Rv1458c: a new diagnostic marker for identification of *Mycobacterium tuberculosis* complex in a novel duplex PCR assay

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**Abstract**

**Purpose.** We explored the efficiency of Rv1458c, the gene encoding a putative ABC drug transporter specific for the *Mycobacterium tuberculosis* complex (MTBC), as a diagnostic marker.

**Methodology.** A 190 bp region of Rv1458c and a 300 bp region of *hsp65* were targeted in a novel duplex PCR assay and the results were compared with those for PCR restriction analysis (PRA) using the restriction enzymes NruI and BamHI. Species identification of a subset of the isolates (n=50) was confirmed by sequencing. Clinical isolates of *M. tuberculosis* (n=426) obtained from clinically suspected patients of pulmonary tuberculosis and mycobacterial (n=13) and non-mycobacterial (n=8) reference strains were included in the study.

**Results.** The duplex PCR assay correctly identified 320/426 isolates as MTBC and 106/426 isolates as non-tuberculous mycobacteria (NTM). The test was 100 % specific and sensitive when compared with NruI/BamHI PCR restriction analysis and highlighted the use of Rv1458c as a diagnostic marker for MTBC.

**Conclusion.** The duplex PCR assay could be developed for use as a screening test to identify MTBC in clinical specimens in peripheral laboratories with limited resources.

**INTRODUCTION**

Conventional diagnosis in patients suspected of having tuberculosis (TB) includes smear microscopy to provide an early indication of infectivity, followed by culture and drug susceptibility testing. Culture testing, although the gold standard, takes up to 6 weeks, sometimes longer for a definitive result [1]. TB control programmes are therefore adopting new diagnostics that are rapid and more sensitive than conventional smear microscopy. The need to improve laboratory diagnosis of tuberculosis has led to innovations in microscopy, such as the light-emitting diode microscope. Microbiological culture techniques have improved with the advent of rapid automated liquid culture systems like the BACTEC MGIT 960 (Becton Dickinson). Nucleic acid amplification assays have altered the landscape of TB diagnosis. While the Xpert MTB/RIF assay (Cepheid) continues to be the most important measurable shift in tuberculosis diagnostics, several others are in the pipeline [2]. However, the expense of these assays limits their use in routine diagnostic work-up in peripheral regions with frugal resources. Hence, despite aggressive programmes for control of TB, among the estimated 9 million people who develop TB, 3 million are not diagnosed, treated or notified to national TB control programmes [3]. The need of the hour is to develop new tools for diagnosis that are rapid and easy to use, but economical. However, rapid assays raise the possibility of false-positive and false-negative results [1]. Current guidelines, therefore, recommend that rapid molecular tests may be used to augment diagnosis along with culture testing, but should not replace culture [1], thus considerably reducing the window period for diagnosis. Culture results may then help the clinicians in confirming the diagnosis. However, even at this stage, a
clinical mycobacteriologist often faces the challenge of differentiating between cultures of Mycobacterium tuberculosis complex (MTBC) and NTM. Moreover, co-infection with different members of MTBC and with other mycobacterial species is not rare in animal and human hosts [4, 5]. This adds to the problems faced by mycobacteriologists and increases the need to differentiate between cultures of MTBC and NTM.

Routinely, identification of MTBC and NTM is achieved by assessing their growth characteristics in culture and biochemical tests, and this is labour intensive, time consuming and often inconclusive [6, 7].

Several techniques have been developed for rapid identification. Direct gene sequencing is highly specific in discriminating between mycobacteria but is usually only used in reference laboratories [8–10]. The high costs of commercially available assays such as Accuprobe (Gen-Probe) and Innolipa Mycobacteria V2 (Innogenetics) have restricted their use in most clinical laboratories, especially in high-burden countries endemic for tuberculosis [10]. Rapid identification of MTBC by detecting the MPT64 antigen in cultures has shown excellent performance with a rapid turnaround time. It is also simple to perform and relatively inexpensive [11, 12]. Although a positive MPT64 assay indicates the presence of MTBC, a negative assay could indicate the presence of any other organism, including NTM.

In our attempt to develop a simple and cost-effective assay to rapidly identify MTBC and NTM and also differentiate between them, we previously reported a simple PCR restriction analysis (PRA) using the restriction enzymes NruI and BamHI [13]. In the present study, we explored a duplex PCR assay using the hsp65 gene and Rv1458c, a putative ABC drug transporter specific for the MTBC, and compared the results for the assay with those for the NruI/BamHI PRA.

**METHODS**

**Clinical isolates**

A total of 449 clinical isolates of Mycobacterium spp. were randomly selected from clinically suspected patients of pulmonary tuberculosis who were admitted to the Rajan Babu Institute of Pulmonary Medicine and Tuberculosis (RBIPMT) and Vallabhbhai Patel Chest Institute (VPCl) during the period 2013 to 2015. RBIPMT serves as a referral centre for patients of tuberculosis in north India, while VPCl serves as a referral hospital in north India for chest diseases. The patients were adults ≥18 years old and were not co-infected with human immunodeficiency virus. The study was approved by the institutional ethical committee, and written and informed consent was obtained from the patients. The clinical isolates obtained were identified by their culture characteristics on Lowenstein–Jensen medium and were also subjected to biochemical identification using niacin, nitrate reduction and semi-quantitative catalase tests by the standard procedure [6].

**Reference strains**

In addition to the clinical isolates of Mycobacterium spp., 21 reference strains were also included in the study: Mycobacterium tuberculosis (H37Rv), M. bovis (ATCC 19210), M. avium (MTCC 1723; IMTECH), M. intracellulare (ATCC 13950), M. fortuitum (MTCC 1023), M. kansasi (MTCC 3058), M. phlei (MTCC 1724), M. smegmatis (ATCC 994), M. terrae (ATCC 15755), M. vaccae (ATCC 997), M. malmoense (ATCC 29571), M. xenopi (ATCC 19250), M. simiae (MTCC 1727), Nocardia brasiliensis (ATCC 19296), Nocardia asteroides (MTCC 274), Escherichia coli (ATCC 35218), Klebsiella pneumoniae (MTCC 3040), Pseudomonas aeruginosa (ATCC 33348), Enterococcus faecalis (MTCC 439), Staphylococcus aureus (ATCC 25923) and Streptococcus pneumoniae (MTCC 2672).

**DNA extraction from cultures**

Chromosomal DNA was extracted from the clinical mycobacterial isolates, H37Rv, M. bovis, the reference strains of NTM and bacteria other than Mycobacterium spp. by the boiling method as described previously [13]. Briefly, a loopful of mycobacterial growth was transferred to a microcentrifuge tube containing 100 µl Triton X-100 (1 %) and 50 µl sterile double distilled water. The suspension was vortexed and boiled at 100°C for 30 min. The suspension was centrifuged in a F2402H Allegra X-22 R rotor (Beckman Coulter) at 8000 r.p.m. for 10 min and the clear supernatant containing mycobacterial DNA was used for PCR.

**Duplex PCR assay**

**PCR**

Amplification of the clinical culture isolates of Mycobacterium spp. and reference strains was performed with the primer set HSP N3 and HSP N4, amplifying a 300 bp region of the hsp65 gene [13]. A second primer set was used to amplify a 190 bp region of the gene Rv1458c using the primer pair ABC T1 (5'–GCAGCATTGAGTACCTGAC-3') and ABC T2 (5'–TCGGTGAACCCAGGTGTC-3'). Rv1458c is a gene encoding a putative ATP binding ABC transporter protein (tuberculist.epfl.ch) [14]. Nucleotide BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed 100% identity (e-value, 5e−91) with most, although not all, members of the MTBC, and no identity with other bacteria, including NTM, Nocardia spp. and Corynebacterium spp. [15]. The members of the MTBC with 100% identity were M. tuberculosis, M. bovis, M. bovis BCG, M. africanum, M. microti and ‘M. canetti’. The 190 bp region of Rv1458c targeted in the study was also scanned for single nucleotide polymorphisms (SNPs) from the genome sequences of 469 clinical isolates available in the public domain [16]. There are 18 SNPs reported within Rv1458c; however, no SNP was detected in the putative conserved region of 190 bp.

A total of 2 µl of the extracted DNA was used as a template for PCR. The 20 µl reaction mixture consisted of 0.4 pM of each primer of hsp65 gene, 0.3 pM of each primer of gene Rv1458c, 300 µM of each deoxynucleoside triphosphate, 3.75 mM MgCl2 and 0.5 U Taq polymerase (Biotools; B and M Labs).
The thermal profile consisted of an initial denaturation for 10 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and primer extension at 72 °C for 1 min. Final extension was performed at 72 °C for 10 min. The PCR product was analysed by electrophoresis on a 1.5 % agarose gel. A 100 bp DNA ladder (Fermentas Life Sciences) was used as a molecular size marker.

Positive and negative controls used in the PCR assay
Each PCR run contained a positive control (H37Rv DNA) and a negative control (double distilled water).

Analytical sensitivity of the assay
The genomic DNA was extracted from the culture of \emph{M. tuberculosis} H37Rv strain, and its concentration was calculated by measuring absorbance at 260 nm using an Infinite F200 Pro spectrophotometer (Tecan). The number of molecules was calculated by multiplying the concentration of DNA by Avogadro’s number \((6.023 \times 10^{23})\) and dividing the whole factor by the molecular weight of the genome of \emph{M. tuberculosis} H37Rv, viz. \(4.4111 \times 10^{9} \text{ng}\) (\(650 \times 10^9\) being the molecular weight of each base pair). Serial dilutions of the genomic DNA of \emph{M. tuberculosis} H37Rv, i.e. \(10^0, 10^1, 10^2, 10^3, 10^4\) and \(10^6\) molecules \(\mu\text{l}^{-1}\), were tested to analyse the analytical sensitivity of the duplex PCR.

Analytical specificity of the assay
The specificity of the primers used for the amplification of \emph{Rv1458c} had been tested bioinformatically, as mentioned earlier. The specificity of the primers was confirmed by amplification with the extracted DNA from 21 reference strains, including 13 mycobacterial species and 8 non-mycobacterial species.

PCR restriction analysis
To test the efficacy of the assay and confirm the test developed by us, all the clinical mycobacterial isolates were also tested by PRA using the enzymes NruI and BamHI, as previously described [13].

Sequencing
PCR products obtained by amplification of the \emph{hsp65} gene with primers HSP N3 and HSP N4 of a subset of clinical isolates \((n=50)\) were subjected to sequencing by an ABI automated sequencer (Ocimum Biosolutions). Sequences were identified by similarity using \emph{BLAST}n available at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Species identification was confirmed if a 97 % match was achieved with any sequence deposited in the database, according to the criteria proposed by McNabb \emph{et al}. [17].

Quality control
Quality laboratory practices were strictly followed at every step. Sterile distilled water was used for reagent preparation. Positive and negative controls were included with every new batch of media and reagents.

Statistical analysis
The performance parameters such as sensitivity and specificity were calculated using the resources at http://ktclearinghouse.ca/cebm/practise/ca/calculators/statscalc (link provided at www.teachepi.org/resources/epict.htm) [18, 19].

RESULTS
Identification of isolates by conventional methods
All 449 isolates were subjected to Ziehl–Neelsen staining and biochemical identification. Of these, 23 isolates were not acid fast and were excluded from the final analysis. Of the remaining 426 isolates, 305 were identified as MTBC and 95 were identified as NTM. The remaining isolates \((n=26)\) could not be identified definitively on the basis of biochemical reactions.

![Fig. 1. Duplex PCR of standard strains. Lane M, 100 bp DNA ladder; lanes 1–21, duplex PCR amplicons of \emph{M. tuberculosis} H37Rv, \emph{M. bovis}, \emph{M. avium}, \emph{M. intracellulare}, \emph{M. fortuitum}, \emph{M. kansasii}, \emph{M. phlei}, \emph{M. smegmatis}, \emph{M. terrae}, \emph{M. vaccae}, \emph{M. malmoense}, \emph{M. simiae}, \emph{N. brasiliensis}, \emph{N. asteroides}, \emph{Escherichia coli}, \emph{K. pneumoniae}, \emph{P. aeruginosa}, \emph{Enterococcus faecalis}, \emph{Staphylococcus aureus} and \emph{Streptococcus pneumoniae}, respectively; lane 22, negative control.](https://www.microbiologyresearch.org)
Identification of mycobacteria by duplex PCR assay

The DNA obtained from 426 cultures of Mycobacterium spp. and 13 mycobacterial reference strains, including H37Rv, were used to amplify a 300 bp region of the hsp65 gene using the primers HSP N3 and HSP N4, and a 190 bp region of Rv1458c using the primers ABC T1 and ABC T2. All of the mycobacterial isolates were amplified using the primers HSP N3 and HSP N4 (Fig. 1). However, the NTM were not amplified with primers ABC T1 and ABC T2. PCR was also carried out with eight non-mycobacterial reference strains (viz. N. brasilensis, N. asteroides, E. coli, K. pneumoniae, P. aeruginosa, E. faecalis, Staphylococcus aureus and Streptococcus pneumoniae). However, none of the non-mycobacterial species were amplified by either primer set (Fig. 1).

We labelled the amplicon obtained with the primers for the hsp65 gene as ‘H’ if amplification occurred and ‘h’ if there was no amplification. If the primers for Rv1458c amplified a 190 bp PCR product, the pattern was labelled as ‘αα’. If no amplification was obtained, the pattern was labelled ‘ββ’ (Table 1). Of all the clinical culture isolates, including the 26 isolates that had not been definitively identified biochemically, 320 were identified as MTBC by duplex PCR and 106 were identified as NTM (Table 2).

Analytical sensitivity of the duplex PCR assay

To analyse the lower limit of detection of the duplex PCR assay, serial dilutions of the DNA sample obtained from H37Rv were tested. The duplex PCR was positive in samples containing ≥10^6 (DNA molecules) µl⁻¹ extracted from the equivalent of ≥10^5 bacilli µl⁻¹ (Fig. 2).

Identification of mycobacteria by Nrul/BamHI PRA and sequencing

To test the efficacy of the duplex PCR assay, all the clinical isolates were subjected to Nrul/BamHI PRA. The method involved amplification of a 300 bp region of hsp65 gene followed by restriction digestion with the restriction enzymes Nrul and BamHI. All of the 320 isolates identified as MTBC by the duplex assay were confirmed by PRA (Table 2). The 106 NTM were also confirmed by PRA (Table 2).

Table 1. Interpretation of duplex PCR assay

<table>
<thead>
<tr>
<th>Band pattern</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>MTBC</td>
</tr>
<tr>
<td>h</td>
<td>Non-mycobacterial species</td>
</tr>
</tbody>
</table>

H, 300 bp band on amplification with primers HSP N3 and HSP N4; h, no amplification with primers HSP N3 and HSP N4; αα, 190 bp band on amplification with primers ABC T1 and ABC T2; ββ, no amplification with primers ABC T1 and ABC T2.

Table 2. Comparison of the performance of biochemical reactions, NruI/BamHI PRA, duplex PCR and/or sequencing in the identification of MTBC and NTM

<table>
<thead>
<tr>
<th>Biochemical identification</th>
<th>PRA</th>
<th>Duplex PCR assay</th>
<th>Sequencing</th>
<th>No. of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTBC</td>
<td>MTBC</td>
<td>MTBC</td>
<td>MTBC</td>
<td>7</td>
</tr>
<tr>
<td>NTM</td>
<td>NTM</td>
<td>NTM</td>
<td>NTM</td>
<td>23</td>
</tr>
<tr>
<td>Not identified</td>
<td>MTBC</td>
<td>MTBC</td>
<td>MTBC</td>
<td>12</td>
</tr>
<tr>
<td>Not identified</td>
<td>NTM</td>
<td>NTM</td>
<td>NTM</td>
<td>8</td>
</tr>
<tr>
<td>MTBC</td>
<td>MTBC</td>
<td>MTBC</td>
<td>ND</td>
<td>298</td>
</tr>
<tr>
<td>NTM</td>
<td>NTM</td>
<td>NTM</td>
<td>ND</td>
<td>72</td>
</tr>
<tr>
<td>Not identified</td>
<td>MTBC</td>
<td>MTBC</td>
<td>ND</td>
<td>3</td>
</tr>
<tr>
<td>Not identified</td>
<td>NTM</td>
<td>NTM</td>
<td>ND</td>
<td>3</td>
</tr>
</tbody>
</table>

ND, Not done.

In addition, the species of a subset of the mycobacterial isolates (n=50) was further confirmed by sequencing. Of the 50 isolates sequenced, 19 had been identified as MTBC by both duplex PCR assay and Nrul/BamHI PRA (Table 3). Of these, 12/19 isolates had not been identified definitively by biochemical methods.

The remaining isolates sequenced (n=31) had been identified as NTM by duplex PCR assay and Nrul/BamHI PRA. These included 23 clinically relevant isolates obtained from repeat respiratory specimens or sterile sites and 8 isolates that had not been identified biochemically [20]. The most common NTM isolated was Mycobacterium avium–intracellulare (17/31; 54.83 %), followed by M. kansasii (7/31; 22.58 %) and Mycobacterium abscessus (4/31; 12.90 %) (Table 3). Of the NTM with potential clinical relevance (n=23), M. avium–intracellulare (11/23; 47.82 %) was the most common, followed by M. kansasii (5/23; 21.73 %) and M. abscessus (4/23; 17.39 %) (Table 3). Single isolates...
Table 3. Comparison between duplex PCR and NruI/BamHI PRA in the sequenced clinical isolates (n=50) of Mycobacterium spp.

<table>
<thead>
<tr>
<th>Species identification of clinical isolates by sequencing (n=50)</th>
<th>Duplex PCR hsp65 Rv1458c</th>
<th>Nru/ BamHI PRA</th>
<th>No. of isolates</th>
<th>No. of clinically relevant isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. tuberculosis</td>
<td>H</td>
<td>H</td>
<td>MTBC</td>
<td>19</td>
</tr>
<tr>
<td>M. avium–intracellulare</td>
<td>H</td>
<td>H</td>
<td>NTM</td>
<td>17</td>
</tr>
<tr>
<td>M. kansasii</td>
<td>H</td>
<td>H</td>
<td>NTM</td>
<td>7</td>
</tr>
<tr>
<td>M. abscessus</td>
<td>H</td>
<td>H</td>
<td>NTM</td>
<td>4</td>
</tr>
<tr>
<td>M. fortuitum</td>
<td>H</td>
<td>H</td>
<td>NTM</td>
<td>1</td>
</tr>
<tr>
<td>M. flavescentis</td>
<td>H</td>
<td>H</td>
<td>NTM</td>
<td>1</td>
</tr>
<tr>
<td>M. mucogenicum</td>
<td>H</td>
<td>H</td>
<td>NTM</td>
<td>1</td>
</tr>
</tbody>
</table>

H, 300 bp band on amplification with primers HSP N3 and HSP N4; \( \infty \), 190 bp band on amplification with primers ABC T1 and ABC T2; \( \beta \), no amplification with primers ABC T1 and ABC T2.

(1/23; 4.34\%) of M. fortuitum, Mycobacterium flavescentis and Mycobacterium mucogenicum were obtained from sterile sites.

Performance indicators of the duplex PCR assay

Of the 426 culture isolates tested by the duplex PCR assay and NruI/BamHI PRA, 320 were identified as MTBC and 106 as NTM. Comparing the results of duplex PCR assay with PRA, the sensitivity of the duplex PCR assay was 100\% (95 \% Confidence Interval (CI), 98.85–100\%). The specificity of the assay was also 100\% (95 \% CI, 96.58–100 \%).

DISCUSSION

Laboratory personnel often presumptively identify cultures of M. tuberculosis on the basis of acid-fast staining and colony characteristics. However, colony morphology may show variations, hence, the need for further identification. Conventional identification of mycobacterial species includes biochemical assays that are cumbersome and time consuming. Inter-assay variations and the inherent limitations of phenotypic assays also hinder the definitive identification of mycobacterial cultures [7].

Moreover, M. tuberculosis is a slow-growing organism and prone to contamination by non-fastidious organisms, which may lead to errors in diagnosis [21]. Molecular assays provide correct identification at this time. However, the high cost of commercial molecular assays precludes their use in several regions endemic for tuberculosis. Hence, several investigators are attempting to use newer technologies for identification of Mycobacterium spp. A number of in-house PCR assays have been used worldwide [22–26]. IS6110 has been the gene targeted in most studies. However, M. tuberculosis strains lacking the IS6110 element have been described previously [27, 28], leading to a search for new targets. devR [29], rpoB [24] and hsp65 [30] are the other gene targets that have been used by various investigators. Of these, rpoB is being widely used to target M. tuberculosis directly in clinical samples in the Xpert MTB/RIF assay. The hsp65 gene has also been targeted directly in clinical specimens by several investigators [31–33].

To our knowledge, this is the first study where Rv1458c has been used as a diagnostic marker for MTBC. The duplex PCR assay with Rv1458c and hsp65 genes successfully identified all 320 MTBC and 106 NTM correctly. Of the 106 NTM, 23 isolates were considered to be clinically relevant, as they were obtained from repeat respiratory specimens or sterile sites. M. avium–intracellulare (47.82\%) was the most common clinically relevant NTM found in the respiratory samples studied (Table 3).

The duplex PCR assay was not designed for species identification of mycobacteria. Rather, it was devised to be used as a screening assay to differentiate between MTBC and NTM in culture specimens. The duplex PCR assay screened out 106 NTM from the 426 mycobacterial cultures included in the study. Species identification was performed by sequencing for all the clinically relevant NTM and only a subset of the contaminating NTM, some of which could not be identified biochemically, thus saving time, effort and funds. A few previous studies also used screening assays to identify NTM and then recommended an RFLP technique or sequencing for species identification [13, 24].

The duplex PCR assay was highly specific as it correctly identified 320 MTBC and 106 NTM when compared with RFLP using NruI/BamHI. In fact, of the subset of isolates that were sequenced (19 MTBC and 31 NTM), all had been correctly identified by the duplex PCR assay. A high degree of specificity was needed as our future aim is to use the assay to differentiate between MTBC and NTM even in mixed cultures. Furthermore, the assay was highly sensitive when performed on cultures, as it could detect 10^5 (DNA molecules) \( \mu l^{-1} \).

In conclusion, the present study targets hsp65 and Rv1458c in a novel duplex PCR assay, which can be developed further to be used directly on clinical specimens as a screening test. It is a simple, cost-effective and rapid method that can be used by laboratory personnel with minimal training. RFLP or sequencing may also be used to supplement species identification.

Funding information

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

The study was approved by the institutional ethical committee, and written and informed consent was obtained from the patients.

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