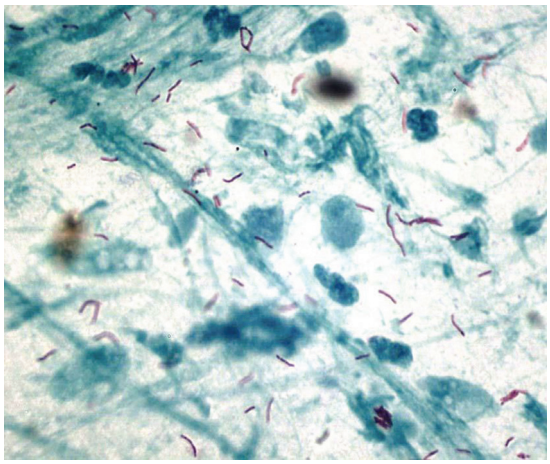

Figure-1: Ziehl Neelsen staining: Direct smear showing Acid fast bacilli. Magnification- 100x

Positive are 22 and 36 respectively. The ZN Smear Negative and Fluorescent negative are 229 and 215 respectively (table-2).

22 samples were both fluorescent and ZN method positive, where 14 samples were fluorescent positive and ZN negative. Out of which 237 samples were both fluorescent and ZN negative. where none of sample was positive and fluorescent negative respectively (table-3).

Scores are definitely high by Fluorescent microscopy: 36 positive against 22 positive by ZN Staining (table-4,5).

DISCUSSION

India has a long history of research and demonstration projects on TB. The detection of Acid fast bacilli is often considered as the evidence of the infected stage. Thus the laboratory plays a critical role in the diagnosis of TB (50). In developing countries microscopy of the specimen is by far the fastest, cheapest and the most reliable method for the detection of AFB. However fluorescent staining has been added in Revised National Tuberculosis Control Program(RNTCP) because of more sensitive and rapid results and can be used in field areas.

The majority of patients i.e. 52 (20.71%) were found to be in the age group of 41- 50 years, followed by 47 (18.72%) patients among 21-30 yrs. So about 53.37% were in the age group 21-50yrs. This age group is active part of the community. Disease in this age group results in reduction of manpower and economic loss to the country. Our study was almost similar to Shivaraman et al who has reported that 40.8% of their study population belonged to this age group. In the study by Narang P and coworkers, at Wardha 26.30% patients belonged to this age group.[8,9] The reasons that make this age group vulnerable to TB are many. They are socially

more active and are exposed to an open case of TB more than any others. In some parts of world HIV pandemic has contributed, as people of this age group are sexually most active.[9]

Out of 251 patients studied, 179(71.31%) were males while 72 (28.68%) were females. Male to female ratio was 2.4:1. Narang. P. et al. have reported that 61.03% of their subjects were male while 38.97% were female. In a male dominating society, usually the male is the earning member. As he goes out for work, he is more likely to come in contact with an active TB case. Men are more likely to acquire habits like smoking, alcoholism etc. The reason behind less number of patients in Females is because they are neglected, dependent, lack of education, lack of awareness. [9]

Different smear microscopy results were achieved by Jain A et al ZN 32.7%, AO 41.6%,Githui et al ZN 65% AO 80%, Ulukanligil et al ZN 67.6% AO 85.7%, Prasanthi k & Kumari AR ZN 50% AO 69% (53-56). It was evident that AO method results scored higher than that of ZN method in all these studies as was the case in this study (ZN 8.76%, AO 14.35%). In this study, AO was found to be more effective than ZN staining. This shows that the fluorochrome staining of sputum smears in comparision to that of ZN staining is a better method of microscopy. (x^2 =149.39,P< 0.001).[10,11,12,13]

Kumar N et al had observed that 85% of culture positive cases could be diagnosed by microscopy alone.[14] In Laifangbam et al study, 59.7% of culture positive cases were diagnosed as ZN stained light microscopy. [15]The figure increased significantly to 97.2% of culture positive cases by AO stained smear fluorescent microscopy (60). In Laifangbam et al it was found that in case of ZN Stain there was agreement in 69.6% cases and disagreement is 30.4% whereas for AO Stain there was agreement in 95.1% cases and disagreement in 4.9% cases. This proves that AO Stain is better method for its close comparability to gold standard technique. Sensitivity of ZN vs Culture, AO vs Culture, ZN + AO vs Culture are 59.72%, 97.22%, 97.22% respectively. specificity of ZN vs Culture, AO vs Culture, ZN+AO vs Culture are 93.33%,90%,86.67% respectively(60). So auramine stain has taken has gold standard method in this study. Cultre facilities are not available, so in this study limited to the stains. In this study positivity difference is 6%,that is fluorochrome staining was found to be 6% more effective than ZN staining. Similar results have reported by studies done by suriya kumar et al.[16] Where as higher smear positivity rates were shown by K. Prashanthi et al2005 and Ulukanligil et al.[12,13]

In the present study, the results showed that from sputum specimen of 273,22 patients had sputum smear positive by ZN staining and fluorescent staining 36 patients gave positive result. The results of present study indicate that the Auramine staining of sputum smears in is a more sensitive method of sputum microscopy for demonstration of AFB in sputum specimen, compared to ZN staining.

The use of Fluorescent microscopy greatly improves the diagnostic value of sputum smear especially in patients with low density of bacilli that are likely to be missed on Zeihl Neelson stained smears. The method is economical in both time and expense and recommended for laboratories handling large number of sputum specimens. Fluorescent staining is superior to that of ZN staining in the presence of low bacterial load as seen in smears with diagnostic cytomorphologial featured tuberculosis, in problem areas like AIE(acute inflammatory exudates alone or with occasional granuloma, AFB positivity by ZN staining is nearly good as the fluorescent method because the bacterial load is high). Using fluorescent microscopy the tubercle bacilli when examined under ultra violet illumination the bacilli appeared as a bright rod against a dark background. Since there was a contrast, the bacilli were readily seen and therefore in very less time large area could be examined. Images were then captured with the digital camera and enhance through image processing techniques. [17]

While in ZN staining acid fast bacilli appeared bright red rods in blue background. In this also image were captured. The potential benefits of automated screening for tubercle bacilli are rapid, acute, inexpensive diagnosis, the ability to screen large number of people; increased resources to monitor patients; and reduction in health risk to staff. Thus, the study reveals that sputum stained by the fluorescent method is useful and reliable for pulmonary Tuberculosis. Since the fluorescent microscopy is costly some laboratories cannot afford to buy fluorescent microscopy, so in these laboratories Ziehl- Neelson staining is most employed. Detection of smear positive cases is the highest priority in any TB control programme, as these cases are infectious and contribute to transmission of disease. Though smear positivity correlates well with infectivity, much of the transmission occurs before the level of bacilli reach 10^5 per ml on the sputum.[13] ZN stain can detect bacilli when they are in the order of 10^5 per ml of the sputum whereas a more sensitive AO stain can detect in the order of 10^4 per ml of sputum.[4]

CONCLUSION

Sputum examination for the tubercle bacilli is usually conducted for patients clinically and radiologically suspected of pulmonary tuberculosis. However, the standard method of sputum examination, that is ZN staining, is not sensitive enough and a large number of the suspected cases can miss diagnosis. Compared to ZN Stain (8.76%) Flurochrome staining(14.35%) was found to be more efficient (P <0.0001) in detection of AFB from cases of Pulmonary tuberculosis.

Fluorescent stain with sensitivity of 100%, specificity of 94.4%, NPV of 100%, is more efficient over ZN stain in detecting Tubercle bacilli in sputum. Since screening is done under low power of magnification (40X), fluorescence has been found to be less time consuming compared to ZN method (100X) in the diagnosis of tuberculosis. Hence it has been advocated to be methods of choice where large numbers of sputum smears are to be examined. The fluorescing bacilli are easily identifiable and cause less eye strain.

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