Peripheral blood based C-PCR assay for diagnosing extra–pulmonary tuberculosis

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Extra pulmonary tuberculosis (EPTB) constitutes around 20% of all tuberculosis cases in India. Conventional methods are of limited use in diagnosing this form of the disease. Polymerase chain reaction (PCR) has emerged as a sensitive and specific tool for documenting the presence of Mycobacterium tuberculosis in clinical samples but lacks quantitative ability. The present study evaluates peripheral blood as an alternative clinical specimen for diagnosing EPTB. Peripheral blood samples from 38 EPTB and 89 non tuberculous subjects were analyzed for the presence of tubercle bacilli by MPB 64 gene based PCR method. The assay gave an overall sensitivity of 60.53% with negative predictive value of 76.92% which is superior to present gold standard of mycobacterial culture (10.53 and 72.36%). Additionally, 43.82% of non tuberculous subjects gave positive results with the PCR, thus mitigating the clinical utility of this test. An in-house Competitive PCR (C-PCR) assay was used to determine the mycobacterial load in peripheral blood from culture positive, culture negative EPTB patients and non tuberculous controls which ranged from 7498 – 12498, 602 – 4797 and 101 – 800 genome equivalent (ge)/mL, respectively. The data clearly demonstrated that C-PCR assay can furnish insightful information in diagnosing extra pulmonary disease.

Keywords: Competitive PCR, Extra-pulmonary tuberculosis, Mycobacterium tuberculosis, PCR

Incidence of extra pulmonary tuberculosis (EPTB) is on the increase world over and the same is higher in Asians than Caucasian populations1,2. Rapid diagnosis followed by immediate initiation of treatment is essential for arresting the progression of this fatal disease not only at individual level but also within the community. The conventional approaches to diagnose pulmonary tuberculosis (TB) either lack sensitivity or are time consuming and these limitations are further accentuated in patients with extra pulmonary presentations. Sputum is the most frequently used specimen for revealing the presence of tubercle bacilli in TB. However, its clinical significance in EPTB is very discouraging3. The diagnosis in such cases poses great challenge and depends upon procuring relevant clinical material from the site of infection that often requires invasive procedures. In view of the mentioned difficulties, the institution of appropriate anti tuberculosis therapy (ATT) is by and large subjective and depends on clinical acumen of the physician4.

Polymerase chain reaction (PCR) has emerged as a promising alternative tool with a high degree of sensitivity and specificity over the conventional methods5. Standard PCR, a qualitative test, fails to differentiate individuals with clinically active disease from the infected ones. Quantitative differentiation is therefore warranted in Indian scenario where approximately 40% of the total adult population is infected with M. tuberculosis bacilli6. Competitive-PCR (CPCR) assay is a sensitive quantitative method for enumerating mycobacterial load in clinical specimens7. Since earlier reports document hematogenous dissemination of M. tuberculosis in TB patients8,9, the present study evaluates the clinical utility of an in-house newly developed MPB 64 gene based C-PCR assay for detection and identification of M. tuberculosis in peripheral blood of EPTB patients.

Materials and Methods

Clinical specimens
Peripheral blood samples (38), along with plural effusion specimens, were collected before the start of
ATT from extra pulmonary TB patients visiting DOTS centers at Sri Guru Ram Das Institute of Medical Sciences and Research, Amritsar, India and TB and Chest Hospital, Govt. Medical College, Amritsar, India. All patients were HIV negative with no history of immunosuppressive conditions such as renal transplantation, diabetes, radiotherapy and cancer. Name, age, sex, history of ATT, family history of ATT and AFB status were recorded of each patient. Additionally, 89 peripheral blood samples were collected as non tuberculous controls. Informed consent was obtained in writing from all the participants and the study was approved by the research degree board of the Guru Nanak Dev University, Amritsar, India.

**Processing of clinical specimens**

**Pleural effusion sample**—Pleural effusion samples collected in the presence of sodium fluoride (10 mg/mL), as an anticoagulant and preservative were centrifuged at 10,000 rpm for 15 min. The pellet obtained was used for microscopic analysis and culture of mycobacteria using Lowenstein – Jensen (L-J) slants following standard mycobacterial procedures.

**Peripheral blood**—Red blood cells were selectively removed by lyses of peripheral blood samples collected from TB patients and control subjects. The remaining leucocytes were pelleted and subjected to mycobacterial DNA isolation employing modified freezing and thawing protocol for PCR analysis.

**PCR analysis**—PCR amplification was performed on isolated DNA samples using specific primers for MPB 64 gene of *M. tuberculosis*. The sequence of the primers used to amplify the 240bp region was:

Forward primer (FW) 5'-TCCGCTGCCAGTCGTCTTCC-3' and Reverse primer (RW) 5'-GTCTCAGCGAGTCTAGGCCA – 3'.

Amplification reaction was performed in 25 μl of master cocktail containing 10 mM Tris (pH 9.0), 50 mM KCl, 0.01% gelatin, 1.5 mM MgCl₂, 50 μM of each dNTP ( dATP, dGTP, dCTP and dTTP), 200 nM of each primer, 25 μg/mL of 8-Methoxypsoralen (Sigma-Aldrich Inc., MO, USA). The content was exposed to UV radiations for 4 min followed by the addition of 0.5 U of Taq polymerase (Bangalore Genei, Bangalore, India). The reaction mixture was subjected to initial denaturation at 94°C for 3 min and then cycled through 35 cycles of denaturation at 94°C for 30s, annealing at 60°C for 30s and extension at 72°C for 30s followed by holding at 72°C for 3 min. PCR products were analyzed on 2% agarose gel stained with 0.5 μg/mL of ethidium bromide.

**C-PCR assay**

*Development of competitor*—Strategy for generating a competitor of MPB 64 gene is shown in Figs 1 and 2. A 30bp modified FW (MFW) primer was designed to have its 5' flanking region similar to the FW primer, and an additional 10bp region (from nt 522 to 531) appended to the 3' end. The MFW and RW primer pair was used to amplify a DNA fragment (competitor construct) of 198bp, which was resolved in agarose gel. Subsequently, it was eluted and purified using gel extraction kit (Bangalore Genei, Bangalore, India) as per the manufacturer’s instructions. The competitor (198bp) and the target (240bp sequence of MPB 64 gene) were initially amplified separately and then co-amplified with the same primer pair (FW and RW) at an optimized annealing temperature of 55°C using the same reaction conditions and cycling parameters as described above.

*Determination of mycobacterial load*—The bacillary load was determined in the peripheral blood samples from EPTB patients and non tuberculous subjects. Constant amount of mycobacterial DNA was coamplified with known amount of competitor constructs and the absolute absorbance of amplified products (240bp and 198bp) were compared. The

![Fig. 1— Strategy to develop competitor of MPB 64 gene of *M. tuberculosis* genome](image-url)
point of equivalence was determined by plotting log of the ratio of target and competitor (Log T/C) against log of competitor (Log C), and the number of tubercle bacilli were calculated.

Statistical analysis—Analysis was carried out using SPSS ver. 10 for windows software (SPSS Inc., Chicago, IL, USA). Sensitivity, specificity, positive predictive value (PPV) and negative predictive values (NPV) were determined.

Results
A total of 127 individuals participated in the present study, of which 38 were extra pulmonary TB patients (tuberculous pleural effusion) while the rest 89 donors were asymptomatic for TB. The distribution of patient and control subjects based on age, gender, ATT history and family history of ATT is summarized in Table 1.

Microbiological analysis—No extra pulmonary specimen yielded positive results with AFB staining, while only 10.53% of them gave positive culture results.

PCR analysis—PCR amplification readily detected MPB 64 gene sequence of *M. tuberculosis* in all the peripheral blood samples of culture positive extra pulmonary TB cases, whereas 55.88% of microbiologically negative clinically diagnosed extra pulmonary TB patients revealed positive amplification results from blood (Table 2). Additionally, 43.82% of the control subjects amplified *M. tuberculosis* specific PCR products with peripheral blood. Overall sensitivity and NPV for peripheral blood based PCR assay in extra pulmonary disease was 60.53% and 76.92% when compared to culture isolation of mycobacteria (10.53% and 72.36%). However, its specificity and PPV were 56.18% and 37.09% as against that of culture (100%) (Table 3). Interestingly, the intensity of PCR products in non tuberculous cases was significantly lower than that obtained from patient population (Fig. 3).

<table>
<thead>
<tr>
<th>Age in year</th>
<th>Extra pulmonary TB (38)</th>
<th>Non tuberculous (89)</th>
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<tbody>
<tr>
<td></td>
<td>02-75 (31.66 ± 15.94)</td>
<td>16-42 (25.42 ± 07.87)</td>
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<th>Sex (Male/Female)</th>
<th>Extra pulmonary TB (38)</th>
<th>Non tuberculous (89)</th>
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<td>21/17</td>
<td>59/30</td>
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<th>History of ATT (Yes/No)</th>
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<th>Non tuberculous (89)</th>
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<td>00/38</td>
<td>00/89</td>
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<tr>
<th>Family history of ATT (Yes/No)</th>
<th>Extra pulmonary TB (38)</th>
<th>Non tuberculous (89)</th>
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<tr>
<td>05/33</td>
<td>03/86</td>
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Table 1—Demographic features of subjects [Values in parentheses are mean ± SD]

<table>
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<tr>
<th>Group of patients</th>
<th>PCR Status</th>
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<tr>
<td>Patient group (n)</td>
<td>PCR positive (%)</td>
</tr>
<tr>
<td>Smear negative culture positive</td>
<td>04 (100)</td>
</tr>
<tr>
<td>Extra pulmonary TB patients (04)</td>
<td>19 (55.88)</td>
</tr>
<tr>
<td>Non tuberculous (89)</td>
<td>39 (43.82)</td>
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Table 2—Amplification of MPB 64 gene of *M. tuberculosis* in the peripheral blood samples of patient population

Fig. 2—Generation and co-amplification of 198bp competitor with *M. tuberculosis* target DNA. [Lane M-100bp DNA ladder; Lane 1- Purified *M. tuberculosis* DNA; Lane 2-198bp competitor generated using MFW and RW primers; Lane 3- Purified 198bp competitor amplified using FW and RW primers and Lane 4- Target and competitor co amplified using FW and RW primers in the same tube]

Fig. 3—Representative agarose gel electrophoresis of PCR products using MBP-64 gene specific primer pair from EPTB patients and controls. [Lane M- 100bp DNA ladder; Lane 1- Negative control; Lane 2-smear negative, culture positive EPTB patients; Lanes 3,4-smear negative, culture negative EPTB patients; and lanes 5,6- non tuberculous controls]
C-PCR assay

Validation of C-PCR assay—Constant amount of DNA was taken and its 10-fold dilution was titrated against serially diluted competitor with its concentration ranging from 10 fg to 1000 fg. Densitometric and computational analyses revealed the point of equivalence to be 195.706 and 20.214 fg, respectively (Figs 4, 5). The bacillary load thereby calculated was $9.02 \times 10^5$ and $9.3 \times 10^4$ copies in the two dilutions.

Determination of mycobacterial load—In order to quantify mycobacterial load in culture positive and culture negative EPTB patients, the dilution range of competitor varying from 10 to 1.25 fg and 2.5 to 0.3125 fg, respectively was titrated with constant amount of DNA (Fig. 6A). Densitometric scanning followed by computational analysis revealed the point of equivalence to be 2.485 and 0.629 fg (Fig. 6B) which corresponds to 11,431 and 2,893 copies of \textit{M. tuberculosis}, respectively. Similarly, in non tuberculous subjects, the dilutions of competitor varied from 0.1 to 0.0125 fg (Fig. 6C). The point of equivalence was revealed to be 0.025 fg which corresponds to 115 copies of \textit{M. tuberculosis} organisms.

Apparently, the mycobacterial load determined by MPB 64 gene based C-PCR assay in peripheral blood samples from smear negative culture positive extra pulmonary TB patients ranged from 1.630 – 2.717 fg which corresponds to 7,498 – 12,498 \textit{M. tuberculosis} organisms, whereas in culture negative patients, the point of equivalence varied between 0.131 – 1.043 fg which is equivalent to 602–4,797 TB bacilli. In non tuberculous controls the point of equivalence ranged from 0.022-0.174 fg which reflected that in asymptomatic patients the detectable TB bacilli by C-PCR varied from 101 – 800 (Table 4).

<table>
<thead>
<tr>
<th>Variables tested</th>
<th>Extra pulmonary TB patients (n=38) %</th>
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<tr>
<td></td>
<td>Culture</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>10.53</td>
</tr>
<tr>
<td>Specificity</td>
<td>100</td>
</tr>
<tr>
<td>PPV</td>
<td>100</td>
</tr>
<tr>
<td>NPV</td>
<td>72.36</td>
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Table 3—Comparison of sensitivity, specificity and predictive values between culture and peripheral blood based PCR in EPTB patients

<table>
<thead>
<tr>
<th>Patient Group (n)</th>
<th>Point of equivalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extra pulmonary TB patients (38)</td>
<td>1.630 – 2.717 fg</td>
</tr>
<tr>
<td>Smear negative culture positive (04)</td>
<td>0.131 – 1.043 fg</td>
</tr>
<tr>
<td>Smear negative culture negative (34)</td>
<td>0.022 – 0.174 fg</td>
</tr>
<tr>
<td>Non tuberculous (39)</td>
<td>0.131 – 1.043 fg</td>
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Fig. 4—Top panel showing a representative agarose gel electrophoretic resolution of co-amplified products of unknown concentration of \textit{M. tuberculosis} DNA. [Lane T- mycobacterial DNA Target; Lane C- competitor. The lower panel shows the determination of point of equivalence by computational analysis following densitometric scanning of agarose gel picture]
Discussion

The conventional approaches to diagnose extra pulmonary TB either lack sensitivity or are time consuming, which is an important impediment to global TB control. The same is apparent from the present investigation, as none of the patients with extra pulmonary presentation was found to be smear positive. Moreover, only 10.53% EPTB specimens could grow on L-J slants. The lower sensitivity of culture in extra pulmonary disease is well accepted and explained by the fact that mycobacteria might be inactivated by immune response of the host\textsuperscript{13}. The average time for detection of \textit{M. tuberculosis} in extra-pulmonary samples was 48.16 ± 13.37 days. (Data not shown).

PCR has been shown to be a promising alternative for establishing rapid diagnosis of tuberculosis with a high degree of sensitivity and specificity. Extra pulmonary TB is usually a paucibacillary disease and patients often present with atypical symptoms as it may involve almost any organ of the body. Appropriate biological sample from such patients is collected employing invasive procedures and in some cases it’s virtually impossible to collect the specimen. These problems warrant less perilous and more accessible clinical specimen. \textit{M. tuberculosis} disseminates into the peripheral blood of TB patients, with or without compromised immune function\textsuperscript{8,9,14}. Therefore, peripheral blood is a good alternative clinical material in patients with EPTB for detecting \textit{M. tuberculosis} by PCR.

PCR yielded high sensitivity as well as NPV but low specificity and PPV when compared to culture isolation of \textit{M. tuberculosis} in extra pulmonary disease (Table 3). High NPV of peripheral blood based PCR test in EPTB patients strongly indicates that the test could help in excluding the presence of TB disease, which is in disagreement to a recent report\textsuperscript{15}. Therefore, this remarkable ability of blood based PCR test to detect EPTB cases can replace the need for more invasive diagnostic approaches. Interestingly, ours is the first investigation where a single copy target (MPB 64 gene) based PCR has been utilized for detecting genome of \textit{M. tuberculosis} in peripheral blood. Other studies on EPTB employed multicopy target, IS 6110, for peripheral blood based PCR assay\textsuperscript{16-18}. However, IS 6110 based assay has a big disadvantage in Indian scenario where a sizable proportion of \textit{M. tuberculosis} isolates are known to lack these elements\textsuperscript{19,20}.

The specificity of PCR assay in present investigation was found to be lower (56.18%) than culture (100%). The low specificity was evidently influenced by positive PCR results (43.82%) among non-TB subjects, which in turn undermines the clinical relevance of this test in diagnosing TB. It is important to mention that the intensity of the PCR products from non tuberculous controls was much lower as compared to their diseased counterparts and was possibly due to lower mycobacterial burden in control population. This finds support from the observation that highest intensity of amplified products was observed among smear negative culture positive patients (Fig. 1). The possibility of contamination was ruled out by assessing the amplification results in the presence of 8-methoxypsoralen; the latter in the presence of UV radiations, is known to intercalate into double stranded nucleic acid thereby forming a covalent interstrand cross-link, which is inhibitory for their amplification\textsuperscript{21}. Additionally, due precautions were taken to avoid contamination by separating the areas where blood samples were processed for DNA isolation, from areas of PCR amplification and analysis of amplified products. The mycobacterial presence in peripheral blood of controls can be explained by the fact that around 40% Indian adults are reported to be infected by \textit{M. tuberculosis} and do not manifest the symptoms of active disease\textsuperscript{6}. Clearly the standard PCR failed to differentiate asymptomatic controls from the paucibacillary EPTB patients.

To address this concern, standard PCR was modified to enable quantification of mycobacterial
load by C-PCR assay and thereby differentiate asymptomatic controls from their active counterparts. C-PCR technique is based on the assumption that amplified product ratio of target and competitor reliably reveals the ratio of their initial copy number. Equimolar concentration of the target and competitor in the reaction resulted in amplification of PCR targets of equal intensity. Given the amount of competitor is known at the point of equivalence; the amount of the target could be determined.

Prerequisite for the C-PCR assay is a competitor which differs in size from the mycobacterial target. A difference, of 42bp, in size of the target and the competitor was created using a simple PCR based strategy (Figs 2, 3). The underline principle of C-PCR assay was validated using an unknown amount of M. tuberculosis DNA from culture biomass and also assaying its 10-fold dilution. Computational analysis of the densitometric scanning of the amplified products revealed the bacterial DNA load of 195.706 fg and 20.214 fg, respectively (Figs. 4, 5). These determinations revealed a very good fit thus verifying the reliability of this technique in determining bacillary load.

C-PCR analysis of DNA samples isolated from peripheral blood of culture positive EPTB patients revealed point of equivalence as 2.485 fg which was equivalent to 11,431 ge/mL bacilli (Fig 6A). Similarly, among culture negative patients, the cPCR assay (Fig. 6B) reflected the mycobacterial burden to be equivalent to 2,893 M. tuberculosis organisms, which was almost one fourth of culture positive individuals. Furthermore, the point of equivalence for asymptomatic controls (Fig. 6C) was 0.025 fg which corresponds to 115 ge/mL of M. tuberculosis.

Based on C-PCR assay in peripheral blood of EPTB patients, the mycobacterial load varied from 7498-12,498 ge/mL in smear negative/culture positive to 602-4797 ge/mL in smear negative/culture negative patients. However, among non tuberculous controls, the mycobacterial load ranged between 101-800 ge/mL. These observations suggested that individuals with bacterial load of <800 ge/mL should be treated as carrying clinically irrelevant number of bacilli, where as those with a threshold value of >7498 bacilli/mL should indicate an active disease (Table 4). Additionally, all those individuals harboring mycobacterial load between these values need to be considered as presumptive TB cases. Keeping in view the enormity of TB burden in India, more detailed investigations are needed to ascertain the significance of mycobacterial load during various clinical stages of M. tuberculosis infection, especially in different Indian populations where such data is totally lacking.

In conclusion, the data generated in the present study clearly exhibits extraordinary sensitivity of C-PCR assay in differentiating between clinically irrelevant and relevant mycobacterial load. This study also points out that the dissemination of M. tuberculosis in peripheral blood is more common than previously thought. This novel armamentarium, in fight against tuberculosis, could help in understanding the dissemination dynamics of tubercle bacilli in circulation. Moreover, such an approach could bring a new dimension in the early detection of M. tuberculosis, in EPTB patients, from a readily accessible clinical specimen and would help in the better management of this ancient scourge.

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