Conventional PCR usage for the detection of Mycobacterium Tuberculosis complex in Cerebrospinal Fluid by MPB64-Target PCR

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Abstract

Tuberculous meningitis is a severe and potentially fatal form of Tuberculosis. Diagnosis of Tuberculous meningitis (TBM) is challenging. Thus rapid, accurate and confirmatory diagnosis is necessary to initiate required therapy. The diagnostic workup involves detection of acid-fast bacilli (AFB) in the cerebrospinal fluid (CSF) by microscopy or culture, however, the difficulty in detecting the organism poses a challenge to diagnosis. The use of the polymerase chain reaction (PCR) in the diagnostic approach to Mycobacterium tuberculosis (MTB) meningitis has been reported as a fast and accurate method using in-house protocols and several commercial kits available. We analysed the performance of PCR for the diagnosis of MTB meningitis in 64 consecutive patients, using MTB culture as gold standard. We used PCR as a tool for detecting mycobacterial mpb64 gene in CSF samples from suspected TB patient. 64 clinical specimens were taken from the patients attending SMI Hospital, Dehradun. 45 were PCR positive, 32 were culture positive and 2 were Zn smear positive. The sensitivity of PCR, culture and microscopy for CSF samples was, thus, 70%, 50% and 3% respectively. Therefore, compared to the conventional culture assay, the PCR can be considered a more useful and advanced technique for the rapid and accurate diagnosis of TBM. Thus PCR is better tool for the diagnosis of Tuberculous meningitis combined with the other concerned tests.

Key words:

Tuberculous meningitis; polymerase chain reaction; Central Nervous System.

How to Cite this Paper:


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Article History:------------------------

Date of Submission: 03-09-2012
Date of Acceptance: 21-09-2012
Conflict of Interest: NIL
Source of Support: NONE

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Introduction

In 2009 the global burden of Tuberculosis (TB) in the given WHO report says that there are 9.4 million incident cases (range, 8.9 million–9.9 million), 14 million prevalent cases (range, 12 million–16 million), 1.3 million deaths among HIV-negative people (range, 1.2 million–1.5 million) and 0.38 million deaths among HIV-positive people (range, 0.32 million–0.45 million). HIV-positives had an estimated 11-13% of these cases, where 80% of these cases accounting in Africa [1]. In India 500,000 people die per year from TB with one person dying every minute [2]. Once TB was thought to be a major contributor to the burden of disease in poor countries, and was nearly completely eradicated in the Western world but now it has resurfaced worldwide and become a global issue. It is the second leading cause of death from infectious diseases (4% of all deaths) and 88 million new cases of TB have occurred throughout the world during the decade gone by (1990-1999) [3]. The focal point in minimizing the risk of TB transmission is rapid diagnosis of the disease, especially in the wake of the emergence of drug-resistant TB and its severe implications for human immunodeficiency virus-infected patients. Early diagnosis plays a vital role in diagnosis of TB [4, 5]. Although acid fast bacilli (AFB) microscopy and Lowenstein–Jensen medium (LJ) culture remain the accepted “gold standard” and most popular among all the methods currently employed worldwide for TB diagnosis, however these traditional methods are either slow or have low sensitivity, especially with the samples that contain low bacterial load which can affect treatment by either delaying or causing inappropriate empiric therapy for TB [6, 7]. In recent times maximum attention has been devoted to developing nucleic acid amplification (NAA) diagnostic technologies owing to their rapidity and sensitivity. Numerous gene targets and several methods for isolating mycobacterium DNA from clinical specimens have been reported with encouraging results [8]. The present study was carried out to selectively target the mpb64 gene in Mycobacterium tuberculosis complex (MTB complex) with 240 bp product for the detection of meningitis tuberculosis. 240bp manase binding protein64 gene may be the most specific sequence for the diagnoses of the MTB complex by PCR, because the lowest number of false positive result was obtained with the mpb64 protein coding gene (10%) in comparison to IS6110 (62%) and heat shock protein (38%) [9].

The mpb64 gene codes for the MPB64 protein and is a 240 bp region (nucleotides 460–700). The sequence of MPB64 gene primers used was MPB64F 5’TCCGCTGCCAGTCGTCTTCC 3’ and MPB64 R-5’GTCCTCGCGAGTCTAGGCA3’.

Processing of clinical specimen:

Cerebrospinal fluid is used as a clinical sample as it typically lack common inhibitors of PCR assay such as haeme, endonucleases and exonucleases that can lead to false negative PCR result. For the proposed study sixty four cerebrospinal fluid were collected from different departments of SMI Hospital, Patel Nagar, Dehradun and were transported properly at 4°C to the Molecular Research Laboratory for further processing. Acid fast staining and culture of MTB was also performed for all the samples. For DNA isolation from the 64 CSF clinical specimens, 200µl of CSF was taken as starting volume. 20µl of ProteinaseK (20mg/ml) were added in all the clinical specimen with the addition of 200 µl of extraction buffer (Lysis buffer 1 and 2). The mixture was vortexed (for 15-20 sec) and incubated at 60°C for 20 minutes thereafter was transferred to 90°C for 10 minutes. 200µl of chilled absolute ethanol was added to each specimen in order to precipitate the DNA. All the contents were vortexed properly and then transferred into prearranged silica columns and centrifuged at 10,000 rpm for 3 minutes at 4°C. Now the columns were transferred to fresh collection tubes. 500µl wash buffer [Reconstituted by 60 ml 98% absolute ethanol
and centrifuged at 10,000 rpm for 3 minutes at 4°C after which the contents were transferred to fresh collection tubes; the procedure was repeated again followed by dry wash at 13000 rpm for 2 minutes. The columns were transferred to autoclaved microfuge tubes and 60 µl of pre heated milli Q water was added to each column which was then centrifuged at 13000 rpm at 4°C for 5min. The column was discarded and eluted DNA was collected in micro centrifuge tube.

**PCR Set up and Amplification**

PCR was carried out for all the CSF specimens including controls. PCR was performed for the amplification of gene mpb64 using primers Forward primer P1-5'-TCCGCTGCCAGTCGTCTTCC and Reverse primer P2 5'-GTCCTCAGGATCTAGGCCA. A reaction mixture of 50 µl containing 10X PCR buffer (250 mM Tris Hcl, 500 mM KCl), 0.2 mM dNTPs, 25µM primers, Taq polymerase (3 units) and Mg²⁺ ions (25mM) as MgSO₄ was prepared. 25µl of DNA template was added in the 25µl of master mix and amplification was done in thermal cycler (Veriti, Applied Biosystems) for 40 cycles. Cycling conditions include; initial denaturation at 94°C for 4 min, denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and primer extension at 72°C for 30 sec were provided with final extension of PCR products at 72°C for 7 minutes.

**Results**

All the sixty four CSF samples were subjected for PCR, acid fast staining and culture. Acid fast staining showed only two positive while 32 samples were culture positive and 45 samples were PCR positive. PCR proved to be a more sensitive method, detecting 45 out of 64 cases (70%), whereas only 32 were culture positive (50%) and for AFB staining two were found positive (3%). The sensitivity of PCR, culture and microscopy was, thus, 70%, 50% and 3% respectively. Tuberculous meningitis (TBM) is a medical emergency that requires urgent treatment because early intervention reduces the risk of mortality.

**Figure 1:** PCR amplification of 240 bp of mpb64 gene of M. tuberculosis on 1.5% agarose gel.

**Discussion**

Diagnostic techniques based on amplification have the potential to increase the sensitivity for detecting mycobacterium as well as to dramatically reduce the time usually necessary to detect and identify these organisms in clinical specimens [10, 11]. Early confirmatory diagnosis of TBM is difficult to establish because of its pleomorphic clinical presentation[12, 13]. Delayed diagnosis and treatment may be associated with many severe CNS complications. Most studies have used IS6110 as a target for PCR-based diagnosis of TBM with variable degrees of success [14, 15, 16, 17, 18, 19, 20]. However, this insertion element is absent in a proportion of M.tuberculosis isolates from Indian strain of mycobacterium tuberculosis which reasons against its utility as a sole target for gene amplification for the detection of tuberculosis in India [21, 22]. In contrast, MPB 64 sequences were universally detected in M. tuberculosis strains in India because 240bp Mpb64 protein gene may be the most specific sequence for the diagnoses of the TB meningitis by PCR, because the lowest number of false positive result was obtained with the mpb64 protein coding gene (10%) in comparison to IS6110 (62%) and heat shock protein (HSP) (38%) [23, 24].
Conclusion:
The mortality and mordiity rate of Tuberculosis meningitis is high in recent year. Early diagnosis of tuberculosis meningitis is very important taking CSF as starting specimen in the treatment of TB meningitis. Direct smear and culture method is most simple method use to diagnosis of Tuberculosis, but is time taking and sometime not give acuarate result. Detection of tuberculosis by PCR is rapid, novel method and it gives result in 2-3 hours and its result is most probably accurate. A molecular method including PCR is thus a specific, sensitive and rapid technique for the detection of tuberculosis meningitis. We here propose that for timely detection and management of Tuberculosis meningitis PCR method can be employed where ever possible as it will cut the time required for detection of the disease and it also help in making the physicians take timely decisions for patient management.

Acknowledgment
The authors are grateful to Honorable Chairman, Shri Guru Ram Rai Education Mission for his kind support and guidance.

Conflict of Interest: None

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9) WHO report 2010 Global tuberculosis control.


