The Brazilian Journal of
INFECTIOUS DISEASES

www.elsevier.com/locate/bjid

Original Article

Utility of multiplex real-time PCR in the diagnosis of extrapulmonary tuberculosis

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ARTICLE INFO

Article history:
Received 26 September 2015
Accepted 5 January 2016
Available online 26 March 2016

Keywords:
Extrapulmonary tuberculosis
Multiplex PCR
IS6110
MPB64

ABSTRACT

Objective: The diagnosis of extrapulmonary tuberculosis is still a challenge because of its pauci-bacillary nature. The aim of the study was to evaluate the role of a multiplex PCR assay in the diagnosis of extrapulmonary tuberculosis and to compare the efficiency of two targets, IS6110 and MPB64 to detect *Mycobacterium tuberculosis*.

Methods: 150 extrapulmonary samples (61 pus/aspirate, 46 tissue, 32 body fluids, and 11 urine) from clinically suspected cases of tuberculosis were included in the study. All the samples were subjected to direct fluorescent microscopy, TB culture (BacT/ALERT 3D, biomerieux, Durham, North Carolina, USA) and a Multiplexed Tandem PCR targeting two mycobacterial DNA sequences, IS6110 and MPB64. Master-Mix reagents and primers were prepared by AusDiagnostics Pvt. Ltd (Alexandria, New South Wales, Australia). The performance of the assay was assessed using a composite gold standard, which included clinical characteristics, microbiology smear as well as culture, histopathology, cytology, radiology, and response to antitubercular therapy.

Results: 20.3%, 23.6%, and 45.3% of specimens were positive by smear, culture, and PCR, respectively. The sensitivity and specificity of the multiplex PCR was 91.9% and 88.4%, respectively, using the composite gold standard. Positive and negative predictive values of the PCR were estimated as 85.1% and 93.8%, respectively. Higher positivity was observed with target IS6110 (44.6%) as compared to target MPB64 (18.9%). The sensitivities of IS6110 and MPB64 individual targets were 90.3% and 64.5%, respectively, and specificities were 88.4% and 97.7%, respectively.

Conclusion: PCR can play an important role in rapid and accurate diagnosis of extrapulmonary tuberculosis. IS6110 alone is an effective target in our part of the country.

Introduction

India is a high tuberculosis (TB) burden country contributing to 26% of the global burden of the disease. While pulmonary TB (PTB) is the most common form of disease, extrapulmonary tuberculosis (EPTB) constitutes around 20% of all TB cases in India.[1] Early diagnosis is essential in instituting effective and timely therapy. Conventional methods like smear and culture are of limited use in diagnosis of EPTB cases due to low

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http://dx.doi.org/10.1016/j.bjid.2016.01.006

sensitivity. Amplification techniques have attracted considerable interest in the diagnosis of tuberculosis particularly with the hope of shortening the time required in detection. Most of the assays are evaluated for pulmonary specimens and smear positive specimens. The diagnosis of extrapulmonary and smear negative tuberculosis is still a challenge especially because of the pauci-bacillary nature. Despite numerous reports in literature, amplification techniques do not yet have an established role in the laboratory diagnosis of tuberculosis, nor have they replaced traditional techniques. Some studies have evaluated the role of PCR in the diagnosis of EPTB using various primers to amplify different targets.[2–4] Highly conserved insertion sequences, IS6110, is the most commonly used target in the detection of *Mycobacterium tuberculosis*. Few Indian studies have pointed out that the most commonly used method targeting insertion sequence IS6110 has a danger of missing at least 10% of Indian tuberculosis patients harboring zero copy number of IS6110.[5] Therefore, there is a need to know which other targets can be used to reliably detect the EPTB cases. The other candidate target used by the researchers in India has been the gene encoding mpt64 (MPB64), a secretary mycobacterial antigen present only in *M. tuberculosis* complex, for the diagnosis of TB.[6,7] In the present study the comparative value of a multiplex PCR system which simultaneously amplifies two targets, for their role in the early detection of EPTB has been analyzed using a multiplex real-time PCR targeting IS6110 and MPB64. Keeping in view the poor rate of culture positivity in EPTB which makes it difficult to evaluate assays when it is considered as the gold standard, we have also made an attempt to evaluate this multiplex PCR using a composite gold standard (CGS) based on clinical presentation along with radiology, histopathology, and response to anti-tuberculous therapy. Further, the present study undertook to evaluate the role of a multiplex real-time PCR assay in the laboratory diagnosis of EPTB in comparison to conventional bacteriological techniques like smear and culture.

Material and methods

Patient population and specimens analyzed

The study was conducted during a period of one year from April 2012 to March 2013. A total of 150 biological specimens were collected from patients with a clinical diagnosis of EPTB, and 20 negative control biological specimens were collected from patients with an alternative diagnosis. The details of the specimens analyzed are given in Table 1.

Table 1 – Clinical specimens and negative controls included in the study.

Type of sample	Clinical specimens ($n = 150$)	Negative control ($n = 20$)
Pus/aspirate	61	5
Tissue	46	5
Body fluid	32	5
Urine	11	5

Sample processing

All the samples were subjected to Direct Fluorescence staining (DF), mycobacterial culture, and multiplex PCR assay. Patient demographics were collected from the case files as well as with the help of respective clinicians.

The diagnosis of tuberculosis was made on the basis of a CGS which included clinical characteristics, microbiology smear as well as culture, histopathology, cytology, radiology, and response to antitubercular therapy. A case was considered true positive if culture was positive or any two of the other criteria mentioned in the CGS.

DF staining was done using Auramine-Rhodamine (Sigma/Himedia) staining and examined using fluorescent microscope.[8] For mycobacterial culture, urine and tissue samples were subjected to digestion and decontamination by N-acetyl-L-cystine NaOH (NALC-NaOH) method.[9] Other samples from sterile sites were subjected to decontamination only if a Gram stain or routine bacterial culture showed the presence of any organism, otherwise were processed directly. All the samples were subjected to culture by BacT/ALERT 3D (biomerieux, Durham, North Carolina, USA) and LJ media as per the standard protocol.[10]

Identification of mycobacteria

Positive growth in either media was identified as *M. tuberculosis* (MTB) using AccuProbe molecular identification system (GenProbe, San Diego, California).[9] AccuProbe culture identification test was performed as described by the manufacturer. For the identification of non-tuberculous mycobacteria (NTM), GenoType Mycobacteria CM (Hain Lifescience GmbH, Nehren, Germany) was used as recommended by the manufacturer.[11] The assay permits the simultaneous molecular genetic identification of the *M. tuberculosis* complex and 14 of the most common NTM species (*M. avium ssp., M. chelonae, M. abscessus, M. fortuitum, M. gordonae, M. intracellulare, M. scrofulaceum, M. interjectum, M. kansasii, M. malmoense, M. peregrinum, M. marinum/M. ulcerans, and M. xenopi*) from cultivated samples.

Real time multiplex PCR

Standardization of PCR

Validation of the real time PCR was done using known positive and negative samples as well as the ATCC standard strain H37 Rv spiked samples. For detection of the analytical sensitivity of the assay, H37 Rv strain was subjected to PCR using both targets (IS6110 and MPB64) in different dilutions. 0.5 McFarland standard was prepared and was serially diluted to achieve different concentrations as 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, and 10^1 bacilli/mL of the sample. Multiplex real-time PCR was run using different dilutions of H37 Rv spiked samples, three times using each dilution.

DNA extraction

Samples after subjecting to decontamination by NALC/NaOH method, the DNA extraction was done using spin column method using DNASure Tissue Mini Kit (Nucleopore, G Brand, Genetix Biotech Asia Pvt. Ltd., New Delhi, India) as per the manufacturer's instructions. Briefly, 25 mg of tissue or 1–2 mL of other samples were placed in a microcentrifuge tube.