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ORIGINAL ARTICLE

Improved Diagnostic Potential of Polymerase Chain Reaction by Amplification of Multiple Gene Targets in Osteoarticular Tuberculosis

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Purpose: Till date, a number of primers have been described for the diagnostic polymerase chain reaction (PCR) assays for tuberculosis (TB). However, most investigators have evaluated PCR's clinical utility using only one primer specific for *Mycobacterium tuberculosis*. The purpose of this study was to evaluate the efficacy of PCR tests targeting two different DNA sequences – insertion sequence 6110 (IS6110) and protein antigen b (Pab), in the same set of clinical samples from osteoarticular TB cases, and to evaluate if the sensitivity of the assay is improved. **Materials and Methods:** Twenty clinical samples obtained from osteoarticular TB cases were subjected to two different PCR assays - 123 base pair (bp) sequence coding for IS6110 and 419 bp sequence coding for Pab. Ten clinical samples from cases of proven septic arthritis were studied as controls. **Results:** The sensitivity of IS6110 PCR and Pab PCR were found to be 75% and 80%, respectively, and the specificity of both IS6110 PCR and Pab PCR was 100%. No significant difference was found between two PCR assays ($P > 0.05$). However, there were two cases which were negative by IS6110 PCR but were positive by Pab PCR. There was one case which was positive by IS6110 but was negative by Pab PCR. Seventeen out of 20 samples showed concordance between the results of two PCR tests, increasing the sensitivity to 85%. **Conclusion:** The diagnostic yield of the PCR test can be improved with the simultaneous amplification of two or more gene targets.

Key words: Polymerase chain reaction, tuberculosis, osteoarticular

INTRODUCTION

Various gene targets have been employed in the diagnostic polymerase chain reaction (PCR) assays for tuberculosis (TB).[1] Most of the past studies have targeted insertion sequence 6110 (IS6110) sequence.[2] However, total absence or presence of only one copy of this sequence has been reported in some clinical isolates.[3-5] Another nucleotide sequence coding for protein antigen b (Pab) specific for *Mycobacterium tuberculosis* (M. tb) complex has been shown to be a useful target in a few studies.[6] The aim was to evaluate the efficacy of PCR assays targeting two different M. tb specific DNA sequences (IS6110 and Pab) in osteoarticular TB cases, with the intention of improving the diagnostic accuracy.

MATERIALS AND METHODS

A prospective study was conducted between July 2013 and December 2014 in our tertiary care health center. The study included two groups: In Group 1 (case group) – Twenty clinical samples were obtained from patients with a strong clinical/radiological/histopathological evidence of TB, including a documented clinical response to antitubercular treatment. These criteria were used as a gold standard, and these were proven cases of tubercular infection. Group 2 (control group) – Ten clinical samples were obtained from patients with the features of septic arthritis with proven bacteriological culture and no evidence of TB. Clinical specimens were collected by arthrocentesis, abscess puncture for pus, curetting for exudation in the sinus, core needle biopsy/open biopsy or fine-needle aspirate (computed tomography-guided/ultrasonography-guided), ensuring strict aseptic precautions.

All pus samples were decontaminated with 4% sodium hydroxide for 10 min and then centrifuged at 6000 rpm for

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20 min. The supernatant was discarded and an equal amount of phosphate buffer was added to the sediment. Tissue samples were homogenenized in pestle and mortar, centrifuged at 6000 rpm for 10 min and 3 ml of Tris buffer was added to the pellet. Synovial fluid samples were centrifuged at 6000 rpm for 10 min and 3 ml of Tris buffer was added to the pellet. DNA was extracted using commercially available QIAGEN DNA extraction kit (QIAGEN GmbH, Hilden, Germany) with one initial modification step of keeping the preliminary processed materials at 80°C for 10 min for inactivation of possible mycobacteria. The material was then processed as per the guidelines of the manufacturer of the kit to obtain the DNA.

Polymerase chain reaction for protein antigen b

The primers used to amplify a 419 base pair (bp) sequence coding for the Pab 38 kDa antigen were

- Forward primer Pabf: 5'-ACCACCGAGCGGTTCGC CTGA-3'
- Reversed primer Pabr: 5'-GATCTGCGGGTC GTCCCAGGT-3'.

Polymerase chain reaction for insertion sequence 6110

DNA amplification of the 123 bp IS6110 insertion element was carried out by two oligonucleotide primers sequence, as follows

- Forward primer IS6110f 5'-CCT GCG AGC GTA GGC GTC GG-3'
- Reversed primer IS6110r 5'-CTC GTC CAG CGC CGC TTC GG-3'.

In each independent PCR assay, the test result was compared with the results for one positive and one negative control. The positive control was the DNA of H_{37} RV strain and negative control was double distilled water. After DNA amplification, the samples were run on 1.5% agarose gel electrophoresis stained with ethidium bromide. The stained gel was examined under ultraviolet light to look for the DNA bands of 419 bp and 123 bp using 100 bp DNA ladder.

RESULTS

The clinical samples from the study cases ($n = 20$) included synovial fluid ($n = 6$), fine-needle aspirate ($n = 6$), pus ($n = 2$), core needle biopsy ($n = 5$), and open biopsy ($n = 1$). Out of the 20 clinical samples from osteoarticular TB group, Pab based PCR showed a positive result in 16 clinical samples, and IS6110 PCR showed a positive result in 15 samples [Table 1]. All the 10 control group samples showed a negative result with both tests.

The sensitivity of IS6110 PCR and Pab PCR were found to be 75% and 80%, respectively, and the specificity of both IS6110 PCR and Pab PCR was 100% [Tables 2 and 3]. The difference between the sensitivity of two PCR assays (IS6110 and Pab) was not found to be statistically significant ($P > 0.05$).

The concordance between two PCRs was calculated using bivariate two-by-two tables. Seventeen out of 20 samples showed concordance between the results of two PCR tests. A moderate agreement between the two methods was found using Kappa statistics ($\kappa = 0.571$). There were two cases (10%) which were negative by IS6110 PCR but were positive by Pab PCR. There was one case (5%) which was positive by IS6110 but was negative by Pab PCR. Thus, using both tests, the sensitivity increases to 85%.

DISCUSSION

TB was declared as a global emergency in 1993 by the World Health Organization (WHO) and still remains a major health problem.[7] It has been estimated that 1%–3% of patients suffering from TB have skeletal involvement.[8] Joint TB occurs most commonly in children and young adults and destroys the joint structure.[9] Early diagnosis and timely institution of antitubercular treatment are thus crucial in these cases, to minimize cartilage destruction.

Accuracy and speed of diagnosis are key factors in the timely management extra-pulmonary tuberculosis (EPTB). The acid-fast bacilli smear examination and radiology have a limited role in the early diagnosis of EPTB.[10] Acid-fast staining requires a large number of bacilli (>10^4 cells/ml) and culture requires 6–8 weeks, which is often negative due to the small number of bacilli in tissue samples.[11] The newer tests like radiometric BACTEC system also lacks sensitivity and may not be cost effective.[12] The *in vitro* T-cell based IFN-gamma release assays have limited utility in diagnosis and treatment in highly endemic countries.[13] Serological antibody detection methods have been widely used; however, due to inconsistent and imprecise estimates, the WHO expert group meeting convened in 2010 has strongly recommended against the use of any of these serological tests for the diagnosis of both pulmonary tuberculosis and EPTB.[14] Thus, the definitive diagnosis of EPTB basically depends on histological evidence, which may also be inconclusive, and often needs a high level of expertise for obtaining and confirming tissue diagnosis.[15] A major breakthrough in the diagnosis of EPTB was achieved by the introduction of nucleic acid amplification tests such as PCR to detect nucleotide sequences unique to M. tb in the tissue samples which give results within a few hours.[16] PCR have been reported to be more sensitive and specific; the detection limit of the PCR is 1–10 bacilli in various clinical samples.[2]

Table 1: Comprehensive demographic data of all the cases involved in the study

Patient number	Age/sex	Diagnosis	Sample	PCR		Remarks
				IS6110	Pab	
1	18/male	TB knee-left	Synovial fluid	Positive	Positive	Good response to ATT
2	50/male	TB knee-right	Synovial fluid	Positive	Positive	Good response to ATT
3	51/male	TB knee-right	Synovial fluid	Positive	Negative	Good response to ATT
4	41/male	TB knee-left	Synovial fluid	Positive	Positive	Good response to ATT
5	24/female	TB spine L1–L2	USG guided aspirate	Negative	Negative	Good response to ATT
6	20/male	TB ankle-left	Synovial fluid	Positive	Positive	Good response to ATT
7	20/female	TB knee-right	Pus (knee joint)	Positive	Positive	Good response to ATT
8	48/male	TB spine L3 with abscess	CT guided FNA	Positive	Positive	Good response to ATT
9	26/male	TB knee-right	Core biopsy	Positive	Positive	HP-epithelioid granuloma; good response to ATT
10	50/male	TB ankle-right	Synovial fluid	Negative	Positive	Good response to ATT
11	28/male	TB knee-right	Core biopsy	Positive	Positive	HP-epithelioid granuloma; good response to ATT
12	70/female	TB shoulder-left	Core biopsy	Negative	Negative	HP-epithelioid granuloma; good response to ATT
13	48/female	Osteomyelitis humerus-left	Open biopsy	Positive	Positive	HP-epithelioid granuloma; good response to ATT
14	50/female	TB spine - L2–L3	CT guided FNA	Positive	Positive	Good response to ATT
15	80/male	TB spine L3–L4 with abscess	USG guided aspirate	Positive	Positive	Good response to ATT
16	29/male	TB spine L3–L4 with abscess	USG guided aspirate	Negative	Positive	Good response to ATT
17	30/male	TB spine L4–L5 with abscess	USG guided aspirate	Positive	Positive	Good response to ATT
18	36/female	Osteomyelitis right distal radius	Pus	Negative	Negative	Good response to ATT
19	28/female	TB knee-right	Core biopsy	Positive	Positive	HP-tubercular synovitis; good response to ATT
20	32/female	TB elbow right	Core biopsy	Positive	Positive	HP-epithelioid granuloma; good response to ATT

TB=Tuberculosis; PCR=Polymerase chain reaction; USG=Ultrasonography; FNA=Fine needle aspiration; CT=Computed tomography; ATT=Anti tubercular therapy; HP=Histopathology; Pab=Protein antigen b; IS6110=Insertion sequence 6110

Table 2: Efficacy of insertion sequence 6110 polymerase chain reaction

IS6110 PCR	Case group	Control group	Total
Positive	15 (true positive)	0 (false positive)	15
Negative	5 (false negative)	10 (true negative)	15
Total	20	10	30

PCR=Polymerase chain reaction; IS6110=Insertion sequence 6110

Table 3: Efficacy of protein antigen b polymerase chain reaction

Pab PCR	Case group	Control group	Total
Positive	16 (true positive)	0 (false positive)	16
Negative	4 (false negative)	10 (true negative)	14
Total	20	10	30

Pab=Protein antigen b; PCR=Polymerase chain reaction

The reported sensitivity of the PCR technique in the diagnosis of osteoarticular TB varies from 53% to 100% and the specificity from 63% to 100%. The reasons for the variations in sensitivity in different studies may be due to variability in PCR protocols (use of different PCR primers and methods of extraction), study setting, reference standards and finally on the expertise of the personnel conducting the assay.[17] Various gene targets such as IS6110, 16Sr RNA gene, 65 kDa protein gene, MPB-64 protein gene, 38 kDa protein gene, TRC4, MTP-40 protein gene, and PPE gene have been employed in these PCR assays. It is evident from the published literature that IS6110 is the most widely used PCR assay, the presumed reason being the presence of multiple copies of IS6110 in the M. tb complex, which confers a high sensitivity to the test. However, Das *et al*.[4] and Yuen *et al*.[5] reported the existence of a group of M. tb strains that appear to have either one or no copy of IS6110 insertion element. Thus, PCR diagnosis based exclusively on the IS6110 sequence could lead to a significant number of false negative results.

To add to the sensitivity, we evaluated and compared two PCR assays involving two different M. tb specific DNA sequences, i.e., IS6110 and Pab for the diagnosis of osteoarticular TB. Both Pab PCR and IS6110 PCR were individually found to have a higher sensitivity of 80% and 75%, respectively. We confirmed that the positive tests obtained by our PCR study were not false positives by repeating the tests and found them to be positive twice. In addition,

we have followed all our patients, and they were found to have a good clinical response to anti-TB chemotherapy thus reconfirming the diagnosis of osteoarticular TB. The study correlated well with a previous study based on Pab PCR test by Sharma *et al*.[18] who reported 82.8% sensitivity and 100% specificity in tuberculous meningitis samples. We obtained a higher sensitivity compared with a study by Negi *et al*.[19] who reported 71.5% sensitivity in EPTB samples using the Pab PCR test.

Comparing the efficacy of two different PCR assays, we found no statistically significant difference between them. Negi *et al*.[20] similarly found no significant difference between the four PCR protocols (IS6110, 38 kDa, 65 kDa and 85B mRNA based PCR) for the early diagnosis of TB in pulmonary and extrapulmonary clinical samples.

The individual sensitivities of IS6110 PCR and Pab PCR were 75% and 80%, respectively. However, the sensitivity of the diagnostic PCR is increased to 85% when both the gene sequences are targeted, instead of only one. This is evident by the concordant results obtained in 17 out of the 20 cases. Thus, we were able to diagnose 10% additional osteoarticular TB cases using "double" PCR assays, which would have been missed otherwise. Since this is a small sample, this may not look significant but may have significant diagnostic implications. Sharma *et al*.[21] have previously shown that the use of more than one target gene (multiplex PCR) increased the diagnostic yield in paucibacillary conditions like osteoarticular TB. In their study, they performed multiplex PCR using two target genes specific for M. tb namely IS6110 and MPB 64. The sensitivity of IS6110 and MPB64 assays in isolation were 73.75% and 80%. Using multiplex PCR, the sensitivity increased to 82%.

Another finding was that a sample from a case of spine TB, who had already received anti-TB therapy for 4 weeks, showed positive result by PCR test and negative result by culture. This confirmed that the DNA amplification methods could detect even the nonviable mycobacteria, making this an important diagnostic tool in patients who have empirically been started ATT, but the diagnosis remains suspicious and not confirmed.

The main limitations of the PCR tests are false positivity and false negativity. The percentage of false positivity in our study was 0% by both PCR tests, compared to the reported rate in the literature ranging from 3% to 20%.[22] Considering the high detection rates by the PCR test, we suggest that repeating the PCR test after some time may further help to reduce the false negative results due to the extremely low bacilli count in some clinical samples. This would be very beneficial in the early stages of the disease when the diagnosis has yet to be established. Performing PCR targeting two or more different gene sequences in the same clinical sample does not add much to the cost of the test since it requires only additional primers specific for M. tb.

CONCLUSION

Using two target genes for PCR in the same sample, the diagnostic accuracy in osteoarticular TB is increased by about 10%, which might be pivotal in clinching an early diagnosis in suspicious cases, especially in endemic areas.

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Conflicts of interest

There are no conflicts of interest.

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