Comparative evaluation of PCR using IS6110 and a new target in the detection of tuberculous lymphadenitis

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We evaluated TRC4 primers using polymerase chain reaction (PCR) which amplify a new target sequence from *Mycobacterium tuberculosis* genome to diagnose tuberculous lymphadenitis and compared the results with PCR using the widely used IS6110 primers. The PCR results were also compared with conventional methods like smear, culture and histopathology. The sensitivity of PCR using both probes is higher than the conventional methods. Out of 101 samples analysed (49 fresh and 52 fixed specimens), PCR using IS6110 and TRC4 primers was positive in 64 and 70 samples, respectively, whereas results with culture and histopathology methods were positive only in 49 and 58 samples, respectively. The problem of false negativity of IS6110 due to the absence of IS6110 copy in 4 *M. tuberculosis* isolates was overcome by using TRC4 primers. The results indicate that with improvement in PCR techniques, PCR using both probes, IS6110 and TRC4 can be a rapid and sensitive adjunct to conventional techniques in the diagnosis of tuberculous lymphadenitis.

TUBERCULOSIS (TB) has been gaining much interest in recent years due to the pandemic of human immunodeficiency virus, which is a significant risk factor for the development of tuberculosis[1]. Conventional methods of detecting mycobacteria in clinical samples, especially in extrapulmonary TB are either low in sensitivity and specificity or are time consuming. There is an urgent need for developing newer tools for the rapid diagnosis of TB.

In India, where the incidence of TB is high, TB lymphadenitis continues to be one of the most frequent causes of lymphadenopathy 30–52% (ref. 2). Cervical lymphnode involvement is the most common form and accounts for 70% of all the lymphnode TB[2]. Diagnosis is usually established by demonstrating mycobacteria either by histopathology or on smears subjected to acid fast stains. Histopathology revealing caseating granulomas is highly suggestive of mycobacterial infection but other factors may produce similar histology. Culture takes 4 to 8 weeks for detection of the organism.

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The detection of *M. tuberculosis* by polymerase chain reaction (PCR) has been found to be useful in the diagnosis of extrapulmonary and pulmonary TB. Several target sequences have been described to detect *M. tuberculosis*[3–8] that include the rRNA genes[2,9], single copy genes encoding structural proteins of 65 and 38 kD (refs 10–12), insertion element IS6110 (ref. 13), and *dnaJ* gene[14]. The sensitivities and specificities using various targets varied from 60 to 100%.

The most widely used primers to detect *M. tuberculosis* in clinical specimens by PCR are from the insertion element IS6110. We previously reported that 40% of the Madras strains carried only a single copy of IS6110 and 4% did not carry even a single copy of IS6110 (ref. 15). Since IS6110-based PCR for diagnosis may in some cases lead to false negative results, we developed a new target for PCR using repetitive element, TRC4. This is a conserved sequence and repeats at least four times in the genome of *M. tuberculosis*[16]. Our previous study using RFLP showed that TRC4 was present in all the 200 *M. tuberculosis* strains analysed[16] (unpublished data). It was also present in the strains which did not harbour the IS6110 copy. Since the TRC4 fragment is a conserved repeat element present in all the strains, we consider it to be a valuable target for diagnostic PCR. From the deduced sequence of 2.126 kb to TRC4 several primer pairs have been designed and one set of primers amplifies a target sequence of 173 bp consistently. The specificity of TRC4 has been analysed using slot blot hybridization[16], Southern blot hybridization with radioactively labelled TRC4, PCR amplification of non-mycobacterial species and atypical mycobacteria using primers which amplify 173 bp product (unpublished observation).

In the present study we evaluated PCR using IS6110 and TRC4 (new target) to detect *M. tuberculosis* from lymphadenitis specimens and compared the results with conventional bacteriological and histopathological methods.

We did a prospective study on 49 fresh lymphnode specimens from 49 patients. The Department of Pathology, Kilpauk Medical College, Chennai, provided the above samples. These fresh specimens were coded and each lymphnode was cut into small pieces. These small pieces were randomly allocated for smear, culture, histopathology and PCR.

A retrospective study was done on 52 formalin-fixed paraffin-embedded tissues from 52 patients included in a study conducted during 1983–1993 by the Department of Pathology, Tuberculosis Research Centre, Chennai.

For histopathological studies, a portion of the fresh lymphnode was fixed in 10% formalin, processed routinely and stained with haematoxylin and eosin (H and E). The sections were also stained by the Ziehl–Nielson (ZN) method for acid fast bacilli.

Histologically TB was diagnosed when a necrotizing, caseating granuloma surrounded by epithelioid cells, lymphocytes, plasma cells and giant cells was seen.

For microscopic studies, lymphnode specimens were cut into small pieces using sterile scissors and forceps. 5.0 ml of sterile distilled water was added and homogenized using an electrical homogenizer. One loopful of this concentrated mixture was taken for smear. The slides were examined by auramine–rhodamine fluorochrome staining.

For culture, the aseptically homogenized lymphnode sample was centrifuged, the supernatant was discarded, the deposit was decontaminated using 5% sulphuric acid and was resuspended in 7 ml of selective Kirchner's liquid medium (KL)[17] and cultured for mycobacteria in multiple media. The culture media used were Lowenstein–Jenson (LJ) medium, LJ medium containing 0.5% sodium pyruvate (LJP), 7H11 oleic acid (Difco Laboratories), albumin agar with malachite green (Difco) and Kirchner's liquid medium (KL).

For the retrospective study, DNA from fresh tissue was extracted using the method of Wilson[18] with slight modification. Thin sections of paraffin-embedded lymphnodes were dewaxed by adding 400 µl of xylene in an eppendorf tube. The specimens were rehydrated sequentially in 100, 80, 70 and 50% ethanol for 10 min with centrifugation after each step of rehydration. After rehydration, the specimen was processed by the CTAB method as mentioned earlier.

A 173 bp region from the repetitive sequence, cloned and identified in our laboratory was selected for amplification on the basis of specificity analysis. Hot start PCR was carried out in a 25 µl volume in 50 mM KCl, 10 mM, Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 0.5 µM of oligonucelotide primers TRC Pri1 and TRC Pri2 (patent pending), or IS6110 primers, (a) 5′CCTGCGAGCGTAGGCGTCGG3′; (b) 5′CTCGTCCAGCGCCGCTTCGG3′, 200 mM of four dNTPS, 0.5 U Taq DNA polymerase (Amersham, UK) and the DNA from clinical specimens. The reactions were subjected to amplification with the following profile 1′ at 96°C, 1′ at 58°C and 1′ at 72°C for 35 cycles. A negative control (consisting of all the reaction components except the DNA template), a processing control (water blank included during the extraction of DNA from the sample) and a positive control (50 ng of *M. tuberculosis* $H_{37}Rv$ DNA) were included in every assay. After amplification, an aliquot of PCR reaction was analysed on 2% agarose gel. After visualization with ethidium bromide, the DNA was blotted onto nylon membrane by the vacuum blotting method (Hoefer Instruments, USA). The amplified product or the whole target sequence of positive control was labelled with horse radish peroxidase and used to probe the immobilized, amplified products.

Stock solutions (including those required for the processing of the sample for DNA extraction) were prepared with millipore filtered, sterile water. These were prepared in a separate room, aliquoted, autoclaved, stored and used only once. Chemical decontamination of surfaces (daily) and equipment (weekly) was done with 0.5% sodium hypochloride and 70% ethanol. Prevention of DNA contamination was further accomplished by physically separating the different steps of the PCR procedure, using separate set of pipettes and tips.

Out of 101 lymphnode specimens included in this study, 49 were fresh lymphnode specimens (unfixed) and 52 were fixed and paraffin-embedded specimens. These samples were coded and taken for PCR analysis.

Positive samples and positive controls produced DNA bands of 123 bp with the IS6110 primers and 173 bp with TRC4 primers (Figure 1). All clinical samples that gave 123 bp and 173 bp fragments on ethidium bromide stained agarose gels were also positive by Southern hybridization with the respective probes. The detection limit of PCR using TRC4 primers was earlier tested using dilutions of genomic DNA purified from *M. tuberculosis*. Reactions were adjusted such that they contained 5×10^7–1 genome equivalent (5 fg of DNA is approximately 1 genome equivalent). PCR was able to detect as few as 1 genome equivalent by agarose gel electrophoresis and Southern hybridization (data not shown). Hybridization increased neither the specificity nor the sensitivity in our study.

Comparison of the results from bacteriological examination, histopathology and PCR is shown in Table 1. Out of 101 samples, only 11 were positive by smear microscopy. Culture and histopathology were positive in 49 and 58 samples, respectively. PCR using IS6110 and TRC4 primers was positive in 64 and 70 samples, respectively. A higher percentage of positives has been obtained with PCR in fresh lymphnode specimens than histopathology, when compared to PCR from fixed specimens.

Figure 1. PCR amplification of clinical samples using TRC4 and IS6110 primers. Lane 1, Negative control (no DNA) with TRC4 primers; Lane 2, positive control (*M. tuberculosis* $H_{37}Rv$ DNA) with TRC4 primers amplifying 173 bp product; Lane 3, positive control (*M. tuberculosis* $H_{37}Rv$ DNA) with IS6110 primers amplifying 123 bp product; Lane 4, negative control (no DNA) with IS6110 primers; Lane 5, molecular weight marker (*Hin*dIII); Lane 6, processing control with TRC4 primers (water blank included during of the clinical sample); Lane 7, a negative clinical sample (with TRC4 primers); Lanes 8 to 11, clinical samples which show 173 bp; Lane 12, processing control with IS6110 primers (water blank included during processing of the clinical sample); Lanes 13 to 16, positive clinical samples which show 123 bp product.

Table 1. Comparison of final diagnosis with results from bacteriological examination, histopathology and PCR

Type of specimen	No. of patients	No. of specimens positive by				
		Smear microscopy	Culture	Histo-pathology	PCR	
					IS6110	TRC4
Fresh lymphnode	49	9	21	22	32	35
Formalin-fixed paraffin-embedded specimens	52	2	28	36	32	35

Among the 101 samples included in the analysis, results for pathology and culture were not available for samples, 1 and 6 respectively. The summary of the analysis is shown in Table 2. Out of these, twenty-eight were positive by culture, histopathology and PCR using two probes. Thirteen samples were negative by all the above criteria, and 12 samples which were positive by culture and histopathology were also positive with either of the probe. Eight samples which were negative by culture and histopathology were also negative by one of the probes. Eight samples which were positive by PCR using both the probes were positive by either culture or histopathology. The agreement of PCR using IS6110 and/or TRC4 with either culture or histopathology was 79%. The two probes classified 14 samples as positive, which were negative by culture and histopathology. Among these, 3 samples were from patients with strong clinical evidence of TB, and two samples were from patients with past history of treatment for TB. Six samples were false negative by both probes and positive by both culture and histopathology. Three samples were negative by PCR and positive by either culture or histopathology. Two samples were negative by PCR using IS6110 and culture, positive by PCR using TRC4 primers and histopathology.

There has been disagreement between the various criteria used for diagnosis. The smear results have not been included in this analysis because they have very low sensitivity. Culture and histopathology differed in 13 samples. Eleven samples were called negative by culture but positive by histopathology and 2 samples vice versa. Similarly there has been discordance between the two probes.

Among the 22 samples in which the PCR results differed between the two sets of primers, 14 were positive using TRC4 primers but were negative by IS6110 primers. Four out of the above 14 samples were negative by culture and histopathology. Hence the actual false negatives by IS6110 are 10. These 10 strains were once again analysed for the presence of IS6110 copy by performing PCR using DNA extracted from the culture of the respective clinical isolate. Out of 10 samples, 4 samples did not have the IS6110 copy. Six false negativity of IS6110 was partially due to the absence of IS6110 from 4 of the TB isolates.

Among the 8 strains which were positive by IS6110 and negative by TRC4 primers, 4 samples were negative by smear, culture and histopathology. Only 4 were false negative by TRC4 primers in contrast to 10 false negatives by IS6110 primers.

Table 2. Comparative evaluation of PCR and conventional methods

Culture	Histo-pathology	PCR		No. of samples
		IS6110	TRC4	
+	+	+	+	28
–	–	–	–	13
+	+	+	–	4
+	+	–	+	8
+	–	+	+	1
–	+	+	+	7
–	–	+	–	4
–	–	–	+	4
–	–	+	+	14
+	+	–	–	6
–	+	–	–	2
+	–	–	–	1
–	+	–	+	2
NA	–	+	+	3
NA	+	+	+	1
+	NA	+	+	1
NA	–	+	ND	1
NA	–	–	+	1

Positive, (+); Negative, (–); NA, not available; ND, not done.

If we use culture as the standard method and calculate sensitivity, TRC4 primers have a sensitivity of 86% with fresh specimens and 78% with all specimens (fresh and fixed) which is better than IS6110 primers with a sensitivity of 76% in fresh and 69% in all specimens. This is not statistically significant. McNemar's test was used for comparison of the probes.

Extrapulmonary manifestations of TB need early and sensitive diagnosis even though it is three times less frequently encountered than pulmonary TB (ref. 19). Conventional procedures to detect *M. tuberculosis* are low in sensitivity due to the low number of infecting organisms. Nucleic acid amplification procedures allow rapid detection of *M. tuberculosis* from clinical specimens. PCR is difficult to be incorporated in clinical laboratories on a routine basis because of the extreme care and precaution demanded by the technical steps involved. Nevertheless, the high degree of sensitivity and rapidity render a positive PCR result clinically useful and there is ample scope to improve the methodology.

In the present study we have used a new target (TRC4) for enzymatic amplification to detect *M. tuberculosis* from suspected lymphnode specimens. The TRC4 primers amplify a 173 bp product. It has already been shown that the longer the amplified fragment, the higher the likelihood of degradation and thus lower the efficacy of the amplification itself[20,21]. Although we designed several primer pairs from the repetitive element (TRC4), which amplify various lengths of the product, we chose Pri1 and Pri2 which gave a consistent 173 bp in a preliminary study. There have been very few studies on PCR in TB lymphadenitis[22,23]. In this study we compared PCR using 2 probes with conventional methods like culture and histopathology and also compared the sensitivity of PCR in fresh versus paraffin-fixed specimens.

Among the conventional tests chosen, the sensitivity of microscopy has been very low and already it is known that this method has a detection limit of 10^4 mycobacteria per sample[8]. Even though culture is considered good standard its sensitivity is low in extrapulmonary-TB due to the paucibacillary nature of the specimen. PCR is able to detect *M. tuberculosis* in such paucibacillary specimens.

Apart from the false negativity problems encountered with PCR techniques, the lack of IS6110 copies in some of the strains also contributed to the false negativity which has been overcome by TRC4 primers. TRC4 has been found to be better in sensitivity (statistically not significant) than IS6110 especially in detecting IS6110 negative strains in this study. The false negative results could have resulted from the presence of inhibitors not detected by control amplification and non homologous distribution of bacteria in the specimen, so that the fraction tested does not contain mycobacteria. These reasons could justify the false negativity observed with both the probes in 6 out of 94 samples. The reason for false negative results in 14 samples with either of the two set of primers is not known. To minimize this discrepancy, more than one specimen from each patient has to be tested just like culture. PCR also should be repeated twice with the same specimen for confirmation when there is a difference in the results of the two probes.

Marchelti *et al.*[24] have reported that the concentration of DNA used affected the outcome of the amplification protocols considerably. It has been reported that the effectiveness of PCR with formalin-fixed paraffin-embedded tissue is impaired by multiple interacting factors including the type of fixative used[25]. Our results also are in agreement with those of Greer *et al.*[25], that the sensitivity of PCR using both probes TRC4 and IS6110 was lower in paraffin-embedded specimens than in fresh lymphnode specimens.

The present study indicates that with improvements in PCR techniques, the PCR with both probes IS6110 and TRC4 can be used as a fast and sensitive adjunct to other conventional techniques in the diagnosis of extrapulmonary TB.

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