TRC4 GENE BASED PCR ASSAY IN DIAGNOSIS OF EXTRAPULMONARY TUBERCULOSIS

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Summary

Diagnosis of Extra-pulmonary tuberculosis (EPTB) is challenging due to atypical presentation and various inadequacies of diagnostic tools. This study aimed to evaluate polymerase chain reaction (PCR) assay by using TRC4 primers in comparison with the IS6110 primers to detect Mycobacterium tuberculosis from extra pulmonary samples. This study was done in Microbiology and Immunology Department of BSMMU from 162 extra-pulmonary samples sent for laboratory investigation of M. tuberculosis during the period of March 2013 to February 2014. All patients were subjected to AFB smear by Z-N staining, culture in L-J media and PCR by two primes IS6110 and TRC4. Among EPTB cases 24 (25.26%) were positive by AFB smear and/or mycobacterial culture and 73(76.84%) cases were positive by PCR test. PCR showed significantly higher detection rate of EPTB cases than that of AFB smear and L-J medium culture (p <0.005). Only IS6110 and only TRC4 were positive in 4.1% and 12.5% cases respectively. The sensitivity of PCR using TRC4 primers was higher (85.71%) than IS6110 primers (80.95%). Repetitive elements TRC4 can be an ideal target for PCR assays to identify M. tuberculosis; in strains carrying no copies or single copy of IS6110 and can play a significant role in diagnose the EPTB cases.

Key words

Extra pulmonary tuberculosis; Polymerase chain reaction; IS6110 primers; TRC4 primers; Mycobacterium tuberculosis.

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Introduction

Tuberculosis (TB) still remains the most important bacterial infectious disease worldwide as approximately 9 million new cases and 1.4 million TB deaths were estimated globally in 2011 [1]. Extrapulmonary Tuberculosis (EPTB) accounts for one fifth of all the cases of tuberculosis in immunocompetent patients. In Bangladesh, the EPTB prevalence was reported to be 4.8% to 21.98% [2,3,4]. EPTB has a wide spectrum of clinical presentation depending upon the anatomical site involved causing a diagnostic dilemma for the physician. Demonstration of granuloma in tissue biopsy or of acid-fast bacilli (AFB) in smears or mycobacterial culture in specimen are less sensitive tools in these cases due to the paucibacillary nature of the specimens [5]. The sensitivity of AFB microscopy in EPTB samples ranges only from <5%- 20% [6,7]. Although mycobacterial culture in Lowenstein-Jensen (LJ) media remains the gold standard for laboratory confirmation of TB disease, the isolation rate in extrapulmonary samples ranged only from 23 to 56% as 10 to 100 viable bacilli are needed for a positive culture [8,9]. Moreover the culture methods are quite slow (requiring 3–8 weeks) that can lead to unacceptable delay in diagnosis. A positive finding of histopathology from extrapulmonary samples coupled with mycobacterial culture is thought to be the most sensitive method available but can miss up to 20% of cases as many times representative tissue/body fluid may not be accessible [8]. Studies have shown that positive Mantoux test, elevated ESR, cytology, elevated adenosine deaminase level in various body fluids may be important components in the diagnosis of EPTB [10,11]. However, all the above mentioned methods has not been established as confirmatory. These inadequacies necessitate the development of an improved, sensitive and specific test for early detection of mycobacterium in EPTB specimen. Polymerase chain reaction (PCR) is highly sensitive and specific assay which may even detect 1-10 organisms within 3-6 hours in different clinical specimens [12,13,14]. Its sensitivity ranges from 42% to 93% depending on the clinical specimens targeting various parts of the genome such as IS6110, 65kDa, devR, TRC4, GCRS, MPB64 etc for the detection of Mycobacterium tuberculosis [6,15,16,17,18,19].

Among them, most of the studies have generally targeted the multi-copy IS6110 sequence of the genome with a sensitivity of 80.5% [20,21,22]. The absence or the presence of only a few copies of this sequence has been reported in some strains, particularly those from Southeast Asia [20]. A large number of clinical isolates of M. tuberculosis from South India had either a single copy (40%) or no copy (4%) of IS6110 resulting false negative results [23]. Also some atypical non pathogenic mycobacterium may contain homologous sequences with and can give false positive results [24,25]. These observation questions the utility of IS6110 as a sole target for mycobacterial gene amplification in diagnostic laboratory and indicating the need to incorporate additional target sites for higher detection. TRC4, a repetitive element with three open reading frames (ORFs) has been demonstrated to be highly sensitive and specific targets for PCR assays to identify M. tuberculosis with sensitivity of 91%; even in strains carrying no copies of IS6110 in tuberculous patients [22,23,26]. This study was aimed to evaluate the PCR assay by using TRC4 primers in comparison with IS6110 in the detection of Mycobacterium tuberculosis from various clinical samples of extrapulmonary tuberculosis.

Materials & methods

The present study was conducted between March 2013 and February 2014 after getting ethical approval. A total of 162 clinical samples like pleural fluid, ascitic fluid, urine, CSF, endometrial tissue, lymphnode and pus collected from clinically suspected cases of extrapulmonary tuberculosis of all age and sex group were enrolled in the present study. The samples were transported to the laboratory immediately after their collection. All samples were subjected to ADA testing and cytological, biochemical, microbiological, histopathological and conventional PCR test examination by two primers TRC4 and IS6110. The tissues were homogenized by using a tissue homogenizer. N-Acetyl-L-Cysteine (NALC) plus 2% NaOH method was used to homogenize the viscous samples. The fluids were centrifuged at 3000g for 20 minutes. The deposits samples were used for the Ziehl-Neelsen staining (ZNS), culture on the Lowenstein-Jensen Medium (LJM) and PCR. DNA extraction was done by using the QIA amp DNA mini kit (QIAGEN) by the spin column method as per the manufacturer's instructions. PCR was performed by conventional gel electrophoresis with the primers targeting IS6110, and TRC4. The cultures were observed for a period of eight weeks before they were declared as negative for growth. The treatment response with anti tubercular drugs were followed up in all patients in the TB group and showed marked improvement.

Diagnostic criteria: EPTB cases were diagnosed by clinical manifestation and/or imaging evidence suggesting TB with one or more of the followings criteria: [10,27,28,29,30]

1) Positive tuberculin test and significant level of ADA value (40 U/L for pleural and ascitic fluid and 10U/L for CSF) in lymphocytic exudative body fluid (protein level 0.45g/l and /or glucose 2.5 mmol), or Presence of granuloma in the biopsy tissue

2) Detection of AFB by microscopy or culture from clinical samples.

3) Positive tuberculin test and / or high ESR and clear response to anti-TB drugs.

A positive treatment response was defined as the improvement of clinical and / or imaging findings after two months of treatment. Using these criteria, the study patients were categorized into two groups- tuberculous (TB) group and non-tuberculous (Non-TB) group.

Data analysis

All data were analyzed by using computer based SPSS (Statistical Package of Social Science) software version: 21.00 with a 95% confidence interval (95% CI). The results of individual tests were compared by Chi square (χ^2) test. The sensitivity, specificity, positive predictive value and negative predictive values were calculated using the standard formulas considering mycobacterial culture as gold standard. Significance was calculated by Z-test of proportion. Statistical significance was assumed at $p < 0.05$.

Results

Among the 162 patients, 58.64% (95/162) were TB patients and 41.36% (67/162) were non- TB according to the case definition in the study. The mean age of the study subjects was 34.17 ± 1.42 years with age ranging from 1- 75 years. 61.7% (100/162) were males and 38.3% (62/162) were females. Among the 95 TB cases, 61.1% (58/95) were male and 38.9% (37/95) were female with male female ratio being 1.57:1. The major presenting clinical features included fever, malaise, chest pain, cough, weight loss and anorexia. The most common samples were pleural fluid 32.63% (31/95) followed by ascitic fluid 14.73% (14/95), urine 13.68% (13/95), pus (9/95) and lymphnode 9.47% (9/95) and the lowest number being endometrial tissue 7.36% (7/95).

Among the tuberculous group, 83.1% (79/95) were tuberculin positive with a mean ESR of 87.46 ± 2.34 mm at first hour. Urine samples were positive by only AFB smear 2.98% (2/16) cases. Non-TB pleural fluid samples 1.49% (1/22) were positive in PCR by using IS6110 primers. None of the non- TB pleural fluid was positive by mycobacterial culture and PCR using TRC4 primers. PCR assay by TRC4 primer was significantly positive (p = < 0.05) among the TB cases 75.78% (72/95) in comparison with PCR assay using IS6110 primer 72.68% (68/95), with AFB 12.63% (12/95) and with culture 22.11% (21/95) (Table-I).

Maximum PCR positivity was found in pleural fluid (87.09% (/)) followed by CSF (83.33% (/12)). Ascetic fluid were positive in 57.14% (/14) cases, urine in 76.92% (/13) and pus in 66.66% (/9) samples. In tissue samples, PCR were positive in 71.43% (/7) endometrial tissue and 77.78% (/9) in lymph node samples. Therefore, 76.84% (73/95) samples were found to be positive by PCR (Table-II).

Among 73 PCR positive cases, 93.15% (68/73) were simultaneously positive by both primers. PCR by only IS6110 primer assay was positive in 1.37% (1/73) cases and PCR by only TRC4 assay was positive in 5.48% (4/73) cases. Among 24 smear and culture positive cases, both IS6110 and TRC4 showed 83.33% (/) PCR positivity and PCR by only IS6110 and only TRC4 showed 4.1% (/) and 12.5% (/) positivity respectively. Among 49 smear and culture negative cases, 97.95% (/) was PCR positive by using both IS6110 and TRC4 primer simultaneously and only TRC4 showed 2.08% (/) positivity. None were positive by only IS6110 primers among bacteriological negative PCR positive cases (Table- III).

The primer was evaluated considering culture as a gold standard in diagnosis of EPTB cases. The sensitivity and specificity of IS6110 were 80.95% and 98.51% respectively .The sensitivity and specificity of TRC4 were 85.71% and 100% respectively (Table-IV).

Table I : Results of AFB smears, mycobacterial culture & PCR assay among EPTB cases (n=95)

Tests	Number of positive cases	Parcentage (%)
AFB smear	12*	12.63
Mycobacterial culture	21**	22.11
PCR assay by IS6110	69	72.63
PCR assay by TRC4	72	75.78
Combined PCR assay	73	76.84

*p < 0.05, compared between AFB smear and PCR
**p < 0.005, compared between Mycobacterial culture and PCR

Table II : Comparison of positivity of Bacteriological examination and PCR assay in different samples among EPTB cases (n=95)

Samples of EPTB cases	Total no of sample	Bacteriological test positive n (%)	PCR positive n (%)
Cerebrospinal Fluid	12	1 (8.33)	10 (83.33)
Ascitic fluid	14	2 (14.28)	8 (57.14)
Pleural fluid	31	7 (22.58)	27 (87.09)
Urine	13	5 (38.46)	10 (76.92)
Pus	9	2 (22.22)	6 (66.66)
Endrometrial tissue	7	3 (42.86)	5 (71.43)
Lympnode	9	4 (44.44)	7 (77.78)
Total	95	24 (25.26)	73 (76.84)

Note: Two different primers were used .Samples positive by any of the primer considered PCR positive.

Table III : Comparison between different combinations of primers with AFB smear and culture result among PCR positive EPTB cases (n = 73)

Test	Number of sample	PCR positive by Both IS6110 & TRC4 n (%)	Only IS6110 n (%)	Only TRC4 n (%)
AFB smear &/or culture positive	24	21 (83.33.0%)	1 (4.1%)	03 (12.5%)
Both AFB smear & culture negative	49	48 (97.95%)	0 (0%)	01 (2.04%)
Total	73	68 (93.15%)	1 (1.37%)	04 (5.48%)

Table IV : Primer evaluation in diagnosis of EPTB among the study population

	IS6110 (%)	TRC4 (%)
Sensitivity	80.95	85.71
Specificity	98.51	100
PPV	85.00	100
NPV	97.06	95.71

Note: Culture was considered as gold standard.

Discussion

The polymerase chain reaction has emerged with the goal of enabling clinicians to make a rapid and accurate diagnosis of tuberculosis patients. In this study, diagnostic efficacy of PCR by using IS6110 and TRC4 primers were evaluated in relation to the conventional techniques. PCR was significantly positive in 76.84% (73/95) of TB cases compared to AFB smear (12.63% (12/95)) (p < 0.05) and culture (22.11% (21/95)) (p < 0.005). This result is comparable with the study of Negi et al (2005) who showed positivity of PCR, AFB smear by Z-N stain and L-J medium culture 74.2%,35.2% and 47.1% respectively [27].

PCR was positive maximum found in pleural fluid (87.09%), followed by CSF (83.33%) and lymph node (77.78%). This is comparable to the study of Trajman et al. (2007) showing PCR positivity in 82.4% cases of pleural fluid and to study in India showing 91% if CSF sample and to study done by Singh et al. (2000) showing 82% positivity in lymphnode samples [22,23].

Among the non-TB samples, a case of malignant pleural effusion was positive by PCR assay using IS6110 primer. The explanation may be the immunosuppression by the malignant lesion caysubg reactivation of a latent tuberculous infection, possible homology of the central sequence (IS) of IS6110 with other atypical mycobacteria causing false positive result and the cross contamination during tube to tube transfer [6,24,31]. Positive AFB smear in the two non-TB urine samples might be due to non-pathogenic mycobacteria as part of the normal flora and those indigenous to water supplies may contaminate staining solutions that may be morphologically indistinguishable from pathogenic species [32,33]. The three false negative PCR compared to conventional methods might be due to sampling errors (non-uniform distribution of microorganisms), low bacterial load, inefficient extraction of DNA, or the presence of PCR inhibitors [34]. PCR was positive in 74.69% (62/83) cases smear negative and in 72.97% (54/74) cases of culture negative cases. This result is comparable to studies done by Ahuja GK et al and Sumi MG et al [35,36]. Cheng et al (2004) stated that patients with clinical and radiological features of active tuberculosis cannot be excluded as the diagnosis of tuberculosis by a negative AFB culture, as demonstrated in our patients [37]. This result may be due to the low organism count at the tissue level or their non-viability but the presence of DNA in tissues could still be easily detected by PCR [38].

In the present study the oligonucleotide primers derived from IS6110, and TRC4 gene, were successfully used to amplify a 123bp and 173bp DNA sequence in M. tuberculosis complex. Among the 73 PCR positive cases, 93.15% cases were positive simultaneously by two primers with 98.63% by TRC4 gene-based PCR and 94.52% by IS6110. Four (5.48%) cases were found to be negative by IS6110 but positive by TRC4. These are false negatives because among these four cases three cases were positive by bacteriological test and one case was responded to antituberculous treatment subsequently. Reason of this may be due to absence or low copy number of IS6110 in genome of some isolates of Mycobacterium tuberculosis mostly reported from south-east India, Tanzania, Malaysia, Oman and Denmark, although no such reports from Bangladesh [23,39,40]. However, the genotyping analysis of the available oligotype data suggests that IS6110 low copy number strains belong to the TbDl+/EAI lineage, which have recently been reported to be in the range from 25% to 50% in Bangladesh and Singapore [41,42,43]. PCR assay based on TRC4 primer in this study showed lowest negativity in only one (1.37%) cases among the PCR positive samples. Considering culture as a gold standard the sensitivity of IS6110 was 80.95% and TRC4 was 85.71 %. Previous studies conducted at the Tuberculosis Research Centre have similarly suggested that the sensitivity of PCR can be increased by using repetitive element like TRC4 primers [26,44]. They assumed that this repetitive element is an ideal target for PCR to identify M tuberculosis from clinical specimens including extrapulmonary tuberculosis, especially to detect carrying no copy or single of IS6110 which is comparable to this study. There is limited data available about the performance of 173 bpTRC4 primer based PCR assay in Bangladesh which necessitates its further evaluation.

Conclusion

Since some strains of MTB may lack the IS6110 element in their genome, using TRC4 primer instead of IS6110 is superior in diagnosing EPTB by PCR assay. In this context it will be also cost effective and rapid if we consider TRC4 as single primer for identification of EPTB especially among hospitalized patients in which atypical presentation is more frequent and where it is sometimes difficult to obtain multiple specimens.

Disclosure

All the authors declared no competing interest.

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