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ORIGINAL ARTICLE

# High resolution melting curve assay for rapid detection of drug-resistant *Mycobacterium tuberculosis*

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**Abstract** We developed and evaluated a high resolution melting (HRM) curve assay by using real-time PCR for the detection of the most frequent mutations of *Mycobacterium tuberculosis*, which are responsible for the resistance of four anti-TB drugs: rifampicin, isoniazid, ethambutol, and streptomycin. The HRM assay was successfully used for the detection of dominant mutations: A516V, H526A, H526T, S531L, L533P, and A516G/S531L in *rpoB*; S315T, and S315A in *katG*; −15C/T, and −8T/C in *mabinhA*; M306I in *embB*; K88Q and K43R in *rpsL*; and 513A/C in *rrs*. We were able to discriminate the mutant from the wild type by analyzing the melting-curve shape in 40 clinical *M. tuberculosis* isolates, and the results of the HRM assay were completely consistent with those of DNA sequencing. This HRM assay is a simple, rapid, and cost-effective method that can be performed in a closed tube. Therefore, our assay is a potentially useful tool for the rapid detection of drug-resistant *M. tuberculosis*.

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## Introduction

Tuberculosis (TB) is one of the major infectious diseases, and it remains a major public health problem worldwide [1]. The situation is further complicated by the emergence of multidrug-resistant (MDR) tuberculosis (TB) and extensively drug-resistant (XDR)-TB strains [2, 3]. Conventionally, the drug resistance of *Mycobacterium tuberculosis* is detected using phenotypic methods. However, the conventional drug susceptibility testing (DST) methods are time consuming because of the slow growth rate of *M. tuberculosis*. Therefore, the development of rapid and efficient DST methods is an urgent priority for the management of drug-resistant tuberculosis.

In contrast to many other bacteria that can acquire antibiotic resistance genes by conjugation, transduction, or transformation, there is no evidence so far of mobile genetic elements, such as plasmids, transposons, or integrons, in *M. tuberculosis*. The drug resistance of *M. tuberculosis* is caused by mutations in relatively restricted regions of the genome.

The mutations associated with rifampicin (RIF) resistance have been detected in the *rpoB* gene, encoding the β-subunit of the DNA-dependent RNA polymerase [4, 5]. Among the RIF-resistant clinical isolates of *M. tuberculosis*, more than 95 % have mutations within an 81-nucleotide RIF resistance-determining region (RRDR) comprising codons 507–533 [6–9]. In contrast, isoniazid (INH) resistance is likely mediated by several molecular mechanisms. The predominant mechanism is the loss of catalase activity associated with mutations in *katG*,

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particularly in codon 315. In addition, approximately 8–30 % of INH-resistant isolates have a mutation located in the *inhA* promoter region [5]. A previous study suggested that approximately 70–80 % of INH-resistant isolates of *M. tuberculosis* could be attributed to mutations in the *katG* gene and *inhA* promoter region [10]. The major mechanism of resistance to ethambutol (EB) is associated with the mutations in the *embCAB* operon, encoding three homologous arabinosyl transferases. In particular, most of the mutations occur at codon 306 of *embB* [11]. The mutations associated with streptomycin (SM) resistance have been identified in the genes encoding ribosomal protein S12 (*rpsL*) and 16SrRNA (*rrs*), which induce alterations in the SM binding site [12, 13].

As already mentioned, more molecular background information on anti-TB drug resistances is accumulating, which has shifted the focus toward the development of reliable molecular-based methods that can rapidly detect drug resistance. Recently, many molecular methods have been developed to detect drug resistance-related mutations, including the microplate hybridization assay, polymerase chain reaction (PCR) single-strand conformational polymorphism (SSCP), and real-time PCR using fluorescent probes [14–16]. Furthermore, commercial methods for the rapid and effective detection of drug resistance in tuberculosis, including the hybridization-based line probe assay (LiPA) and Xpert MTB/RIF assay, are now available [17, 18]. However, these methods are costly and are unable to detect mutations other than a few specific mutations. On the other hand, the recently developed high resolution melting (HRM) curve assay is a simple and rapid PCR-based method for detection of DNA sequence variation by using saturating DNA dyes. In contrast to other molecular methods, the HRM assay offers the advantage of the ability of detecting any mutation in the target gene sequence.

In this study, we developed and evaluated the HRM curve assay by using real-time PCR to detect the most frequent mutations in *M. tuberculosis* isolates that showed resistance to four anti-TB drugs: RIF, INH, EB, and SM. Here we report an optimized one-step method for scanning the *rpoB*, *katG*, *mab-inhA*, *embB*, *rpsL*, and *rrs* genes of *M. tuberculosis* by using the HRM assay.

## Materials and methods

### Sample collection

In this study, 27 DNA samples were used to develop the HRM assay. Two types of mycobacterial DNA samples were collected. A total of 21 *M. tuberculosis* isolates were collected from patients with bacteriologically confirmed pulmonary TB from the National Hospital Organization (NHO) Mie-Chuo Medical Center during the period from 2000 to 2009. In addition, six DNA samples with drug-resistant mutations (strain no. ref 14-7, 14-8, 14-10, 14-11, 15-18, and 2X-1-14) were provided by the Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association (JATA) and used as the positive reference.

To further validate the HRM assay with a blinded series of isolates, 40 clinical *M. tuberculosis* isolates (strain no. 2011029–2011068) were collected from the NHO Mie-Chuo Medical Center in 2011. This study followed the guidelines of clinical study in Japan, and no information regarding patients linked to the clinical isolates was obtained.

### DNA extraction

Genomic DNA of the clinical *M. tuberculosis* isolates used for this study was extracted using an ISOPLANT kit (Nippon Gene, Japan). The extracted DNAs were diluted tenfold with TE buffer and were used as the template DNA for PCR and the HRM assay. DNA concentrations for reference DNA samples were measured using the GeneQuant pro (GE Health Care, USA).

### Drug susceptibility test

DST was performed at three different institutions: 15 isolates were tested at the NHO Mie-Chuo Medical Center, 6 isolates at the Research Institute of Tuberculosis (RIT), and 3 isolates at the Mie Prefecture Health and Environment Research Institute. A standard proportion method with 1 % Ogawa medium, which is equivalent to Lowenstein–Jensen medium, was used at the NHO Mie-Chuo Medical Center and RIT according to the Japanese guideline. A broth dilution method using BrothMIC MTB-I (Kyokuto, Japan) was used at the Mie Prefecture Health and Environment Research Institute to measure the minimum inhibitory concentrations (MICs) of each drug.

### PCR amplification and sequence analysis

Primers used for PCR and sequencing are listed in Table 1 [19–24]. PCR amplicons were purified using the QIA quick PCR purification kit (Qiagen, Germany). The nucleotide sequences of the amplified fragments were directly determined using the Terminator cycle sequencing kit (version 3.1) and the ABI PRISM 3130 Genetic Analyzer (ABI, USA). Sequence analysis was performed using GENETYX software for Windows, version 8 (Genetyx, Japan), using *M. tuberculosis* H37Rv DNA as the reference sequence.