Rapid identification of multidrug-resistant Mycobacterium tuberculosis isolates by rpoB gene scanning using high-resolution melting curve PCR analysis

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Background: Multidrug-resistant (MDR) Mycobacterium tuberculosis poses a serious threat to the control of tuberculosis (TB) and constitutes an increasing public health problem. The availability of rapid in vitro susceptibility tests is a prerequisite for optimal patient treatment. Rifampicin resistance caused by diverse mutations in the rpoB gene is an established and widely used surrogate marker for MDR-TB. We used a high-resolution melting (HRM) curve analysis approach to scan for mutations in the rpoB gene.

Methods: A total of 49 MDR-TB and 19 fully susceptible non-MDR-TB isolates, as determined by conventional drug susceptibility testing using the BACTEC-MGIT960 system, were used to evaluate the suitability of HRM curve analysis as a rapid and accurate screening system for rifampicin resistance.

Results: HRM analysis of the rpoB cluster I site allowed the correct allocation of 44 of the 49 MDR-TB isolates and all non-MDR-TB isolates. Three of the five MDR-TB isolates (60%) falsely identified as non-MDR-TB harboured the V176F mutation that could be specifically detected by an additional HRM assay. The combined HRM analysis of all strains and isolates exhibited 95.9% sensitivity and 100% specificity.

Conclusions: With a positive predictive value of 100% and a negative predictive value of at least 99.9%, this combined HRM curve analysis is an ideal screening method for the TB laboratory, with minimal requirements of cost and time. The method is a closed-tube assay that can be performed in an interchangeable 96- or 384-well microplate format enabling a rapid, reliable, simple and cost-effective handling of even large sample numbers.

Keywords: HRM, rifampicin resistance, MDR-TB, SNP analysis, mutation detection

Introduction

The increasing proportion of multidrug-resistant tuberculosis (MDR-TB) (by definition Mycobacterium tuberculosis resistant to at least the two major anti-TB drugs, isoniazid and rifampicin) has become one of the major problems throughout the world. In Austria, MDR-TB accounted for 1.6% of all cases of TB documented at the National Tuberculosis Reference Laboratory between 2001 and 2007, with a minimum in 2002 (0.3%) and a maximum in 2004 (3.0%). Treatment of MDR-TB requires prolonged and expensive chemotherapy using second-line drugs of increased toxicity.

A key element in the control of drug-resistant TB is the early institution of an effective therapy coupled with surveillance and monitoring activities that enable timely intervention to reduce the transmission and spread of the disease. While conventional drug susceptibility testing for TB is a process that can take several weeks, the use of molecular techniques based on PCR amplification of genes involved in resistance mechanisms, followed by the detection of key mutations associated with resistance, provides faster results.

It is estimated that >90% of the rifampicin-resistant TB isolates are also resistant to isoniazid, making rifampicin resistance a good surrogate marker for MDR-TB. More than 95% of the rifampicin-resistant M. tuberculosis isolates have mutations...
within cluster I of the \( \text{rpoB} \) gene encoding the \( \beta \)-subunit of the RNA polymerase.\(^\text{10}\) Therefore, this region is an ideal target for molecular rifampicin resistance testing. Many commercially available assays identify only the most important mutations within this core region, making these kits unreliable in geographic areas where less common or novel mutations are more frequent. High-resolution melting (HRM) curve analysis surpasses specific probes-dependent classic genotyping methods since it identifies the whole amplification product, thus allowing the detection of unknown sequence alterations in addition to known mutations. HRM curve analysis for accurate identification of mutations in PCR products has been recently described.\(^\text{11–14}\)

The use of new instruments combined with DNA intercalating dyes that can be used at saturating concentrations permits discrimination of virtually all types of sequence changes in PCR amplicons without manual handling of PCR products. Several applications of HRM curve analysis including DNA methylation studies, mutation scanning and single-nucleotide polymorphism (SNP) genotyping have been described.\(^\text{13,15–18}\) Single-nucleotide changes represent the smallest genetic change and are divided into four classes distinguished by a different melting temperature shift (\( T_m \)).\(^\text{19,20}\) SNP class 1 involves C/T and G/A, and SNP class 2 involves C/A and G/T base exchanges that can easily be genotyped by HRM due to \( T_m \) differences of \( \geq 0.5 \) °C.\(^\text{20}\) In contrast, bases switch only the strand in the SNP class 3 C/G base exchange and the SNP class 4 A/T base exchange, producing very small \( T_m \) differences of \( <0.4 \) °C for SNP class 3 and \( <0.2 \) °C for SNP class 4. The separation of melting curves can be improved by adding a known genotype to the unknown samples.\(^\text{20}\) In general, HRM curve analysis is a simple and rapid scanning method for known and unknown mutations and can dramatically reduce the turnaround time for mutation screening and testing. The aim of the study was to develop and evaluate HRM curve analysis as a suitable PCR-based screening method for the rapid identification of multidrug resistance in \( M. \) tuberculosis isolates by \( \text{rpoB} \) gene scanning.

Materials and methods

Microorganisms

DNA from a total of 68 \( M. \) tuberculosis isolates (19 fully susceptible isolates including the H37Rv ATCC 27294 strain; 49 MDR-TB isolates including one XDR-TB isolate) was provided by the Austrian National Reference Laboratory for Tuberculosis. The 67 isolates including the H37Rv ATCC 27294 strain; 49 MDR-TB isolates accounted for 25 different spoligotypes; 21 isolates yielded the Beijing type. An alkaline lysis procedure was used to extract DNA from samples in accordance with the instructions for the COBAS AMPLICOR assay (Respiratory Specimen Preparation Kit, Roche Diagnostics). Conventional drug susceptibility was tested both phenotypically and genotypically as recommended by the manufacturers. In brief, isolates were grown in MGIT medium at \( 8 \) °C in the BACTEC MGIT 960 instrument (Becton, Dickinson and Company, Sparks, MD, USA). Susceptibility testing was performed after the instrument flagged a positive signal. On day 1 or 2 following positivity, cell suspensions were used undiluted. Tubes positive for \( >2 \) days were subcultured in a new MGIT medium. A 100 \( \mu \)L aliquot from a positive MGIT 960 broth was pipetted into 10 \( \text{mL} \) of sterile saline to prepare a 1:100 dilution of the growth suspension; 500 \( \mu \)L of this dilution was used as inoculum for the growth control tube. BACTEC MGIT 960 drug susceptibility testing supplement (0.8 \( \text{mL} \) oleic/albumin/dextrose/catalase), 100 \( \mu \)L of the drug stock solution and 0.5 \( \text{mL} \) of the positive MGIT 960 broth were added to MGIT medium. Drug susceptibility was continuously monitored for 10 days or until resistance was indicated.

GenoType MTBDRplus kits (Hain Lifescience, Nehren, Germany) were used for genotypic susceptibility testing. In brief, for amplification, 35 \( \mu \)L of a primer-nucleotide mixture, amplification buffer containing 2.5 mM MgCl\(_2\), 1.25 U of hot-start Taq polymerase (QIAGEN, Hilden, Germany) and 5 \( \mu \)L of a preparation of chromosomal DNA in a final volume of 50 \( \mu \)L was used. The amplification protocol consisted of: a denaturation step at 95 °C for 15 min, followed by 10 cycles of 95 °C for 30 s and 58 °C for 120 s, then an additional 20 cycles of 95 °C for 25 s, 53 °C for 40 s and 70 °C for 40 s and 30 s and 58 °C for 120 s. The hybridization protocol was performed at 45 °C for 30 min and was followed by washing steps and colorimetric detection of the hybridized amplicons. After a final wash, the strips were air dried and fixed on paper.

HRM curve PCR analysis and DNA sequencing

A 193 bp fragment containing cluster I of the \( \text{rpoB} \) gene was amplified for subsequent HRM analysis using the forward primer \( \text{rpo105} \) (5’-CGTGGAGGCCATCACCCGAGACGT-3’) and the reverse primer \( \text{rpo273} \) (5’-GACCTCCAGCGCGACGCTCAGC-3’).\(^\text{21}\) A 125 bp fragment containing the V176F mutation was amplified using the forward primer \( \text{Cd176HRM-F} \) (5’-GTCATGGGTTACCTCCGATGACCGAGAGA-3’) and the reverse primer \( \text{Cd176HRM-R} \) (5’-ACTTTGCTAATGTCGTCGAACTGACCCC-3’). A homology search in GenBank revealed 100% specificity of the used primers for \( M. \) tuberculosis complex strains. PCR and HRM were performed in a single run on a LightCycler LC480 instrument (Roche Diagnostics, Penzberg, Germany) in a reaction mixture containing 10 ng of genomic DNA, 0.25 pmol of each primer and 3 mM MgCl\(_2\) in the LightCycler\(^\text{\textregistered}\) 480 High-Resolution Melting Master mixture containing ResoLight dye (Roche Diagnostics) with PCR grade water adjusted to a final volume of 20 \( \mu \)L. Genomic \( H37Rv \) DNA (1 ng) was added to the reaction mixture for the amplification and HRM analysis of the cluster I-containing PCR product. Reaction conditions included an activation step at 95 °C for 10 min followed by 45 cycles of 95 °C for 10 s, 60 °C for 10 s and 72 °C for 10 s. Prior to HRM, the products were heated to 95 °C for 1 min and then cooled to 40 °C for 1 min. HRM was performed from 60 to 95 °C, rising at 1 °C/s with 25 acquisitions per degree. All reactions were performed in quadruplicate using an epMotion work station (Eppendorf, Hamburg, Germany) for automatic sample preparation in 384-well microtitre plates (LC plate; Roche Diagnostics, Vienna, Austria).

LightCycler 480 Software version 1.5 was used for HRM curve analysis. The melting curves were normalized and temperature shifted to allow samples to be directly compared. Difference plots were generated by selecting a negative control, converting the melting profile to a horizontal line and normalizing the melting profiles of the other samples against this sample. Significant differences in fluorescence from the horizontal baseline were indicative of mutations. Differences were judged as significant if the replicates fell outside the range of variation seen in the wild-type samples.

The \( \text{rpoB} \) gene of all samples was subsequently sequenced from position 1 to 3041 of the coding region for the confirmation and determination of sequences different from the \( H37Rv \) sequence. Overlapping sequences were obtained for subsequent assembly using the following primer combinations: \( \text{rpoB-F} \) (5’-TACGGTCG
GCGAGCTGATCC) and rpoB-R (5’-TCGTTTTCGACGATGTCGACGACG); rpoB3041-R (5’-TCTCGGTCATCATCGGGAAGTC); rpoBr-Fmyk (5’-GTTCATCGAAACGCCGTA) and rpoB3041-R (5’-TCTCGGTCATCATCGGGAAGTC); rpoB1038-F (5’-TCTAAGGGCTCTCGTTGGTC) and rpoB1551-R (5’-ACGTCTGCGGTGTGATCGCCTC) and rpoB105-R (5’-TCGTTTCGACGATGTCAAGGCAC) and rpoB2015-R (5’-TGCATCACAGTGATGTAGTCG). All primers had a respective M13 sequence attached to the 5’-end of the gene-specific priming sequence. Sequence analysis was performed using a SequiTerm Excel II Cycle Sequencing Kit (Epicentre, Madison, WI, USA) with fluorescent-labelled primers M13 universal (5’-TGTAAAACGACGGCCAGT) and M13 reverse (5’-CAGGAAACAGCTATGACC) (MWG-Biotech, Ebersberg, Germany) in a Licor 4300 automated DNA sequencer (LI-COR Bioscience, Lincoln, NE, USA), according to the manufacturer’s instructions. All obtained sequences were assembled and edited and compared with the GenBank database rpoB sequence of the strain H37Rv to determine sequence variations.

Results

Gene scanning of a 193 bp amplification product of cluster I of the rpoB gene was performed on 49 MDR-TB and 19 non-MDR-TB isolates (including strain H37Rv). All samples were spiked with a 10th of genomic DNA of the H37Rv control strain to improve and facilitate the discrimination of SNP classes 3 and 4 mutations. After normalization, shifting and difference plotting of the melting curves, different sets of differential melting curves were obtained (Figure 1). One characteristic HRM curve profile was obtained for all the non-MDR-TB isolates as well as for 5 of the 49 MDR-TB isolates. Nine further melting curve profiles were found for the remaining 44 MDR isolates (Figure 1). HRM results were scrutinized by sequencing cluster I of all isolates and revealed that the 18 clinical non-MDR-TB isolates lacked any mutation: in relation to cluster I of the rpoB gene, they were indistinguishable from the H37Rv strain. Sequence analysis of cluster I of the five MDR isolates showing melting curve profiles indistinguishable from the non-MDR-TB isolates revealed that one isolate had a double mutation, CTG436CGG and GAC441TAC, and that the remaining four MDR isolates lacked any mutations within the scanned region of cluster I.

Further sequencing of the rpoB gene of these four MDR isolates revealed a GTC176TTC mutation in three of them and an ATC497TTC mutation within cluster II of the rpoB gene in the fourth. For the V176F mutation, a second HRM analysis assay was established that allowed the specific detection of this mutation in all three isolates (Figure 2).

Thirty-three of the 49 MDR isolates showed a unique melting curve profile indistinguishable from each other but different from that of the non-MDR-TB isolates (Figure 1). This specific melting curve profile was associated with an SNP class 1 TCG456TTG mutation in 30 isolates, a deletion of an AAC in position 444 in one isolate and with a TCG456TTT double transversion in another single isolate (Table 1). Three of the 30 isolates with the TCG456TTG mutation had an additional mutation outside cluster I: an ATC494GTC mutation within cluster II, a GCG504GTG and a TTC509TCC mutation (Table 1).

The second most frequent MDR-specific melting curve profile was found in six isolates: in four isolates this melting curve profile was associated with an SNP class 2 mutation GAC441TAC, in one isolate with an SNP class 2 mutation CAA438AAA and in one isolate with a TTC insertion between

Figure 1. Normalized and temperature-shifted difference plot of amplification products of cluster I of the rpoB gene of the 68 TB isolates. The baseline (blue) represents the H37Rv sequence comprising all the non-MDR-TB isolates as well as the four MDR-TB isolates. The mutations of the respective MDR-TB isolates are indicated for the different melting curve profiles. An isolate with a neutralizing double mutation yielded a melting curve profile indistinguishable from that of the non-MDR-TB isolates. All reactions were performed in quadruplicate.
Discussion

Re-appraisal of global drug resistance data suggests that the problem of drug-resistant TB is more critical than previously perceived. Patients with MDR-TB are at high risk of treatment failure. Methods for the rapid identification of resistance in *M. tuberculosis* enable an earlier appropriate treatment of patients and an optimized disease control. In this study, a gene scanning method was evaluated for the rapid and simple identification of rifampicin resistance in *M. tuberculosis*. Rifampicin resistance is associated with MDR-TB, making resistance to this drug an important marker for genotypic drug susceptibility testing. Molecular detection of rifampicin-resistant *M. tuberculosis* usually involves the detection of mutations within cluster I of the *rpoB* gene covering codons 432 to 458 according to the *M. tuberculosis* nomenclature. Although various molecular assays for the rapid detection of rifampicin resistance have been described, all of them are hampered by the multitude of test steps necessary to detect the different non-synonymous SNPs conferring resistance. Therefore, we evaluated the feasibility of genotypic MDR-TB testing using a gene scanning method for resistance to rifampicin. Mutation scanning using HRM curve analysis limits the number of reactions essential for mutation detection within an amplification product to a single step by targeting the whole sequence. This makes the method very cost-effective and accurate, giving results similar to those of sequence analysis. In our study, 45 of the 49 (92%) Austrian MDR-TB isolates tested had mutations within cluster I of the *rpoB* gene. This is a lower frequency than that observed in previous studies, where up to 98% of rifampicin-resistant *M. tuberculosis* isolates had mutations within cluster I. We were able to demonstrate mutations in the *rpoB*-coding region (inside or outside cluster I) that resulted in a net amino acid exchange for all the 49 MDR-TB isolates tested.

Overall, 63 of the 68 (93%) isolates were correctly identified as MDR-TB or non-MDR-TB by mutation scanning of cluster I of the *rpoB*. HRM curve analysis allowed the accurate detection of SNP classes 1 and 2 deletions and insertions. In SNP classes 3 and 4 mutations, bases switch only the strand. This results in very low melting temperature differences (T_m) that are more difficult to detect. The detection of SNP classes 3 and 4 mutations can be improved by the addition of reference DNA (in our study genomic DNA of H37Rv). This modification results in the formation of heteroduplex amplicons exhibiting melting curve profiles different from those of homozygous amplicons. For the intended use of the assay as a solid mutation screening tool, spiking sharpens the detection of demanding SNP classes 3 and 4 mutations. Nevertheless, in our study, one MDR-TB isolate was incorrectly identified as non-MDR-TB despite spiking. This false-negative result was due to an erasing effect on the melting curve profile caused by two mutations (T to G and G to T) in different codons (CTG436CGG and GAC441TAC). However, direct comparison of the two melting curve profiles (MDR-TB double mutant and H37Rv), a refinement in normalization, temperature shift setting and sensitivity allowed the differentiation of these rather similar melting curves (data not shown).

The remaining incorrect results of HRM curve analysis, where the four MDR-TB isolates were falsely identified as non-MDR-TB, lacked any mutations within cluster I of the *rpoB* gene. Sequencing confirmed the HRM results but also revealed that three of the four MDR-TB isolates had a V176F (GTC176TTC) mutation and one isolate an I497F (ATC497TTC) mutation within the *rpoB* gene. The V176F mutation is known to confer high rifampicin resistance and has been reported as one of the most frequent *rpoB* mutations outside cluster I. The V176F mutation was detectable in an additional HRM PCR assay.

![Figure 2. Normalized and temperature-shifted difference plot for the V176F mutation within the *rpoB* gene. (a) Isolates with the wild-type GTC codon and (b) the three isolates with the mutated TTC codon. All reactions were performed in quadruplicate. This figure appears in colour in the online version of JAC.](https://academic.oup.com/jac/article-abstract/63/6/1121/750504)
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**Table 1.** Mutations detected among the 68 investigated *M. tuberculosis* isolates from position 1 to 3041 of the coding region of the *rpoB* gene; codon and amino acid numbering for *M. tuberculosis* is based on strain H37Rv.
The use of HRM curve analysis for mutation scanning of the cluster I site within the rpoB gene of TB isolates allowed the rapid and cost-effective identification of 44 of 49 the MDR-TB isolates. In comparison with the gold standard culture-based phenotyping method, the assay gave a sensitivity of 89.8% and a specificity of 100%. A combination of both assays increased the sensitivity to 95.9%. Thus, the sensitivity and the general performance of this HRM curve analysis assay are correlated to the ratio of isolates yielding mutations within cluster I of the rpoB gene to isolates with mutations outside cluster I. Therefore, the higher sensitivity of 98% recently reported for a two-step HRM protocol can be explained by the fact that all tested isolates harboured mutations inside cluster I. In addition, the performance of the assay will be negatively influenced when applied in geographic areas with a high prevalence of MDR-TB. In Austria, a country with no more than 3% of Mycobacterium tuberculosis isolates a year being MDR, the positive predictive value of the combined HRM assay would be 100% and the negative predictive value at least 99.9%, making this assay an ideal screening method for the TB laboratory, with minimal requirements of cost and time. An improvement on our assay is the combined single-step closed-tube design in an interchangeable 96- or 384-well microplate format that enables rapid, reliable, simple and cost-effective handling of large numbers of samples.

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Transparency declarations

None to declare.

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