Development of a rapid and cost effective molecular diagnostic assay for species identification of MOTT culture isolates

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Abstract: Disease caused by Mycobacteria other than tuberculosis (MOTT) frequently causes sickness and death. These bacteria are found in water sources and soil and are particularly concentrated in biofilms. Species identification is important in those cases, of patients positive for AFB and do not improve clinically. Present study includes development of High Resolution Melt Curve Analysis to characterize the mycobacterium species. Six thousand nine hundred thirty three samples were processed and 1386 isolates were used for the development of HRM curve analysis for differentiating MOTT. An HRM curve was generated for each isolate to identify the MOTT species. Most specific technique for species identification is sequencing. The sequencing of the strains was also evaluated using the same isolates. The results of both techniques were compared to the gold standard proportional method. With the use of this assay we can identify 27 clinically relevant Mycobacterial species. Sequencing is good technique but it is an expensive for resource limited settings and needs expertise for analysis, while the HRM curve makes this assay an ideal identification method for the TB laboratory.
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1. Introduction

Mycobacterium tuberculosis infection is an important health concern for developing countries. Other species of Mycobacteria are usually saprophytes, but can be opportunistic and at times deadly pathogens. These other Mycobacteria are referred to as Mycobacteria other than tuberculosis (MOTT). (Katoch VM, 2004 and Portaels F, 1995)

Different MOTT species have different antibiotic susceptibility patterns, and their resistance to anti-tuberculosis drugs is of particular importance. For these reasons, the accurate and early differential diagnosis of MOTT is required for optimal outcomes. Currently, the identification of clinical isolates of Mycobacteria at the species level is primarily based on the characteristics of the cultured bacteria and the bio-chemical test results. These conventional tests can take several weeks to perform and cannot always precisely identify the species (Bang HI, 2011). Furthermore, these testing procedures are complex and laborious, and they are usually impeded by the slow growth of Mycobacteria in culture. There are numerous species of MOTT, and recently developed molecular methods have enabled the recognition of many of these species (Koh WJ et al., 2003). In this study, polymerase chain reaction (PCR) - High resolution Melt curve analysis was used for MOTT species identification.

2.Materials and Methods

Six thousand nine hundred thirty three samples were received from various hospitals in Delhi NCR for Mycobacterium spp. culture during the month of January 2009 to December 2012. From these samples 1386 strains were isolated. These isolates were tested by BACTEC 460 NAP TB Differentiation Test to differentiate MTB complex from MOTT strains. Speciation of MOTT isolates was done by PCR-HRM assay using universal PCR primers, targeting 16S rRNA gene hyper variable region and compared with conventional identification and PCR- gene sequencing methods.

Microbiological analysis: For decontamination of specimens from normal bacterial flora, specimens were treated by N-acetyl- L- cysteine-NaOH Method.(Scott CP et al., 2002) Smears were stained with Ziehl-Neelsen (ZN) for detection of acid-fast bacilli (AFB). For the BACTEC method (Becton Dickinson, Cockeysville, MD, USA), 0.5 ml of processed specimen was inoculated into the BACTEC 12B vial supplemented with PANTA (a mixture of five different antibiotics: polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin) and incubated at 37^0C. Readings were taken twice a week for the first two week and once a week thereafter for culture positivity for 8 week. After 8 week if the growth index (GI) was zero, the specimens were considered as negative.

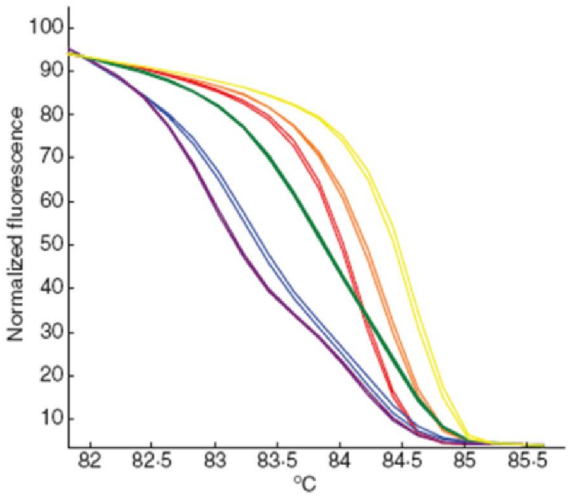
AFB smears were made from vials with a GI of 50-100, and further identification of MTBC was done by the BACTEC NAP (p-nitro-α-acetylamino-β-hydroxy propiophenone) differentiation test (Becton Dickinson,Sparks, MD, USA) (Siddiqi SH, 1996)

DNA Extraction: Total genomic DNA was extracted by following protocol of Life Tech, India Pvt Ltd. DNA Isolation kit and DNA was stored at -20^{0}C for further processing.

Amplification of 16S rRNA gene: The target was amplification by conventional PCR. A reaction mixture of 50 µl contains, 5µl of 10X PCR buffer (250 mM Tris Hcl, 500 mM KCl), 5µl of 2 mM dNTPs, 1 µl each of forward and reverse primers (25µM primers), 0.5 µl *of Taq polymerase* (5 units/µl) 4µL of 25mM MgCl and 8.5µl of nuclease free water. 25µl of DNA template was added in the master mix. Cyclic conditions used were, initial denaturation at 94^{0}C for 6m, followed by 35 repetitive cycles of denaturation at 94^{0}C for 1 minute, annealing at 58^{0}C for 1minute and primer extension at 72^{0}C for 1 minute. Final extension at 72^{0}C for 7 minutes with final storage of amplicons at 4^{0}C, which was later resolved on 2% agarose to check the size of amplified product.

Sequencing: PCR amplified product for 16S rRNA gene of mycobacterium was sent to Ocimum Biosolutions Pvt Ltd, Bangalore for sequencing.

PCR-HRM: High Resolution Melt (HRM) is a closed tube, post-PCR analysis method. PCR products can be discriminated according to sequence, length, GC content, and single base pair change (Herrman MG et al., 2006 and Wojdacz TK et al., 2007). For the present study Applied Biosystem 7500 Fast Real Time PCR machine with HRM Analysis software v2.0, and Melt Doctor HRM reagents were used (Zhou L et al., 2008). Pair of primers was designed to amplify 500 bp products. Reference strain (from *Mycobacterium tuberculosis* H37Rv) was used as positive control, and a sample without template DNA was used as a negative control, which is significant for measuring any false positive signal caused by contamination. In a sterile micro centrifuge tube, 10 µl of Melt doctor HRM Master Mix (from Applied Biosystem) ,1µl each of forward and reverse primers, 8 µl nuclease free water, and 5µl of the template DNA were added to make the total reaction volume 25µl. Reaction conditions for HRM, Holding Stage, the first step for the enzyme deactivation carried out was at 95^{0}C for 10 min followed by forty cycles, each of denaturation, extension and annealing at 95^{0}C for 15 sec, 60^{0}C for 1 min and 60^{0}C for 1 min respectively. Further for dissociation steps cycling conditions were, denaturation at 95^{0}C for 10 sec, annealing at 60^{0}C for 1 min, and high resolution melting at 95^{0}C for 15 sec. High resolution melting step should be done with 1 % ramp rate of thermal cycler. Results are interoperated by reviewing High Resolution melting data. HRM Analysis software v2.0. (Available in Applied Biosystem 7500 Fast Real-Time PCR System) (Wittwer CT et al., 2003) was employed a three-step analysis. The first step was to normalize the raw melting-curve data by setting the pre-melt (initial fluorescence) and post melt (final fluorescence) signals of all samples to uniform values. The second step was to shift the temperature along the x-axis of the normalized melting curves at the point where the entire double-stranded DNA is completely denatured. The final step was to further analyze the differences in melting-curve shape by subtracting the curves from a reference curve (also called the "base curve"), thus generating a difference plot curve, which helps to cluster samples into groups that have similar melting curves (Liew M et al., 2004). Each sample was visually verified by analyzing the generated PCR curves (Herrmann MG et al., 2006). Software analyzes the HRM curve data to spot changes in the shape of the curve that indicate different species. (Fig-1).



Normalized high resolution melt curve of 16S rRNA amplicons generated from mycobacterium spp; (——) M. *tuberculosis*, (——) M.*avium*, (——) M. *triples*, (——) M. *kansasii*, (——) M. *fortuitum*, (——) M. *intracellulare*.

Fig-1. Normalized HRM curves of 16S rRNA amplicons generated from Mycobacterium spp.

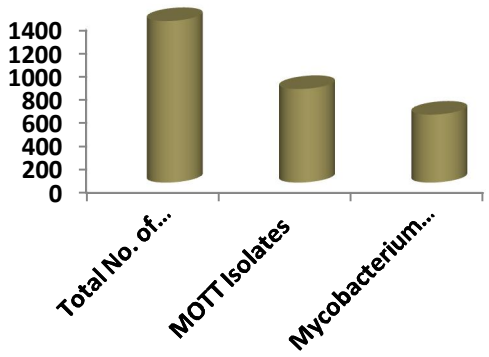


Fig-2. NAP differentiation result of Clinical Isolates

3.Results

Among the 1386 Mycobacterial isolates tested 803 (58%) were *M. tuberculosis* and 583 (42%) were MOTT. MOTT includes commensal organisms and pathogenic species. Currently available Mycobacterial species identification assays are time consuming, expensive and numerous MOTT strains well known to cause disease in humans are not included in these assays. Thus there is a need for a rapid, comprehensive and cost effective Mycobacterial species identification assay like the PCR-HRM Mycobacterial species identification assay, which can identify 27 clinically relevant Mycobacterial species.

4.Discussion

PCR–HRM curve analysis using the 16S rRNA gene produces a rapid, cost effective and reliable diagnostic technique to detect and differentiate at least 27 species of the Mycobacteriaceae family. In addition, the technique may be useful in detection of unknown Mycobacteriaceae species and identification of them to the closest species used as reference. Unlike other methods, it prevents carry-over contamination of PCR products as opening of the PCR tube is avoided or any post amplification procedures are not required in this method. To the best of our knowledge no such data has been published, involving development of high throughput genotype screening technology to distinguish mycobacterium other than tuberculosis species. Thus, in India, this technology can be utilized for the screening of government as well as private TB laboratories.

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