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Evaluation of PCR Using MPB64 Primers for Rapid Diagnosis of Tuberculosis Meningitis

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Abstract

Purpose: Diagnosis of tuberculosis (TB) is largely based on microscopy and culture, which either lack sensitivity or are time consuming. In the present study a PCR test based on DNA sequence coding for MPB64, specific for *Mycobacterium tuberculosis* was compared with Ziehl-Nelson (ZN) stained acid fast bacilli (AFB) smear examination, culture based on conventional Lowenstein Jensen (LJ) medium for diagnosis of tuberculosis using cerebro-spinal fluid (CSF) samples from tuberculosis meningitis (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. The sensitivity of microscopy, culture and PCR using MPB64 were 1.11%, 10%, 82.22% respectively and specificity was 100% for each.

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 tuberculosis 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. Although, conventional modalities such as microscopy and culture, are considered as gold standard tests, they have low sensitivity and limited role in diagnosis of pauci-bacilliary TB especially TBM.

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

Nucleic acid amplification techniques (NAAT) such as polymerase chain reaction (PCR) have been reported to be more sensitive and specific. Several *M. tuberculosis* specific sequences such as IS6110, Protein antigen b [8], MPB64 and 65kDa have been evaluated [9,10]. Most of the earlier PCR based studies have used IS6110 because of its repetitive nature [9,11]. However, complete absence, or presence of a few copies of this sequence have 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 *M. tuberculosis* complex [18,19] and some studies have reported sensitivity of 75 – 90% and specificity of 100%. There are few reports, with small sample size, on evaluation of MPB64 for the diagnosis of TBM patients in our region. Therefore, in the present study, for the first time, we evaluated PCR based assay using MPB64 primers specific for

M. tuberculosis complex in CSF samples of patients with TBM and non TBM control group and its comparison with conventional microscopy and culture in a large number of subjects [20].

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 to 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 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*, H_{37} 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 5M NaCl and 80 µl of high salt CTAB (cetyl- 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 H 37 RV 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 240bp. 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), 10pmole of each primer, 2.5 units Taq DNA Polymerase (Banglore Genei, Banglore, India) and 5µl of extracted DNA. Amplification was carried in a thermal cycler, which involved 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 products were separated on 1.5% agarose gel and samples showing the presence of 240bp band under ultraviolet transillumination were considered positive.

Statistical methods

The sensitivity, specificity, positive predictive and negative predictive values were calculated using the standard formulae [21].

Results

Of the 130 CSF samples studied, 9 were samples from patients with confirmed TBM, 81 were from patients with clinically suspected TBM, 20 were from patients with non-TBM infections, and 20 were from patients with non-infectious neurological diseases. Figure 1 shows the 240bp amplification product (MPB64) of *M. tuberculosis* by PCR.
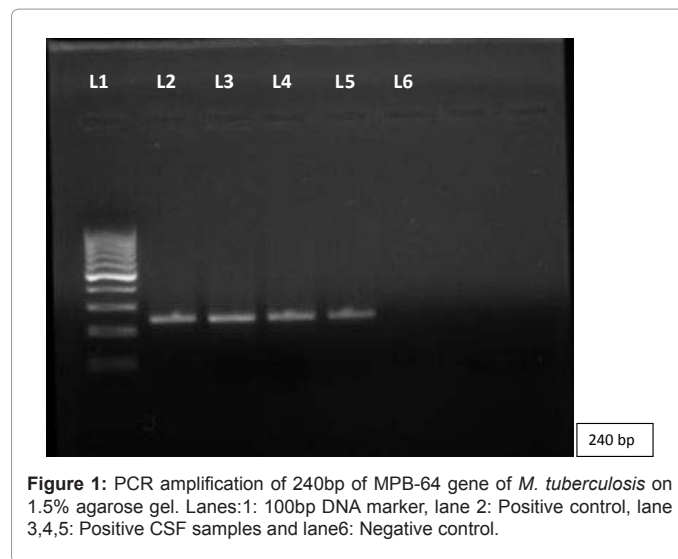

Figure 1: PCR amplification of 240bp of MPB-64 gene of *M. tuberculosis* on 1.5% agarose gel. Lanes:1: 100bp DNA marker, lane 2: Positive control, lane 3,4,5: Positive CSF samples and lane6: Negative control.

On microscopic examination, AFB was positive in one CSF sample, which was also positive by culture and MPB64 PCR. Out of 9 confirmed TBM (culture positive) cases, 8(88.8 %) were positive by MPB64 PCR. Of 81 suspected TBM patients, MPB64 PCR was positive in 66(81.48%) patients and all 66 patients showed good response to anti-tuberculosis drug therapy. 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 tuberculosis drug therapy. 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 were 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

Tuberculosis meningitis is one of the most serious manifestation of extra-pulmonary tuberculosis and prompt diagnosis and treatment is required for better clinical outcome. The conventional methods such as microscopy and culture are not sensitive in pauci-bacilliary TB. Therefore the present study was carried out to evaluate the utility of PCR targeting MPB64 gene in comparison with conventional techniques such as microscopy and culture for the rapid diagnosis of TBM.

Though microscopy is very economical, it has limitation of low sensitivity in extra-pulmonary pauci-bacilliary TB such as 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 10^4/ml. Our results are similar to previous studies, which showed sensitivity in the range of 0 – 10% [22-28]. 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 [29].

Though culture has been the gold standard test, it is time consuming. In the present study culture showed a sensitivity and specificity of 10% and 100% respectively. Other case series have also reported similar low CSF culture sensitivities which varies from 25 to 70% [30,31].

Therefore, culture takes too long time and cannot contribute in rapid diagnosis and management of TBM patients.

Among the various methods studied, PCR is considered the most sensitive and specific diagnostic method especially in cases where clinical suspicion is high but AFB staining is negative [32]. The majority of PCR based studies have targeted insertion sequence IS6110, as multiple copies are present in *M. tuberculosis* genome [33-35]. It has however been shown that there are *M. tuberculosis* strains prevailing in India which do not contain IS6110 [34]. It is believed that more studies are required to establish its utility in the diagnosis of TBM [36].

In our study, the MPB-64 PCR detected the presence of *M. tuberculosis* DNA in 88.8% of patients with confirmed TBM and 81.48% patients with clinically suspected TBM whose smear and culture results were negative. Seth et al[36] showed a similar rate of 85% in confirmed TBM cases where as Afroze et al. [37] showed a slightly lower rate of 77.7% using similar MBP64 target. Some other studies have reported similar sensitivity rates ranging from 75-85% [36,38,39]. In terms of specificity it was 100% which agreed with other studies that showed similar specificity. Manjunath et al. [15] evaluated PCR assay targeting MPB64 on extra-pulmonary TB specimens with high sensitivity and specificity [40]. 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 [41].

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 TBM in other studies [41,42]. Sometimes the tough cell wall of *M. tuberculosis* makes the isolation of target DNA difficult [41].

We have evaluated MPB-64 primer for the first time in large number of TBM and non-TBM CSF samples. A number of other studies have used IS6110 as target for amplification, but this may not be appropriate in our population due to previous reports that isolates in our environment lack IS6110 [33]. The sensitivity and specificity in the present study was much higher than earlier studies and suggest the utility of MPB-64 PCR as an important tool in the diagnosis of MTB, where conventional methods fail. Dar et al. [18] showed that PCR for MPB64 gene also provides a useful alternative for the diagnosis of pulmonary tuberculosis from sputum and pauci-bacillary samples such as Broncho Alveolar Lavage (BAL) and pleural fluid in which conventional methods show low sensitivity, and in areas where strains show low copy number of IS6110 targets.

In conclusion, MPB-64 PCR can play potentially important role in strengthening the diagnosis of TBM especially in pauci-bacillary cases. And in those in whom IS6110 is absent or present in low copy number. MPB-64 PCR needs further evaluation in large group of TBM patients before we can use it for routine diagnosis. In future, multiplex PCR should be carried out for diagnosis of TBM patients using different targets together.

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