International Journal of Microbiology Research
ISSN: 0975-5276 & E-ISSN: 0975-9174, Volume 5, Issue 1, 2013, pp.-336-340.
Available online at http://www.bioinfopublication.org/jouarchive.php?opt=&jouid=BPJ0000234
DOI: http://dx.doi.org/10.9735/0975-5276.5.1.336-340

THE VALUE OF FLUORESCENT MICROSCOPY AND BLEACH SEDIMENTATION METHOD FOR DETECTION OF SMEAR NEGATIVE TUBERCULOSIS

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Received: October 23, 2012; Accepted: January 03, 2013

Abstract- The diagnosis of tuberculosis in developing countries has been done by direct microscopic examination which has low sensitivity. Most of the patients with TB are detected as free of TB. For the low sensitivity of the directed microscopy, a new high sensitive fluorescent microscope was developed. In addition bleach sedimentation technique increased the sensitivity of both direct and fluorescent techniques. This investigation was undertaken to evaluate the value of fluorescent microscopy and bleach sedimentation method for the detection of smear negative tuberculosis cases in sputum sample in comparison with direct microscopy.

A total of 132 sputum samples from patients aged 7 to 75 years were examined during the study period. Culture (Gold standard) result identified 75 subjects as positive. Among 75 cultures positive sputum samples direct Ziehl Neilson technique identified 50 samples as positive with sensitivity of 66% and specificity of 98.2%. In fluorescent microscopy 55 of them were identified as positive with sensitivity of 78.6% and specificity of 80.7% in comparison with culture. On the other hand over night bleach sedimentation in Ziehl Neilson technique has increased the sensitivity to 76% and resulted in specificity of 91.2%. Bleach fluorescent microscopy has increased the sensitivity to 82.7% and resulted in specificity of 80.7% in comparison with culture. From the total of 132 samples 81 were smear negative samples as confirmed by direct microscopy. From these 81 samples direct fluorescent microscopy has identified 12 samples as positive out of 25 culture positive samples with sensitivity of 48% and specificity of 80.3%. Bleach direct microscopy has identified 8 positive with sensitivity of 32% and specificity of 89.3%. Bleach fluorescent microscopy identified 13 as positive with sensitivity of 52% and specificity of 82%. Therefore, the combination of bleach sedimentation and florescent microscopy can be used as a diagnostic tool for the reduction of morbidity and mortality occurring from Tuberculosis and can be used for early diagnosis and treatment of the disease.

Keywords- Bleach sedimentation, fluorescent microscopy, smear negative TB

Citation: Amare G., et al. (2013) The Value of Fluorescent Microscopy and Bleach Sedimentation Method for Detection of Smear Negative Tuberculosis. International Journal of Microbiology Research, ISSN: 0975-5276 & E-ISSN: 0975-9174, Volume 5, Issue 1, pp.-336-340. *DOI: 10.9735/0975-5276.5.1.336-340.*

Introduction

Pulmonary tuberculosis (PTB), an infectious disease caused by the *Mycobacterium tuberculosis* (MTB), a slender, rod and an obligate aerobe. It is chronic infection of the lung. MTB belongs to the genus Mycobacterium which comprises the acid fast bacilli that are difficult to satin. Mycobacterium is neither gram positive nor gram negative i.e., they are stained poorly with the dye used in Gram stain [1].

The disease is transmitted by means of invisible droplet nuclei containing the organisms that have left the reservoir during breathing, sneezing or coughing. Transmission generally occurs indoors where droplet nuclei can stay in air for long time. Its initial site of infection is lung. The disease generally manifested with low grade persistent fever, night sweating, significant weight loss, fatigue and generalized weakness [2,3].

Diagnosis is generally done by demonstrating the presence of tubercle bacilli in clinical specimen. The specimen most commonly examined for the presence of tubercle bacilli is sputum. In most countries TB detection is made by using microscopy for smear made from sputum sample on slides using Ziehl Neilson staining technique. In the recent days there are different advanced techniques like fluorescent techniques which use flourochrome stains (auramine rhodamine stains), light emitting diode (LED) fluorescent microscopy, polymerase chain reaction (PCR), radiometric assay as well as culture isolation [4,5]. Direct microscopy takes significant labor and training as well as time which resulted in the workload to be intensified. In addition the main drawback of this method is its poor sensitivity as compared to other methods [6].

The use of conventional fluorescent microscopy increase the sensi-

tivity since the fluorochrome stained bacilli stands out brightly against the background and more fields can examined in short periods of time. It has also reduced the amount of labor force requirement. This form of illumination is suitable for the detection of auramine O- stained bacilli and may become an affordable alternative for improving diagnostic microscopy in laboratories serving poor income settings with high load of smear examinations. The main advantage of fluorescence staining is that slides can be examined at a lower magnification, allowing the examination of much larger area per unit of time. Fluorescence microscopy requires only two minutes examining an area that bright field microscopy requires ten minutes [7].

Although the above mentioned advantage of conventional fluorescent microscopy there has been little information on increment of sensitivity on smear negative TB patients. Therefore, the present study is designed to evaluate the value of fluorescent microscopy and bleach sedimentation method for the detection of smear negative TB cases in sputum sample in comparison with direct microscopy.

Materials and Methods

Study Design

A cross-sectional study was conducted to assess the values of fluorescent microscopy and blanch sedimentation for the detection of smear negative tuberculosis.

Study Site

This study was conducted at EHNRI TB Laboratory, Addis Ababa Ethiopia. The institute currently gives various activities like researches, trainings, proficiency testing, and panel testing, standardizations and product management. TB laboratory is one of the specialized departments of the institute. It is equipped with plenty of facilities like LJ culture and BACTEC Culture for the isolation of *Mycobacterium tuberculosis* and drug sensitivity testing. Additionally the laboratory performs numerous activities like simple and sophisticate diagnostic technique for TB, as confirmatory and external quality assurance for samples referred from different institutions in the country, Provide training for health professionals, also performing various researches on the emergency of drug resistance TB by the staffs and other post graduate and undergraduates.

Populations

Source Population

All types of sputum specimen referred to EHNRI TB laboratory from the different region and zones of the country during the study period.

Study Population

Smear negative and suspected samples for smear positive referred to EHNRI TB laboratory from different part of the country during the study period.

Sampling Method and Sample Size

One hundred thirty two sputum samples were include in the study using convenient sampling method. These samples were collected from patients referred to EHNRI for diagnosis and drug sensitivity test during the study period.

Collection, Handling and Transportation of Sample

Early morning sputum was collected in sterile universal disposable container. The specimen labeled with an appropriate code and delivered to EHNRI TB laboratory and stored at 2-8 degree centigrade until it was processed.

Laboratory Methods

Five different examinations were performed: direct microscopy using direct and bleach concentrated sputum smear, standard fluorescent microscopy using direct and bleach concentrated sputum smear and TB culture as gold standard method.

Zeihl Neilson Technique

The standard Acid fast staining procedure was used [8].

Fluorescent Microscope for Diagnosis of TB

In the fluorescent microscope the bacteria has been stained with fluorescing dyes called flourochrome. The fluorescent stained bacteria were seen glowing against dark background. Auramine was used as staining dye to demonstrate AFB since it binds to the nucleic acid in the mycobacterium nucleus. After being stained with auramine the smear was decolorized with acid alcohol which removes the dye from the background and finally potassium permanganate was used as a counter stain to cover the background. The bacilli fluoresce white-yellow against the dark background [8].

Mycobacterium Culture

Mycobacterium bacilli was grown an aerobically on protein enriched Lowenstein Jenson (LJ) egg medium. The optimal temperature for growth was maintained at 35-37°C. MTB grows 2 up to 3 week after incubation but culture was incubated for 6 weeks before being discarded.

Bleach (NaOCL)

Sputum sample and Bleach (NaOCL) were mixed in equal proportion so that the bleach emulsified all the mucous substances and concentrated the bacterium for easy diagnosis in different microscopic methods.

Data Analysis

Data was analyzed using SPSS version 16 and the statistical tests between dependent and independent variables was done using chi-square (χ^2) test. Where the numbers in cell was less than five, a Fisher's exact test was used. P values $\leq$ 0.05 were considered statistically significant.

Results and Discussion

Tuberculosis diagnosis in most parts of the world is done by method which lacks both sensitivity and specificity. With the emergence of new diagnostic methods like fluorescent microscopy and sputum concentration techniques using bleach sedimentation method, the sensitivity has improved considerably as compared to direct microscopic method with Ziehl Neilson staining method [1].

In this study a total of 132 sputum samples from patients aged 7 to 75 years were examined in EHNRI TB laboratory. The mean age of study subjects was 33 years with standard deviation of 12 years. Out of the total, 71(53.7%) were male and 61 (46.2%) were female. Based on sputum consistency, samples were classified as salivary, Mucoid, purulent and bloody sputum, which accounts for 62 (46.9%), 28(21.2%), 34(25.7%), 8(6%) respectively out of the total. The socio-demographic characteristics, sputum consistency and the subject's disease status with each method of examination are given in [Table-1] and [Table-2].

The p values in [Table-1] and [Table-2] indicate that there is signifi-

cant correlation between the culture results and the age of patients as well between consistency and all test methods results (culture and the microscopic methods). On the other hand, there was no significant correlation between consistency and all test methods results (culture and the microscopic methods). Also, there was no significant correlation between the microscopy methods results with age and sex of patients (p>0.05).

The number of positive cases of *M. tuberculosis* among the study group was 75(56.8%) case with culture (gold standard method), 51 (38.6%) with direct ZN (DZN), 62(46.9%) with bleach treated ZN (BTZN), 70(53%) with direct fluorescent microscopy (DFM) and 73 (55.3%) with bleach treated fluorescent microscopy (BTFM) [Table-1], [Table-2].

When comparison was made with the gold standard (culture) the direct ZN technique has correctly identified 50 (37.8%) as positive out of 75(56.8%) culture positive cases with the sensitivity (SN) of 66% and positive predictive value (PPV) 98%. Also, ZN has correctly identified 56 (42.4%) as negative out of 57(43.2%) culture negative cases with specificity (SP) of 98.2% and negative predictive value (NPV) 30.6% and test efficiency (TE) 80.3% [Table-3].

Table 1- Socio demographic characteristics and sputum consistency as compared with culture, DZN and DFM

Characteristics	Total	Culture		Chi sq.	P value	DZN		Chi sq.	P value	DFM		Chi sq.	P value
Sex		Positive	Negative			Positive	Negative			Positive	Negative		
M	71(53.7%)	42 (59.1%)	29 (40.8%)	0.343	> 0.05	28 (39.4%)	43 (60.6%)			42 (59%)	29 (41%)		
F	61 (46.2%)	33(4%)	28 (46%)			23 (37.7%)	38 (62.3%)	0.04	>0.05	27 (44.3%)	34 (55.7%)	2.914	>0.05
Age (yrs.)													
< 20	10 (7.5%)	7 (70%)	3 (30 %)			4 (40%)	6 (60%)			6 (60%)	4 (40 %)		
21-30	54 (40.9%)	37 (68.5%)	17 (31.4%)	12.75	< 0.05	26 (48%)	28 (52%)			34 (63%)	20 (37%)		
31-40	38 (28.7%)	14 (36.8%)	24 (16.2%)			10 (26.3%)	28 (73.7%)	7.3	>0.05	16 (42%)	22 (57.8%)	7.614	>0.05
41-50	21 (15.9%)	14 (66.7%)	7 (33.3%)			9 (42.8%)	12 (57.2%)			12 (57.2%)	9 (42.8%)		
> 50	9 (6.8%)	3 (33.3%)	6 (66.7%)			2 (22.2%)	7 (77.8%)			2 (22.2%)	7 (7.8%)		
Consistency													
Salivary	62 (46.9%)	23 (37%)	39 (63%)	19.4	< 0.05	8 (13%)	54 (87%)			21 (33.2%)	41 (66.7%)		
Mucoid	28 (21.2%)	20 (71.4%)	8 (28.6%)			17 (60.7%)	11 (39.2%)	32.6	< 0.05	19 (67.8%)	9 (32.2%)		
Purulent	34 (25.7%)	26 (76.5%)	8 (23.5%)			21 (61.7%)	13 (38.2%)			24 (70.5%)	10 (29.5%)	17.37	< 0.05
Bloody	8 (6%)	6 (75%)	2 (25%)			5 (62.3%)	3 (37.7%)			6 (75%)	2 (25%)		

Table 2- Socio demographic characteristics and sputum consistency as compared with culture, BTZN and BTFM

Characteristics	Total	Culture		Chi sq.	P value	BTZN		Chi sq.	P value	BTFM		Chi sq.	P value
sex		Positive	Negative			Positive	Negative			Positive	Negative		
M	71(53.7%)	42 (59.1%)	29 (40.8%)			33 (46.4%)	38 (53.6%)			42 (59%)	29 (41%)		
F	61 (46.2%)	33(54%)	28 (46%)	0.343	> 0.05	29 (47.5%)	32 (52.5%)	0.015	> 0.05	31 (50.8%)	30 (49.2%)	0.926	> 0.05
Age (yrs.)													
< 20	10 (7.5%)	7 (70%)	3 (30 %)			6 (60%)	4 (40%)			6 (60%)	4 (40%)		
21-30	54 (40.9%)	37 (68.5%)	17 (31.4%)			27 (50%)	27 (50%)			34 (63%)	20 (20%)		
31-40	38 (28.7%)	14 (36.8%)	24 (16.2%)	12.75	< 0.05	14 (36.8%)	24 (63.2%)	7.31	> 0.05	16 (42%)	22 (58%)	6.9	> 0.05
41-50	21 (15.9%)	14 (66.7%)	7 (33.3%)			12 (57%)	9 (42.8%)			14 (66.7%)	7 (33%)		
> 50	9 (6.8%)	3 (33.3%)	6 (66.7%)			3 (33%)	6 (66.7%)			3 (33.3%)	6 (66.7%)		
Consistency													
Salivary	62 (46.9%)	23 (37%)	39 (63%)			13 (21%)	49 (79%)			23 (37%)	39 (63%)		
Mucoid	28 (21.2%)	20 (71.4%)	8 (28.6%)	19.4	< 0.05	19 (67.8%)	9 (32.2%)	32.13	< 0.05	17 (60.7%)	11 (39.3%)	18.28	< 0.05
Purulent	34 (25.7%)	26 (76.5%)	8 (23.5%)			25 (73.5%)	9 (26.5%)			27 (79.4%)	7 (20.6%)		
Bloody	8 (6%)	6 (75%)	2 (25%)			5 (62.3%)	3 (37.7%)			6 (75%)	2 (25%)		

Table 3- Comparison of the different microscopic method (DZN, DFM, BTZN, BTFM) taking culture as gold standard method

	Parameters				
Method	SN	SP	PPV	NPV	TE
DZN	66%	98.20%	98%	30.80%	80.30%
DFM	78.60%	80.70%	84.30%	74.20%	79.50%
BTZN	76%	91.20%	91.90%	74.30%	82.50%
BTFM	82.70%	80.70%	84.90%	77.90%	81.80%

The direct fluorescent microscopy (DFM) technique identified 59 (44.7%) as positive out of 75(56.8%) culture positive subjects with sensitivity of 78.6% and PPV of 84.3% and 46 (34.8%) as negative out of 57(43.2%) culture negative subjects with specificity of 80.7% and NPV of 74.2% and test efficiency of 79.5%. The result showed that there is an increase in sensitivity form 66% in DZN to 78.8% in DFM, which showed improvement of 12.8% [Table-3]. Also as indicated by other studies, the direct microscopy is a technique of low sensitivity and there is a need to improve the detection rate of TB with improved methods of diagnosis like use of fluorescent microscopy and bleach sedimentation techniques [10-13].

The bleach treated ZN (BTZN) technique identified 57(43.2%) as positive out of 75 (56.8%) culture positive samples with a sensitivity of 76% and PPV of 91.9%. whereas, 52 (39.4%) as negative out of 57 (43.2%) culture negative samples with specificity of 91.2% and NPV of 74.3% as well as test efficiency of 82.5%. Meanwhile bleach treated fluorescent microscopy (BTFM) technique identified 62 (47%) as positive out of 75 (56.8%) culture positive samples with sensitivity of 82.7% and PPV of 84.9% and 46 as negative out of 57 culture negative cases with specificity of 80.7% and NPV of 77.9% and test efficiency of 81.8%. The results showed that there is an increase in sensitivity from 76% in BTZN to 82.7% in BTFM, which showed an improvement of 6.7% and an increase in sensitivity from 78.6% in DFM to 82.7% BTZN showing an improvement of 4.1% [Table-3].

Subjects identified as culture negative but positive in the four techniques may be due to that 10% of patients with anti –TB treatments can continue to cough up non-viable, but stainable bacilli with-in few weeks of the therapy and also most of our source populations suspected were referred for drug sensitivity test.

The result obtained from the total 132 subjects indicates that there are 81(61%) smear negative samples by the DZN method [Table-1], [Table-2]. The DZN smear negative samples were again re-examined by the other four techniques. Culture has identified 25 (31%) as positive, while DFM, BTZN, BTFM methods identified 23 (28%), 14(17.3%) and 23(28%) as positive out of the 81 smear negative samples by DZN technique respectively [Table-4].

Comparison was made on DZN smear negative samples for the three techniques (BTZN, BTFM and DFM) by taking culture as gold standard. The result showed that DFM identified 12 (14.8%) correctly as positive out of 25 (30.8%) culture positive DZN smear negative samples with sensitivity of 48% and PPV of 52.2% while 13(16%) as negative out of 56 (69.1%) culture negative smear negative samples with specificity 80.3% and NPV of 77.6% and test efficiency of 70.4%.

Bleach treated direct microscopy identified 8 (9.8%) correctly as positive out of 25 (30.8%) culture positive samples with sensitivity of 32% and PPV of 57.1% and 17 (21%) as negative out of 56 (69.1%) culture negative with specificity 89.3% and NPV of 25.4% and test efficiency of 71.6%.

Bleach treated fluorescent microscopy identified 13 (16%) correctly as positive out 25 (30.8%) culture positive samples with sensitivity of 52% and PPV of 56.5% and 12 (14.8%) as negative out of 56 (69.1%) culture negative samples with specificity 82.1% and NPV of 79.3% and sensitivity of bleach treated fluorescent microscopy was higher than the other two ($P < 0.05$), while bleach treated DZN showed higher sensitivity when compared with DZN ($p<0.05$) [Table -4].

Table 4- Comparison of the different microscopic method (DFM, BTZN, BTFM) taking DZN as a gold standard method

	Parameters				
Method	SN	SP	PPV	NPV	TE
DFM	48%	80.30%	52.20%	77.60%	70.40%
BTZN	32%	89.30%	57.10%	25.40%	71.60%
BTFM	52%	82.10%	56.50%	79.30%	72.80%

Direct ZN technique identified 51 (38.7%) out of 132 sputum samples as positive, These positive ZN smears were compared by their degree and the result showed that 13 (25.4%) as scanty (1- 9 bacilli in 100 fields), 11 (25.5%) as +1 (10-100 bacilli 100 fields), 13 (25.4%) as +2 (1-9 bacilli in 1 field) and 14 92.4%) as +3(more than 10 bacilli in 1 field). Results graded as scanty and +1 in DZN were compared with four techniques (culture, DFM, BFM and BZN) for improvement of grade results; Culture identified 1 (7.6%) as negative, 0% as scanty, 1(7.6%) as +1, 1 (7.6%) as +2 and 10 (77%) as +3 out of the 13 scanty results in DZN. This result indicates there is a general improvement among scanty samples when graded by culture. One subject identified scanty in DZN technique was negative in culture, this is probably due to non-viable bacilli that could not grow on culture media [Table-5].

Table 5- Grade comparison of 13 scantly samples in direct ZN technique in different microscopic methods (DZN, DFM, BTFM).

	Grade result in different micro, and cult methods				
Method	NEG[1]	SC[2]	13	24	35
DFM	3 (23%)	---	1(7.6%)	8(61%)	1(7.6%)
BZN	---	1(7.6%)	7(53.8%)	2(15.3%)	3(23%)
BFM	1(7.6%)	---	---	3(23%)	9(69%)
CUL	1(7.6%)	---	1(7.6%)	1(7.6%)	10(77%)

Culture result also identified 0% as negative, 9% as scanty, 0% as +1, 45.4% as +2, 45.4% as +3out of 11 results which were graded as +1 in DZN [Table- 6].

In a prospective study done in, Magahi District Hospital, Kenya, a total of 204 specimens were diagnosed by fluorescent microscopy and cultured on LJ culture media. Fluorescent microscopy sensitivity was 22.2% after sedimentation with 3.5% NaOCl. In our study the sensitivity of fluorescent microscopy after sedimentation with 3.5% NaOCl was 82.7%. The result between these two studies shows that there is an increase in sensitivity in our study. This might be attributed to most of our samples being referred for drug sensitivity test and their samples having more false negative rates, in addition they might have low number of bacilli in their sputum samples than in this study sample [11].

Table 6- Grade comparisons of 11 samples graded as +1 in direct ZN technique in different microscopic methods (DZN, DFM, BTZN and BTFM)

	Grade result in different micro, and cult methods				
Method	NEG	SC	1	2	3
DFM	3(27.2%)	2(18%)	1(9%)	2(18%)	3(27.2%)
BTZN	---	2(18%)	4(36%)	2(18%)	3(27.2%)
BTFM	1(9%)	---	3(27%)	2(18%)	5(45.4%)
CUL	---	1(9%)	---	5(45.4%)	5(45.4%)

Conclusion

Bleach sedimentation technique and fluorescent microscopy can significantly increase the detection rate of Tuberculosis; especially for smear negative samples reported by direct microscopy. Combination of these methods can be used as a diagnostic tool for the reduction of morbidity and mortality occurring from Tuberculosis and can be used for early diagnosis and treatment of the disease.

Acknowledgement

The authors are grateful to the authorities of EHNRI TB Laboratory, Addis Ababa, and School of Medical Laboratory Sciences, Addis Ababa University, Ethiopia.

Conflict of Interest

The authors declare that there is no conflict of interest.

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