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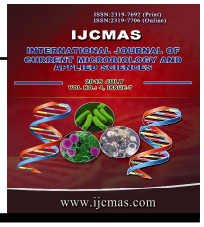


**Original Research Article**

# Study of Target gene IS 6110 and MPB 64 in Diagnosis of Pulmonary Tuberculosis

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*Mycobacterium tuberculosis*, IS6110, MPB64, PCR Tuberculosis (TB)

**A B S T R A C T**

Tuberculosis (TB), one of the most common infectious disease, captured one-third of world's population. Early detection of disease is an important task. Conventional methods are time consuming. Polymerase Chain Reaction (PCR) is a rapid diagnostic test but selection of target genes is an important game. The aim of our study is to compare the efficiency of two target genes IS6110 & MPB64 in routine diagnosis of *Mycobacterium tuberculosis*. 100 samples of clinically suspected cases of pulmonary tuberculosis were collected and processed immediately for Ziehl Neelsen staining, Cultured on Lowinstein Jensen (LJ) media followed by biochemical tests and PCR for detection of target genes IS6110 & MPB64 individually and together both. In total, 30% were AFB positive and 21% were culture positive on LJ. Positivity for both IS6110 & MPB64 were 77%, while 70% by IS6110 and 48% by MPB64 only. We concluded that both target genes IS6110 & MPB64 should be used together to avoid missing of any positive case.

## Introduction

Tuberculosis, second most common infectious disease spreading their wings. Roughly one-third of the world's population has been infected with *M. tuberculosis* with new infections occurring in about 1% of the population each year (Poongulali *et al*., 2014). As per WHO Global TB report 2013, About 8.6 million people develop TB and 1.3 millions died from TB (including 320000 deaths among HIV positive people). India alone accounted for 2.0-2.5 million cases in 2010, thus contributing approximately 26% of all cases worldwide.

According to National Tuberculosis Control Programmes, 2.6 million new cases of sputum smear positive pulmonary tuberculosis; 2.0 million new cases of sputum smear negative pulmonary tuberculosis were observed in 2010 worldwide (Pingle *et al*., 2014).

Early detection of tuberculosis by conventional method is time consuming because culturing can take 4 -8 weeks. Direct staining and microscopy has low sensitivity and specificity and can provide

only a preliminary diagnosis. PCR is the rapid detection technique with high sensitivity (Balakrishnan *et al*., 2010). PCR uses oligonucleotide primers to direct the amplification of target nucleic acid sequences via repeated rounds of denaturation, primer annealing and primer extension.

The choice of target genes is important step for sensitivity and specificity. Some of the target genes include the genes for IS6110, 38 kDa antigen (PhoS, CIE Ag78 or Pab), MPB64 (23 KDa), MPB70 (18kDa), 23SrRNA and 16SrRNA. Among these various conserved regions the most commonly used regions for indentifying mycobacterial DNA include (1) amplification of repetitive insertion sequences IS6110, (ii) amplification of genes encoding mycobacterial antigens such as 23kDa protein, MPB64 protein and (iii) amplification of ribosomal RNA (Shankar *et al*., 1990, Thierry *et al*., 1090, Boddinghaus *et al*., 1990).

IS6110 is widely used target genes, due to the presence of its multiple copies in *M. tuberculosis* complex genome which is believed to confer higher sensitivity (Lima *et al*., 2003, Rafi *et al*., 2007, Jin *et al*., 2010). However, few studies from different geographical regions of the world have reported that some clinical isolates have either a single copy or no copy if IS6110 which leads to false negative results (Dale *et al*., 2003, Thangappah *et al.*, 2003).

MPB64 is a major secreted protein specific to *M. tuberculosis* complex having a molecular weight of about 23,000. The protein was initially isolated from culture fluid preparations from *M. bovis* BCG Tokyo and designated as MPB64. In some studies, MPB64 primer were used to detect *M. tuberculosis* in clinical samples other than sputum and found highly specific for the *M. tuberculosis* complex

Therefore, this study was carried out to compare the efficiency of these two primers in routine diagnosis of *Mycobacterium tuberculosis*.

## Material and Methods

Fresh, clinically suspected 100 cases of pulmonary tuberculosis, which come to our lab for investigation, were included in this study. Suspected signs and symptoms were history of fever, weight loss, cough, lymphadenopathy suggestive of pulmonary tuberculosis, with or without evidence in chest x-ray, ESR, CT scan, positive history of contact/ family history or past history of tuberculosis, Positive Mantoux test/BCG vaccination evidence. These are usually come from the chest clinic, outdoor and indoor patients from T.B. & Chest department, CSSH, Meerut. 20 healthy controls were also included in the study, these controls were individuals which do not have any T.B. history or any related symptoms.

Sputum/ gastric aspirate/ pleural fluid were included in this study. 5 – 10 ml of exudative material, brought up from lings after a deep productive cough were collected in a clean sterile universal container and transported immediately to the laboratory.

All samples will decontaminate and concentrate by the modified Petroff's method (Tripathi *et al*., 2014). Sediments were divided into three parts, one part was used for smear preparation followed by Ziehl neelsen staining and second part was used for culture on Lowinstein Jensen media and identification by Niacin test, Nitrate reduction test, catalase test (Hamed *et al*., 2013). Third part was used for DNA

extraction for PCR. Smear was examined as per RNTCP protocol (RNTCP, 2009).

**DNA extraction:** Mycobacterial DNA was extracted by using automated nucleic acid extractor "G-Xtracter" (Genetix Asia Pvt. Ltd.) as per manufacturer's guidelines (Genetix India).

**Amplification of M. tuberculosis DNA using IS6110 primer**: First round of PCR was done on all extracted DNA samples. First PCR was carried out using the sequence for IS6110 gene (Tang *et al*., 2004).

Primer 1: 5'-CCT GCG AGC GTA GGC GTC GG-3'

Primer 2: 5'-CTC GTC CAG CGC CGC TTC GG-3'

Each PCR reaction contained 1x buffer, 200µM dNTPs, 1.5 U Taq Polymerase, 200 ng of each Primers and 5 µl of sample DNA per 50 µl of reaction volume. DNA amplification was performed for 35 cycles following an initial denaturation at $94^0$C for 5 min. in a thermal cycler (Applied Biosystem) by using following programme: denaturation at $94^0$C for 1.5 min., annealing at $70^0$C and extension at $72^0$C for 1.5 min with a final extension of 10 min at $72^0$C. The amplified product was stored at $4^0$C till the detection by electrophoresis.

**Amplification of M. tuberculosis DNA using MPB 64 primer**: Second round of PCR was also done on all extracted samples by using primer for MPB 64 gene (Parekh *et al*., 2006):

Primer 1: 5'- TCC GCT GCC AGT CGT CTT CC-3'

Primer 2: 5'- GTC CTC GCG AGT CTA GGC CA-3'

Each reaction contained 1X buffer, 250 µM dNTPs, 1.5 U Taq Polymerase 200 ng of each of primer and 5 µl of sample DNA per 50 µl of reaction. DNA amplification was performed for 30 cycle following an initial denaturation step at $94^0$C for 5 min in the thermal cycler by using the following programme: denaturation at $94^0$C for 2 min, annealing at $50^0$C for 2 min and extension at $72^0$C for 2 min with a final extension of 10 min at $72^0$C. The amplified product was stored at $4^0$C till the detection.

**Detection of PCR products**

Amplified products of each PCR assay performed, 25 µl of the reaction solutions of amplification were resolved on 2% agarose gels containing 1 µg ethidium bromide per 20 ml and product were visualized by Gel Documentation System (Genie, India). IS6110 primer showed band at 123 bp position (Fig 2) and MPB64 showed band at 240 bp position (Fig 3). Positive and negative controls were also processed with each batch of clinical samples.

**Results and Discussion**

Out of 100 samples processed, 30 (30%) were positive for AFB, whereas 21 (21%) were positive for mycobacterial culture (Figure 1). Among the culture positive specimens 14 (14%) were *M. tuberculosis* and 7 (7%) were non tuberculosis mycobacteria. All 7 (7%) non-tuberculosis mycobacteria positive samples were negative by both PCR systems.

Various degrees of reactivity in each of PCRs were found by applying two PCR assays to these samples from patients with clinical suspicion of tuberculosis. The overall higher proportion of positive results were detected with PCR assay 1 (targeting IS6110) by which 70 (70%) showed positive results as compared to PCR assay 2

(targeting MPB64) by which 48% samples showed positive results (Table-1). When combined in 77 (77%) samples, at least one of PCRs made it possible for us to detect *M. tuberculosis* DNA in samples (Table 1).

The usefulness, priority and scope of various techniques used in TB diagnosis depend on the epidemiological situation prevailing in individual countries and on the resources available. In most low income countries, the only practically available bacteriological method for diagnosing tuberculosis is direct smear microscopy for AFB.

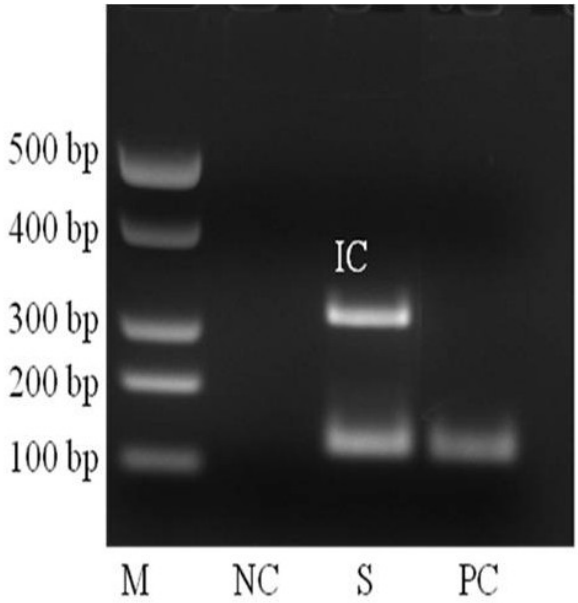
PCR with its rapidity and higher sensitivity facilitates the early and accurate identification of the causative organism of tuberculosis (i.e. *M. tuberculosis*) and thus very helpful for treatment, prevention and control of this chronic infectious disease. PCR as molecular diagnostic, had been using for many years for the detection of Mycobacterial DNA. However, PCR of mycobacterium DNA with one type of primer sometime may cause ambiguity in the detection of MTB-DNA and therefore, should be checked with other primers specific for mycobacterium tuberculosis complex. Primer specific for the most conserved region IS6110 was used for the diagnosis of tuberculosis by PCR. MPB64 primer specific for *groEL* gene was used in other study to detect *M. tuberculosis* in clinical samples and found highly specific for the *M. tuberculosis* complex (Seth *et al*., 1996). Therefore, this study was carried out to determine whether single step PCR technique could be used to the routine diagnosis of mycobacterium tuberculosis on samples by comparing the efficiency of these two primers.

**Table.1** Culture, microscopy and PCR positivity

| Method | Number of samples | % Positivity |
|---|---|---|
| Culture | 100 | 21 % (n=21)<br>(14 M. tb + 7 NTM) |
| AFB smear Microscopy | 100 | 30% (30) |
| PCR (IS6110 + MPB 64) | 100 | 77% (77) |
| PCR 1 (IS 6110) only | 100 | 70% (70) |
| PCR 2 (MPB 64) only | 100 | 48% (48) |

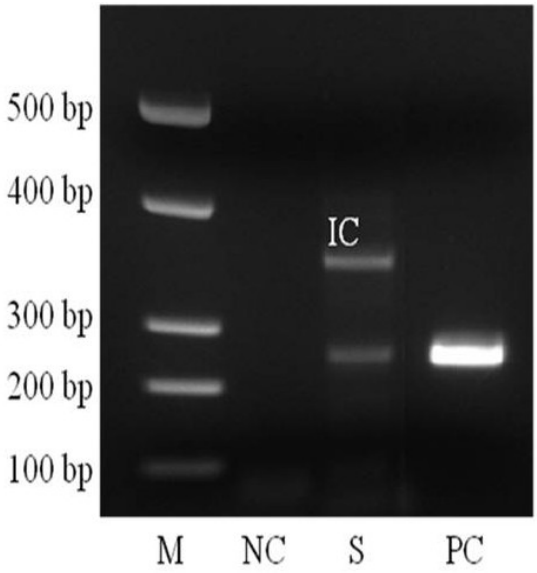
**Figure.1** Growth of Mycobacterium tuberculosis on Lowenstein Jensen media

**Figure.2** Amplified product of M. tuberculosis using primer IS6110




M= Markers, NC=Negative control, IN= Internal control,for Mycobacterial sp. As per kit manufacturers protocol (350 bp), bp= Base Pair

**Figure.3** Amplified product of M. tuberculosis using primer MPB 64




M= Markers, NC=Negative control, IN= Internal control,for Mycobacterial sp. As per kit manufacturers protocol (350 bp), bp= Base Pair, S=Sample, PC= Positive Control

Carry-over contamination is a major problem in PCR based detection. However, in this study, all necessary precautions were maintained to avoid contamination. Several other factors play an important role in efficacy of PCR protocol. The molecular mass of the amplification product is important. It has already been shown that the longer the amplified fragment, the higher the likelihood of degradation and thus the lower the efficacy of the amplification itself. Our results confirm this concept where we found higher number of positives by IS6110 system than MPB64 system. *M tuberculosis* is reported to have 1-10 copies of IS6110, though it is predominantly used as a target for PCR (Narayan *et al*., 2002). This suggests the need to choose the correct primers, such as targeting repetitive elements and those amplifying relatively shorter DNA sequences, which are thus less prone to fragmentation. In the present study, we used two PCR techniques to identify the causative agent. When results of both the systems were combined positive signals were obtained from 77 (77%) of 100 patients. The positive signals obtained in these cases were higher with IS6110 system 70/100 (70%) than with MPB64 system 48/100 (48%). Although 8 (8%) samples were negative by IS6110 but were positive by MPB64 method. This could be due to absence of IS6110 copies, which is known to exist in Indian *M. tuberculosis* strains. Thus our data strongly support the use of one additional PCR other than IS6110 system so as to reduce false negativity in the samples harboring zero copy of IS6110 element which is known to exist in Indian *M. tuberculosis* strains. PCR positivity results on such samples have been reported between 83-87% using IS6110 systems (Totsch *et al*., 1996, Kidane *et al*., 2002).

**Conflict of Interest:** Nil

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