Evaluation of Multiplex PCR for Rapid Diagnosis of Female Genital Tuberculosis

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Abstract

Background: Female genital tract tuberculosis (FGTB) is a very common disease in developing countries. Rapid and specific diagnosis is of paramount importance.

Purpose: To evaluate Multiplex PCR using MPB 64 and IS6110 primers directed against M. tuberculosis for the diagnosis of FGTB and to compare the different methods available for diagnosis like histopathology, smear microscopy and TB culture.

Materials and Methods: Multiplex PCR was performed on endometrial biopsy samples of 21 FGTB confirmed cases, 49 clinically suspected FGTB cases and 25 Non TB (control group) patients.

Results: Multiplex PCR had sensitivity of 95.23% for confirmed cases and specificity of 100% for confirmed FGTB cases. In 49 clinically diagnosed, but unconfirmed FGTB cases multiplex PCR was positive in 61.22% cases. The overall sensitivity of microscopy, culture, Histopathology and multiplex PCR were 1.42%, 8.57%, 21.42%, 72.85% and specificity was 100%, 100%, 100% and 100% respectively.

Conclusion: Multiplex PCR using MPB 64 and IS6110 primers has a high sensitivity and specificity in diagnosis of FGTB.

Introduction

Tuberculosis is an important cause of morbidity and mortality with 9.4 million cases and 1.8 million deaths reported globally in 2008. India alone accounts for over 20% of the world TB cases. The prevalence of female genital tuberculosis (FGTB) in infertility clinics varies between 15 to 20% among different countries. FGTB is responsible for 5-16% cases of infertility among

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young Indian women in age group of 18 to 40 years. Prompt diagnosis followed by timely treatment is important as FGTB is mostly asymptomatic and cause extensive damage leading to infertility. The laboratory diagnosis of TB is primarily based on microscopy and culture, the techniques which lack sensitivity and are time consuming. Even the newer tests like BACTEC and serological tests are not cost effective. Hence the development of rapid, sensitive and specific test for detection of mycobacteria has been a long standing need. Polymerase chain reaction is highly promising nucleic acid amplification technique for early diagnosis of M. tuberculosis.

Most of the PCR based studies have used single target like IS6110 or protein b for amplification. However single gene target can result in false negative results as some of these target genes may be absent in 9-10% of M. tuberculosis isolates from India. Thus it argues against its utility as a sole target for amplification in PCR. In studies where two or more gene targets were amplified the results with respect to sensitivity were much better. An alternative approach is to use multiplex PCR (MPCR), in which several target genes are amplified simultaneously. We had earlier evaluated MPCR for diagnosis of tuberculous meningitis (TBM) and osteoarticular tuberculosis (OATB), it had shown good sensitivity of 86.6% for diagnosis of TBM and 82% for OATB respectively. However, the specificity was 100% in both the cases. To the best of our knowledge, role of multiplex PCR using IS6110 and MPB64 for early diagnosis of FGTB has not been evaluated. Hence we studied the utility of MPCR using IS6110 and MPB64 for rapid diagnosis of M. tuberculosis in endometrial biopsy samples of patients of genital tuberculosis. We have chosen insertion sequence as one of the targets in MPCR because of multiple copy number. MPB64 was chosen as a second target because it had shown a good sensitivity and specificity for diagnosis of tuberculosis in our previous study.

**Materials and Methods**

Endometrial biopsy samples from total 95 patients received in Mycobacteriology laboratory for acid fast staining and culture were tested. Patients were in age range of 18 to 40 years. The relevant history and other details of patients were noted and they were divided into two groups on the bases of following criteria.

**Group I: FGTB (n=70)**

a. Confirmed FGTB cases (n = 21): culture / smear positive / Histopathology Positive
b. Suspected FGTB cases (n = 49): smear / culture / Histopathology negative but Suspected of having genital TB on clinical grounds like infertility and laparoscopic findings and where other causes of infertility were ruled out were included in the study.

**Group II: Control Group**

Endometrial biopsy samples were taken due to some other problems other than tuberculosis like carcinoma.

**Specimen Collection**

Endometrial biopsy samples were collected taking full aseptic precautions and send to laboratory within 1 h in normal saline for AFB staining, culture, Multiplex PCR and part of endometrial biopsy was kept in 10% formalin that was sent for histopathology.

**Sample Processing**

All samples were processed in class II biosafety cabinet. The endometrial samples were decontaminated and concentrated by using N-acetyl-L-cystine sodium hydroxide (NALC-NaOH) method followed by mixing with equal amount of decontamination solution and kept for 15-20 minutes at room temperature. The samples were vortexes for 5-20 seconds. Equal volumes of phosphate buffer were added to the samples and were centrifuged at 3000 rpm for 20 min. The supernatant was discarded and the sediment re-suspended in 1-3 ml of phosphate buffer. The re-suspended sample was processed for preparation of smear, culture, M PCR and part of sample was stored at -20°C.

**AFB staining and culture**

Decontaminated samples were examined for AFB by Ziehl-Neelsen method and culture was done on two LJ slants using standard procedures and incubated for 6 weeks.

**Histopathology**

Paraffin-embedded tissue section were prepared and stained with Hematoxylin-eosin and examined for granulomatous reactions suggestive of Mycobacterium tuberculosis.

**Multiplex PCR**

DNA was extracted from tissue samples as previously described by Van Soolingen using Chloroform: Isoamyl alcohol extraction method and was stored at -20°C. Multiplex PCR was standardized and was found to have quantitative sensitivity to detect the DNA equivalent to 2-3 organisms. It tested positive with standard strain of M. tuberculosis, H₃₇Rv. In each independent MPCR assay, test results were compared with the results for one positive and one negative control. The positive control included was the DNA of H₃₇Rv and negative control included was the PCR grade water. Identification of M. tuberculosis was done using a specific pair of primers designed to amplify IS6110 and MPB64 in the M. tuberculosis complex and the expected band size was about 123bp for IS6110 and 240 bp for MPB64. The sequence of primers used for IS6110 was ISI: 5’-CCTGCGAGCGTAGGCGT 3, IS2: 5’-CTCGTCAGGCCTCTTCCG 3’. Primers used for MPB64 were MPB1:5’TCC GCT GCC AGT CGT CTT CC-3’, MPB2:5’-GTC CTC GCG AGT CTA GGC CA-3’ respectively.

Following components were added to eppendorf (for 50 µl reaction). PCR buffer 10X, dNTPs (Mix) 10mM, Primer IS1 (10pm / µl), IS2 (10pm / µl), MPB1 (10pm / µl) and MPB2 (10pm / µl), Taq polymerase 5U / µl, DNA template and water. DNA amplification was performed for 40 cycles following an initial Denaturation step at 95°C for 5 min in thermo cycler by using the following program: Denaturation at 94°C for 1 min, annealing at 65°C for 1.5 min, extension at 72°C for 1.5 min and final Extension: 72°C for 10 min.

The amplified product was stored at 4°C till the detection. For detection of amplification samples were run on 1.5% agarose gel stained with ethidium bromide. The stained gel was examined under UV light to look for bands 123 bp of IS6110 and 240 bp of MPB64 using molecular weight marker of 100 bp ladder. The samples showing the presence of these bands under ultraviolet transillumination were considered positive (Figure 1).

**Specificity and sensitivity of the MPCR Assay**

MPCR was highly specific for M. tuberculosis as no amplification
biopsy samples for diagnosis of female genital tuberculosis (FGTB) with multiplex PCR using IS6110 and MPB64 as target specific for M. tuberculosis complex. The subjects were grouped as confirmed FGTB (n = 21) cases, suspected FGTB (n = 49) and control groups (n = 25). Figure 1 shows the representative photograph of multiplex PCR (MPCR).

Out of 21 confirmed FGTB cases, 6 were culture positive and 15 were positive on histopathology (Table 1), 20 (95.23%) were MPCR positive respectively. Only one case was positive by AFB staining and that was also positive by culture, histopathology and MPCR. Out of 20 MPCR positive cases, MPB64 and IS6110 band were positive in 19 (90.47%) cases. In the confirmed FGTB cases, there was 1 case which was missed by IS6110 and 1 was missed by MPB64 as shown in gel photograph in lane 6 there is only MPB64 band and no IS6110 band. Similarly in lane 7 of gel photograph shows band for IS6110 but band for MPB64 is missing. Out of 6 cultures positive cases, MPCR was positive in 5 cases. All of them were positive for MPB64 and IS6110 respectively. Out of 15 histopathology positive cases, MPCR was positive in 15 cases. MPB64 and IS6110 were positive in 14 cases and there was one case which was picked by MPB64 and missed by IS6110 and one cases was missed by MPB64 and picked by IS6110 (Table 1).

In 49 clinically suspected cases of FGTB, MPCR was positive in 31 (63.26%) cases and out of these 31 cases MPB64 was positive in 30 (61.22%) cases and IS6110 was positive in 28 (57.14%) cases respectively. MPCR was negative in control group thus giving specificity of 100%. A final diagnosis of FGTB was made for 70 patients based on result of culture, microscopy, histopathology and clinical examination. MPCR was positive in 51 cases, histopathology in 15 and culture in 6 and AFB smear in one case respectively. Thus sensitivity of MPCR, histopathology, culture and microscopy was 72.85%, 21.42%, 8.57% and 1.42% respectively. However the sensitivity of MPCR in confirmed FGTB cases and suspected FGTB was 95.23% and 63.26% respectively. There were total 3 (4.28%) cases out of 70, which were missed by IS6110 but were picked by MPB64. Similarly there were 2 (2.85%) of 70 cases which were only IS6110 positive. By using two primers together in MPCR there was an increase in sensitivity to 72.85% for MPCR whereas sensitivity of MPB64 and IS6110 alone was 70% and 67.14% respectively. In the control group all the tests were negative thus giving

### Results

The study evaluated 95 endometrial biopsy samples for diagnosis of female

- **Table 1: Comparison of MPCR with smear culture and histopathology**

<table>
<thead>
<tr>
<th>Type</th>
<th>Subtype</th>
<th>Smear +n (%)</th>
<th>Culture +n (%)</th>
<th>Histopath +n (%)</th>
<th>mPCR +n (%)</th>
<th>MPB64 +n (%)</th>
<th>IS6110 +n (%)</th>
<th>Only MPB64 +n (%)</th>
<th>Only IS6110+ n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group-1</td>
<td>Confirmed FGTB cases</td>
<td>1 (4.76)</td>
<td>6 (28.57)</td>
<td>15 (71.42)</td>
<td>20 (95.23)</td>
<td>19 (90.47)</td>
<td>19 (90.47)</td>
<td>1 (4.76)</td>
<td>1 (4.76)</td>
</tr>
<tr>
<td></td>
<td>Suspected FGTB cases</td>
<td>-</td>
<td>-</td>
<td>31 (62.36)</td>
<td>30 (61.22)</td>
<td>28 (57.14)</td>
<td>2 (4.08)</td>
<td>1 (1.42)</td>
<td></td>
</tr>
<tr>
<td>Total (n = 70)</td>
<td></td>
<td>1 (1.42)</td>
<td>6 (8.57)</td>
<td>15 (21.42)</td>
<td>51 (72.85)</td>
<td>49 (70)</td>
<td>47 (67.14)</td>
<td>3 (4.28)</td>
<td>2 (2.85)</td>
</tr>
<tr>
<td>Group-2 Non-TB</td>
<td>Non-TB control group</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

- **Table 2: Sensitivity and specificity of multiplex PCR for diagnosis of FGTB**

<table>
<thead>
<tr>
<th>Test</th>
<th>FGTB cases (n = 21)</th>
<th>Control group (n = 25)</th>
<th>Sensitivity %</th>
<th>Specificity %</th>
<th>PPV %</th>
<th>NPV %</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPCR</td>
<td>Positive</td>
<td>51</td>
<td>-</td>
<td>72.85%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>19</td>
<td>25</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPB 64</td>
<td>Positive</td>
<td>49</td>
<td>-</td>
<td>70%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>21</td>
<td>25</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IS6110</td>
<td>Positive</td>
<td>47</td>
<td>-</td>
<td>67.14%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>23</td>
<td>25</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Histopathology</td>
<td>Positive</td>
<td>15</td>
<td>-</td>
<td>21.42%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>55</td>
<td>25</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Culture</td>
<td>Positive</td>
<td>6</td>
<td>-</td>
<td>8.57%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>64</td>
<td>25</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microscopy</td>
<td>Positive</td>
<td>1</td>
<td>-</td>
<td>1.42%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>69</td>
<td>25</td>
<td></td>
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</tr>
</tbody>
</table>

- **Fig. 1: L1: MM, L2: positive culture (both MPB64 and IS6110 band), L3, L4 shows the clinical samples picked by both primers, L5: Clinical sample picked by only MPB64 band, L6: Clinical sample picked by insertion sequence only, L7: Clinical sample which was negative, L8: Negative control**

product were produced with other mycobacterium species, such as *M. avium, M. fortuitum, or M. kansasi* (data not shown). Sensitivity was estimated by serial dilutions of M. tuberculosis DNA. The MPCR detected 10fg, which is equivalent to two mycobacterial genomes.

**MPCR Quality control**

To avoid contamination during DNA extraction and amplification strict precautions were taken, including separate areas for DNA extraction, reagent preparation, amplification and product detection and regular meticulous cleaning of surfaces with 10% hypochlorite. In addition all the reagents were aliquots upon arrival in the laboratory. Positive and negative control were included with each set of reaction. The positive control was DNA extracted from *H. RV* whereas negative control was PCR grade water. To demonstrate the presence of inhibitors in MPCR all negative samples were spiked with positive control DNA and no inhibitors were detected on spiked samples as all were positive with spiked DNA.

The study evaluated 95 endometrial biopsy samples for diagnosis of female
Female genital tract tuberculosis (FGTB) is a very common disease in developing countries. It can have various clinical manifestations like menstrual irregularities, infertility and lower abdominal pain. The reported prevalence of FGTB in different studies varies from 1-19%. However, it is very well recognized that a large number of FGTB patients can be asymptomatic and may only present with infertility. Hence an early diagnosis of FGTB is of paramount importance as the delay in institution of therapy may result in irreversible damage. The tests like ESR and Mantoux are very nonspecific and conventional techniques like acid fast staining and Mycobacterial culture have a very low sensitivity and are also time consuming more so in paucibacillary conditions like FGTB. Nucleic acid amplification techniques like PCR have increased the diagnostic yield in various other paucibacillary tuberculous infections like tubercular meningitis and even FGTB. Most of the PCR based studies have reported use of only a single primer (IS6110) as a target for amplification for diagnosis of Mycobacterium tuberculosis (MTB) infection. However, it has been demonstrated that some strains of Mycobacterium tuberculosis lack this sequence in up to 9-10% cases. Use of two or more gene targets for amplification has been demonstrated to increase the diagnostic yield of MTB infections in other clinical settings like tuberculosis meningitis and even FGTB.

We evaluated the role of multiplex PCR (MPCR) using the two different primers for diagnosis of FGTB in proven and clinically suspected cases. To the best of our knowledge, the role of MPCR for diagnosis of FGTB has not been reported earlier. The overall sensitivity of this MPCR was 72.85% and specificity was 100%. The sensitivity in culture and histopathology proven cases was 95.23% and in clinically suspected cases was 63.26%. The specificity of PCR in diagnosis of FGTB has been reported to be between 22-56% in other single gene amplification PCR studies.

The overall sensitivity of MPCR in our study is higher than that reported in those studies. Our study also shows that the MPCR has a very high sensitivity and specificity in culture and histopathology proven cases. The low sensitivity in suspected cases could be due to inadequate/non representative specimens, technical failure in processing of specimen, single sample, period of specimen collection as endometrium gets sloughed every month and the presence of PCR inhibitors in the sample. The other cause of low sensitivity in clinically suspected cases may be due to inclusion of patients without MTB infection but a higher clinical suspicion of tubercular infection in an endemic area. The other studies have also reported similar factors for low sensitivity of PCR in diagnosis of MTB infection.

In our study, IS6110 was positive in 90.47% of confirmed cases and 57.14% of suspected cases; whereas MPB 64 was positive in 90.47% of confirmed cases and 61.22% of suspected cases. MPB64 picked up 3 cases which were missed by IS6110 and two cases were picked by IS6110 but missed by MPB64. The sensitivity of IS6110 in our study is similar to that reported by Roya et al.

This again highlights the fact that there are strains of MTB in India which lack not only IS6110 (n = 4) but MPB64 (n = 2) also. Thus, MPCR has a definite advantage of utilizing this fact to increase the overall sensitivity of PCR over single / individual PCR tests for diagnosing MTB infection. The other advantage of MPCR over doing two PCR’s using different primers is the low cost, lesser chances of contamination, less time consumption and better utilization of manpower in resource constraint settings.

Conclusions

MPCR using IS6110 and MPB 64 has a good sensitivity and specificity in diagnosis of FGTB and it is higher than that reported in the earlier single gene PCR studies.

References

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