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DIAGNOSTICS

Evaluation of multiplex PCR using *MPB64* and *IS6110* primers for rapid diagnosis of tuberculous meningitis

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SUMMARY

Tuberculous meningitis (TBM) is one of those most serious manifestations of extra-pulmonary tuberculosis and prompt diagnosis and treatment is required for better clinical outcome. It is difficult to diagnose due to lack of rapid, sensitive, and specific tests. Newer methods, which are easy and reliable, are required to diagnose TBM at an early stage. Thus our aim was to evaluate the Multiplex polymerase chain reaction (PCR) technique, using primers directed against the insertion sequence *IS6110* and *MPB64* gene for the detection of *Mycobacterium tuberculosis* in Cerebrospinal fluid (CSF), for rapid diagnosis of TBM patients.

102 CSF samples were analyzed from patients suspected with TBM along with a control group of 10 patients having other neurological disorders. CSF sediments were analyzed individually for *M. tuberculosis* DNA by Multiplex PCR using two set of primers targeting insertion sequence *IS6110* and gene MBp64, which is very specific for MTBC.

Out of 37 patients diagnosed with TBM clinically, *MPB64* PCR was positive in 22, *IS6110* PCR was positive in 28, both PCR using Multiplex were positive in 34 and Microscopy was positive in one. Thus Sensitivity of *MPB64* PCR, *IS6110* PCR, Multiplex PCR and Microscopy were found to be 62.3%, 75.4%, 91.8% and 2.7% respectively. In non TBM group PCR was negative in all cases hence, the specificity was 100%.

Multiplex PCR system using primers targeting *IS6110* and *MPB64*, for the detection of *M. tuberculosis* DNA in CSF samples, has high sensitivity than any one of them alone, and could be used for the early detection of TBM in CSF samples.

1. Introduction

Tuberculosis (TB) remains a major global health problem, responsible for ill health among millions of people each year. TB ranks as the second leading cause of death from an infectious disease worldwide, after the human immunodeficiency virus (HIV) [1]. The world Health Organization has estimated that there were 9 million new TB cases in 2013 and 1.5 million deaths [1]. TB mortality is unacceptably high given that most deaths are preventable if people can access health care for a diagnosis and correct treatment is provided [1]. The early and rapid diagnosis of *Mycobacterium Tuberculosis* (MTB) is very important for controlling and initiation of drug treatment regimen. The laboratory diagnosis of TB is mainly based on the microscopic examination by the Ziehl – Neelsen (ZN) staining and MTB culture, which are widely used in the laboratories of public health centers for the routine analysis. ZN staining is a cheap used in the laboratories of public health centers for the routine analysis [2]. ZN staining is a cheap technique and easy to perform but lacks sensitivity and is unable to distinguish between MTB members and other atypical mycobacteria. Moreover, ZN staining can only detect acid-fast bacilli in concentrations exceeding 10,000 organisms per ml [1,2]. MTB culture is the gold standard method but it requires viable microorganisms and long time incubation (up to 4 weeks), representing a problem especially for patients with critical situations such as immunocompromised

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or AIDS patients [2–5]. To overcome these limitations, molecular approaches have been introduced into clinical mycobacteriology laboratories. In this field, the most common technique used is the DNA amplification by the Polymerase Chain Reaction (PCR). The opportunity to use PCR for the detection of MTB in clinical samples has been reported [6–12]. Many MTB DNA sequences; including "*MPB64*" and "*IS6110*" are used as targets for MTB detection by PCR [1,2,6–12].

The insertion sequence "*IS6110*" is a transposable element which is present in the members of MTB complex in multiple copies (up to 25 copies), except *Mycobacterium bovis* BCG which harbors a single copy and absent in other mycobacteria [6,7,13–15]. For those reasons the insertion sequence "*IS6110*" has been reported as the most common target used for the MTB diagnosis in the clinical samples and demonstrated that the detection rate of MTB complex targeting "*IS6110*" was higher than that of microscopy or MTB culturing with a considerable time [3,4,6,13,15–19]. Furthermore, due to its high numerical and positional polymorphism, "*IS6110*" sequence has become a widely used marker in the epidemiological studies [16,17,20,21] and the fingerprinting of this transposable element has been used for the strain identification and phylogenetic analysis. Consequently, "*IS6110*" sequence is a useful and reliable tool for the diagnosis of mycobacterial strains in clinical specimens [6,10,14,16,19,22].

Similarly, *MPB64* has been demonstrated to be highly specific for *Mycobacterium tuberculosis* complex [23] and other studies have reported sensitivity and specificity of 75–90% and 100% respectively [9,10,19,23]. The literature regarding the evaluation of *MPB64* in patients of TBM in our region is scanty [9,24].

Thus, this study was planned to evaluate the use of Multiplex PCR using insertion sequence "*IS6110*" and "*MPB64*" gene for rapid diagnosis of MTB in clinical samples to improve the TB management in Nepal.

2. Methods

A total of 112 CSF samples received for AFB smear and PCR at Microbiology laboratory of tertiary care hospital of Nepal, in between Feb 2013 to June 2015, were evaluated. The age of patients ranged from 7 to 89 years with average age of 39. A relevant history and other details of the patients were noted from the case records. Ethical approval for the study was obtained from the Institutional review Board (IRB) of Annapurna Neurological Institute and Allied Sciences. Clinical Diagnosis of tuberculous meningitis was made on the basis of following criteria; (i) clinical symptoms including headache, vomiting, fever, photophobia and (ii) Radiological finding including tuberculous legions [25,26].

2.1. Processing of CSF samples

CSF samples were obtained using lumbar puncture method by experienced Neurosurgeons. All the samples were processed immediately without any storage. All the 112 CSF samples were subjected to two microbiological tests: ZN staining and Multiplex PCR, using primers MPB64 and *IS6110*. Most of the samples were processed immediately after collection and if needed, were stored at −20°C. Using 300 μl of centrifuge deposit for PCR, the rest of the deposit was used for acid fast microscopy by Ziehl–Neelson (ZN) staining. PCR was standardized and was found to have sensitivity of detecting DNA equivalent to 2–3 organisms. It tested positive with standard strain of *M. tuberculosis*, H37RV DNA.

2.2. DNA extraction

300 μl of CSF sample was used for the extraction of DNA. Genomic DNA was isolated from the H37RV strain of *M. tuberculosis*, which was used as positive control, and from clinical specimens using the QiAMP mini kit (Qiagen, Chicago, USA), following manufacturer's instruction.

2.3. Amplification of DNA

Multiplex PCR was optimized and performed using two set of primers, *MPB64* and *IS6110*. *MPB64* primer targets and amplifies the gene *MPB64*, producing 240 bp product and *IS6110* primer amplifies insertion sequence *IS6110* of *M. tuberculosis* complex, which produces 123 bp product. Primers for both sequences were opted from previous studies [6,9,15] and the PCR conditions were optimized for multiplex PCR. The primers were analyzed using NCBI BLAST with insertion sequence IS6110 (Gene bank ID KP844721.1) and MPB64 gene (Gene bank ID 885925) before using them. The sequences of primers (Macrogen, Seoul, Korea) and amplicon sizes are given in Table 1 below:

Positive control containing DNA of H37RV and negative control containing all components except template DNA were included in each PCR reaction. Amplification of DNA was carried out with PCR Master mix (Solis Biodyne, Estonia) in thermal cycler 5 Prime/02 (Bibby Scientific Ltd. UK).

PCR program for amplification of both targets includes initial denaturation at 94 °C for 5 min and then 35 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min and extension at 72 °C for 1.5 min. This was followed by final extension at 72 °C for 2 min.

2.4. Data analysis

Data analysis and kappa correlation was made using data analysis software SPSS Version 20.1.

3. Result

Out of 112 CSF samples analyzed, 102 were from the patients clinically suspected with TBM, while 10 were from patients with non-infectious neurological diseases. Figure 1 shows, 240 bp amplified product of *MPB64* sequence and 123 bp amplified product of *IS6110* insertion sequence of *M. tuberculosis* by Multiplex PCR.

On microscopic examination, one CSF sample was positive for AFB, which was also positive for *IS6110* and *MPB64* PCR. Out of 102 CSF samples from patients suspected with TBM, 52 were male and 50 were female. 34 samples were positive for either of primers while using multiplex PCR. Out of 34 samples, 23 were positive for *MPB64* while 28 were positive for *IS6110* insertion sequence and 17 samples were positive for both *IS6110* and *MPB64* PCR. Out of 28 *IS6110* positive PCR samples, 11 were negative for *MPB64* and out of 22 *MPB64* positive samples 6 were negative for *IS6110*. In a control group of 10 patients, all showed negative result for all three tests resulting 100% specificity.

37 patients were clinically diagnosed with TBM based on results of clinical symptoms, Radiology, Surgery and response to anti-

Table 1
Primer sequences for *IS6110* and *MPB64*.

S.N.	Primer	Sequence (5′–3′)	Band size
1	*IS6110*	CCTGCGAGCGTAGGCGTCGG CTCGTCCAGCGCCGCTTCGG	123 bp
2	*MPB64*	TCCGCTGCCAGTCGTCTTCC GTCCTCGCGAGTCTAGGCCA	240 bp

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