THE UTILITY OF IS6110 SEQUENCE BASED POLYMERASE CHAIN REACTION IN COMPARISON TO CONVENTIONAL METHODS IN THE DIAGNOSIS OF EXTRA-PULMONARY TUBERCULOSIS

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Abstract

IS6110 sequence based polymerase chain reaction (PCR) was compared with conventional bacteriological techniques in the laboratory diagnosis of extra-pulmonary tuberculosis (EPTB). One hundred and ninety one non-repeated clinical samples of EPTB and 17 samples from non-tuberculous cases as controls were included. All the samples were processed for Ziehl-Neelsen staining for acid fast bacilli (AFB) and 143 samples were processed by culture for M. tuberculosis. All the samples were processed for PCR amplification with primers targeting 123 bp fragment of insertion element IS6110 of M. tuberculosis complex. Of the total 191 samples processed, 34 (18%) were positive by smear for AFB. Culture for AFB was positive in 31(22%) samples among the 143 samples processed. Either smear or culture for AFB was found positive in 51(27%) samples. Of the total 191 samples processed 120 (63%) were positive by PCR. In 140 samples, wherein both the conventional techniques were found negative, 74 (53%) samples were positive by PCR alone. Among 51 samples positive by conventional techniques, 46 (90%) were found positive by PCR. PCR assay targeting IS6110 is useful in establishing the diagnosis of EPTB, where there is strong clinical suspicion, especially when the conventional techniques are negative.

Key words: Extra-pulmonary tuberculosis, polymerase chain reaction, IS6110 sequence.

Materials and Methods

Clinical specimens and conventional bacteriological techniques

One hundred and ninety one, non-repeated clinical samples from suspected cases of EPTB, were collected with due informed consent of the patients. All the samples were apportioned for conventional bacteriological techniques and for PCR procedure (Table 1). All fluid samples were centrifuged at 3000 rpm for 15 minutes and the deposit was processed by Ziehl-Neelsen staining for AFB. Culture for M. tuberculosis was carried as per the standard bacteriological procedure. In 140 samples, wherein both the conventional techniques were found negative, 74 (53%) samples were positive by PCR alone. Among 51 samples positive by conventional techniques, 46 (90%) were found positive by PCR. PCR assay targeting IS6110 is useful in establishing the diagnosis of EPTB, where there is strong clinical suspicion, especially when the conventional techniques are negative.

Table 1: Details of samples investigated in this study

<table>
<thead>
<tr>
<th>Samples</th>
<th>No. of samples collected</th>
<th>No. of samples investigated PCR</th>
<th>No. of samples investigated AFB smear</th>
<th>No. of samples investigated AFB culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphnode aspirates</td>
<td>78</td>
<td>78</td>
<td>78</td>
<td>78</td>
</tr>
<tr>
<td>Cerebrospinal fluid</td>
<td>46</td>
<td>46</td>
<td>46</td>
<td>46</td>
</tr>
<tr>
<td>Ascitic fluid</td>
<td>41</td>
<td>41</td>
<td>41</td>
<td>13</td>
</tr>
<tr>
<td>Pleural fluid</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>66</td>
</tr>
<tr>
<td>Synovial fluid</td>
<td>02</td>
<td>02</td>
<td>02</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>191</strong></td>
<td><strong>191</strong></td>
<td><strong>191</strong></td>
<td><strong>143</strong></td>
</tr>
</tbody>
</table>

ND- Not Done
medium and Lowenstein-Jensen medium with sodium pyruvate (LJ-P). Another portion was inoculated into Kirchner’s liquid medium and after incubation for six weeks was subcultured onto LJ and LJ-P media. All lymphnode aspirates were inoculated directly into Kirchner’s liquid medium and later subcultured onto LJ and LJ-P media. The growth after eight weeks of incubation, if appeared was confirmed to be *M. tuberculosis*, based on slow growth rate, absence of pigmentation, Niacin test positivity and absence of growth on LJ medium with p-nitrobenzoic acid.

**Extraction of DNA from clinical samples**

All the samples were processed for DNA extraction as per the standard method of Herman *et al*. Essentially the cells were lysed with lysozyme followed by treatment with proteinase K and sodium dodecyl sulphate. Proteins and macromolecules were precipitated using NaCl and hexadecyltrimethylammonium bromide -NaCl solutions. Nucleic acids were recovered from aqueous phase after extraction with chloroform and isoamyl alcohol. DNA was further precipitated overnight with isopropanol at -20°C. The pellet was washed with ethanol and later reconstituted in TE buffer.

**PCR amplification and documentation**

Amplification of DNA was performed with primers IS-F- 5’-CTCGCACGGAGTGACGTCCGG-3’and IS-R- 5’CTCGACCCAGCCGCTTGAGG-3’, to amplify 123 bp fragment of insertion element IS6110 of *M. tuberculosis* complex as reported earlier,[4] with some modifications. Briefly PCR was carried out in 50 μL volume, using 200μM dNTPs, 20pM of each primer and 1U of Taq polymerase, followed by template. Conditions followed were initial denaturation at 94°C for four minutes, followed by 35 cycles at 90°C for one minute, 60°C for one minute, and 72°C for one minute, and a final extension at 72°C for 10 minutes. The amplified products were subjected to electrophoresis on a 2% agarose gel containing ethidium bromide and the results were documented (Figure). Throughout the PCR processing the three room procedure and other recommended stringent precautions were followed and the results were evaluated in the light of the performance of appropriate positive and negative controls, to avoid cross-contamination and false positive reactions.

Along with 191 test samples, 17 fine needle aspirates from non-tuberculous cases were also included as controls. They were: pyogenic abscess - 10, malignancy - 4, non-specific lymphadenitis - 2 and reactive lymphadenitis - 1.

Statistical analysis: The sensitivity of the conventional tests and PCR assay was calculated keeping each one of them as gold standard against another and the significance of difference was determined by proportion test; probability value of 0.05 was taken as significant value (p < 0.05).

**Results**

Thirty four out of 191 samples (18%) were positive by smear for AFB. Culture for AFB showed positive in 31(22%) samples among the 143 samples processed. Either smear or culture for AFB was found positive in 51(27%) of the total 191 samples investigated. Among the different samples processed lymphnode aspirates showed higher proportion of positivity (41%) by either smear or culture for AFB (Table 2).

PCR was positive in 120 out of 191 samples (63%). PCR alone was positive in 74 (53%) among 140 samples that were negative by both the conventional bacteriological techniques - smear and culture for AFB. Among these samples found negative by conventional bacteriological techniques (Table 2), the contribution of PCR was observed high among CSF - 71% (the data of synovial fluid was ignored, considering smaller sample), but low among pleural fluids (38%).

Analysis of PCR results among samples positive by conventional methods showed that out of the 34 samples positive by AFB smear 31(91%) were positive by PCR and out of 31 samples positive by culture, 29 (94%) were positive by PCR. Thus, of the total 51 samples positive by either smear or culture for AFB, PCR was positive in 46 (90%) samples. Among those five samples negative for PCR, four were lymphnode aspirates and one was CSF (Table 3).

Thus conventional bacteriological techniques were positive in 27% of samples whereas PCR was positive in 63% of samples. This difference was observed to be statistically significant (p < 0.001). In addition, 53% of samples showed positive only by PCR, where in conventional tests were found negative. All the non-tuberculous fine needle aspirates control samples were found negative by PCR and conventional techniques.

When conventional tests were taken as gold standard, the sensitivity of PCR assay was 90%, however when PCR assay was taken as gold standard the sensitivity of

![Figure: PCR amplification of IS6110 sequence of *M. tuberculosis* complex among the samples of extra-pulmonary tuberculosis, PC-Positive control; NC- Negative control; Lane-1- Molecular weight marker (100 bp) Lanes-3 to 7 - samples positive for IS6110 sequence (1-5)](https://www.ijmm.org)
This suggested that among the cases of suspected EPTB the PCR assay was more sensitive compared to conventional tests.

**Discussion**

EPTB encounters many problems like the pauci-bacillary nature of the samples, inadequate sample amount or volume; apportioning of the samples for various diagnostic tests resulting in ununiform distribution of microorganism etc. All these limitations reflect in the poor contribution of conventional bacteriological techniques in the establishment of diagnosis of EPTB. This has stimulated the application of polymerase chain reaction in the laboratory diagnosis of EPTB.

Our in-house PCR assay was based on the amplification of a fragment of the IS6110, which is specific for the *M. tuberculosis* complex. The amplification of IS6110 insertion sequence, which belongs to IS3 family and is found in almost all members of the *M. tuberculosis* complex. Most strains of *M. tuberculosis* carry 10-15 copies, which are present in a wide variety of chromosomal sites.

In our study, conventional bacteriological techniques were positive in 51 (27%) samples, where as PCR showed 120 (63%) samples positive, out of total 191 samples processed. This difference was found to be statistically significant (*p* < 0.001). Earlier Indian studies also documented increased positivity by PCR targeting IS6110 elements in samples of EPTB. Negi *et al.*[5] showed 77% of positivity among EPTB samples. Tiwari *et al.*[10] showed 62% of total positivity rate among EPTB samples and detection of *M. tuberculosis* DNA in 57% of AFB negative EPTB samples.

In this study, all samples (except five) found positive by conventional techniques, were positive by PCR (90%). Of the five samples three were positive by smear alone and two were positive by culture alone. All these five specimens were tested for the presence of substances inhibiting Taq polymerase by repeating PCR, diluting duplicate samples and found to be negative for presence of inhibitors. Four of these were fine needle aspirates of lymphnodes and were collected from highly suspected cases of tuberculosis. Such false negativity has been encountered by other workers. Tiwari *et al.*[10] reported false negative in 5 out of 133 samples. Kesarvani *et al.*[11] reported 1 out of 65 samples. Most of them were lymphnode aspirates, similar to this study. The only possible reason for false negative result by PCR may be unequal distribution of AFB in aspirated material in these samples.

Although absence or the presence of fewer copies of target sequence IS6110, in some strains of *M. tuberculosis* has been reported[12,13] some of the earlier studies reported that PCR assay targeting IS6110 sequence were more sensitive.[10,11] Also a recent Indian study by Negi *et al.*[5] on comparison of different PCR protocols, targeting different gene sequences of *M. tuberculosis*, showed higher positivity (77%) in PCR targeting IS6110 compared to other targets like 65kDa (75%), 38 kDa (72%) and 85B protein (73%). Among samples found negative by conventional techniques, PCR targeting IS6110 has shown higher positivity (26%) than PCR for other targets. Also the methodology of PCR
for IS6110 has been widely carried out in different technical set ups and has been proven to be simple and reproducible, compared to methodologies for PCR targeting other gene sequences.

Thus PCR assay targeting IS6110 is highly useful in the establishment of the diagnosis of EPTB. This can be applied where there is strong clinical suspicion, especially when the conventional techniques are negative. The rapidity, high sensitivity and simplicity of PCR targeting IS6110 gene sequence, may even compensate the higher cost of the test compared with less sensitive conventional tests in the diagnosis of EPTB.

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References


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