High Resolution Melting Curve Analysis for Rapid Detection of Streptomycin and Ethambutol Resistance in *Mycobacterium tuberculosis*

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\textbf{ABSTRACT}

**Objectives:** Development of molecular techniques for rapid detection of drug resistant tuberculosis allows for the prompt initiation of appropriate anti-TB treatment. We aimed to assess high-resolution melting (HRM) analysis for the detection of rpsL, rrs and embB mutations to identify streptomycin and ethambutol resistance in *Mycobacterium tuberculosis*.

**Materials and Methods:** A total of 76 clinical isolates of *M. tuberculosis* including 25 SM-R, 21 EB-R and 30 drug susceptible – determined by the proportion method of drug susceptibility testing (DST) – were analyzed by HRM analysis, and the results were confirmed using DNA sequencing.

**Results:** The sensitivity and specificity of the HRMA compared to phenotypic DST were 88\% and 100.0\%, respectively for the detection of streptomycin resistance (SM-R), and 90.4\% and 96.6\%, respectively for ethambutol resistance (EB-R). Three SM-R and two EB-R isolates had no mutations in the studied regions of rpsL, rrs and embB genes determined by DNA sequencing and therefore were not identified as resistant by HRM assay. Interestingly, one phenotypic EM-S isolate was found by sequencing to have a mutation at codon 423 (Met $\rightarrow$ Ilu) of embB gene and was clustered as resistant by HRM as well.

**Conclusions:** The sensitivity and specificity of HRM curve assay was consistent with DNA sequencing, which is the gold standard method for genotypic DST. This assay can be utilized as a screening method for detection of drug-resistant tuberculosis, offering the advantages of a high throughput, single step, cost effectiveness, and rapid work flow method.

**Keywords:** HRM analysis; *Mycobacterium tuberculosis*; rpsL; rrs; embB.

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INTRODUCTION

The fact remains that tuberculosis (TB), the cause of ill health among millions of people each year, is still a considerable problem. It ranks as the second leading cause of death from an infectious disease, after the human immunodeficiency virus (HIV). The latest estimates are 9.0 million new TB cases in 2013 and 1.5 million TB deaths (1).

The association of streptomycin resistance (SM-R) in Mycobacterium tuberculosis with mutations in rpsL and rrs, encoding the ribosomal protein S12 and 16S rRNA, respectively is evident (2). Within the rpsL gene, mutations at codon 88 and – the most commonly occurring mutation – at codon 43 (K43R) in SM-R M. tuberculosis have been described. Mutations within rrs have been found in the 530 loop and the 912 loop, but these are less common compared to mutations within the rpsL gene (3-5).

The major mechanism of resistance to ethambutol (EB) is related to mutations in the embCAB operon, encoding three homologous arabinosyl transferases, mostly at codon 306 of embB (6, 7).

Iran, with an estimated TB incidence rate of 21 per 100,000, shares geographical borders with high TB-burden countries, including Azerbaijan, Armenia, Pakistan and Afghanistan; the reported rate of resistance to any anti-TB drug was 23% and 65% in new and previously treated cases, respectively. Also, 19% of new TB cases and 44% of previously treated patients were found to be resistant to SM. These amounts were 4% and 31% for new cases and previously treated patients in the case of EB (8, 9).

Conventional phenotypic methods are used to investigate M. tuberculosis drug resistance. However, the conventional drug susceptibility testing (DST) methods are time consuming due to the slow growth rate of M. tuberculosis. Therefore, the rapid identification of drug-resistant M. tuberculosis using molecular methods is a useful aid in the appropriate treatment given earlier, and decreases transmission of resistant strains. In addition, the development of a rapid, low cost and sensitive assay could potentially be used in settings where cost effectiveness is essential (10).

High resolution melting (HRM) curve analysis assay has been investigated for rapid detection of point mutations, single-nucleotide polymorphism (SNP), internal tandem duplications, simultaneous mutation scanning and genotyping in various bacteria. This method needs only the usual unlabeled primers and a dsDNA binding dye and detects sequence variants based on differences in the melting profiles between test and reference DNA. Also, this method is a closed-tube system, thereby minimizing cross-contamination from amplified DNA. Furthermore, it can be used for analyzing a large number of samples per run in a short time, which makes it a good candidate tool for mutation scanning (11, 12).

In the present study, we aimed to evaluate HRM assay for detection of mutations in rpsL, rrs and embB genes, to identify SM-R and EB-R in M. tuberculosis. To our knowledge, this is the first report to describe HRM analysis application for the detection of ethambutol and streptomycin resistance in clinical isolates of M. tuberculosis from Iran.

MATERIALS AND METHODS

Clinical isolates of M. tuberculosis strains

Samples including 20 M. tuberculosis clinical isolates were obtained from patients with bacteriologically confirmed pulmonary TB from hospitals in Tehran during the period between 2010 and 2012. These samples which consisted of 5 SM-R and 5 EB-R, and 10 drug susceptible isolates were used as the reference samples for initial development of the HRM assay. To validate the HRM assay, a blinded series of clinical M. tuberculosis isolates comprising 25 SM-R, 21 EB-R and 30 drug susceptible collected between 2012-2014 from six different provinces of Iran (including Tehran, Alborz, Sistan-Baluchestan, Lurestan, Khorasan and Kermanshah) were examined.

Drug susceptibility testing

Drug susceptibility testing (DST) was performed in three different institutions, including Tehran University of Medical Sciences, Kermanshah University of Medical Sciences and Mashhad University of Medical Sciences. It was carried out using the proportion method on Lowenstein-Jensen (LJ) medium containing 4.0 µg/mL for SM or 2.0 µg/mL for EB. M. tuberculosis H37Rv ATCC 27294 strain (susceptible to both SM and EB) was considered as a control (13).

DNA extraction and primer design

The isolates were subcultured on Lowenstein-Jensen solid medium and incubated at 37°C for
two to four weeks. Then, genomic DNA was extracted from the isolates according to a method described previously, and DNA concentration obtained from each sample was measured using Nano Drop (Thermo Scientific, USA). The DNA samples were stored at –20°C for subsequent experiments (14). The primers used for HRM analysis of the rpsL, rrs and embB genes were designed using Primer3Plus web tool (available online http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/). HPLC-purified primers were used to achieve the best performance. The nucleotide sequences of primers used for PCR and product sizes of amplicons analyzed by HRM assay are mentioned in Table 1.

**PCR and high resolution melting curve analysis**

The PCR reaction was performed using mi-real-time EvaGreen® Master kit (Metabion, Martinsried, Germany) including the following components per reaction: DNA template (2 µL), 2X master mix (12.5 µL), each primer (1.5 µL), and PCR grade water (Metabion) adjusted to a final volume of 25 µL.

PCR and HRM curve analysis was performed using a Rotor-Gene 6000 apparatus (Qiagen). The PCR condition used for generating amplicons for HRM analysis consisted of one cycle of 95°C for 2 min, 40 cycles of 95°C for 15 s and 60°C for 40 s. The post-PCR HRM analysis was carried out following the amplifications by heating the samples from 80°C to 95°C at a rate of 0.1°C per step, with continuous fluorescence detection. All samples were examined in duplicate to ensure reproducibility of the melting curves. HRM curve data analysis was performed using Rotor-Gene 6000 Series Software (Version 1.7) to calculate the derivative of the intensity of fluorescence at different temperatures (dF/dT).

The software analyzes the differences in the shape of the melting curve between a sample and the wild-type control strain (M. tuberculosis H37Rv) by generating a difference plot curve. This plot helps with clustering samples into groups having similar melting curves; hence, sequence polymorphisms can be detected.

**DNA sequencing**

Another set of primers were designed for determination of the nucleotide sequences of an extended region of all gene analyzed by HRM to assess the mutations conferring resistance and to confirm the results obtained by HRM (Table 2).

Amplification reactions were carried out in a final volume of 50 µL of PCR master kit (Ampliqon, Denmark), 0.2 µM of each primer and 10 ng of template DNA. The PCR cycling was run under the following conditions: 5 min at 94°C, followed by 30 cycles of 30 s at 94°C, 45 s at 58°C and 45 s at 72°C, followed by a final extension at 72°C for 5 min. Then, the PCR products were used as templates for targeted DNA sequencing that was performed by Macrogen Company (Korea). All sequencing reactions were carried out in both forward and reverse directions using the primer

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Gene</th>
<th>Sequence (5’-3’)</th>
<th>Product size (bp)</th>
<th>Tm</th>
<th>Nucleotide positions</th>
</tr>
</thead>
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<tr>
<td>rpsl_F</td>
<td>rpsL</td>
<td>TATGCACCCCGGTGTACA</td>
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<td>59</td>
<td>98-115</td>
</tr>
<tr>
<td>rpsl_R</td>
<td>rpsL</td>
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<td>56</td>
<td>279-296</td>
<td></td>
</tr>
<tr>
<td>rrs1_F</td>
<td>rrs</td>
<td>CGGGTTCTCTCGGATTGACG</td>
<td>128</td>
<td>60</td>
<td>456-475</td>
</tr>
<tr>
<td>rrs1_R</td>
<td>rrs</td>
<td>CAAACCACCTACGAGCTCTT</td>
<td>57</td>
<td>583-564</td>
<td></td>
</tr>
<tr>
<td>rrs2_F</td>
<td>rrs</td>
<td>GGGTTTCTCTTGAGATC</td>
<td>130</td>
<td>58</td>
<td>825-844</td>
</tr>
<tr>
<td>rrs2_R</td>
<td>rrs</td>
<td>AATTAATCCACATGCTCCGC</td>
<td>60</td>
<td>954-935</td>
<td></td>
</tr>
<tr>
<td>embB1_F</td>
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<td>TCGTCGGACGACGCTACAT</td>
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<td>62</td>
<td>867-884</td>
</tr>
<tr>
<td>embB1_R</td>
<td>embB</td>
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<td>63</td>
<td>1143-1126</td>
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<tr>
<td>embB2_F</td>
<td>embB</td>
<td>ATGCCGCTTTCAACAACCGC</td>
<td>333</td>
<td>59</td>
<td>1186-1203</td>
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<tr>
<td>embB2_R</td>
<td>embB</td>
<td>GGTGCGCTTCCAACACCGT</td>
<td>60</td>
<td>1518-1501</td>
<td></td>
</tr>
</tbody>
</table>

F=Forward, R=Reverse

**Table 1.** Primer sequences used for HRM assay
pairs that were similar to the primers for PCR amplification. The sequences were analyzed using ChromasProver 1.7.1 Software (Technelysium), and mutations were determined by comparing our sequencing data with the nucleotide sequences of rpsL, rrs and embB genes of M. tuberculosis H37Rv deposited at Tuberculist (http://genolist.pasteur.fr/Tuberculist/) and the GenBank databases (http://www.ncbi.nih.gov/gene).

Statistical analysis

Sensitivity was determined as [Number of drug-resistant isolates with mutations]/ [number of drug-resistant isolates with mutations+number of drug-resistant isolates with wild type].

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>No of isolates</th>
<th>HRM assay</th>
<th>DNA sequencing</th>
<th>Nucleotid changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptomycin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Susceptible (30)</td>
<td>30</td>
<td>NM</td>
<td>NM</td>
<td>WT</td>
</tr>
<tr>
<td>Resistant (25)</td>
<td>7</td>
<td>M</td>
<td>NM</td>
<td>AAG→AGG</td>
</tr>
<tr>
<td>Resistant</td>
<td>5</td>
<td>M</td>
<td>NM</td>
<td>AAG→ATG</td>
</tr>
<tr>
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<td>1</td>
<td>M</td>
<td>NM</td>
<td>AAG→AGG</td>
</tr>
<tr>
<td>Resistant</td>
<td>4</td>
<td>NM</td>
<td>M</td>
<td>WT nt907A→C</td>
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<tr>
<td>Resistant</td>
<td>3</td>
<td>NM</td>
<td>M</td>
<td>WT nt516C→T</td>
</tr>
<tr>
<td>Resistant</td>
<td>1</td>
<td>NM</td>
<td>M</td>
<td>WT nt526C→T</td>
</tr>
<tr>
<td>Resistant</td>
<td>1</td>
<td>NM</td>
<td>M</td>
<td>WT nt856A→T</td>
</tr>
<tr>
<td>Etambutol</td>
<td></td>
<td>EmbB</td>
<td>EmbB</td>
<td>-</td>
</tr>
<tr>
<td>Susceptible (30)</td>
<td>29</td>
<td>NM</td>
<td>WT</td>
<td>-</td>
</tr>
<tr>
<td>Resistant (21)</td>
<td>11</td>
<td>M</td>
<td>ATG→ATA</td>
<td>Met→Ile codon423</td>
</tr>
<tr>
<td>Resistant</td>
<td>5</td>
<td>M</td>
<td>ATG→GTG</td>
<td>Met→Val codon306</td>
</tr>
<tr>
<td>Resistant</td>
<td>2</td>
<td>M</td>
<td>CAG→CGG</td>
<td>Gln→Arg codon497</td>
</tr>
<tr>
<td>Resistant</td>
<td>1</td>
<td>M</td>
<td>CAG→CGG</td>
<td>Gly→Asp codon406</td>
</tr>
<tr>
<td>Resistant</td>
<td>2</td>
<td>NM</td>
<td>TCG→TCT</td>
<td>Ser→Leu codon366</td>
</tr>
</tbody>
</table>

NM=no mutation; M=mutation; WT=wild type; S=susceptible; R=resistant

**TABLE 3.** Results of HRM assay to detect the mutations conferring resistance in SM and EB in blinded series of M. tuberculosis samples
drug-resistant isolates without mutation); and specificity as [Number of drug-susceptible isolates without mutations]/ [number of drug susceptible isolates with mutations+number of drug-susceptible isolates without mutations]. Calculation of the 95% confidence interval was done using the Adjusted Wald method (http://www.measuringusability.com/wald.htm).

RESULTS

Initial development of HRM assay for detection of streptomycin and ethambutol resistance

The HRM assay developed in this study was designed to identify dominant mutations related to drug resistance in \( rpsL \), \( rrs \) and \( embB \) genes by using 20 \( M. \) tuberculosis DNA samples. By the HRM assay, the mutated isolates were easily differentiated from the susceptible (wild-type) isolates by comparing the differences in the melting-curve shapes.

The results obtained by HRM assay were consistent with those of phenotypic DST in all reference samples. The HRM assay used for scanning of the \( rpsL \) and \( rrs \) genes in five clinical SM-R reference samples, correctly identified corresponding mutations including K43R (AAG → AGG) and K88Q (AAG → ATG) alterations in \( rpsL \) and 516C/T and 907A/C alterations in \( rrs \) gene. Similarly, all five EMB resistant reference strains harboring M306I (ATG → GTG) mutation determined by sequencing was correctly clustered as resistant by HRM.

Validation of HRM assay with blinded samples

A series of 76 samples was blindly analyzed to validate the HRM assay (Table 3). By HRM analysis of A fragment of 199 pb of \( rpsL \) gene and two fragments of 128 pb and 130 pb of \( rrs \) gene in 25 SM-R strains, 22 isolates were accurately identified as resistant (Table 3). However, three streptomycin-resistant isolates had no detectable mutation in the studied regions of \( rpsL \) and \( rrs \) using HRM. Importantly, DNA sequencing results of the regions encompassed by the HRM assay showed concordance with the HRM assay results for these isolates.

Similarly, HRM analysis correctly recognized \( embB \) mutations in 19/21 of phenotypically EB-R strains. The remaining two phenotypically EB-R isolates which had been falsely clustered as susceptible (wild-type) by HRM, were confirmed for lacking any mutation in the studied regions of \( embB \) by sequencing. All 30 SM-S and 29 EB-S strains showed the wild-type HRM curve pattern and sequencing data confirmed the absence of mutation in the target regions of \( rpsL \), \( rrs \) and \( embB \) genes in these isolates. However, one phenotypically EB-S isolate showed a melting curve distinct from susceptible isolates but similar to those of resistant ones (Figure 1). Nucleotide sequencing

FIGURE 1. Representative normalized and difference plots of HRM curve analysis for mutant discrimination in \( rpsL \) genes (A and a, respectively), \( rrs1 \) (B and b, respectively) \( rrs2 \) genes (C and c, respectively, \( embB1 \)genes (D and d, respectively) and \( embB2 \)genes (F and f, respectively).
a. Normalized and temp-shifted difference plot of rpsL.

b. Normalized and temp-shifted difference plot of rrs1.

B. Normalized and shifted melting curves of rrs1.
C. Normalized and shifted melting curves of rrs2

D. Normalized and shifted melting curves of embB1
d. Normalized and temp-shifted difference plot of embB1

F. Normalized and shifted melting curves of embB2

f. Normalized and temp-shifted difference plot of embB2
identified a mutation at codon 423 of embB gene for this isolate.

**DNA sequencing**

Nucleotide sequences of three genes analyzed by HRM were determined to study the type and frequency of mutations and also to confirm the HRM results.

Twenty eight percent (7/25) of SM-R isolates was found to have a mutation (AAG → AGG, Lys → Arg) at codon 43, 20% (5/25) of isolates had a mutation (AAG → ATG, Lys → Met) at codon 88, and 4% (1/25) of isolates had a mutation (AAG → AGG, Lys → Arg) at codon 44 of rpsL. In total, 52% (13/25) and 36% (9/25) of SM-R isolates harbored mutations at rpsL, and rrs genes respectively. No mutation was found within the rpsl and rrs genes in the remaining 12% (3/25) of the SM-R isolates by direct sequencing method (Table 3).

Among 21 EB-R isolates, 11 were found to harbor a mutation at codon 306 (ATG → GTG, Met → Val), five at codon 497 (CAG → CGG, Cln → Arg), two at codon 406 (GCC → GAC, Gly → Asp) and one at codon 366 (TCG → TCT, Ser → Leu) of the embB gene. However, the remaining 9.5% (2/21) of the EB-R isolates lacked any mutation within the embB gene determined by direct sequencing method. Interestingly, one EB-S isolate had a mutation at codon 423 (ATG → ATA, Met → Ile) of embB gene (Table 3).

**Sensitivity and specificity**

In order to evaluate the sensitivity and specificity, the HRM curve analysis for SM-R and EB-R was compared to the conventional DST results. The sensitivity for identification of resistance was found to be 88% for streptomycin (95% confidence interval 69.2-96.6) and 90.4% for ethambutol (95% CI 69.8-98.5), while the specificity was found to be 100% (95% CI 90.1-100.0) and 96.6% (95% CI 81.9-99.9) for streptomycin and ethambutol, respectively. Altogether, there was a 100% concordance between HRM analysis and DNA sequencing for the detection of resistance conferring mutations in all genes.

**DISCUSSION**

Drug resistance in tuberculosis is currently determined using culture-based methods such as the aga proportion method, or liquid media methods like the BACTEC MGIT 960. Although these methods are more rapid it requires at least one week for the determination of drug susceptibility (15). Getting an early drug susceptibility result is clinically essential for patients to initiate an appropriate TB treatment regimen leading to better outcomes. It also allows surveillance of antibiotic resistance rates, which are relevant to TB control (16). HRM has been recognized as a relatively new post-PCR method providing the detection of subtle sequence variations by analyzing the melting-curve shape after PCR amplification using saturating DNA dye, which produces an amplicon-specific melting curve. This technique has been successful in mutation scanning, single nucleotide polymorphism genotyping, and identification of many bacterial species, including screening for drug resistance in *M. tuberculosis* (17-19). Usually, it is recommended that the size of PCR product should be less than 400 bp for HRM, but shorter amplicons are more sensitive for the detection of minor changes and offer higher resolution. Therefore, we designed primers which got the amplicons with this length.

In order to expedite the detection of SM-R and EB-R in *M. tuberculosis*, we have developed HRM assay to search for mutations in the corresponding rpsl, rrs and embB genes. To our knowledge, this is the first report of the use of HRM for rapid identification of streptomycin and ethambutol resistance by analyzing rpsl, rrs and embB genes mutations in the clinical samples of *M. tuberculosis* from Iran.

In our study, the concordance of HRM curve analysis with DNA sequencing was found to be 100%. The assay was successfully used for the detection of dominant rpsl (codons 43 and 88) and rrs mutations (nucleotides 513 to 907).

In previous studies, the association of rpsl and rrs mutations with SM-R varied, but it was likely that it accounted for approximately 25% to 80.4% of SM-genotypes (20-22). In this study, sequencing data revealed that 88% of SM-R isolates had mutations in rpsl or rrs, or both, which is consistent with the results obtained by previous studies. All of these strains were correctly identified by the current HRM curve analysis. However, HRM was unable to detect resistance when the studied gene regions lacked a nucleotide alteration. Indeed, three phenotypically SM-R strains not harboring any mutation in rpsl or rrs were not identified in the HRM curve analysis. This can be due to the
involvement of other additional genes in streptomycin resistance such as gidB gene, which encodes a 7-methylguanosine methyltransferase specific for 16S rRNA. Although mutations in the gidB gene have been demonstrated to be associated with low level streptomycin resistance, mutations in these genes have been reported for streptomycin-susceptible clinical isolates as well (23, 24). Thus, further investigations are needed to confirm the association of gidB with streptomycin resistance. However, we have only studied the most commonly involved genes, while other genes and mechanisms involved in streptomycin resistance were not traced. All SM-S isolates generated a wild-type HRM curve and were correctly classified as susceptible (specificity, 100%).

Our HRM assay detected embB mutations in 19 of 21 (90%) EB-R isolates among the 52.38% (11/21) harbored embB 306 mutations. This level is much lower compared to Cuba and the Dominican Republic (70%) and Germany (68%), but similar to the level estimated in China (55%) and Russia (48%) (25, 26), which shows that geographic distribution of embB mutations among the EB-R M. tuberculosis isolates differs around the world. Two EB-R isolates had no detectable mutations in regions not encompassed by our assay. It has been suggested that genetic alterations in other drug target genes such as embA and embC may be involved in drug resistance development in these isolates. One discordant isolate had a mutation at codon 423 of embB (Met→Ile) that was detected by both HRM and sequencing methods, but it was found to be susceptible to EB by phenotypic DST. It is assumed that the cause of this discrepancy is the failure of the phenotypic test by deterioration of drugs during storage. In addition, it is possible that the low or intermediate level resistance due to this mutation makes it difficult to be diagnosed by the phenotypic test and all mutations found in embB are not necessarily associated with ethambutol resistance. Unfortunately, this sample was not viable for repeating the susceptibility testing.

Compared to phenotypic DST, streptomycin resistance was identified by our HRMA with specificities of 100% and sensitivities of 88%. Wang reported that HRM had a sensitivity and specificity of 100% for the detection of SM resistance (27). A study from Singapore showed a sensitivity and specificity of 87.5% and 100%, respectively for the detection of SM-R by HRM (28). HRM analysis performed by Sethi et al. was found to have a sensitivity and specificity of 61.8% and 100%, respectively for SM-R identification (29). Also, Negai et al. reported that sensitivity and specificity of HRM were 95.9% and 100%, respectively for genotyping drug resistance among SM-R and SM-S isolates (10).

On the other hand, the calculated sensitivity and specificity for HRM assay used in the current study for the detection of EB resistance was found to be 90.4% and 96.6%, respectively. Lately, just one study has evaluated the usefulness of an HRM analysis for the detection of drug resistant M. tuberculosis; Negai et al. reported 100% sensitivity and specificity for the identification of EB-R in M. tuberculosis isolates; this can be attributed to the fact that samples studied by them were more likely to harbor mutations within the regions studied by HRM, while – as confirmed by direct sequencing results – in our study, the inability of HRM to detect resistance in a few isolates was mainly due to the absence of mutation within the studied genes. Indeed, we did not have false negative results compared to sequencing, which is considered to be the gold standard method for genotypic DST.

Concordance between the results of HRM assay and DNA sequencing for all genes analyzed in this study was found to be 100%, suggesting that this assay is extremely accurate for the rapid detection of drug-resistant M. tuberculosis. However, there is a difference in sensitivity of molecular diagnostics due to the variation in frequency and type of mutations between different geographic regions.

The HRM assay has several advantages compared to other molecular methods. Firstly, it is simple and rapid, because the amplification and melting analysis can be done as a single procedure on a real-time PCR device. Secondly, the potential risk of cross-contamination from subsequent reactions can decrease, because this assay is performed in a single closed tube in just one step. Thirdly, there is no need to costly fluorescent probes or specialized reagents.

There are also some disadvantages related to the use of HRM assay for the detection of drug resistance. Firstly, it can be performed only on culture samples – therefore, if this method could be practicable for clinical specimens and the diagnostic interval in detection of drug resistant M. tuberculosis would be further shortened. Secondly, the accuracy of HRM assay depends on some factors such as quality of the template DNA, instrument...
and dye, which is limiting its extensive application. Thirdly, the genetic basis of resistance is still not completely understood; therefore, similarly to other genotypic DST methods, the HRM assay cannot detect drug resistance conferred by unknown mechanisms and it may not be able to fully replace the conventional culture based DST method (30).

CONCLUSION

In conclusion, we developed a specific HRM assay for the detection of mutations in M. tuberculosis, conferring the targets for resistance to streptomycin and ethambutol. In order to screen the gene mutations associated with M. tuberculosis drug resistance, the rapid and sensitive HRM assay could be used routinely at low costs, which would be a suitable approach for laboratories located in less economically developed countries. One limitation of the present study is that this assay cannot yet replace conventional DST because all kinds of mutations related to SM and EB resistance were not explored.

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