Evaluation of PCR using MPB64 Primers for Rapid Diagnosis of Tuberculous Meningitis

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Abstract

Purpose: Diagnosis of Tuberculosis (TB) is largely based on microscopy and culture, which either lack of sensitivity or time consuming. In the present study a PCR test based on DNA sequence coding for MPB64, specific for Mycobacterium tuberculosis was compared with Ziehl-Neelson (ZN) stained AFB smear examination, culture based on conventional LJ medium for diagnosis of tuberculosis using clinical samples obtained from Cerebro-Spinal Fluid (CSF) samples from TBM patients.

Methods: PCR using MPB64 primers was performed on 9 TBM confirmed (culture was positive), 81TBM suspected and 40 Non TBM (control group) patients.

Results: MPB64 PCR had sensitivity of 88.8% and specificity of 100% for confirmed TBM cases, where as in 81 clinically diagnosed but unconfirmed TBM cases MPB64 PCR was positive in 81.48% cases respectively. The overall sensitivity of microscopy, culture and PCR using MPB64 were 1.11%, 10%, 82.22% and specificity was 100%, 100% and 100% respectively.

Conclusion: PCR is an important diagnostic tool for rapid diagnosis of TBM.

Keywords: PCR; Tubercular meningitis

Introduction

Tuberculosis is a major cause of morbidity and mortality worldwide. The World Health Organization has noted that the global incidence of TB is increasing by 0.4% per annum [1]. Central nervous system TB accounts for about 5% of all extra-pulmonary TB and Tuberculous Meningitis (TBM) is the most serious complication [2]. Delay in the diagnosis and institution of therapy can result in neurological sequelae in 20-25% of cases [3]. Prompt and accurate diagnosis is of paramount importance for better patient outcome. Conventional modalities like microscopy and culture, although considered as gold standard have low sensitivity and limited role in diagnosis of pauci-bacillary condition like TBM.

The development of rapid, sensitive and specific test for detection of mycobacterium has been a long standing need. A number of mycobacterial antigen [4,5] and antibody kits [6,7] have been developed but are quite inadequate and needs validation.

Nucleic Acid Amplification Techniques (NAAT) such as Polymerase Chain Reaction (PCR) has been reported to be more sensitive and specific. Several Mycobacterium tuberculosis specific sequences like IS6110, Protein antigen b [8], MPB64 and 65 kDa have been evaluated [9,10]. Most of the earlier PCR based studies have used IS6110 because of its repetitive nature [9,11]. However, either absence or presence of a few copies of this sequence have already been reported in some isolates [12-15]. Studies from India have also reported that a large number of clinical isolates (11-40%) had either a single copy or no copy of insertion sequence [16,17]. MPB64 has been demonstrated to be highly specific for Mycobacterium tuberculosis complex [18,19] and other studies have reported sensitivity and specificity of 75-90% and 100% respectively. The literature regarding the evaluation of MPB64 in patients of TBM in our region is scanty. Therefore, in the present study, we evaluated PCR based assay using MPB64 primers specific for Mycobacterium tuberculosis complex in CSF samples of patients with TBM and non TBM control group and its comparison with conventional techniques like microscopy and culture.

Materials and Methods

A total of 130 CSF samples received for AFB smear and culture in laboratory of tertiary care hospital of India, between September 2008 and December 2009 were evaluated. Patient's age ranged from 12-90 years. The relevant history and other details of the patients were noted from the case records. The patients were divided into 3 groups: Group I: TBM (n=90): (a) confirmed TBM-culture/smear positive (n=9), and (b) suspected TBM: smear/culture negative, clinical and laboratory features suggestive of TBM and response to anti-tuberculosis therapy [20] (n=81); Group II: Non-TBM infectious meningitis (n=20): (a) pyogenic meningitis (n=8), (b) viral meningitis (n=10), and (c) fungal meningitis (n=2); Group III: Non-infectious neurological disorders(n=20): head injury (HI: n=10), Landry-Guillain-Barre syndrome (LGBS: n=2), multiple sclerosis (n=2) and tumors (n=6). The present study is a part of project approved by institute ethic committee.

Processing of CSF sample

All the 130 CSF samples were subjected to three microbiological tests: Ziehl-Neelsen staining (ZN), culture on Lowenstein-Jensen medium and PCR with MPB-64. The CSF samples of the subjects were

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processed in a biosafety cabinet placed in a specially assigned room. About 200-300 μl of specimens aliquots were stored at 20°C. Using 200 μl of centrifuge deposit for PCR, the rest of the deposit was used for acid fast microscopy by Ziehl-Neelsen method and culture was performed on 2 slopes of Lowenstein-Jensen medium. PCR was standardized and was found to have sensitivity to detect the DNA equivalent to 2-3 organisms. It tested positive with standard strain of M. tuberculosis, H37 RV DNA.

DNA extraction

DNA was extracted according to the CTAB-phenol-chloroform extraction method. Briefly 0.2 ml of CSF was centrifuged at 10,000 rpm for 10 min. The supernatant was discarded and pellet was suspended in 500 μl of TE Buffer (Tris -EDTA,) 30 μl 10% SDS and 3 μl proteinase k (20 mg/ml), mixed and incubated at 37°C for 1 hr. After incubation, 100 μl of 5 M NaCl and 80 μl of high salt CTAB (cetyle- trimethyl ammonium bromide) were added and mixed followed by incubation at 65°C for 10 min. An approximate equal volume (0.7-0.8 μl) of chloroform-isoamyl alcohol (24:1) were added, mixed thoroughly and centrifuged for 5 min at 10,000 rpm.

The aqueous viscous supernatant was carefully decanted and transferred to a new tube. An equal volume of phenol: chloroform-isoamylalcohol (25:24:1) was added followed by a 5 min spin at 10,000 rpm. The supernatant was separated and then mixed with 0.6 volume of isopropanol to get a precipitate. The precipitated nucleic acids were washed with 75% ethanol, dried and re-suspended in 100 μl of TE buffer.

Polymerase chain reaction

In each independent PCR assay, positive control and one negative control were included. The positive control was the DNA of H37Rv and negative control included the PCR grade water. Identification of M. tuberculosis was done using a specific pair of primers designed to amplify MPB-64 in the M. tuberculosis complex and the expected band size was about 240 bp. The sequence of primers used P1ff and P2r were:

5'- ACC AGG GAG CGG TTC GCC 'TGG '3' and 5'- GAT CTG GGG GTC GTC GGA GCT -3' respectively.

A 25 μl reaction contained 10X assay buffer (Banglore Genei, Banglore, India), 10 mM dNTPs (Banglore Genei), 10 pmole of each primers, 2.5 units Taq DNA Polymerase (Banglore Genei, Banglore, India) and 5 μl of extracted DNA. Amplification was carried in a thermal cycler, which involves 35 cycles including denaturation at 95°C for 4 min, annealing of primers at 63°C for 1 min. and primer extension at 72°C for one min. the amplification product were separated on 1.5% agarose gel. Lane 1: 100bp DNA marker, lane 2: Positive control, lane 3: 240 bp amplification product of MPB64 of M. tuberculosis by PCR.

On microscopic examination, AFB was positive in one CSF sample, this was also positive by culture and MPB64 PCR which emphasize on the paucibacillary nature of TBM. Out of 9 confirmed TBM (culture positive) cases, 8 (88.8 %) were positive by MPB64 PCR. Whereas out of 81 suspected TBM patients, MPB64 PCR was positive in 66 (81.48%) patients (Table 1) and all 66 patients had shown good response to anti-tuberculous drug therapy. The response to treatment was judged by the experienced neurologists in treating TBM. In control group, all 40 patients showed negative result in all the three tests, thus giving 100 percent specificity.

A final diagnosis of TBM was made in 90 patients, based on results of culture, microscopy, PCR and response to anti tuberculous drug therapy (Table 2). Of these 90 patients, PCR was positive in 74, culture in 9, and microscopy in one. Thus the sensitivity of MPB64 PCR, culture and microscopy was 82.22%, 10% and 1.11% respectively. However the sensitivity of PCR in the confirmed TBM and suspected TBM group was 88.8% and 81.46% respectively. In non TBM group PCR was negative in all cases. Hence, the specificity was 100%.

Discussion

Tuberculous meningitis is one of the most serious manifestations of extra-pulmonary tuberculosis and prompt diagnosis and treatment is required for better clinical outcome. The conventional methods like microscopy and culture are quite insensitive in paucibacillary conditions. So, the present study was carried out to evaluate the utility of PCR targeting MPB64 gene in comparison with conventional techniques like microscopy and culture for the rapid diagnosis of TBM.

Though microscopy is very economical, it has limitation of low sensitivity in extra-pulmonary paucibacillary conditions like TBM. In the present study AFB smear was positive in only one patient, which was also positive for culture and PCR. The number of bacilli required for positive acid-fast staining is 10/ml. Our results are similar to previous studies, which had shown sensitivity in range of 0-10% [21-27]. A recent study established that both CSF volume and duration of the microscopic evaluation are independently associated with bacteriological confirmation of CNS tuberculosis, suggesting that a minimum of 6 ml of CSF fluid should be examined microscopically for a period of 30 min [28].

Though culture has been the gold standard, it is time consuming. In

![Figure 1: PCR amplification of 240 bp of MPB64 gene of M. tuberculosis on 1.5% agarose gel. Lane 1: 100bp DNA marker, lane 2: Positive control, lane 3: 240 bp amplification product of MPB64 of M. tuberculosis by PCR.

the present study, culture has shown a sensitivity and specificity of 10% and 100% respectively. Other case series have also reported the similar low CSF culture sensitivities which varies from 25 to 70% [29,30]. So culture takes too long time and cannot contribute in rapid diagnosis and management of TB patients.

Among the various methods studied, PCR is considered to be most sensitive and specific diagnostic method especially in cases where suspicion is high but AFB staining is negative [31]. The majority of PCR based studies have targeted insertion sequence IS6110, as multiple copies are present in M. tuberculosis genome [32-34]. It has however been shown that there are M. tuberculosis strains prevailing in India which do not contain IS6110 [33]. It is believed that more studies are required to establish its utility in the diagnosis of TB [35].

In our study, the MPB-64 PCR detected the presence of M. tuberculosis DNA in 88.8% of patients with confirmed TB and 81.48% patients with clinically suspected TB whose smear and culture results were negative as shown in table 1. Seth et al. [35] had shown a slightly lower positivity of 77.7% by using the similar target MPB64. The other studies have also reported a similar sensitivity ranging from 75-85% [35,37,38]. In terms of specificity it is 100% and other studies have also shown a similar specificity. Manjunath et al. has evaluated the PCR targeting MPB64 on extra-pulmonary TB specimens with high sensitivity and specificity [39]. The reason for variable sensitivity in many studies could be due to low volume of CSF available, insufficient lyses of cells, loss of DNA during purification or different target used for amplification [40].

In our study, there was one patient who was culture positive but PCR negative and there were 15 clinically suspected patients who were also PCR negative. All these 15 patients responded to anti-tubercular therapy. The various reasons for PCR negativity in the culture positive case and the clinically suspected cases which responded to anti-tuberculosis drug therapy could be small volume of CSF received in the microbiology laboratory, low number of bacteria in the CSF, poor lyses of bacteria in the CSF samples or the presence of some PCR inhibitors like bacterial contaminants, phenol etc., in the CSF samples. Similar factors have also been reported to be responsible for PCR negativity in culture confirmed as well as clinically suspected cases of TB in other studies [40,41]. Sometimes the tough cell wall of M. tuberculosis makes the isolation of target DNA difficult [40].

We have evaluated this MPB64 primer for the first time in large number of TB and non-TB CSF samples. A number of other studies have used IS6110 as target for amplification, but this IS6110 may be missing in our population as mentioned above. The sensitivity and specificity in the present study was much higher than earlier studies and suggest the utility of MPB64 PCR as an important tool to guide a clinician in the diagnosis, where conventional methods fail. Dar et al. [18] has shown that PCR for MPB64 gene also provides a useful alternative for the diagnosis of pulmonary tuberculosis from sputum and paucibacillary samples like BAL and pleural fluid in which conventional methods show low sensitivity, especially in areas from which strains show a low copy number of targets like the IS6110 insertion sequence.

According to our study, MPB64 PCR could make a considerable impact in the diagnosis of TB, which is often missed by conventional tests producing negative results or cause an unacceptable delay in diagnosis. This is especially true in TB cases in which early diagnosis is essential for better outcome of the disease. In conclusion, MPB64 PCR can play potentially important role in strengthening the diagnosis of TB especially in paucibacillary cases and in those in whom IS6110 is absent or present in low copy number. In future, multiplex PCR should be carried out for diagnosis of TB patients using different targets together.

Table 1: Results of AFB smear/culture and MPB64 PCR.

<table>
<thead>
<tr>
<th>TYPE</th>
<th>SUBTYPE</th>
<th>NO. OF PATIENTS</th>
<th>SMEAR (+) %</th>
<th>CULTURE (+)%</th>
<th>MPB64PCR (+) %</th>
</tr>
</thead>
<tbody>
<tr>
<td>GROUP-1</td>
<td>Confirmed TBM Patients</td>
<td>9</td>
<td>1 (11.11%)</td>
<td>9 (100%)</td>
<td>8 (88.8)</td>
</tr>
<tr>
<td></td>
<td>Suspended TBM Patients</td>
<td>81</td>
<td>-</td>
<td>-</td>
<td>66 (81.48)</td>
</tr>
<tr>
<td>Total</td>
<td>90</td>
<td>2 (2.25%)</td>
<td>19 (22.35%)</td>
<td>65 (76.4%)</td>
<td></td>
</tr>
<tr>
<td>GROUP-2</td>
<td>Non-TBM Infectious Meningitis</td>
<td>8</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>PYOGENIC MENINGITIS</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>VIRAL MENINGITIS</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GROUP-3</td>
<td>Control Group Non-infectious</td>
<td>20</td>
<td>-</td>
<td>-</td>
<td>-</td>
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Table 2: Sensitivity and specificity of PCR test compared to culture/AFB smear.

<table>
<thead>
<tr>
<th>Test Result</th>
<th>Final Diagnosis</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>TBM (N=90)</td>
<td>Non TBM (N=40)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPB 64</td>
<td>Positive</td>
<td>74</td>
<td>-</td>
<td>82.22%</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>16</td>
<td>40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Culture</td>
<td>Positive</td>
<td>9</td>
<td>0</td>
<td>10%</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>81</td>
<td>40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microscopy</td>
<td>Positive</td>
<td>1</td>
<td>0</td>
<td>1.11%</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>89</td>
<td>40</td>
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References


