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Sensitivity and specificity of direct and concentrated smear microscopy using culture and PCR based on *IS6110* analysis for the detection of acid-fast Bacilli in suspected and having pulmonary tuberculosis

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Abstract

Sensitivity and specificity of direct and concentrated AFB smear microscopy has been investigated by PCR analysis and culture methods to determine a rapid and cheap detection of pulmonary tuberculosis. In this study 305 patients were selected and 915 specimens were collected from suspected and hospital admitted patients. Patients were taken from Dhaka Central Jail Hospital and National TB Hospital, Dhaka, Bangladesh. All samples were smeared and Ziehl-Neelsen method and Lowenstein-Jensen (L-J) method were applied. PCR analysis and culture method was used to confirm the detection. Isolated DNA was used in PCR analysis. In this detection study PCR based IS6110 analysis was developed to identify the pulmonary tuberculosis rapidly and cheaply. A total of 915 samples were prepared for analysis and found 70.47% and 82.85% sensitivity in direct and concentrated smears respectively. Both type of smears (direct and concentrated) showed 100% sensitivity and specificity in culture and PCR IS6110 analysis. Since the direct and concentrated smears with the developed method of PCR IS6110 analysis showed significant detection, so that the proposed method was suggested for the detection analysis of *Mycobacterium tuberculosis* in pulmonary for the developing and poor countries.

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Introduction

The Incident rate of Tuberculosis (TB) is increasing day by day around the world. It is thought that one third population of the world is infected with *M. tuberculosis* and new infections occurring at a rate of about one per second (WHO report 2010). The world Health organization has estimated 13.7 million chronic active cases globally in 2007, while in 2010 there were an estimated 8.8 million new cases with 1.5 million associated deaths, mostly occurring in developing countries (WHO report 2009 and 2011). Bangladesh is one of the five high burden countries for tuberculosis in the South-East Asia Region. The estimated prevalence of all forms of tuberculosis and incidence rate in the country was 391 and 225 per 100 000 population, respectively in 2006 (WHO report 2008).

Pulmonary tuberculosis is preventable and treatable but the developing countries cannot achieve a complete eradication. Developing countries like Bangladesh faces this public health problem for the diagnosis of the Tuberculosis using various methods. Among the developing countries, Bangladesh ranks the 6th highest number of TB patients (WHO report 1996). *M. tuberculosis* is the most frequent member for the cause of TB among *M. tuberculosis complex* (MTC) members worldwide (Albert 2004). Direct and Concentrated Smear Microscopy is used for the diagnosis of Tuberculosis but the direct smear microscopy is inexpensive, simplest and relatively easy to perform for the poor staffed (Suárez *et al.,* 2002, van Cleeff *et al., 2005*, Eisenach *et al.,* 1990) and equipped laboratories. This method reports within hours of receipt of the sample and provides reliable epidemiological indicators needed for the evaluation of the National Tuberculosis Control Program. The limitation is, for detection at least 5x10^3 Bacilli per ml of sputum is required. Smear microscopy is useful for initial diagnosis of tuberculosis from respiratory specimens of Acid-Fast Bacilli. On the other hand, detection of Pulmonary Tuberculosis by concentrated smear microscopy needs well equipped setting in the laboratories with proper staff. Centrifuge machine is required for concentrating the sample, but the machine is not properly set up in low income countries like Bangladesh for diagnosis.

In Bangladesh, there are 250 DOTS centers are working for TB diagnosis but they are not properly equipped with centrifuge machine. Unprocessed sputum samples are used to prepare the smears in these laboratories.

M. tuberculosis is identified from sputum (lung secreted) directly under a microscope or by culture methods and indirectly through different molecular technique such as *IS6110* PCR analysis (Tevere *et al.,* 1996). In acid-Fast Bacilli, staining method is used to characterize the *M. tuberculosis*, treated with acidic solution. Ziehl-Neelsen staining method is widely used which is stained the AFB with bright red on the blue background. Lowenstein-Jensen, Middle-Brook 7H11 are used as a solid media and BECTEC radiometric system as a liquid media can be useful technique for Mycobacterium identification within two weeks but this method is the slower detection for diagnosis TB.

Recently, a new test method is developed for the detection of Mycobacterium at a cheap, fast and more accurate way, which is defined as a polymerase chain reaction (PCR) detection of bacterial DNA. PCR allows rapid nucleic acid amplification and direct detection of the *M. tuberculosis* (Hopewell 1994). Rapid and cheap diagnosis will be useful for developing countries. The objective of this study is to compare the sensitivity and specificity of direct and concentrated AFB smear microscopy using culture and PCR analysis for the rapid and cheap detection for developing world.

Methods and materials

Sampling and study population

It is reported that 85% TB is associated with Pulmonary and therefore sputum is a choice of specimen to investigate the TB. A total of 915 sputum samples were aseptically collected from patients of Dhaka Central Jail Hospital and National TB

Hospital, Dhaka, Bangladesh those were clinically suspected Pulmonary Tuberculosis disease. Samples were processed in biological safety cabinet in the laboratory, after collection. Early morning sputum samples were considered for better result from the patient as it contains *M. tuberculosis*, than one taken at later in the day. Three sputum samples were collected from each patient in the consecutive three days. Then these specimens were stored in a refrigerator for consecutive testing. A study out line (Fig. 1) and characteristics of study population (Table 1.) are presented below.

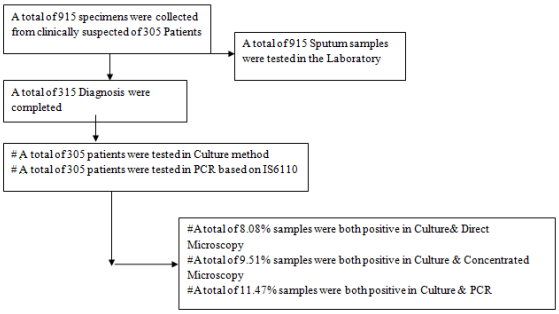

Fig. 1. Study frame.

Sample preparation

Sputum samples were used for the analysis of both direct and concentrated smear microscopy which is also performed with the culture method and PCR based *IS6110* analysis. Unprocessed specimens were used to prepare smear which was kept on 3 cm×2 cm slides. Both direct & concentrated smears were prepared by Ziehl-Neelsen staining method using carbol fuchsin solution. For staining, the slides were arranged on a staining rack over a sink. The smear was flooded with carbol fuchsin. The carbol fuchsin was heated by using a metal stick with a piece of cotton wool, which was lighted after putting a bit of burning spirit on it and stopped when the first vapor was seen and kept it for 5 minutes. The slides were then washed with distilled water. The washed slides were decolorized with 25% H_2S0_4 for 2 to 3 minutes & again washed the slides with distilled water. After washing the slides were counter stained with methylene blue and kept at room temperature for 2 to 3 minutes. After washing with distilled water, the slides were dried by electrical heater.

Carbol fuchsin solution preparation:

Carbol fuchsin solution was prepared by dissolving 3.0 gm of basic fuchsin in 100 ml 96% ethanol; this was solution (1). Phenol solution was prepared by dissolving 5 gm of phenol crystals in 100 ml of distilled water; this was solution (2). For 100 ml of carbol fuchsin solution 10 ml of solution (1) was mixed with 90 ml of solution (2).

Decoloring agents preparation

H_2SO_4 (25%, v/v) was used as decoloring agents. For 100 ml of solution, 25 ml of H2SO4 acid was mixed with 100 mL of distilled water.

Counter staining solution preparation

Methylene blue was used as counter stain. For 100 ml of methylene blue solution 0.3 gm of methylene blue chloride was dissolved in 100 ml distilled water

The samples were diluted with the 4% NaOH and 0.5% N-acetyl-L-Cysteine (NALC, Sigma), and decontaminated the samples. Samples were vortexed for 2 minutes and incubated at 37°C for 30 minutes. Incubated samples were diluted and centrifuged at 4000 rpm for 15 minutes. Pellets were collected from this supernatants for the experiment of AFB microscopy, culture method and extraction of DNA for PCR analysis.

Culture method

M. tuberculosis was detected using the culture method of egg based Lowenstein-Jensen (L-J) selective media. Culture media was usually made in bottles rather than Petri dishes because mycobacteria, especially tubercle bacilli, were present in very small numbers in most specimens. This necessitates comparatively large inocula that were spread evenly over the surface of the medium.

Culture media preparation

Homogenized eggs solution:

Fresh hen eggs, not more than one week old, were cleaned with soap solution and soaked in 70% ethanol for 30 minutes. The eggs were broken and the whole eggs (yellow and white) were taken into

sterile flask. The eggs were homogenized by using a magnetic stirrer for at least 30 minutes and filtered through the four layers of sterile gauze into a sterile cylinder.

Table 1. Characteristics of study population.

Characteristics	Total	Positive in Culture	Positive in PCR
Male, gender	305	105	105
Age, Median (IQR)	31	32	32
Previously treated/on going treatment	4	1	4
New case	301	104	105
Mortality (Within 6 months)	2	2	2
Clinical Presentation	Present (%); Absent (%)		
Fever	65.59 ; 34.49		
Cough	92.89 ; 11.11		
Night Sweating	48.19 ; 51.81		
Weight loss	55.08 ; 44.92		
Shortness of breath	44.26 ; 55.74		
Chest pain	69.83 ; 30.16		
Smoking habit	86.23 ; 13.77		

Table 2. Primers used for the amplification of IS6110

Primers	Sequences	Tm
IS6110 F	5′-CCTGCGAGCGTAGGCGTCGG-3	70
IS6110 R	3′-TTCCHCTCGCATCCGCAGCC-5	70

Note-F-Forward, R- Reverse.

Table 3. grading of AFB microscopy (WHO recommended):

AFB (-) ve	AFB (+) ve	Scanty+	1+	2+	3+
If no Bacilli in 100 field	If Bacilli available in 100 fields	1-9 AFB per 100 fields	10-99 AFB per 100 fields	1-10 AFB per field	>10 AFB per field

Table 4. Density of AFB in Direct smear microscopy and Concentrated AFB microscopy

Direct Smear Results	Concentrated Smear results					Total
	Negative	Scanty	1+	2+	3+	
Negative	828 (98.4%)	10 (1.2%)	3 (0.36%)	0 (0%)	0 (0%)	841
Scanty	1 (11.1%)	2 (22.2%)	6 (66.6%)	0 (0%)	0 (0%)	9
1+	0 (0%)	0 (0%)	8 (42.1%)	10 (52.6%)	1 (5.2%)	19
2+	0 (0%)	0 (0%)	1 (3.7%)	16 (59.2%)	10 (37.1%)	27
3+	0 (0%)	0 (0%)	0 (0%)	0 (0%)	19 (100%)	19
Total	829	12	18	26	30	915

Salt solution:

Salt solution was prepared by dissolving 6.2 g of Lowenstein-Jensen base to 100 mL with dH_2O containing 2 mL of glycerol. The salt solution was autoclaved in 121 to 124 ^{o} C for 15 minutes, and finally cooled to 45 to 60 ^{o} C. Salt solution always kept below 30 ^{o} C. Lowenstein-Jensen base is a salt composition of 4.80 g of KH_2PO_4, 0.48 g og $MgSO_4$, 1.20 g of Mg citrate, 7.20 g of L-aspergine, 24 mL of Glycerol & 0.80 g of Malachite green.

The L-J media was prepared aseptically by mixing salt solution with eggs suspension at 9:1 ratio. The L-J media (5.0 mL) were transferred into universal container or culture bottle. The bottles were slanted by inspissations at 85° C for 50 minutes and subsequently incubated with loosen cap at 37°C for at least 48 hours to check the sterility. The prepared L-J bottles were either immediately used or stored in the incubator by keeping the caps tighten enough to prevent evaporation for further use.

DNA extraction and PCR

Phenol Chloroform-Isoamyl alcohol method was used for DNA extraction (Amita *et al.,* 2002). Culture samples were diluted with dH_2O and centrifuged at 4000g for 15 minutes to extract the DNA. These suspended samples were processed for incubation at 37°C using 10 mg/mL Lysozyme, 10% Sodium dodosyl sulfate (SDS), 15 μL of ProteinaseK, preheated CTAB (N, Cetyl –N,N,N-Trimethyl ammonium bromide) and Chloroform-isoamyl alcohol. This solution then centrifuged at 1200 rpm for 15 min and discarded the supernatant. This suspension was dried and dissolved the pellet in dH_2O which contained the DNA. This isolated DNA was used in PCR analysis.

PCR based *IS6110* analysis was developed to identify rapidly the *M. tuberculosis*. In this analysis, genomes of the isolates were analyzed by PCR to detect the *IS6110* region. Primers were designed on the basis of internal region of the *IS6110* region presented in the *M. tuberculosis* genome (Table 2).

2.5 μL isolated DNA was used as template for amplification in 15 μL reaction mixture. This reaction mixture was prepared with the composition of deionized water, 10x PCR buffer, 50 mM $MgCl_2$, 10 mM dNTP, 5 µM forward primer, 5 µM reverse primer, 5 unit/ μL taq polymerase at the amount of 7.175 μL, 1.5 μL, 0.45 μL, 0.30 μL, 1.50 μL, 1.50 μL, 0.075 μL and 2.5 μL respectively. The reaction mixtures and the isolated DNA were then heated in thermal cycler (MJ Research, PTC-200 Peltier thermal cycler) according to the following cycles: Step 1 (95 °C for 1 minutes), Step 2 (95 °C for 30 seconds, 56 °C for 1 minutes 30 seconds, 72 °C for 4 minutes), Go to step 2 for 35 times, Step 3 (72 °C for 5 minutes), End. The annealing temperature of each primer set was calculated by following equation:

Tm = 4(G+C) +2(A+T)

In the gel electrophoresis, 10 μL PCR analytes were run at 150 V, 1.5% Agarase gel for 1 hour and stained with Ehtidium Bromide. The band size was determined by the comparison of 100 bp DNA ladder (Invitrogen).

Results

Out of 305 patients that submitted a total of 915 specimens for AFB smear, culture and PCR investigation, most of the patients were suffered with cough (93%), fever (66%) and smoking habit (86%) (Table 1). No gender distributions were observed with 100% male patients having the age of 14 to 65. A total of 4 patients were under previously treatment or ongoing treatment condition where rest of the patients were either highly suspected or having TB.

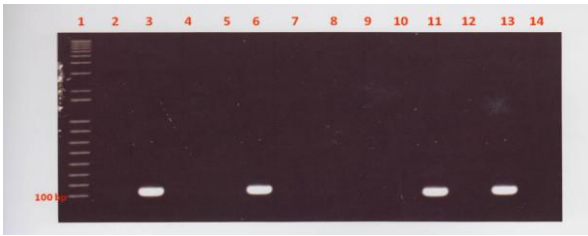

Fig. 2. Agarose Gel Electrophoresis of IS6110 internal region amplified with Primer pair IS6110F and IS6110R that amplified 123 bp fragments. In the figure the slot no. 6,11,13, indicate the presence of amplified region; slot no. 4,5,7,8,9,10,12,14 indicate the absence of amplified region; slot no. 3,2,1 represents positive control, negative control, 100bp ladder respectively.

From the studied specimens, 74 were found as AFB positive and AFB negative, Scanty negative, 1+, 2+, 3+ were found at the number of 9, 19, 27 and 19 respectively. It was conferred that 92% specimens were identified as negative by direct smear

microscopy. In the concentrated AFB smear microscopy 87 and 828 were found as positive and negative AFB respectively. Others specimens were determined as scanty positive, 1+, 2+, 3+ at the number of 13, 18, 26, and 30 respectively (Table 4).

Table 5. Direct and Concentrated smear microscopy of 1st, 2nd, and 3rd specimens.

Specimen	Direct AFB Smear Microscopy	Concentrated AFB Smear Microscopy
1st (n=305)	20	28
2nd (n=305)	26	29
3rd (n=305)	28	30

Table 6. Direct smear microscopy, Concentrated smear microscopy and Culture test results

No of Specimens					
+++	-++	+-+	- -+	++-	-+-
73	14	1	17	8	3
Total Specimens	Positive specimens in Culture test				
915	Specimens	Direct	Concentrated		
	105	74(70%)	87(83%)		

Table 7. Comparison of AFB smears performance with various method

Smear	Direct	Concentrated	Culture	PCR *IS6110*
Positive	74	87	105	105
Negative	841	828	810	810
Sensitivity (%)	70.47	82.85	100	100
Specificity (%)	96.31	97.82	100	100

Table 8. Sensitivity and Specificity of Direct and Concentrated Smear Microscopy

Gold Standard: Sputum Culture /PCR *IS6110*

Sensitivity for Direct smear microscopy True positive (TP): 74 False negative (FN): 31 Sensitivity = TP/(TP+FN)100 = 70.47%	Sensitivity for Concentrated smear microscopy True positive (TP): 87 False negative (FN): 18 Sensitivity = TP/(TP+FN)100 = 82.85%
Specificity Direct smear microscopy True Negative (TN) : 810 False Positive (FP) :31 Specificity = TN/(TN+ FP)100 = 96.31%	Specificity concentrated smear microscopy True Negative (TN) : 810 False Positive (FP) :18 Specificity = TN/(TN+ FP)100 = 97.82%

Table 5. shows the comparison of direct and concentrated smear microscopy for multiple specimens. A total of 28 samples were found as positive AFB in the concentrated smear microscopy and 20 samples were found in direct smear microscopy. Identification number was increased in another two consecutive specimens for the both methods. This number was significant for concentrated smear microcopy than direct smear microscopy.

Table 6. shows the results obtained from culture method in which 105 samples were found AFB positive in direct and concentrated smear

microscopy from the 105 culture positive cases, 16% culture positive were found as negative in both direct and concentrated AFB microscopy. 0.98% specimens were found positive in AFB smear microscopy of culture negative cases.

Discussion

The findings in this study showed that about 73(8%) were positive both on direct and concentrated methods. Also, 13 (2%) sputum samples were found positive in concentrated method. More than 90% results were found as negative AFB in both direct and concentrated smear microscopy. 13 samples were found as positive in concentrated AFB smear microscopy from the 841 negative AFB specimens obtained in direct AFB smear microscopy. 66% of scanty positive, 53% of 1+, and 37% of 2+ AFB were found in direct smear microscopy and these were migrated to 1+, 2+, and 3+ respectively in concentrated smear microscopy. No significant difference was found in both methods for high positive, i.e. 3+. Multiple specimen analysis is the correct way to stabilize a result to be validated. Therefore, in this study the results were analyzed separately for the first three specimens from a suspected prisoner. Among the first specimens, 28 were found to be positive by concentrated AFB smear microscopy whereas 20 were positive in direct AFB smear microscopy. It was clearly observed that identification number was significantly higher in concentrated AFB smear microscopy than direct AFB smear microscopy (Table 5).

In this study, culture and PCR based *IS6110* analysis was simultaneously done beside the AFB microscopic examination. For culture sputum specimens were incubated into L-J media and observed at weekly for efficient growth of *M. tuberculosis*. Positive AFB formed a small colony with buff color in L-J media and considered them for positive detection. Contaminated cultures having moulds, liquefied or dark green form were discarded. Besides PCR based *IS6110* analysis was done on the presence or absence of insertion sequence *(IS6110)* region. In the *IS6110* analysis, the region was amplified using two primers named *IS6110* F and *IS6110* R. This amplified product was identified by 1.5% Agarose gel electrophoresis. The presence of IS6110 region strictly conserved in *M. tuberculosis complex* and indicated positive results for AFB. 105 specimens were identified by the PCR based *IS6110* analysis. These were confirmed by the presence of the band (123 bp) on gel image (Fig. 2).

Results of culture method on L-J media and *IS6110* analysis were comparable because both the methods were considered as gold standard for identification of *M. tuberculosis* complex in the sputum specimen of pulmonary (Table 7). The PCR based analysis is very quick and sensitive for AFB detection but culture method need 8 weeks for a positive/negative result.

While the major objective of the present study was to establish the overall sensitivity and specificity of direct and concentrated AFB smear microscopy considering culture or *IS6110* PCR as gold standard method. Although AFB microscopy is believed to be the most practical and fastest technique in establishing a presumptive diagnosis of pulmonary tuberculosis but in order for tubercle bacilli to be seen by microscopic examination of sputum smears, there must be approximately 10^4 organisms per ml of sputum (Contreras A *et al.,* 1988).If false positives are very high then unnecessary therapy or prolonged hospital stay occurs, with further delay in the arrival at a correct diagnosis and proper treatment. The direct AFB smear method which is widely used in most of the laboratories for confirmation of pulmonary TB applicable to control programs in low income countries because of the less time involved in the preparation which is not without risk of infection to personnel handling sputum specimens. The concentrated technique which is used either NaOH or /and NALC for digestion and decontamination of the sample.

The sensitivity and specificity of the tested method were calculated as,

$$\text{Sensitivity} = \frac{TP}{TP + FN}$$

$$\text{Specificity} = \frac{TN}{TN + FP}$$

Where, TP = true positive, FN = false negative, TN = true negative, FP = false positive

The sensitivity of direct and concentrated smear microscopy was different on the use of positive culture and PCR analysis as a gold standard (70% vs. 83%) (Table 8). The concentrated smear microscopy can increase the sensitivity upto 12% but in negative culture and PCR analysis, the specificity was found similar at a rate of 96.31% and 97.81% respectively for direct and concentrated smear microscopy. However, the direct smear was always less sensitive than the smear made from the concentrated specimens overall. Therefore, caution must be exerted in laboratory settings when a direct smear is substituted for a smear made from a concentrated specimen. During those times when a laboratory is unable to adhere to the requirement to submit within 24 hour a smear report obtained by using a concentrated method, it should explore alternate methods such as that described by Saceanu *et al* .,(1993*)* or at least consider the direct AFB smear report to be preliminary and to confirm the result once the specimen is concentrated.

Conclusion

Taken together, direct smear microscopy is less sensitive than smear made from the concentrated specimens and furthers these two methods in combination with the PCR based *IS6110* analysis will be more sensitive at 100% for direct and concentrated smear microscopy respectively and specific upto 12% in both methods but the culture method needs more time than PCR analysis. The previously published study of (Marie Yvette *et al.,* 1995) showed no sensitivity for direct and concentrated smear microscopy and also Cattamanchi *et al.,* (2009) showed the same. In another studied results from Peterson *et al.*, (1999) showed a lower significance for direct smear microscopy than concentrated smear microscopy. There was no significant difference of sensitivity in direct and concentrated smear microscopy found from the published study of Cattamanchi *et al,* 2009 (10). 97% Specificity was found in both direct and concentrated microscopy. 70% and 82% sensitivity was found in direct and concentrated microscopy respectively. The present study was able to show a significant difference between the sensitivity and specificity of direct and concentrated smear microscopy which was confirmed by PCR based *IS6110* analysis in a cheap, fast and more accurately.

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Declaration of Interest

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