

Detection of *Mycobacterium tuberculosis* from Clinical Specimens by Conventional and Molecular Technique in Punjab, Pakistan

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Abstract: Pakistan is one of the high burden countries of *Mycobacterium tuberculosis* (MTB) infection globally, with high incidence and mortality. In this study, the conserved sequence, IS6110 was evaluated for the detection of MTB in respiratory specimens in Pakistan, where no such report is available earlier. A total of 1970 sputum samples in duplicate (on spot and morning) were collected. DNA was extracted from clinical specimens and H37Rv strain. Amplification of 135bp fragment of “IS6110” conserved sequence of MTB was carried out by PCR. Of 1970 samples, 452 (23%) were culture positive and 1518 (77%) were negative, 366 (19%) were smear positive and 1604 (81%) were smear negative. PCR successfully detected all smear positive and culture positive specimens except 8 smears positive and 26 culture positive specimens. While 77 smear negative and 9 culture negative specimens were positive in PCR. The overall diagnostic specificity of PCR was 99% and sensitivity was 94% taken culture as gold standard. PCR method targeting IS6110 sequence was of value in the identification of TB where there is strong clinical suspicion, particularly when the conventional techniques are negative. This study showed that IS6110 conserved sequence of MTB detection is a suitable method for rapid diagnosis in highly burden country such as Pakistan. This proposed study would help us for further research in order to reduce the burden of TB and reduce the mortality and morbidity by improving the treatment success rate.

Key words: *Mycobacterium tuberculosis* • Tuberculosis • Polymerase Chain Reaction • IS6110

INTRODUCTION

Tuberculosis (TB) is one of the oldest known diseases to human beings. It spreads through aerosols from person to person [1]. MTB is the major causative agent of tuberculosis (TB), a chronic infectious disease that remains an important public health problem throughout the world [2].

Pakistan is included in the nations having high prevalence of TB. TB infection is increasingly affecting the young generation widely, most prone are 15 to 45 years of age, in their maximum fruitful years of life [3]. The poverty, increasing population density, unhygienic life style, rapid industrialization, urbanization, illiteracy and environmental pollution are the principle affecting aspects for the incidence and spread of TB [4, 5]. Pakistan was 8th among the 22 highest TB burden countries [6], but now Pakistan is 5th among 22 highest TB burden countries [7]. Prevalence of all cases is 329 per 100,000 population.

Presently, the conventional TB diagnostic tests like Ziehl-Neelsen staining and MTB culture is less sensitive and time consuming, respectively [8, 9]. In published studies, 35% to 65% Acid Fast Bacilli (AFB) culture were reported as positive [10, 11]. Culturing is time demanding, requires 2 to 8 weeks for final results that leads to late treatment. Molecular techniques such as PCR, although requires technical skills and cost-effective are used in detection of MTB due to its higher sensitivity and time reduction [11]. Several reports have described the use of PCR in the analysis of pulmonary TB from sputum specimens [11, 12].

A specific nucleotide transposon, known as “IS6110” belongs to IS3 family is a part of MTB complex members and usually appears in multiple copies (up to 25 copies), having single copy number in *Mycobacterium bovis* BCG and is totally missing from the remaining mycobacterial strains [13, 14]. Due to this reason, “IS6110” sequence is the most common target used for the MTB detection in

clinical samples and confirmed that diagnostic rate of MTB complex targeting “IS6110” sequence has higher efficiency and sensitivity in diagnostics than microscopy or culturing techniques with significant time [15,16]. Various mycobacterial strains can be effectively diagnosed by using “IS6110” as a target sequence [13, 17]. IS6110 is highly polymorphic sequence therefore it is used as a genetic marker in epidemiological studies [18].

The present study was conducted to assess the usefulness of modern molecular techniques such as PCR to confirm the presence of MTB-DNA directly in the clinical specimen in Pakistan for the first time. Some of the studies reported lower efficiency of PCR in detection of MTB by amplification of 16S rDNA and hsp65 genes and IS6110. Although, using 16S rDNA gene sequencing is the common practice [19], but it requires subsequent sequencing and cannot differentiate between closely laying species of mycobacteria [19], therefore it is not a very good choice. Same is the case with hsp65; it also requires sequencing for proper differentiation between the non- tuberculosis mycobacterial strains and complex MTB [20, 21]. Therefore, the marker sequence like “IS6110” is the best choice, which exists only in MTB complex members, is the mainly used target for MTB DNA amplification [15]. The insertion sequence “IS6110” can also be used for distinguishing of the members of MTB complex from other mycobacteria in routine tests [13]. IS1081 has been used for the finding of MTB complex in the paucibacillary specimens [22].

This proposed study would help us to reduce the burden of TB and also reduce the mortality and morbidity by improving the treatment success rate, finally leading to better health.

According to the requirement of fast, specific and sensitive diagnosis of tuberculosis, the study was conducted having the following objectives:

- To evaluate the usefulness of PCR test for the detection of conserved sequence of MTB and to compare it with the culture as the laboratory gold standard.
- To compare the sensitivity of the PCR test on same samples with that of microscopy and culturing.
- To evaluate the role of PCR in the detection of *M. tuberculosis* DNA in clinical specimens.

MATERIALS AND METHODS

Patients were selected with a history of cough for more than two weeks with or without other symptoms,

pulmonary X-ray abnormalities and patients on TB treatment at the time. Permission to conduct the present study was provided by the members of ethical committee of Quaid-i- Azam University, Islamabad, Pakistan. Written consent for use of clinical record was taken from suspected patients who visited department of Bacteriology, Institute of Public Health, Lahore, Punjab for their treatment before the beginning of the study.

Sample Collection: A total 1970 sputum specimen (in duplicate) (on spot and morning) were collected in 50 ml sputum containers from Tuberculosis suspected individuals of general population of different areas of Punjab province.

Laboratory Diagnosis of Tuberculosis

Smear Microscopy: Sputum smear preparation and Ziehl-Neelsen (ZN) staining was done [23, 24].

Decontamination: All the pulmonary specimens were decontaminated with mucolytic agent *N*-acetyl *L*- cysteine (NALC- NaOH) method [25, 26].

Processing of Sputum for Culture: The sputum was poured in centrifuge tubes (at least 2 ml, not more than 5 ml) and added an equal volume of the NALC- NaOH solution to the tube and tightened the screw cap. Then vortexed for 20 seconds and kept at 20- 25°C for 15 minutes. After that, the tube was filled with phosphate buffer and vortexed. Then centrifuge was done at 3000 g for 15 minutes. Supernatant was poured off carefully and the deposit was resuspended in approximately 0.5 ml phosphate buffer. The sediment of processed specimen was inoculated onto two slopes of Lowenstein Jenson (LJ) medium with help of sterilized wire loop and placed in incubator at 37°C up to eight weeks or appearance of any visible colonies, whichever was earlier. Smear was prepared by applying one drop on a slide, marked with the ID number, for microscopy examination. In first week, cultures were observed twice to check contamination so that a timely request for other samples may be possible and later on cultures were observed after one week. The cultures, which were contaminated or show growth before one week, were discarded.

Identification of *M. Tuberculosis* by Biochemical Tests:

M. tuberculosis colonies on LJ medium were identified by para- nitrobenzoic acid (PNB) test and Nitrate reductase test (NRT) [27, 28].

DNA Extraction for PCR Assay: The DNA extraction was performed by the procedure given by Aldous *et al.* [29].

Quantitative Analysis of *Mycobacterium* Genomic DNA

Gel Estimation Method: DNA quantification was carried out using 0.8% Agarose gel. About 2 µl of Bromophenol blue dye was used, which acts as tracking agent to load 1 µl DNA on Agarose gel. The gel was run for 30 min at 100 volts maintained in 0.5 X TBE buffer. Standard DNA ladder of known concentration (50ng/µl) was used to run along with samples as a reference. DNA bands were observed in UV light through BIO-RAD gel documentation system. The quantification analysis of all the DNA samples was done and all samples were brought at same concentration level i.e. 50 ng/µl.

Direct Detection of MTB in Clinical Specimens by PCR

Assay: The MTB insertion sequence “IS6110” identification in respiratory specimens was used as target sequence for MTB detection through nested PCR [30, 12].

Confirmation of Mtbstrains: PCR was run with commercially designed primers F-1 and R-1 (Table 1) specifically for the 135bp IS6110 insertion sequence of MTB (it is a conserved sequence found only in MTB). It further allowed the detection and specific identification of the extracted MTB DNA.

The amplification reaction was performed in total volume of 20 µl in the 0.2 ml PCR reaction tube. An 18 µl of the above mixture was added into the tube and 2 µl of isolated DNA of each sample was added and 2 µl dH₂O, as negative control, was added into the corresponding tubes and was mixed thoroughly.

PCR Amplification: PCR reactions were performed in 4 steps. The mixture was first denatured at 95°C for 4 min. Then, in the next 35 cycles of PCR were performed with denaturation at 95°C for 30 seconds, primer annealing for 45 seconds at 50°C and elongation at 72°C for one min. In the last phase a final extension was done at 72°C for 10 minutes. For every reaction, a positive control, containing DNA from H37Rv MTB strain and negative control, in which DNA template was absent from the amplification mixture were included.

Detection of PCR Products: Amplification products were analyzed by 1.2% agarose gel electrophoresis. Conditions of electrophoresis were set to 80 volts for half an hour. Results of the gel were then viewed under UV light in Gel documentation system (Bio Rad).

Significance was determined by the χ^2 test with Yates's correction. The PCR results were classified as true positive (Tp), true negatives (Tn), false positive (Fp) and false negative (Fn). Sensitivity was calculated as: $Tp / (Tp + Fn) \times 100$ and Specificity was calculated as: $Tn / (Tn + Fp) \times 100$ [31]. A positive predictive value was calculated as $Tp / (Tp + Fp)$ and a negative predictive value was calculated as $Tn / (Tn + Fn)$.

RESULTS

Out of 1970 specimens subjected for the mycobacterium identification by ZN staining, culture and PCR (Table 4), a total of positive results were obtained in 366, 452 and 435 specimens by microscopy, culture and PCR, respectively (Fig. 1).

A total number of 1970 isolates were studied by microscopy and used for culturing. A total of 366 specimens were ZN positive, of these 164 were smear positive in both spot and morning specimens. A total of 144 specimens were positive in morning samples only and 58 were positive in spot specimens only. A total of 452 culture specimens were positive. Similar results obtained for 347 specimens were positive by both microscopy and culture. However, discordance was present in 124 cases. A total of 105 were positive in culture and negative by microscopy and 19 were positive by microscopy and negative in culture. A total of 11 samples were positive in microscopy but culture contaminated.

Comparison of MTB detection methods in 1970 specimens by both Molecular technique and smear microscopy is reported in Table 5 and considerable differences are shown. In reality, 77 smear negative specimens were detected by PCR and 8 smear positive specimens with low number of seen AFB were not detected by molecular technique. Furthermore, of the 1970 specimens, 1527 were negative by both microscopy and PCR and 358 were by microscopy positive and confirmed by PCR technique. When microscopy was used as reference, PCR sensitivity was 97.8% and specificity was 95 %. A positive predictive value was 0.82 and a negative predictive value was 0.99.

Comparison of results obtained in the 1970 specimens by molecular technique (PCR) and culture is reported in Table 5. The results obtained in the 1935 specimens were similar. Out of these, 1509 specimens were negative by culture and confirmed by PCR testing and 426 specimens were positive by both culture and PCR testing. Discordance was obtained for 33 cases in the specimens.

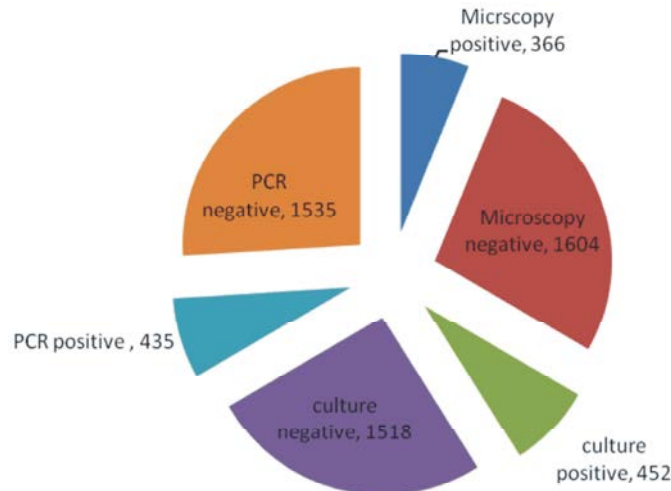


Fig. 1: Total number of negative and positive specimens by Microscopy, culture and PCR

Table 1: Primers used for the conserved sequence of *M. tuberculosis*

IS6110 –F	5- ACCTGAAAGACGTTATCCACCAT- 3
IS6110 –R	5- CGGCTAGTGCATTGTCATAGGA- 3

Table 2: Preparation of reaction mixture for PCR

No.	Reaction components	Volume / reaction (µl)	Concentration
1	PCR buffer	2	10X
2	MgCl ₂	2	25 mM/µl
3	dNTPs	2	2.5 mM/ µl
4	IS6110- Forward primer	0.75	10 mM/ µl
5	IS6110- Reverse primer	0.75	10 mM/ µl
6	Taq – polymerase	0.2	5U/ µl
7	Isolated DNA sample	2	50 ng/ µl
8	Double distilled water	10.3	Nil
9	Total reaction volume	20	

Table 3: PCR conditions

Step	Temperature (°C)	Time
Initial denaturation	95	4 min
Denaturation	95	30 sec
Annealing	50	45 sec
Extension	72	1 min
Final extension	72	10 min

Table 4: Comparison of results obtained by ZN Microscopy, culture and PCR

Total specimen	ZN staining		Culture technique		Nested PCR	
	Positive	Negative	Positive	Negative	Positive	Negative
1970	366	1604	452	1518	435	1535

Table 5: 2x2 table for PCR values for sensitivity and specificity on microscopy and culture

	+ve Microscopy	-ve microscopy	+ve Culture	-ve culture
+ve PCR	358	77	426	9
-ve PCR	8	1527	26	1509

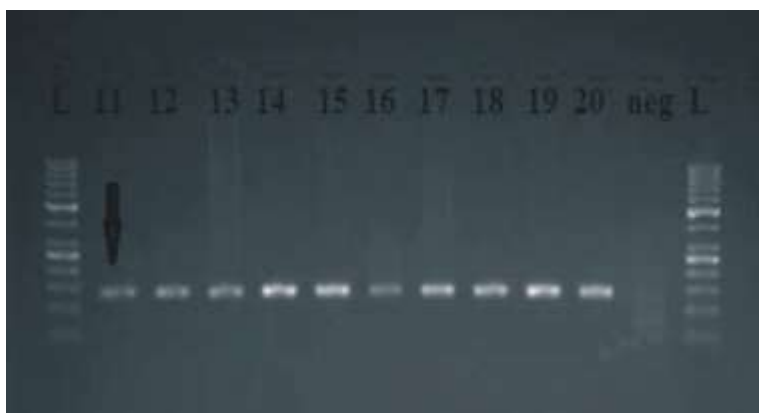


Fig. 2: Agarose gel electrophoresis of PCR technique for the confirmation of IS6110 sequence. 11, positive control; 12- 20, positive samples (135 bp); 21, negative control; and 50 bp DNA ladder on both side of gel.

A total of 9 specimens were negative in culture and positive in PCR. A total of 26 samples were negative in PCR and positive in culture. Amongst these 9 cases, four were positive in smear microscopy seen low number of AFB (Figure 2 and Table 5). When culture was used as reference, PCR sensitivity was 94% and specificity was 99 %. A positive predictive value was 0.97 and a negative predictive value was 0.98.

The presence of insertion sequence in MTB was tested by PCR using primers design based on IS6110 sequence (Table 1). In these results, the sensitivity and specificity of the direct PCR technique in clinical specimens to culture as “Gold standard” were calculated of the 1970 samples. For that reason, the PCR technique in these results has a fine sensitivity (94%) and excellent specificity (99%). The results showed the positive predictive value (0.97), negative predictive values (0.98). Thus rapid testing using PCR targeting a conservative region specific for MTB which presence only in MTB, suggesting this sequence is a reliable source for the rapid diagnosis and intolerance of MTB isolates in Pakistan.

DISCUSSION

All over the world the timely diagnosis of MTB, drug resistance confirmation is the most challenging issue nowadays, especially in the case of smear negative sputum, body fluids, pus and biopsies [32]. The diagnosis of Tuberculosis (TB) is a big problem for the developing countries because conventional techniques are less sensitive and time demanding and modern and sensitive techniques are very much expensive. Definitive diagnosis of tuberculosis depends on isolation and identification of

the causative agent. Growing of *M. tuberculosis* is time consuming, so rapid detection is necessary for better treatment. It is necessary to find new method for early and rapid detection of MTB. Microscopy is fast and rapid method for diagnosis, but it is suitable only for the preliminary diagnosis due to low sensitivity and specificity. It can only detect 60 to 70 % acid-fast bacilli (AFBs) in culture positive specimens [33, 34]. Although microscopy is insensitive, its fast and simplicity make it widely diagnostic test for many laboratories. It can be used to assess the infectivity of a patient with pulmonary tuberculosis. Patients generates smear positive specimen are the most infectious and quick treatment can be given before the culture result obtained. Microscopy cannot distinguish between non tuberculosis and tuberculosis. Additionally ZN microscopy can detect AFB, if more than 10, 000 organism per ml present in sample [8].

Diagnosis of TB on culture based still well in spite of its inherent limitations such as take long time and requirement of specialized facility. Culture required 6 to 8 weeks for final results for TB diagnosis, appropriate treatments of individuals infected by MTB are often delayed. Although MTB culture is time consuming technique but its high sensitivity is reported in this study in comparison with direct examination, 452 sputum cultures were positive versus 366 microscopy positive. These results are in agreement with the worldwide use of MTB culture as a “gold standard” technique [35]. In some previous studies false positive cultures have been reported due to cross contamination in laboratories and the mean false positive rate has been 3.1% [36]. The sensitivity of smear microscopy can be affect by many factors, counting sputum quality, smearing grounding, staining procedures, assessment time and the amount of

training received in correct smear examination. The sensitivity of microscopy increases due to concentration of sputum specimens, e.g. by centrifugation [37]. The results obtained in this study of 1970 isolates by microscopy and cultures are reported in table 5. A total of 366 specimens were positive by ZN microscopy versus 452 cultures positive. In the smear positive specimens, 164 was smearing positive in both spot and morning specimens and 144 were positive in morning samples only and 58 were positive in spot specimens only. Similar results obtained for 347 specimens were positive by both microscopy and culture. However, discordance was present in 124 cases. 105 were positive in culture and negative by microscopy and 19 were positive by microscopy and negative in culture. A total of 11 samples were positive in microscopy but culture contaminated. Thus there is an urgent need of the new techniques, for safe, rapid and correct diagnosis of TB. Molecular techniques has becomes trends in rapid diagnosis of tuberculosis. Some of the studies reported lower efficiency of PCR in detection of MTB by amplification of 16S rDNA and hsp65 genes and IS6110. Although using 16S rDNA gene is the common practice [19] but it requires subsequent sequencing and can't differentiate between closely laying species of mycobacteria [19], therefore it is not a very good choice infect. Same is the case with hsp65; it also requires sequencing for proper differentiation between the non tuberculosis mycobacterial strains and complex MTB [20, 12]. Therefore the marker sequence like "IS6110" is the best choice, which exists only in MTB complex members, is the mainly used target for MTB DNA amplification [15]. The insertion sequence "IS6110" can also be used for distinguishing of the members of MTB complex from other mycobacteria in routine tests [13]. IS1081 has been used for the finding of MTB complex in the paucibacillary specimens [22]. Early diagnosis of tuberculosis is essential for clinical management and public health control measures. A significant part of this study was devoted to the development and evaluation of rapid diagnostic protocols for MTB. The present study was conducted to assess the usefulness of modern molecular techniques such as PCR to confirm the presence of MTB DNA directly in the clinical specimen.

In the present study, we compared the results of PCR with the cultures and microscopy. Fast DNA preparation method was used for PCR for rapid detection of *M. tuberculosis*. A total number of 1970 isolates were studied. All the isolates were tested by microscopy, cultures and by PCR.

The core drawback of primer- based PCR is false negative results in regarding 20% of sputum samples due to the existence of Taq DNA polymerase inhibitors [38], or false positive results due to the PCR amplicons or due to the contamination of the samples with mycobacterial DNA.

In the previous study reported that the presence of low or missing copy number of insertion sequence "IS6110" in *M. tuberculosis* strains from other regions such as in Denmark 50% of strains showed 11- 15 copies of IS6110 [39]. In the 75% of the strains showed 6- 10 copies in the Tunisia [40] and 11- 20 % of the strains showed zero or 1- 2 copies of IS6110 in the different geographical region of India [41]. In the less than 1% in San Francisco [42] and 2 % in the Vietnam [43] showed IS6110 lacking strains.

The diagnostic sensitivity of the PCR was 97.8% and specificity was 95% for the respiratory specimens. In smear negative specimens the bacterial load was low, often challenging for the rapid diagnostic methods such as PCR. This PCR protocol involves less manual handling procedures so that minimum risk of cross contamination.

The 99% specificity of the carefully designed primers perfectly distinguishes MTB from other mycobacterial species in culture. PCR based method could reduce the turn-around-time from 6-8 weeks to 24 hours in routine service. PCR would be used to supplement conventional diagnostic methods.

RECOMMENDATIONS AND CONCLUSIONS

Under no circumstances PCR can replace conventional culture techniques since rare isolates with no IS6110 insertion sequence will be missed. To implement proper public health control measures, mycobacterial culture is also essential for surveillance of epidemiology and drug resistant MTB in the community. We proposed (recommended) that great care should be taken in designing primer pairs for the insertion sequence IS6110 to avoid false negative or false positive results. For diagnostic PCRs, multiple targeting regions such as IS6110 and hsp65 or IS6110 and TRC4 could be fine policy to enhance correctness of *M. tuberculosis* detection in clinical specimens.

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