Original Article

Improved detection of *Mycobacterium tuberculosis* using two independent PCR targets in a tertiary care centre in South India

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Abstract

Introduction: Tuberculosis (TB) causes significant morbidity and mortality worldwide as one of the leading infectious diseases. In India, more than 1.8 million new cases occur every year. Rapid and accurate diagnosis of TB would improve patient care and limit its transmission. This study aimed to evaluate a dual target polymerase chain reaction (PCR) diagnostic assay to detect *Mycobacterium tuberculosis* from pulmonary and extra-pulmonary samples at a tertiary care centre in South India.

Methodology: Samples were collected from patients with a low index of suspicion of TB. Acid-fast smears were performed by Auramine O fluorescent microscopy and PCR was performed by using two site-specific primer pairs targeting IS*6110* by nested PCR and TRC_4 by conventional PCR. Amplified products for IS*6110* and/or TRC_4 were indicative of *M. tuberculosis*.

Results: Among 114 (19 pulmonary and 95 extra-pulmonary) samples tested by PCR assay, 12 (11%) were positive for both IS*6110* and TRC_4, of which 11 (10%) were non-respiratory and one was (1%) respiratory in origin. PCR for TRC_4 alone was positive for eight (7%) non-respiratory and two (2%) respiratory samples, while IS*6110* alone tested positive for six (5%) non-respiratory samples and one (1%) respiratory sample. Of a total of 29 PCR positive samples, 17 (15 %) were acid-fast smear positive.

Conclusion: Although the target site of IS*6110* is specific for *M. tuberculosis*, some strains from South India may lack this region. Therefore, the use of an additional target site (TRC_4) is required for improved detection of *M. tuberculosis*.

Key words: *Mycobacterium tuberculosis*; polymerase chain reaction; IS6110; TRC_4; South India

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Introduction

Tuberculosis (TB) is the major cause of death by a bacterial infectious disease worldwide. The World Health Organization (WHO) estimates that there were approximately 8.9 to 9.9 million incident cases of TB and about 1.1 to 1.7 million TB deaths globally in 2008. India contributes the highest number of new cases, accounting for 20% of the global burden, of which approximately 1.2 % occur in persons with HIV [1]. In India, pulmonary TB is the most common form of the disease; however, extra-pulmonary TB comprises 20% of cases [2]. Early diagnosis and identification of TB are essential in instituting effective, timely therapy. Diagnosis of TB is mainly based on clinical presentation, histopathology, and the demonstration of acid-fast bacilli (AFB) in smears and the isolation of *Mycobacterium tuberculosis* from culture. These diagnostic criteria have limitations that include atypical clinical presentations of disease, and poor sensitivity and specificity of AFB microscopy, particularly with paucibacillary specimens. Culture for *M. tuberculosis* usually takes four to six weeks to grow on solid media, delaying time to results. The diagnosis of extra-pulmonary TB is particularly difficult to establish, especially in developing countries because of the paucibacillary nature of extra-pulmonary specimens and because the signs and symptoms of disease can be non-specific [3,4].

Polymerase Chain Reaction (PCR) allows detection of *M. tuberculosis* directly from clinical specimens [5] and, given the limitations of conventional culture methods, PCR is currently the most sensitive, rapid diagnostic laboratory method [6]. However, considering the number of cases diagnosed with TB in India, there is an urgent need to use multiple diagnostic modalities for rapid detection of *M. tuberculosis* to control the transmission of TB. In the United States, molecular tests are routinely performed to detect *M. tuberculosis*. In India,

however, molecular methods such as PCR are under-utilized, even when there are no cost constraints.

Some of the targets used to amplify *M. tuberculosis* for detection include *IS6110*, 65kDa, and 38kDa antigen coding regions [7]. Most studies have generally targeted the multi-copy IS*6110* sequence of the genome [7,8,9,10,11]. However, the absence or the presence of only a few copies of this sequence has been reported in some strains, particularly those from Southeast Asia [12]. A large number of clinical isolates of *M. tuberculosis* from South India [13] had either a single copy (40%) or no copy (4%) of IS*6110*, thus indicating the need to incorporate additional target sites for improved detection. TRC_4, which is specific for *M. tuberculosis* complex, was sequenced and patented by the Tuberculosis Research Centre (TRC-ICMR), Chennai, India, as an ideal target for PCR assays to identify *M. tuberculosis*; especially in strains carrying no copies of IS*6110* in tuberculous meningitis and tubercular pleuritis patients [13,14,15] .

The present study was undertaken to detect *M. tuberculosis* by a PCR assay that combined targets IS*6110* and TRC_4 as well as by acid-fast fluorescent microscopy from pulmonary and extra-pulmonary specimens obtained from patients with undiagnosed fever who attended specialty clinics at a tertiary care center in South India. Tuberculosis may be under-diagnosed in these patients as the primary problems are specialty related.

Methodology

Patient population

This study was conducted from January 2007 to August 2008, utilizing patients seen in specialty care clinics for nephrology, neurology, general medicine, obstetrics and gynaecology, gastro-enterology and cardiology of a tertiary referral centre. The clinical samples collected from the clinic patients who had undiagnosed fever and co-morbidities were tested for detection of *M. tuberculosis* using a PCR assay.

Specimens analyzed

Among the 114 specimens analyzed, 95 (83%) were extra-pulmonary, consisting of 40 urine samples, 18 cerebrospinal fluids (CSF), 14 body fluids, seven pus, five tissues, five continuous ambulatory peritoneal dialysis fluid (CAPD), two endometrial aspirates, and one specimen from each of the following: gastric aspirate, bone marrow aspirate, fine needle aspirate, and semen. The 19 (17%) pulmonary specimens tested consisted of 13 pleural fluids, four sputum samples, and one from each of the following: endo-tracheal secretion (ET), and bronchoalveolar lavage (BAL).

Sample processing

All specimens were sent to the laboratory in sterile containers and were processed per the manufacturer's instructions (commercially available kit; GeNei, Bangalore, India). In brief, the specimens were processed and decontaminated by N-acetyl L-Cysteine (NALC)-NaOH method and then centrifuged at 8,000 rpm for 15 minutes. All collected sediments were aliquoted into multiple vials and the remaining were processed for PCR within 24 hour or stored at -20°C in a freezer until tested. Fluorescent smear microscopy by auramine-O method was performed from each specimen using standardized procedures [16]. Tissue biopsy specimens were homogenized in a sterile container (GeNei, Bangalore) before testing. With a view to rapid diagnosis, the clinicians sent the samples only for PCR; therefore, culture for *M. tuberculosis* was not performed.

Quality control

Reagents were aliquoted and each aliquot was used only once. Sterile microfuge tubes and PCR tubes were used for the PCR assay. Reagent preparation, DNA extraction, DNA amplification and detection were performed in separate rooms to avoid cross-contamination of amplicons. A positive control was included in each test and distilled water was included as a negative test control. Uracil-N-glycosylase (UNG) was used in the amplification process to avoid post PCR DNA contamination.

Amplification of mycobacterial DNA

DNA was extracted from specimens per the manufacturer's instructions (GeNei, Bangalore, India).

(a) Nested polymerase chain reaction (nPCR): A single tube nested PCR was performed using the proprietary IS6110 primer sequences (GeNei, Bangalore, India) targeting *M. tuberculosis*. The DNA was amplified with forward and reverse primers of the outer region of *M. tuberculosis* and the first product was amplified with the inner primer in the second amplification per the manufacturer's instructions (GeNei, Bangalore, India).

The PCR conditions for outer sense primers for the first round of amplification were as follows: initial denaturation at 22°C for 10 minutes, 94°C for 5

Table 1. Comparison of PCR targeting IS*6110* and *TRC*$_4$ in clinical samples from various specialty clinics wth smear microscopy

Origin of sample	Speciality clinics	Clinical Sample	AFB Smear		Number of PCR positives	PCR Positive		
			Positive	Negative		IS*6110* alone	*TRC*$_4$ alone	IS*6110* + *TRC*$_4$
Extra-pulmonary	Nephrology	Urine	5	6	11	4	3	4
	Obstetrics Gynaecology	Peritoneal fulid	-	1	1	1	-	-
		peritoneal nodule	1	-	1	-	-	1
		Urine	1	-	1	1	-	-
		Endometrial aspirate	1	1	2	-	2	-
	Neurology	Pus	4	-	4	-	2	2
	Gastro enterology	Pus	1	1	2	-	-	2
		Urine	-	1	1	-	1	-
	Cardiology	Colonoscopy	1	-	1	-	-	1
	General medicine	Urine	1	-	1	-	-	1
Pulmonary	Nephrology	Sputum	-	1	1	-	1	-
	Neurology	Endotracheal secretion	-	1	1	-	1	-
	Cardiology	Sputum	1	-	1	-	-	1
	General medicine	pleural fluid	1	-	1	1	-	-
Totals			17	12	29	7	10	12

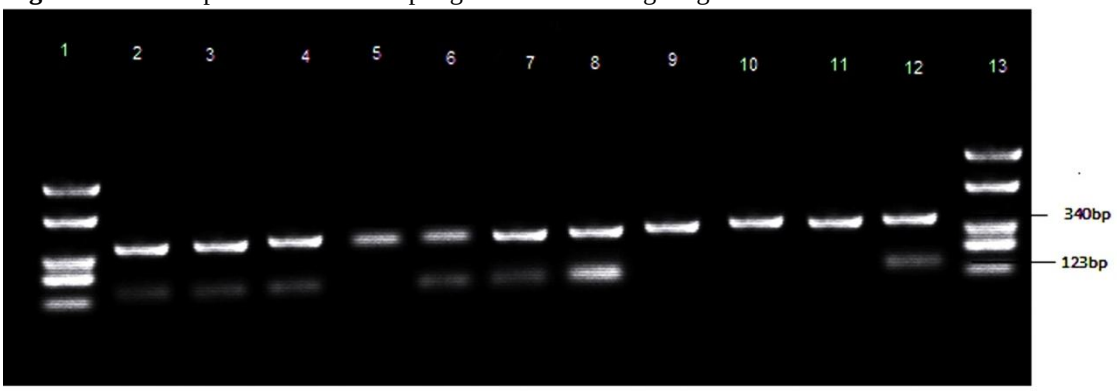
minutes, 20 cycles of 94^{o}C for 30 seconds, 68^{o}C for one minute, 72^{o}C for one minute, and a final extension of 72^{o}C for 7 minutes. The PCR conditions using the inner set of primers for the second amplification consisted of initial denaturation step at 94^{o}C for 5 minutes, 30 cycles of 94^{o}C for 30 seconds, 68^{o}C for 30 seconds, 72^{o}C for 30 seconds and a final extension of 72^{o}C for 7 minutes.

(b) Conventional PCR targeting *TRC*$_4$: The primer and red dye Master Mix kit were obtained from Bangalore Genei. Samples were amplified using *TRC*$_4$ Primer-1 (5'-GACAACGACGTGCGCCTACT-3') and *TRC*$_4$ primer-2 (5'-ACCGAATTAGCGTAGCTCC-3'). The amplification cycles were performed on an automated thermal cycler (MJ Research Corp, Encino, CA, USA). The initial step of denaturation was not performed. The amplification consisted of 35 cycles of 94^{o}C for one minute, 58^{o}C for one minute, 72^{o}C for one minute and the final primer extension step at 72^{o}C for 10 minutes.

The detection of amplified PCR products was determined using agarose gel (2%) electrophoresis stained with ethidium bromide (0.5 µg/ml) and subsequently visualized on the 260 nm wavelength UV transilluminator of the gel documentation system (BIO-RAD, Hercules, CA,USA). A result was considered positive for the target when a well-defined DNA band corresponding to the sample was observed along with the controls and molecular weight marker. Samples which had amplified products measuring 123 bp for IS*6110* and 173 bp for *TRC*$_4$ were considered positive (Figures 1 and 2).

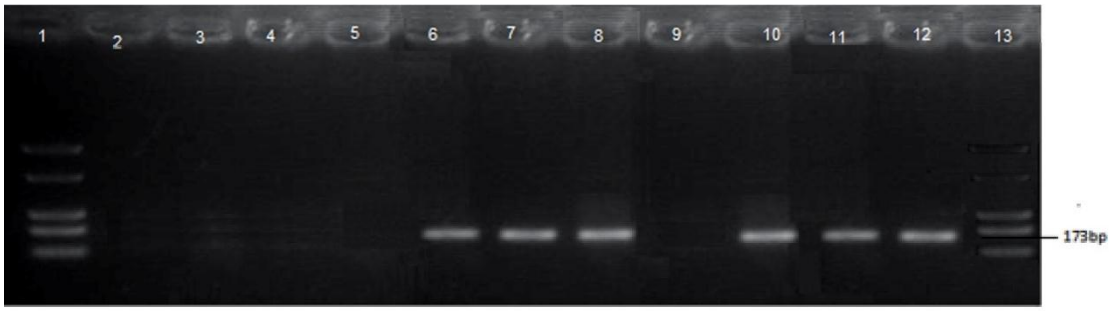
The PCR tests were performed by investigator (RB) from pulmonary and extra-pulmonary samples. These samples were retested by another investigator (GS) who was blinded to the initial test results. The same techniques were used as outlined previously and the tests were performed in the same laboratory.

Figure 1. PCR amplification of 123 bp region in IS*6110* targeting PCR for *M. tuberculosis*



Lanes 1 and 13: molecular weight marker; Lanes 2, 3, 4: positive for 123 bp; Lanes 5 and 9: negative control; Lanes 6, 7, 8: positive for 123 bp; Lanes 10 and 11: negative for 123 bp; Lane 12: positive control

Figure 2. PCR amplification of 173 bp region in *TRC4* gene of *M. tuberculosis*



Lanes 1 and 13: molecular weight marker; Lanes 2, 3, 4: negative for 173 bp; Lanes 5 and 9: negative control; Lanes 6, 7, 8, 10, 11: positive for 173 bp; Lane 12: positive control

Results

Of the 114 specimens analyzed, 17 (15%; 15 non-pulmonary and 2 pulmonary) were AFB smear-positive. These included seven urine samples, five pus samples, and one of the following: peritoneal nodule, colonoscopy fluid, endometrial aspirate, pleural fluid, and sputum. All 17 AFB smear-positive specimens were also PCR-positive for *M. tuberculosis*. With regard to PCR target detection, four samples were IS*6110* positive, five were *TRC*$_4$ positive and eight were positive for both IS*6110* and *TRC*$_4$. Table 1 shows the distribution of non-pulmonary and pulmonary specimens, their PCR reactions**,** and their AFB smear results.

In addition, there were 12 AFB smear-negative specimens that were also PCR positive. These included seven urine samples, one peritoneal fluid, one sputum, one endometrial aspirate, one pus sample, and one endotracheal secretion. Of these, three specimens were PCR-positive for *IS6110* alone, five for *TRC*$_4$ alone, and four were positive for both *IS6110* and *TRC*$_4$.

Overall, 25 non-pulmonary and four pulmonary specimens (29/114, 25% of all samples) were positive using the dual target PCR assay. Therefore, as compared to smear microscopy, the PCR method showed a 71% improvement in detection of *M. tuberculosis*.

Analysis of samples by PCR using IS*6110* and *TRC*$_4$ showed seven samples (6.1% of all specimens) to be positive for IS*6110* but negative for *TRC*$_4$ (six non-respiratory and one pleural fluid), while 10 samples (9%) were positive for *TRC*$_4$ but negative for IS*6110* (four urine samples, two endometrial aspirates, two pus samples, one sputum and one endotracheal secretion). Twelve specimens were positive for both IS*6110* and *TRC*$_4$ (11%). These included five urine samples, four pus samples, one peritoneal nodule, one colonoscopy fluid, and one sputum.

In summary, sample analysis by PCR using IS6110 and TRC$_4$ resulted in seven samples positive for IS6110 and negative for TRC4 and 10 samples positive for TRC$_4$ and negative for IS6110. Seventeen

samples were positive by smear microscopy and were also PCR positive.

Discussion

TB remains a major global public health problem, especially in India. The clinical utility of detecting *M. tuberculosis* by PCR is its reduction in the time to detection and its accuracy in detecting the pathogen in AFB smear-negative paucibacillary specimens. A recent meta-analysis [17] concluded that fluorescence microscopy was approximately 10% more sensitive than conventional carbol fuchsin AFB stains and takes less time to read, but its limitations include the expense of purchasing a fluorescent microscope and the requirement for reading slides in a darkroom.

In our study, PCR was performed by amplification of the *IS6110* insertion sequence, which belongs to the IS3 family and is found in almost all members of the *M. tuberculosis* complex. Most strains carry 10 to 15 copies, which are present in a wide variety of chromosomal sites [18]. Previous studies have documented increased positivity using the IS*6110* target in extra-pulmonary samples [7]. Negi *et al.* analyzed various targets specific for *M. tuberculosis* and reported the highest PCR positivity rates for pulmonary (90%) and extra-pulmonary (77%) samples when using IS6110; their results further confirmed the poor sensitivity of smear microscopy for pulmonary (49%) and extra-pulmonary (24%) samples. Tiwari *et al.* reported 62% PCR positivity in 133 extra-pulmonary samples and 22% positives by smear microscopy [7,19]. A potential problem with using this target is that some strains from certain parts of the world lack the IS*6110* insertion sequence [20]. Even though PCR has been used in various laboratories; the sensitivity of PCR can vary from 11% to 81% [21]. Of the 114 samples analysed, 10 samples were IS*6110* negative and TRC_4 positive, and seven samples were IS*6110* positive and TRC_4 negative. Therefore, PCR using one target alone cannot detect all strains of *M. tuberculosis* but the use of two targets can improve pathogen detection. Previous studies conducted at the Tuberculosis Research Centre have similarly suggested that the sensitivity of PCR can be increased by using two sets of targets (IS*6110* and TRC_4) to detect tuberculous meningitis and tubercular pleuritis [14,15]. Our study confirms the utility of dual targets to detect *M. tuberculosis* from extra-pulmonary sites.

Patients presenting to the nephrology clinic had chronic kidney disease (CKD), or were on dialysis or were post-transplant. Preliminary reports suggest that patients with hemodialysis, or CKD are more prone to genitourinary TB [22, 23]. In India, genitourinary TB comprises 20% of all extra-pulmonary cases [24]. Since patients attending specialty clinics are receiving therapy for their primary disease, the symptoms of TB may be inconsistent or atypical in presentation. Very often the clinicians need evidence to aid them in the decision-making process to start anti-tuberculous drugs. Though culture remains the gold standard for TB diagnosis, solid culture media would require four to six weeks for growth. This delay would negatively affect patient care. While time to positivity with liquid culture media could be reduced to approximately one to four weeks, the financial investment and the need to subculture to solid media for identification pre-empts the advantage.

The majority of the clinical samples for testing were obtained from nephrology patients with CKD, renal failure, ongoing dialysis or post transplant. Extra-pulmonary specimens were the majority (83%) analysed in our study and these cases may be missed had the PCR assay not been performed. In addition, the exclusion of the TRC_4 primers in PCR assays would have resulted in samples being reported as falsely negative. A major limitation of our study is that the results of PCR could not be compared with those of culture to obtain the performance characteristics of the dual-targeted molecular assay.

We suggest that PCR and other culture methods for *M. tuberculosis* detection be used in conjunction with clinical parameters to obtain a diagnosis of TB. PCR is a powerful diagnostic tool to detect *M. tuberculosis*, in extra-pulmonary TB cases [25,26]. Other rapid tests such as serology have at present no defined role in TB diagnosis [27]. While culture remains the gold standard, clinicians prefer to use rapid tests to solve clinical dilemmas. It is therefore important to optimize techniques to improve PCR assays to detect *M. tuberculosis*. Currently, there is an urgent need for diagnostic laboratories that utilize PCR methods to incorporate two primers (IS6110 & TRC_4) to increase accuracy of *M. tuberculosis* detection. Additional, rapid assays that are inexpensive are needed in resource poor areas to assist in curtailing the transmission of TB.

Conclusion

The data presented here support the development of PCR assays with dual targets to improve *M. tuberculosis* detection in clinical specimens. Since

some strains from South India may lack the IS6110 element, PCR assays using primers from a repetitive element such as TRC_4 will improve test sensitivity. There is an urgent need to increase awareness of the importance of incorporating TRC_4 in the molecular diagnostic assays used for detection of *M. tuberculosis*. Our study focused on paucibacillary specimens from specialty care clinic patients, who often present with atypical clinical symptoms and AFB smear-negative specimens. We have demonstrated increased PCR sensitivity using dual targets for *M. tuberculosis* detection.

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