Molecular typing and differentiation of *Mycobacterium tuberculosis* clinical isolates using Double Repetitive Element PCR and Duplex PCR

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**Abstract**

**Background:** To date, the advancements in polymerase chain reaction (PCR) assures accurate, fast identification and mycobacterial speciation in clinical settings, which promotes a better tuberculosis (TB) treatment regimen.

**Methods:** In this study, a total of 78 clinically suspected cases of TB were processed for the detection of Mycobacterial infections by standard Ziehl Neelsen (ZN) staining, conventional Lowenstein–Jensen (LJ) and BACTEC MGIT-960™ liquid culture. Strain typing was performed by using Double Repetitive Element PCR (DRE-PCR) and Duplex PCR (DPCR) to differentiate *Mycobacterium tuberculosis* complex (MTB) from non-tuberculous mycobacteria (NTM), respectively.

**Results:** Of 78 clinical isolates, 25 (32%) were drug-susceptible, and 53 (68%) were resistant to at least one drug. The BACTEC MGIT-960™ showed the highest (88.5%) positivity rate, compared with conventional LJ (82%) and ZN smear (61.5%). The mean time detection and drug susceptibility for MTB was 28 and 40 days in LJ culture, and 10 and 13 days in BACTEC MGIT-960™ culture. Using DPCR, *Mycobacterium avium* infection was identified in HIV-positive (2.56%) and MTB in HIV-negative patients (97.4%), and the DRE-PCR system divulged 15 unique genotype patterns, and an institutional-based epidemiology database was created.

**Conclusions:** The combination of an in-house DRE–DPCR system could possibly identify and differentiate MTB from other mycobacterial species in a single reaction. In addition, restriction polymorphism analysis and DNA sequencing of NTM could assist in species identification directly from clinical isolates.

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Introduction

Tuberculosis (TB) remains a global pandemic and kills millions of people worldwide even though effective treatment is available [1]. Approximately 40% of the world’s TB cases are from India and China [4] (WHO 2012). The increased prevalence and mortality of TB made an urgent call to enhance rapid diagnosis, as delayed diagnosis of mycobacterial infections leads to late and improper anti-TB treatment; differentiation of mycobacteria is also essential to facilitate epidemiology [2,3]. Up to now, conventional procedures like X-ray filming, Ziehl Neelsen (ZN) sputum microscopy and solid culturing on Lowenstein–Jensen (LJ) for TB diagnosis is being followed in developing countries, all of which fail to differentiate mycobacterial species in clinical specimens. The problem associated with LJ culturing is that it takes months to carry out drug sensitivity testing (DST). Most importantly, the success rate depends on an experienced person’s investigation [4–7].

Advancements in molecular biology like various polymerase chain reaction (PCR) procedures and automated liquid culture systems allow for rapid identification and speciation of mycobacteria alongside the clinical specimen with significant sensitivity than conventional procedures [4,8,9]. Regardless of the false positivity (i.e., detection of dead bacilli in the specimen) of the PCR application in TB diagnosis, it allows for a timely and accurate identification of mycobacteria in a variety of clinical specimens, but it fails to meet the requirement of precise speciation of *Mycobacterium tuberculosis* complex (MTB) infection from non-tubercular mycobacteria [7,9,10] in certain circumstances. It is important to identify and differentiate the causative mycobacteria and non-tuberculous mycobacterium (NTM) infection, as NTM can cause problems in clinical settings, as its pathogenic potential varies in susceptibility tests towards anti-tuberculous drugs [11]. A simplified multiplex PCR assay was developed to differentiate MTB from NTM based on the specific nucleotide variation in the RNA polymerase β-subunit encoding gene (rpoB) [12].

Therefore, in this study, a combination of PCR procedures was adapted using specific gene targets viz DRE-PCR (amplification of DNA segments located between two repetitive elements IS6110 and the polymorphic GC-rich repetitive sequence [PGRS]) and Duplex PCR (DPCR) (amplify the rpoB DNAs of MTB and NTM) from clinical specimens. The DPCR products were further digested with *MspI* enzyme and subjected to DNA sequencing. On the whole, PCR results were evaluated with the results of ZN sputum smear microscopy, LJ culture and BACTEC MGIT-960™ culture systems.

Materials and methods

Study settings

A panel of 78 MTB clinical isolates were obtained from TB patients. Patients with clinical symptoms suspected for TB infection were enrolled in this study and hospitalized on the physician’s advice for TB diagnosis, and hospitalized treatment-failure TB patients’ samples were also analyzed. The clinical specimens were obtained for diagnosis as per the routine laboratory investigation.

Sample processing procedure

Fig. S1 illustrates the detailed sample testing procedure that was followed for the diagnosis to identify and to differentiate MTB complex from NTM species from the clinical specimen. For culture, clinical specimens were processed with NaOH–NALC and were inoculated onto LJ culture slants. All inoculated culture tubes were incubated at 37 °C and contamination was checked daily for the first week and was observed for colony formation once in every week for 8 weeks [7,13,14]. For BACTEC MGIT-960™ culture, cerebrospinal fluid (CSF), lymph node tissue extract, endometrial tissue extract and pleural fluid were directly inoculated into MGIT-960 tubes and remaining samples were carried out for DNA isolation. All samples were confirmed for acid fast bacilli (AFB) by ZN staining and further subjected for identification of MTB complex using p-nitro benzoic acid (PNB) assay [15].

Identification of mycobacteria

Originally, identification of mycobacteria at the species level was done based on colony morphology, pigmentation profile, and growth rate on conventional solid media. In addition, the biochemical test results were performed, such as niacin test, nitrate reductase test and heat-stable catalase test (pH 7.0, 68 °C). Identification procedures attempted only to differentiate MTB and NTM infection from clinical specimens to aid results with DRE-PCR and DPCR procedures.

In vitro phenotypic drug susceptibility testing

Conventional LJ [13] and BACTEC MGIT 960™ [14] were used to determine the drug susceptibility profile of the clinical isolates against first-line anti-tuberculosis drugs. The standard reference strain MTB H37Rv ATCC 27294 was used as the DST control.

Mycobacterial genomic DNA isolation

Phenol: Chloroform extraction method was used to extract genomic DNA directly from the clinical specimen, as well as from well-grown cultures of both drug resistant and drug susceptible as described previously [16]. DNA samples were stored at −20 °C for further PCR analysis.

PCR amplification

The complete list of primers used in this study is summarized in Table S1. Identification of mycobacterium at the species level was done by in-house PCR amplification of IS6110 element directly from clinical specimens and grown cultures with primers IS1 and IS2 as described previously [17]. Strains with no IS6110 element were further amplified with mtp40 gene primers PT3 and PT5 as described previously [17]. The DRE-PCR was performed as described previously [18]. Briefly, the DRE-PCR step was performed in a final volume of 50 µl. Each reaction tube contained 15 µl of 5× PCR Master mix.
Amplification of rpoB (Eppendorf) was used for all amplification reactions. To increase the intensity of bands and the discrimination power of DRE-PCR technique, the amount of template DNA (2–5 µl) and Taq polymerase (1–1.5 U) were increased in an extension step to 2 min instead of the usual 1 min extension.

Differential identification of MTB isolates

Amplification of rpoB gene-specific nucleotide variation by DPCR was performed to distinguish MTB from NTM isolates as described previously [12]. Essentially, the DPCR step was performed in a final volume of 50 µl. Each reaction tube contained 15 µl of 5x PCR Master mix solution (New England Biolabs, USA) that contained 10 mM Tris–HCl (pH 8.6), 25 units/ml Taq DNA polymerase, 50 mM KCl, 5% glycerol, 0.2 mM each of dNTPs, final buffer 1× 1.5 mM MgCl2, 0.08% IGEPAL® CA-630, 0.05% Tween® 20 and 10 pmol of each primers: Ris 1, Ris 2, Pntb 1, Pntb 2 per µl. To increase the intensity of bands and the discrimination power of DRE-PCR technique, the amount of template DNA (2–5 µl) and Taq polymerase (1–1.5 U) were increased in an extension step to 2 min instead of the usual 1 min extension.

Restriction digestion analysis of NTM strains

Restriction enzyme MspI was selected based on the rpoB gene sequence and directly applied to the PCR products of the suspected specimen. Ten micro liters of PCR amplified products of NTM isolate; 1–2 U of restriction enzyme (New England Biolabs); and restriction buffer 4™ were added to the 0.5 ml fresh micro centrifuge tube and finally nuclease free water was added to a final volume of 25 µl reaction. Restriction digestion was performed at 37 °C for 1–2 h in a controlled heat environment (Thermal cycler) and was electrophoresed in 2% agarose gel. The suspected NTM isolate’s digested DNA products were subjected to automated DNA sequencing with primers M5 and RM3 separately as previously described [12].

Agarose gel electrophoresis

DRE-PCR, DPCR amplified and restriction digested products were resolved on 2% agarose gel with 100-bp DNA ladder (HiMedia, India). DNA fingerprints were analyzed visually, and the position and pattern similarity were observed and used for the strain differentiation. Variations in band intensity were not taken to constitute strain differences.

Results

A total of 78 patients, 64 (82%) males and 14 (18%) females were included in this study. Of these, 76 (97.4%) were HIV-negative and 2 (2.6%) were HIV-positive, who were male patients aged 47 and 38 also included in the study. The common clinical manifestations in these patients were weight loss, fever, and severe cough. Of the 78 clinically suspected patients, 61 (78.2%) patients had pulmonary TB infection and 17 (21.8%) had extrapulmonary TB (ETB) (5 CSF, 3 lymph node tissue, 5 blood, 3 endometrial tissue and 1 pleural fluid).

The isolation and drug susceptibility profile of mycobacteria from clinical specimens were studied and summarized in Tables 1 and 2. The primary culture recovery mean time detection and drug susceptibility for MTB was 28 and 40 days in solid LJ culture and 10 and 13 days in BACTEC MGIT-960™ culture. The total positivity for BACTEC MGIT-960™ was 88.4%, LJ was 82% and ZN smear had 61.5% (Table 1). The low positivity rate obtained in ZN smear and LJ was due to low bacilli loads in the clinical specimen. Table 2 summarizes the drug susceptibility results obtained from conventional LJ and BACTEC MGIT-960™ methods.

Both phenotypic and genotypic procedures were followed for differential identification of MTB and NTM from clinical specimens in this study setting. Conventional biochemical assays viz. PBN test, niacin production, nitrate reductase and catalase-peroxidase tests, along with various PCR procedures viz. I56110, mtp40 were performed, and tests results were summarized in Table 3. An NTM strain Mycobacterium avium was identified from 2 (2.56%) HIV-positive patients by biochemical assays, and the results were confirmed with DPCR and DNA sequencing methods.

In total, 15 different DRE-PCR patterns were obtained and separated into 6 categories based on the number of bands, position and their similarity (Figs. 1 and 2a–c). Category I (10.3%) was comprised of 8 strains with 5 bands (Fig. 1c lanes 2 and 3). Category II (18%) was comprised of 14 strains with 4 bands (Fig. 1c lane 4). Category III (46.2%) was observed in 36 strains with 3 bands (Fig. 1b, lanes 1–6). Category IV (16.6%) contained 13 strains with 2 bands (Fig. 1a, lanes 6, 10, 11, 16 and 18). Category V contained 5 strains (6.4%) with 1 band (Fig. 1a lane 2), and Category VI (2.5%) contained 2 strains with no band (Fig. 1a, lanes 7, 17 and 19).

Bands were obtained with amplification of genome position in MTB, and their pattern was observed and analyzed in agarose gel (examples shown in Fig. 1 DRE-PCR assay). Twenty-five MDR and 14 RIF mono-resistant strains were observed with 4 bands, in which the majority of the strains demonstrated a similar banding pattern. Among them, 22 strains demonstrated the same banding pattern, 15 strains constituted another category and the remaining strains showed a difference in position of a single or more of amplicons (Fig. 1b and c). One strain showed 1 band after two times of PCR amplification; 20 strains (80%) among the 25 susceptible strains showed 5 bands completely different from the others (Fig. 1c). To evaluate the clinical applicability of the DRE-PCR, samples were subjected to two rounds of amplification reactions, and most of the strains demonstrated the same profile, and the position of the stronger bands were identical; therefore, they were designated as signature bands (Fig. 1a).

The diversification of MTB complex strains from NTM was confirmed further by DPCR assay. A clear band at 235-bp amplicon (Fig. 2) in agarose gel depicted the presence of MTB strains, and the band at 136-bp amplicon (Fig. 3) in agarose gel indicates the presence of M. avium strain, an NTM obtained from HIV-positive patients. After amplification
of the DPCR procedure, clinical specimens showed 136-bp DNA amplicon in agarose gel was further analyzed by restriction fragment length polymorphism (RFLP) and by direct sequencing for species identification (IIT, Chennai, India).

**Discussion**

In clinical settings, it is difficult to identify and differentiate the infection caused by MTB and NTM by conventional procedures because of the similar clinical manifestations. Thus, it delays making a definitive diagnosis, and negatively impairs TB treatment [3,19]. To date, several PCR-based procedures are available for rapid identification and differentiation of MTB, but high priority is recommended to identify mycobacterial infections at the species level [20]. In this preliminary study, the prevalence of strain type among this setting was studied and found that drug-resistant strains were predominant in this clinical setting [21]. Hence, this study is extended to identify and differentiate the speciation of mycobacterial infection with reference to the prevalence of drug-resistant strains among the hospitalized patients. In addition, 19 MDR-TB (24.3%) hospitalized treatment-failure patients were included in this study setting.

Among the 78 isolates studied, the majority of the strains (46.1%) were identified as Genotype C (Category III), which was observed with 3 bands, and shared a common banding pattern [18,21]. Category III consists of 7 strains from 4 drug-resistant isolates, 13 strains from MDR-TB, and 9 from INH-resistant isolates. The majority of the strains in Category III/drug-resistant demonstrated similar epidemiology patterns and similar banding patterns (Fig. 1b). Interestingly, clinical strains with drug susceptibility showed a maximum number of bands (5 bands) than other counterparts (Fig. 1c). A different DRE-PCR pattern was obtained from epidemiologically unrelated MTB strains from patients, whereas the same DRE-PCR pattern was observed with epidemiologically related MTB strains from patients.

In recent years, there has been an increase in the incidence of TB cases due to mycobacteria other than MTB complex (MOTT) reported in clinical settings. This is important in the clinical treatment view, especially in terms of treatment strategies and rapid differentiation of causative organisms [8,12,22]. In this study, identification and differentiation of MTB and NTM was performed by the PCR amplification of rpoB gene. Each primer in the DPCR primer sets was assessed for its specificity in a separate PCR reaction in the same condition and an amplicon of either 235-bp from MTB complex or 136-bp from NTM was observed. In total, 78 clinical strains were analyzed by DPCR and 2 were identified as (136-bp for NTM strains) M. avium (a non-mycobacteria), and all of the remaining strains were phylogenetically MTB complex and observed with 235-bp amplicon in agarose gel. Biochemical and PCR procedures (Table 3) were found to have satisfactory results to successfully identify and differentiate M. avium in this clinical setting. In addition, the reference MTB H37Rv ATCC 27294 and Mycobacterium smegmatis mc²155 ATCC 19420 strains were also included in this study to avoid inconsistency of the DPCR method. As expected, 235-bp product was obtained for MTB H37Rv ATCC 27294 and 136-bp for M. smegmatis mc²155 ATCC 19420. Therefore, the DPCR assay can be used for the successful differential identification of mycobacterial infections in clinical specimen.

**Table 1 – Rates of recovery of mycobacteria and relationship of concentrated specimen microscopy with mycobacterial culture on individual system/medium.**

<table>
<thead>
<tr>
<th>Culture system/Media</th>
<th>ZN smear microscopy</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Smear positive</td>
<td>Smear negative</td>
</tr>
<tr>
<td></td>
<td>Culture positive</td>
<td>Culture negative</td>
</tr>
<tr>
<td>MGIT-960™</td>
<td>48 (61.5%)</td>
<td>21 (26.9%)</td>
</tr>
<tr>
<td>L–J</td>
<td>45 (57.7%)</td>
<td>19 (24.3%)</td>
</tr>
</tbody>
</table>

|                                                                 |
| Smear positive Culture positive | Smear negative Culture negative | Smear positive Culture negative |
|                                 | 0                              | 2 (2.6%)                        |
|                                 | 9 (11.5%)                      | 12 (15.4%)                      |

**Table 2 – Drug resistance profile of M. tuberculosis clinical isolates.**

<table>
<thead>
<tr>
<th>Drugs</th>
<th>No. of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>H + E + R + S</td>
<td>9 (11.5%)</td>
</tr>
<tr>
<td>H + E + R</td>
<td>2 (2.6%)</td>
</tr>
<tr>
<td>H + R</td>
<td>16 (20.5%)</td>
</tr>
<tr>
<td>R + E</td>
<td>2 (2.6%)</td>
</tr>
<tr>
<td>R</td>
<td>14 (17.9%)</td>
</tr>
<tr>
<td>H</td>
<td>10 (12.8%)</td>
</tr>
<tr>
<td>Susceptible</td>
<td>25 (32.1%)</td>
</tr>
<tr>
<td>Total</td>
<td>78 (100%)</td>
</tr>
</tbody>
</table>

H: Isoniazid; S: Streptomycin; E: Ethambutol; R: Rifampicin.

**Table 3 – Detection of mycobacterial infections by conventional biochemical and molecular procedures in clinical specimen.**

<table>
<thead>
<tr>
<th>Organism/Species</th>
<th>PNB test</th>
<th>Niacin test</th>
<th>Nitrate reductase test</th>
<th>Heat-stable catalase test @ 68°C</th>
<th>PCR IS6110</th>
<th>mtp40</th>
<th>DPCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycobacterium tuberculosis</td>
<td>S</td>
<td>+ve</td>
<td>+ve</td>
<td>Inhibition</td>
<td>+ve</td>
<td>+ve</td>
<td>235-bp</td>
</tr>
<tr>
<td>Mycobacterium avium</td>
<td>R</td>
<td>−ve</td>
<td>−ve</td>
<td>No inhibition</td>
<td>−ve</td>
<td>−ve</td>
<td>136-bp</td>
</tr>
</tbody>
</table>

S: Susceptible; R: Resistant; PNB: p-nitro benzoic acid; PCR: Polymerase chain reaction; DPCR: Duplex polymerase chain reaction.
MTB complex and NTM in the clinical settings. In addition to the identification of NTM by DPCR, further confirmation was carried out by RFLP analysis and by direct sequencing for NTM species identification. Auspiciously, DPCR yielded only one product at 235-bp for MTB strains, and 136-bp product for \textit{M. avium} strains from HIV-positive patients; no \textit{Mycobacterium bovis} or \textit{Mycobacterium africanum} were identified in this study setting irrespective of the clinical isolates tested. Moreover, the (235-bp) \textit{rpoB} DNAs of MTB have confirmed that all the isolates were phylogenetically related, and similar results were also obtained by conventional biochemical testing (niacin production, PNB test and nitrate reductase reaction).

The lower sensitivity of the sputum smear microscopy not only makes it difficult to identify NTM, it also makes it difficult to implement the DOTS strategy in a clinical setting [7]. To date, the impact of PCR technology in TB-control programs is unavoidable. Therefore, it is necessary to analyze the mycobacterial species responsible for dissemination within the hospitalized patients. In this study setting, it was identified that \textit{M. avium} (2.56%) of NTM found in HIV-negative clinical isolates.

**Fig. 1** – DRE-PCR assay examples. (a) Lane 1: \textit{M. tuberculosis} H37Rv, lane 2–18: DRE-PCR amplified products banding pattern, lane 9: 100-bp molecular marker. Variations were observed in the positions of the bands based on different MWs of the amplified products. (b) An example of Category III clinical strains. For example, the commonly isolated category strain designated C yields three bands. (c) Category of all sensitive strains isolated from different patients generated indistinguishable patterns by the DRE-PCR method.

**Fig. 2** – DPCR assay (Representative examples of MTB isolates). Lane 1: Marker DNA (100-bp ladder), lane 2: Positive control of \textit{M. tuberculosis} H37Rv ATCC 27294, lanes 3–8: Amplicon of size 235-bp was amplified from \textit{M. tuberculosis} complex from HIV-negative clinical isolates.
patients were often indicated as non-responders to anti-TB drugs and labeled as MDR-TB cases. Therefore, it is recommended to exploit DPCR assay to identify and differentiate the causative mycobacterial species from a clinical specimen as an alternative to avoid over compliance with anti-TB drugs and to avoid its side effects.

A local institutional-based epidemiology database has been created to stratify the strains and prevalence of drug resistance among the patients studied. The combined usage of DRE and DPCR protocols introduced here resulted in the amplification of bands and hence helps clinicians to determine and discriminate causative mycobacterial species to avert further spread [23,24]. It is important to note that 68% of the isolates from patients displayed drug resistance at least one drug. This greater difference could be partially attributed to the smaller sample size of patients hospitalized at Puducherry, South India; however, it did not change the national average significantly.

In this study, the primers Tbc1, Tbc2 & M5, RM3 were selected from the rpoB gene of MTB and NTM to differentiate MTB from NTM. Pinsky and Banaei [9] developed a cost-effective, real-time PCR assay targeted to identify MTB complex members; however, in most of the laboratories the implementation of real-time PCR remains difficult due to its cost and standardization of protocol. Hence, the usage of conventional PCR is still unavoidable in resource-poor settings, but it should be instituted to meet the technological innovations to increase its efficiency.

Conclusions

The usage of DRE-PCR and DPCR PCR of rpoB gene methods possibly identify and differentiate the MTB complexes and the M. avium species directly from clinical specimens in a single reaction. In addition, DRE-PCR could be used as a simple and rapid screening method to stratify a large number of isolates into clusters and to create a database based on the banding pattern of MTB isolates in agarose gel electrophoresis. This can help the physician to identify a range of drug-resistant strains.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijmyco.2014.11.061.

REFERENCES


