SHORT COMMUNICATION

POLYMERASE CHAIN REACTION USING THE MPB64 FRAGMENT FOR DETECTION OF MYCOBACTERIUM TUBERCULOSIS COMPLEX DNA IN SUSPECTED TB CASES

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ABSTRACT

Background: Various primers have been used for detection of *Mycobacterium tuberculosis* complex (MTBC) DNA using Polymerase chain reaction (PCR). The MPB64 gene has been demonstrated to be a highly specific target for MTBC detection.

Aims & Objectives: To detect MTBC DNA using MPB64 primers in clinical specimens of Nepalese patients suspected to have tuberculosis (TB).

Materials and Methods: DNA was extracted from 44 clinical specimens of patients suspected of having TB and MPB64 targeted PCR was performed.

Results: Bands comprising 240 base pair (bp) of MPB64 sequence were present in 15 of the 44 clinical samples, of which 4 were pulmonary and the remainders were extra-pulmonary samples. The overall positivity of MPB64 targeted PCR was 34.1%.

Conclusion: PCR targeting the MPB64 fragment has a potential of detecting the MTBC DNA and has potentially valuable clinical applications in early detection of TB in Nepal.

Key Words: MPB64; Tuberculosis; Extra- Pulmonary Tuberculosis; Polymerase Chain Reaction

Introduction

Tuberculosis (TB) is an infectious disease caused by Mycobacterium tuberculosis complex (MTBC), which includes *M. tuberculosis*, *M. bovis*, *M. africanum, M. canetti* and *M. microti*.[1] TB has become a serious global health issue. Out of 8.6 million cases of TB reported in 2012, 1.3 million people died.[2] The increasing impact of TB is partly attributable to the advent of multidrug resistant (MDR) and extensively drug resistant (XDR) strains of *M. tuberculosis* and rise in cases of HIV.[3] Moreover, the timely diagnosis of this disease presents practical difficulties and this has made the management of TB difficult in developing countries

Laboratory diagnosis of TB in such regions is mainly based on microscopic examination of Acid Fast Bacilli (AFB) smears and culture of the organism.[4,5] Both of these tests have low efficiency in diagnosis of paucibacillary conditions like pleural TB or meningeal TB.[6] Although considered as a gold standard, mycobacterial culture methods also have a mean incubation period of at least 4 weeks, a serious disadvantage.[7]

As reviewed elsewhere, nucleic acid amplification techniques (NAAT) such as polymerase chain reaction (PCR) have been demonstrated to provide fast, sensitive and specific identification of the causative agent of TB.[1,5] Most studies have targeted the MTBC specific insertion sequence IS6110, because of its repetitive nature,[8] however absence of this sequence has already been reported in some isolates.[9-11] Hence other target sequences have been used in place of or in conjugation with IS6110. These include MPB64, GroEL, PhoS, CIE Ag78 and Pab[12] and TRC4.[13]

The MPB64 gene is highly specific for MTBC[14] and has been used in the diagnosis of TB.[15-18] Reported sensitivity and specificity ranges are 70-94.4% and 88-100% respectively.[6,19,20] The literature regarding the use of MPB64 PCR is scanty in our country. Therefore in the present study, we employed PCR to detect the MPB64 gene instead of IS6110 in suspected TB cases.

Materials and Methods

A total of 44 clinical specimens were analyzed: 12 pulmonary samples (sputum) and 32 extrapulmonary samples (urine, cerebrospinal fluid, pleural fluid, synovial fluid, aspirated fluid, ascetic fluid, pus, biopsy and endometrial fluid) from patients (2 confirmed and 42 suspected for TB) visiting Annapurna Neurological Institute and Allied Sciences from October 2013 to March 2014. Informed consent was obtained from the patients and the Institutional Ethics Committee of Annapurna Neurological Institute and Allied Sciences, Kathmandu, approved the study. The procedures followed for the

human subjects were in accordance with the ethical standards as laid down by the ICMR-Ethical Guidelines for Biomedical Research on Human Participants.

DNA Extraction

For pulmonary samples, concentration and decontamination procedures were carried out using Petroff's method before DNA extraction[21] whereas extrapulmonary samples were used directly. Genomic DNA was isolated from the H37Rv strain of *M. tuberculosis* which was used as a positive control and from clinical specimens using the Wizard Genomic DNA Purification kit (Promega Co, USA).

Amplification of DNA

PCR using primers targeting the MPB64 gene sequence of MTBC was performed as described by Kusum et al.[19] The sequence of primers were MPB1: 5'-TCC GCT GCC AGT CGT CTT CC-3' (Forward primer) and MPB2: 5'-GTC CTC GCG AGT CTA GGC CA-3' (Reverse primer) (Macrogen,USA) which amplified a 240-bp fragment from MTBC DNA.

For 25 µl reaction, these components were added: 2X Red dye PCR Master Mix (Promega Co, USA), Primer MPB1 (10 pm/µl), MPB2 (10 pm/µl), nuclease free water and template DNA.A positive control containing the extracted DNA of H37Rv and a negative control containing all of the components except template DNA were included in each PCR reaction. Amplification of DNA was carried out in the thermal cycler 5PRIME/02 (Bibby Scientific Ltd, UK) and comprised initial denaturation at 94^0C for 10 min and then 35 cycles of denaturation at 94^0C for 45 sec, annealing at 59^0C for 45 sec and extension at 72^0C for 45 sec. This was followed by final extension at 72^0C for 7 min. The amplification products were analysed on 2% agarose gels (Fisher Scientific, India) using a 100 bp DNA ladder (Promega Co, USA), visualized on the UV- light transilluminator Firereader (UVItec , UK) and then photographed.

Results

The presence of 240bp bands confirmed the presence of MPB64 sequence in 15 of 44 clinical samples, of which 4 were pulmonary samples and 11 extrapulmonary samples. The overall positivity of MPB64 targeted PCR was 34.1% (Table 1). Specifically, as detailed in Table 2 below, of the three MPB64 positive pleural fluid specimens, one had normal ADA activity (10 U/L) while the other two were strong suspects for TB (55 and 60 U/L). Likewise, one MPB64 positive CSF specimen had a normal ADA level (6.2 U/L) and the other was ADA positive (15 U/L). [MicroExpress ADA-MTB diagnostic kit reference ranges: normal < 30 U/L; strong suspect >40 U/L to 60 U/L for PF; normal: <10 U/L; positive: >10 U/L)].

Table 1: Results of AFB smear, culture and MPB64 PCR

Type	Subtype	No. of cases	Smear (+)	Culture (+)	MPB64 PCR (+)
Group 1	Confirmed TB cases	2	2 (100%)	2 (100%)	2 (100%)
	Suspected TB cases	42	-	-	13 (30.95%)
	Total	44	2 (100%)	2 (100%)	15 (34.1%)

Table-2: Characteristics of cases testing positive for TB by MPB64 PCR

Code	Sample type	ATT History	X-RAY/CT/MRI findings	ADA activity (U/L)	Co-diagnosis
TB07	Sputum	No	Chest abnormality	N/A	No
TB25	PF	5 months	Marrow changes in multiple vertebra, mainly D11, Right side pleural effusion	60	Gastric cancer with liver, spinal and lung metastases, b/l LLP
TB17	Urine	No	N/A	N/A	Hematuria
TB18	PF	No	Right side pleural effusion	10	CKD V
TB16	PF	No	Right side pleural effusion	55	CKD V
TB42	Sputum	5 months	Chest lesion	N/A	No
TB41	CSF	No	Sub-acute combined degeneration of spinal cord	6.2	Transverse myelitis, meningitis b/l LLP,
TB43	TF	No	Cerebral and lacunar infarction	N/A	Left ischemic stroke
TB34	Sputum	No	Chest abnormality	N/A	No
TB56	SF	No	Erosion of parts of metacarpal bone, lytic lesion of distal radius	N/A	Osteoporosis
TB14	CSF	No	Mild hydrocephalous	15	HIV infection (CD4 count-39 cells/µL)
TB10	Bone marrowbiopsy	No	Marrow signal changes in C3-C4 vertebral bodies	N/A	Tubercular spondylitis (cervica) with Discitis

Information was available for 12 of the 15 MPB64 PCR positive cases. ADA test: Adenosine Deaminase Activity test; ATT: Anti-Tuberculosis Treatment; PF: Pleural Fluid; CSF: Cerebrospinal Fluid; SF: Synovial Fluid; TF: Tissue Fluid; CKD V: Chronic Kidney Disease stage 5; b/l LLP: Bilateral Lower Limb Paresis

Discussion

In our study, MPB64 bands were observed for known cases of TB (comprising positive controls) and cases

previously treated for TB, as well as cases considered to have high likelihood of TB based on ADA levels, but not for the negative control in each PCR reaction, providing evidence that MPB64 PCR can be used to detect TB cases in Nepalese clinical samples.

TB is the most common cause of pleural effusion when bacterial load is very low, making it difficult to detect by conventional techniques.[22] Furthermore, it is also quite hard to detect bacteria in small volumes of paucibacillary fluids such as CSF specimens, particularly as bacteria will often be irregularly dispersed within body compartments.[23] It has been reported that positive results for MPB64 PCR are obtained only at concentrations of $3x10^2$ bacilli/ml or above.[12] Nonetheless, positive results were obtained for three pleural fluid specimens and two CSF specimens, including samples with normal ADA levels, in which bacilli concentrations are likely to be particularly low. These results provide evidence that MPB64 targeted PCR is able to detect apparent TB in patients who would not have been identified quickly by the ADA test and PCR could also be useful in screening neurological cases to check for unsuspected TB.

The test was also positive for two patients with a history of anti-tuberculosis treatment (ATT), suggesting MPB64 PCR may be useful to detect low levels of persistent infection. It will be important to confirm this by additional studies. This may require checking such patients for treatment compliance as well as drug susceptibility testing for MDR and XDR TB.

About one third of the MPB64 PCR positive cases in this study presented with neurological symptoms. For such cases, there is a paramount value in enabling clinicians to obtain a fast answer from PCR rather than wasting time on slower or less accurate diagnostic tests to determine if TB is (or isn't) the problem, as rapid diagnosis and treatment of TB is of even greater importance when the central nervous system is affected.

One constraint of this study is the small sample size and the results obtained here need to be further validated with larger sample sizes in the future. In addition, the occurrence of TB cannot be solely determined by the presence or absence of bands for MPB64 PCR but needs to be confirmed by one or more conventional diagnostic tests. Concordant with other authors, absence of a gold standard test, notably the mycobacterial culture which could otherwise have been used to assess the accuracy of the PCR, is a major limitation of our study.[15,20,24] Nonetheless, the radiological findings were corroborative with the results for most of the cases positive for MPB64 PCR. As described by Therese and colleagues,[10] PCR results should be interpreted in combination with the radiological and clinical findings of patients in cases where results of conventional procedures are unclear or are not available.

For patients not picked up by MPB64 PCR, the standard PCR using the IS6110 fragment as target may help to detect TB, however false negative results of PCR might have arisen from the presence of inhibitors in the sample, poor lysis of the bacterial cell wall or loss of DNA during the purification step.[19] Any of these factors has the potential to limit any PCR, independent of the gene targeted. This will require investigation in future research.

Conclusion

Detection of the MPB64 gene by targeted PCR appears to be able to detect TB rapidly in at least one third of potential cases, including some where bacterial levels may be low or past treatment has been ineffective. Once confirmed with additional studies, this test has potentially valuable clinical applications in the early detection of TB cases in Nepal, in particular those with CNS involvement.

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