ORIGINAL ARTICLE

High-resolution melt curve analysis for rapid detection of rifampicin resistance in *Mycobacterium tuberculosis*: a single-centre study in Iran

S. Arefzadeh[1], T. Azimi[2], M. J. Nasiri[1], Z. Nikpor[1], H. Dabiri[1], F. Doustdar[1], H. Goudarzi[1] and M. Allahyartorkaman[3]

1) *Department of Microbiology, School of Medicine, Shahid Beheshti University of Medical Sciences,* 2) *Department of Pathobiology, School of Medicine, Tehran University of Medical Sciences and* 3) *Regional Tuberculosis Reference laboratory, Tehran University of Medical Sciences, Tehran, Iran*

Abstract

This study aimed to compare the diagnostic accuracy of high-resolution melting (HRM) analysis in comparison with Xpert MTB/RIF as well as conventional drug susceptibility testing (DST) for the detection of rifampicin (RIF) resistance in *Mycobacterium tuberculosis* in Iran. A comparative cross-sectional study was carried out from April 2017 to September 2018. A total of 80 culture-positive clinical samples selected during the study period were analysed for detection of RIF-resistant TB by conventional DST, Xpert MTB/RIF, and sequencing. Sensitivity and specificity of the HRM calculated according to DST was our reference standard test in this study. The overall sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of HRM assay were found to be 100%, 89.33%, 38.46%, and 100% respectively. The analysis demonstrated that the diagnostic accuracy of HRM tests is insufficient to replace Xpert MTB/RIF and conventional DST. HRM tests have the advantage of time to result and may be used in combination with culture. Further work to improve molecular tests would benefit from standardized reference standards and the methodology.

Keywords: High-resolution melting analysis, Iran, rifampicin resistance, tuberculosis, Xpert MTB/RIF
Original Submission: 16 December 2019; **Accepted:** 5 March 2020
Article published online: 12 March 2020

Corresponding author: M. J. Nasiri, Department of Microbiology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
E-mail: mj.nasiri@hotmail.com

T. Azimi is equally first author.

Introduction

Tuberculosis (TB) is one of the most serious public health problems worldwide. Approximately 10.4 million people were infected with TB in 2016, and 90% of these were adults [1]. The emergence of multidrug-resistant TB (MDR-TB) has had a significant negative effect on TB control strategies. In 2016 there were 600 000 new cases with resistance to rifampicin (RIF), of which 490 000 had MDR-TB [1]. In Iran, a country with moderate TB incidence, it was reported that MDR-TB accounted for 12% of previously treated TB cases and 1% of new TB cases [2]. MDR-TB has been associated with worse treatment outcomes than drug-susceptible TB [3–8]. RIF resistance is frequently associated with concomitant isoniazid resistance and thus is considered as a proxy for MDR-TB [9]. Thus, for better management of drug-resistant TB, early detection of RIF resistance and starting adequate treatment are extremely important to reduce the risk of death [10–13]. Although the conventional drug susceptibility testing (DST) method is the reference standard test to diagnose drug-resistant TB, it is a time-consuming process that takes up to several weeks to show results [14]. The Xpert MTB/RIF is an automated molecular assay endorsed by the WHO for the early diagnosis of both *Mycobacterium tuberculosis* infection and RIF resistance [15]. The assay has been in use in Iran for detection of RIF resistance. However, implementation of this method in all regional laboratories of Iran is not affordable. High-resolution melting (HRM) curve analysis is a simple and robust method for detecting drug-resistant TB [16–21]. The advantages of HRM include rapid turnaround times, it is a closed-tube method that greatly reduces contamination risk, and, unlike other methods, it requires no post-PCR

https://doi.org/10.1016/j.nmni.2020.100665

handling [22,23]. The method is based on the analysis of fluorescence curves produced by a DNA-binding dye during strand dissociation events in the melting phase following real-time PCR [23–25]. In the present study, we aimed to compare the diagnostic accuracy of HRM in comparison with Xpert MTB/RIF and DST for the detection of RIF resistance in *M. tuberculosis* in Iran.

Materials and methods

M. tuberculosis clinical isolates

Twenty clinical isolates of *M. tuberculosis* (ten RIF-resistant TB and ten drug-susceptible isolates) were selected as the reference samples for the initial development of the HRM assay (the specimens used were culture isolates). To validate the HRM assay, 80 clinical isolates (five RIF-resistant TB and 75 drug-susceptible isolates) collected between April 2017 and March 2018 from the regional TB reference laboratory of Tehran, Iran, were used for the screening in a blind manner. The reference strain H37Rv, susceptible to RIF, was included in each run as a wild-type positive control, and nuclease-free water was used as a negative control. The ethics committee of Shahid Beheshti University of Medical Sciences approved the study, and all patients provided written informed consent (IR.SBMU.MSP.REC.1396.107).

Identification of *M. tuberculosis*

Clinical specimens were processed by the standard sodium hydroxide method, and smears were prepared by the Ziehl–Neelsen staining method [26]. After decontamination, specimens were inoculated onto Lowenstein–Jensen (LJ) solid medium. For the identification of mycobacteria, the slope cultures were incubated at 37°C and examined for growth once weekly for up to 6 weeks. Bacterial isolates identified as *M. tuberculosis* using standard biochemical tests (i.e. production of niacin and catalase, nitrate reduction) and molecular methods (*IS6110* based PCR assay) [26,27]. Only one culture isolated per study subject was considered for further analysis.

Conventional DST of *M. tuberculosis*

Conventional DST for RIF was performed for all culture-positive samples with the proportion method on LJ solid medium with a standard critical concentration of 40 μg/mL for RIF as previously described [26]. *M. tuberculosis* H37Rv strain (ATCC 27294) was used for quality control in DST.

Xpert MTB/RIF assay

Xpert MTB/RIF procedure was used according to the manufacturer's instructions for the molecular detection of RIF resistance [15]. Briefly, Xpert sample reagent was added to 1 mL of specimens in the ratio 1:2, and then the mixture was transferred to the Xpert test cartridge. Cartridges were inserted into the Xpert machine, and the automatically generated results were read after 90 min.

DNA extraction

DNA from the clinical isolates of *M. tuberculosis* was extracted from bacterial colonies grown on LJ slants as described previously [28].

HRM assay

The EvaGreen® Master kit (Metabion, Martinsried, Germany) was used for HRM assay; it contained the following components per reaction mixture: 10 μL of the 5X master mix, 2 μL of the template, 1 μL each primer and PCR grade water adjusted to a final volume of 20 μL. The RIF primers used for this experiment have been reported previously [17]. All assays were run on the Rotor-Gene 6000 instrument (Qiagen, USA) using the following cycling parameters: initial denaturation at 95°C for 10 min, 40 cycles of 94°C for 30 s and 60°C for 30 s. When a simple PCR amplification was complete, the HRM analysis was performed from 80–94°C with 0.1°C increments every 2 s. The reference strain H37Rv, susceptible to RIF, was included in each run as a wild-type positive control, and nuclease-free water was used as a negative control. Rotor-Gene 6000 Series Software 1.7 was used for HRM analysis.

DNA sequencing

Sequencing was used to confirm resistance in all phenotypically RIF-resistant strains using the primers reported previously [24].

Statistical analysis

Data were analysed with MedCalc 14 statistical software. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the HRM were calculated according to DST as our reference standard in this study.

Results

DST

Resistant and susceptible isolates were confirmed by both Xpert MTB/RIF and conventional DST. The concordance between the Xpert MTB/RIF and conventional DST for RIF resistance was 100%.

Initial development of HRM assay for detection of RIF resistance

For the initial assay development, we tested the assay for mutations within the rpoB gene from ten RIF-resistant M.

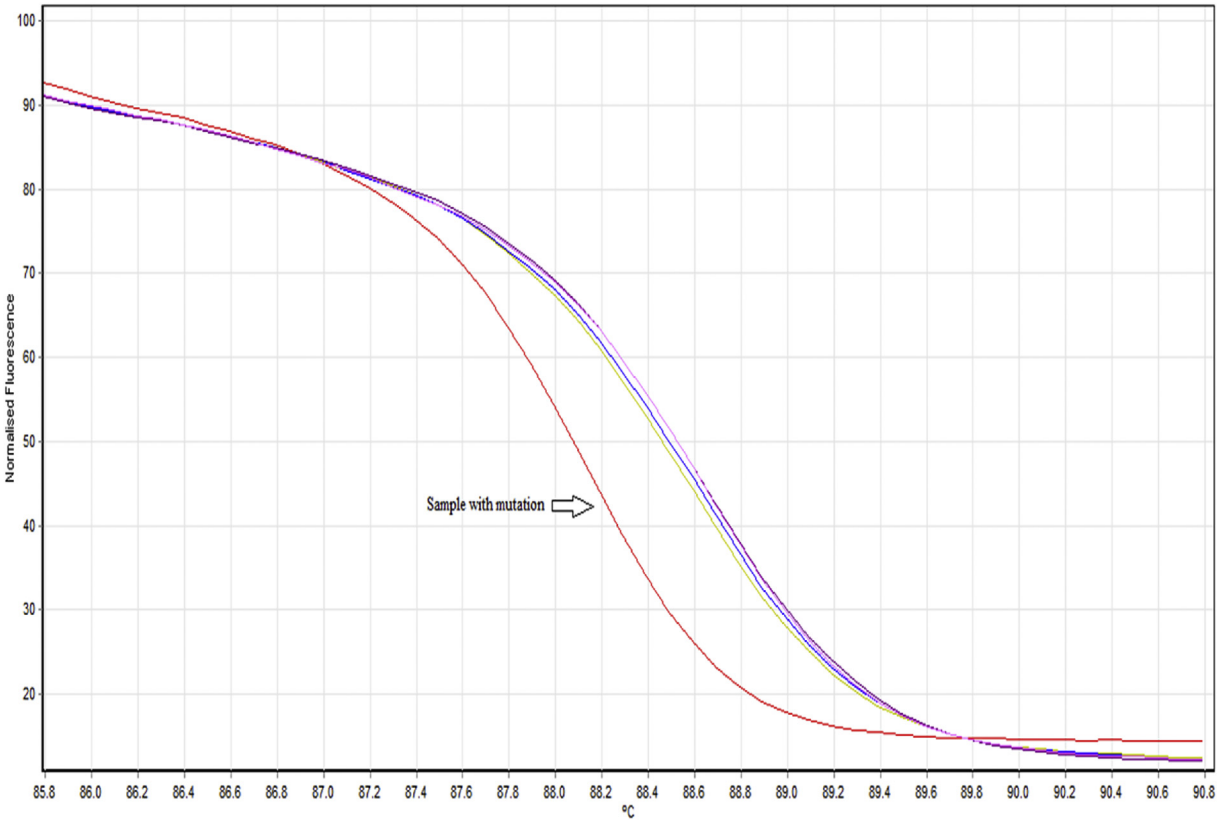

FIG. 1. Normalized melting curves of *rpoB*, illustrating the high-resolution melting (HRM) profile separation between wild-type strains and strains containing mutations (red).

tuberculosis isolates. All RIF-resistant M. tuberculosis isolates were detected by our HRM assay. We also analysed ten drug-susceptible isolates to check for false-positive results. All ten isolates were correctly identified as wild-type. The normalized melt curves of PCR products of isolates with different mutations are shown in Fig. 1. Visually, samples with mutations (red line) are easily differentiated from the wild type by the distinct differences in the shape of the melt curves.

Validation of the HRM assay with a blinded series of strains

All five RIF-resistant isolates in our blinded samples were observed to have mutations in *rpoB* by HRM (Table 1). However, eight susceptible isolates (confirmed by Xpert MTB/RIF and conventional DST) were falsely clustered as resistant by HRM. These isolates were confirmed by sequencing to lack any mutation in the studied regions of *rpoB*.

DNA sequencing

Single-point mutations in a 118-bp region of the *rpoB* gene were observed in all five phenotypically resistant isolates.

Sensitivity and specificity

In order to evaluate the sensitivity and specificity, the HRM curve analysis was compared to the Xpert MTB/RIF and conventional DST results for the detection of RIF-resistant M. tuberculosis isolates. The overall sensitivity, specificity, PPV and NPV of the HRM assay were found to be 100%, 89.33%, 38.46%, and 100% respectively.

Discussion

In the current study we have evaluated HRM for the detection of RIF resistance in *M. tuberculosis*. We found that the sensitivity of HRM was slightly higher than was found in previous studies [20,23,24,29]. In the study conducted by Galarza et al. the sensitivity for detection of RIF resistance was found to be 98.7% and the specificity was 97.75% [24]. Likewise, based on a meta-analysis in the year 2013, a pooled sensitivity of the HRM curve analysis was 94%, and the pooled specificity was very high at 99% [23]. One potential reason for a lower sensitivity of HRM in other studies is that 5% of RIF resistance is due to mutations

TABLE 1. Performance of high-resolution melting (HRM) compared to conventional drug susceptibility testing (DST)

	Drug susceptibility test (rifampicin)[a]				
HRM	**Resistant (5)**	**Sensitive (75)**	**Total**	**Sensitivity (95%CI)**	**Specificity (95%CI)**
Resistant	5	8	13	100.0 (50.0–100.0)	89.3 (80.0–95.2)
Sensitive	0	67	67		
Total	5	75	80		

[a]The concordance between the Xpert MTB/RIF and conventional DST for rifampicin resistance was 100%.

at DNA sites other than the *rpoB* region [30]. In our study the obtained sensitivity of 100% may be due to the fact that none of the RIF-resistant isolates had mutations outside the *rpoB* core.

Our results also indicated that the specificity of HRM was slightly lower than that found in previous studies [20,23,24,29]. The possible reasons for false-positive results might be as follows: it is possible that these mutations are silent or confer low-level resistance, and it may also occur due to the cross-contamination.

In recent years, several rapid molecular assays—including commercial kits and Xpert MTB/RIF—have been used for the diagnosis of drug resistance in *M. tuberculosis* [31–34]. Although rapid, these commercial methods are too expensive and too technically demanding for limited-resource countries like Iran.

HRM has advantages over current molecular techniques. For example, the assay can determine the RIF resistance in a large number of specimens simultaneously, within approximately 2 h of obtaining DNA. Furthermore, HRM is able to detect mutations without the need for labelled primers, product fractionation, DNA restriction or specific sequence analyses, providing options for meeting the needs of the laboratory [17]. An additional appealing feature of HRM is its adaptability. Commercial molecular techniques (i.e. real-time PCR and HRM) cannot detect drug resistance conferred by unknown mutations, and this limitation is common to all of them. However, by HRM, as new mutations are identified, additional probes could easily be designed and added to the assay [17]. This is an important feature because patterns of drug resistance can vary widely in a particular geographic area.

However, results from melting analysis depend on several factors. An important disadvantage of the HRM assay is that the accuracy of the assay critically depends on the sample source, preparation, quality of the extracted DNA, concentration, amplicon length, GC content, equipment, and the dye [22,23,35]. A previous study showed that a higher sensitivity could be achieved when fresh samples were used for HRM because the DNA might have degenerated during sampling [22]. Wittwer et al. indicated that the method is more sensitive for fragments smaller than 600 bp [36]. Likewise, in their study Taylor et al. suggested that lower GC content might have an association with false-negative results [37]. Moreover, dye and instruments are also very important in the accuracy of HRM. All of these parameters may to some degree limit the extensive application of HRM. Furthermore, standard optimization procedures must be established before HRM is used in clinical laboratories worldwide [23]. Furthermore, the human intervention required with HRM can increase the risk of contamination.

Our systematic review has some limitations. First, HRM was performed only on culture isolates. If this method could be used for the detection of RIF resistance in *M. tuberculosis* from clinical specimens, the diagnostic time would be further shortened. Second, HRM assay may detect silent or other mutations which are not actually associated with drug resistance. Third, we did not study all kinds of mutations related to RIF resistance, and thus the technique cannot yet replace conventional DST. Fourth, our sample size was small, and at a larger sample size there would be an increased chance of finding non-*rpoB* mutated resistant strains.

In conclusion, the analysis has demonstrated that the diagnostic accuracy of HRM tests is insufficient to replace Xpert MTB/RIF and conventional DST. HRM testing may be used in combination with culture due to the advantage of the time to result. Evaluation of new molecular DST would be an important next step to further expedite drug-resistant TB detection.

Author contributions

T. Azimi and **S. Arefzadeh** are joint first authors of this paper. All authors have made substantial contributions to all of the following: the conception and design of the study, acquisition of data, analysis and interpretation of data; drafting the article or revising it critically for important intellectual content; and final approval of the version to be submitted.

Conflict of interest

All of the authors declare that there are no commercial, personal, political, or any other potentially conflicting interests related to this paper. This study was financially supported by

Research Department of the School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran (Grant number:10347).

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