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Evaluation of D-PCR using the RNA polymerase Gene (*rpoB*) Primers for Rapid Differential identification of *Mycobacterium tuberculosis* Complex and Nontuberculous Mycobacteria

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ABSTRACT

Tuberculosis caused by Mycobacterium tuberculosis complex (MTC) is the leading cause of death worldwide. Detection of these bacteria is generally done by acid fast staining which also stains Non-tuberculous mycobacterium (NTM). It is significant to that this identification and differentiation between MTC and NMT be made before time, as the drug sensitivity profile of NTM is different from that of Mycobacterium tuberculosis complex and NTM will not responds to standard TB chemotherapy. In this study, we have evaluated a duplex polymerase chain reaction (D-PCR) to simultaneously detection and differentiation of Mycobacterium tuberculosis complex (MTC) and Non-tuberculous mycobacterium (NTM). The D-PCR described here is a rapid, important, and cost-effective tool for determining whether the causative organism is Mycobacterium tuberculosis complex or Non-tuberculous mycobacterium, and will be helpful for disease surveillance.

Key words: Duplex Polymerase Chain Reaction (D-PCR), Non- tuberculous mycobacterium (NTM), Amplicon

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INTRODUCTION

Fresh cases of TB have been decreasing for several years and fell at a rate of 2.2% between 2010 and 2011. The TB mortality rate has reduced 41% since 1990 and the world is on track to accomplish the global target of a 50% decline by 2015. However, the overall problem of TB remains vast (1). As Detection of these bacteria is generally done by acid fast staining which also stains Non-tuberculous mycobacterium (NTM). The non-tuberculous mycobacteria (NTM) are the mycobacteria other than members of the Mycobacterium tuberculosis complex and M. leprae, have been acknowledged since the 1950s as organisms capable of causing human disease (2). NTM infection can cause many clinical complications, Strategies used for the disease management of patients with *Mycobacterium tuberculosis* complex (MTBC) and non tuberculous mycobacteria (NTM) are different, Therefore, it has become important to differentiate between the two during the early stage of the diagnostic procedure (3).

Although conventional biochemical methods are able to identify mycobacterial species; but these are slow, laborious and require intricate safety precautions. However, recent methodological advances in molecular biology have

provided substitute fast approaches, like PCR and PCR-linked methods. For the fast and accurate diagnosis of M. tuberculosis and NTM, target genes specific to mycobacteria are used in a PCR (4). Because the prevalence of NTM infection is increasing, any methods competent of simultaneously determining the presence of M. tuberculosis and /or NTM would be valuable. For this purpose, multiplex PCR, has been regularly used, as this can be specifically detect and identify different species of the genus Mycobacterium (5) and differentiate members of the M. tuberculosis complex in the routine diagnostic laboratory by using Mycobacterium genus- and species-specific genes. Though, some of these genes have been found to lack specificity for M. tuberculosis strains found in India subcontinent. The mtp40 gene is not present in all MTB complex strains (6). In addition, IS6110 PCR has been reported to produce false-negative and false-positive results, and these reports advised that the multiplex PCR targeting of these genes has linked problems. Thus, there is an increasing demand for quick, specific, and sensitive diagnostic methods for the detection and identification of Mycobacterium tuberculosis and NTM for effective treatment of the disease (7, 8, 9). In addition, mixed infections of MTB and NTM have been reported (10). In the present study, we evaluate the usefulness of a duplex PCR assay, to differentiate M. tuberculosis complex and NTM by using RNA polymerase β-subunit-encoding gene (rpoB). To demonstrate the efficiency and usefulness of the rpoB gene based D-PCR, we used it to identify the clinical isolates of mycobacteria which were confirmed culture positive by BACTE 460 TB system.

MATERIALS AND METHODS

Sample processing:
This prospective study was undertaken at Auroprobe laboratories NH-58, Muradnagar, Ghaziabad Delhi-NCR India. A total of 3166 samples received for diagnosis of Mycobacterium by AFB smear and culture at auroprobe Laboratory, from Nov 2009 to December 2011 were evaluated. Patient's age ranged from 1 – 90 years. The relevant history and other details of the patients were noted from test requisition form (TRF). All specimens were examined microscopically using the ZN stain for the presence of acid fast bacilli as per the standard protocol. All clinical specimens were inoculated into BACTEC 12 B vial, after digestion and decontamination by modified petroff method (11). The medium used in the BACTEC 460 TB system was 4 mL of Middlebrook 7 H12 broth with carbon 14 (14C) labeled palmitic acid. The clinical specimen was inoculated along with an antibiotic mixture containing solution - PANTA. All the inoculated bottles were incubated at 37 ^{0}C and observed for growth. BACTEC 12 B bottles were read every first day of every week, up to six weeks using the BACTEC 460 instrument. Total numbers of positive cultures recovered by both the methods were recorded. All the mycobacterial isolates from the culture media were differentiated by the NAP (para Nitro-α- acetyl amino-β-hydroxy propiophenone) test. The average time for identification/differentiation between MTB and NTM was four days. Ethical approval was not needed for the current study as all the samples from the subjects were received for clinical diagnosis from Different collection points and we had not disclosed any identification of the subjects.

DNA extraction: Silica adsorption based column method was used for the extraction of bacterial DNA from the all culture positive samples. DNA extraction was performed according to manufacturer's instructions by nucleopore DNA extraction kit (12, 13).

Duplex PCR: Two pairs of primers were used as follows: Tbc1 (5'-CGT ACG GTC GGC GAG CTG ATC CAA-3')-TbcR5 (5'-C CAC CAG TCG GCG CTT GTG GGT CAA-3') and M5 (5'-G GAG CGG ATG ACC ACC CAG GAC GTC-3')-RM3 (5'-CAG CGG GTT GTT CTG GTC CAT GAA C-3') (14). DNA amplification with two pair of primers was performed in Applied Biosystems® Veriti® 96-Well Thermal Cycler in a 50 µl reaction mixture containing 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM MgCl , 25 pmol of each primer, 200 µM dNTP, 1 U of Taq polymerase and 5 µl of DNA template. The amplification parameters were as follows PCR was performed with an initial denaturation of 95°C for 5 min, 30 cycles of amplification (30s at 95°C, 60s at 72°C), and a final elongation at 72°C for 5 min. The PCR products were analyzed in 1.6 % agarose gel by electrophoresis (~2.5 V/cm) with 100 bp DNA ladder for size determination, stained with 0.5 µg/ ml of ethidium bromide and photographed under an UV-transilluminator. After completion of the D-PCR, all NTM isolates showing a 136-bp DNA amplicon and all MTB isolates were showing a 235-bp DNA amplicon, respectively (figure 1).

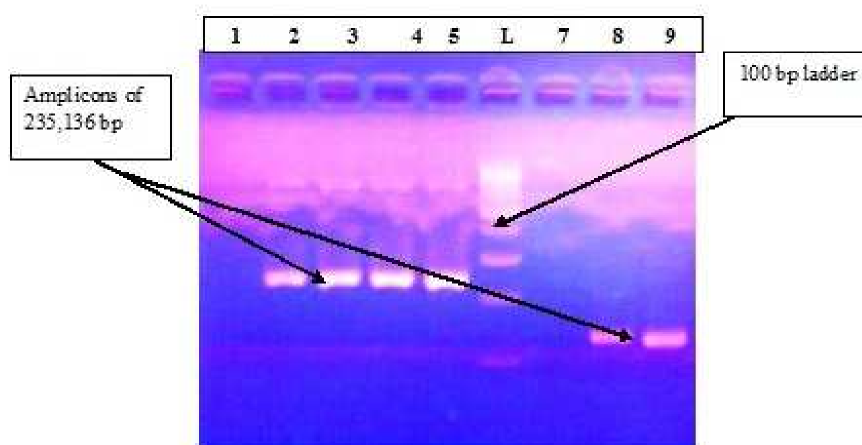

Figure: 1 D-PCR assay results performed with culture isolates of M. tuberculosis complex and MOTT. Two amplicons of different sizes (235 and 136 bp) were amplified from M. tuberculosis complex (lanes 2 to 5) and MOTT (lanes 8 to 9) strains by a single D-PCR. Lanes: L, ladder DNA (100-bp ladder). Lane 1 and 7 NC Samples.

RESULTS

Of the 3166 specimens, 100 specimens got contaminated by other microorganisms and other two gave non interpretable results when tested by BACTEC 460 TB system. These were removed prior to further analysis. Of these, 641 (20.90%) Mycobacterium isolates were detected by BACTEC 460 culture method. These isolates were tested by BACTEC 460 NAP TB Differentiation test to differentiate MTB complex from NTM strains. Among the 641 mycobacterium isolates tested 455 (70.98%) were *M. tuberculosis* and 186 (29.01%) were NTM given in (Table 1).

Table1: No of MTB Complex and NTM differentiation by NAP test (N=3066)

Types of Mycobacterial identified by NAP test (N=3066)	
Species	**No of Positive samples**
M. tuberculosis	455
MOTT	186
Unidentified	2425
Total	3066

D-PCR assay with a mixture of the two primer sets was performed on all 186 NTM isolates confirmed by NAP test earlier. While the 235-bp DNA was amplified all culture positives of *M. tuberculosis* complex (455), whereas the 136-bp DNA was amplified from all of all NTM isolates (Table 2). Therefore, D-PCR assay allowed the differential identification of *M. tuberculosis* complex and NTM in a single reaction.

Table: 2 No of mycobacterium isolate identified by D-PCR

D-PCR product (size in base pairs)	**No. of isolates identified as**	
	M. tuberculosis	**NTM**
	(n =455)	**(n =186)**
***M. tuberculosis* complex (235)**	455	0
NTM specific (136)	0	186

DISCUSSION

Tuberculosis is still one of the most severe problems in the world and timely diagnosis and treatment is required for better clinical findings (15). The conventional methods of diagnosis like microscopy and culture are not sensitive in those cases in which sample contains low bacterial load. Differentiation between M. tuberculosis and NTM in those patients who are an AFB smear positive has been a challenge for clinicians; however, it is tough to properly differentiate between MTB and NTM diseases based on clinical findings (16).

We evaluated a duplex PCR-based method to Differentiation between M. tuberculosis and NTM based on amplification of rpoB gene sequences. Through this study, we differentiate mycobacteria from culture positive samples with a duplex PCR-based method. Our data show that the duplex PCR for rpoB gene is promising for identifying and differentiation between M. tuberculosis and NTM mycobacterial. Therefore the present study was carried out to evaluate the utility of D-PCR targeting rpoB gene in comparison with conventional techniques such as microscopy and culture for the rapid diagnosis and differentiation of MTB and MOTT.

Though microscopy is very economical, it has limitation of sensitivity and the AFB positive is not the confirmation of Mycobacterium Tuberculosis and NTM. While culture method has been the gold standard test, but it is time consuming. In the present study D-PCR showed a sensitivity and specificity of 100% and 100% respectively culture positive samples. In addition to its advantages of simplicity and sensitivity, D-PCR based identification method provides a evidence for the differentiation of these two most important mycobacterial groups, which have dissimilar modes of infection and need different treatments.

CONCLUSION

In conclusion, the D-PCR assay based on rpoB provides a rapid and reliable means for the differential identification of M. tuberculosis and NTM in culture with a single reaction.

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