Triplex real-time PCR melting curve analysis for detecting *Mycobacterium tuberculosis* mutations associated with resistance to second-line drugs in a single reaction

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Objectives: Resistance to anti-tuberculosis (TB) drugs has been a great challenge for global TB control. Limited tools have been developed to detect resistance to second-line drugs that are associated with extensively drug-resistant (XDR) TB. In this study we aimed to develop a simple and widely applicable assay for detecting mutations associated with second-line drug resistance in *Mycobacterium tuberculosis*.

Methods: Three dually labelled probes targeting *gyrA*, *rrs* and the promoter of *eis* were designed to detect resistance to fluoroquinolones and second-line injectable agents (capreomycin, amikacin and kanamycin). A triplex reaction with all three probes and corresponding primers was first tested against 13 isolates with different mutations in the targeted regions. Then, the triplex assay was applied to 109 second-line drug-resistant isolates in a blind manner and the results were compared with the sequencing data.

Results: All mutations in the targeted regions of 13 representative isolates could be detected through significant Tm reductions of the corresponding probe compared with the wild-type control. The detection results with 109 isolates were 100% concordant with sequencing data. Twelve ofloxacin-resistant isolates were detected as heteroresistant, indicating the coexistence of mutant and wild-type strains or the existence of different *gyrA* mutations.

Conclusions: We have developed a simple and widely applicable assay to detect second-line drug resistance of *M. tuberculosis*. This method, combined with assays for detecting first-line drug resistance, provides an efficient and reliable tool to diagnose multidrug-resistant TB and XDR-TB.

Keywords: *M. tuberculosis*, TB, drug resistance, rapid diagnosis

Introduction

Drug-resistant tuberculosis (TB), especially multidrug-resistant (MDR) TB, defined as resistance to at least isoniazid and rifampicin, represents the most urgent challenge for global TB control. MDR-TB typically requires 2 years of treatment with more expensive and toxic second-line drugs that mainly include fluoroquinolones, injectable aminoglycosides and capreomycin.¹ ² The misuse of these second-line drugs or the poor adherence of MDR patients during this long treatment can lead to the acquisition of secondary resistance and the emergence of extensively drug-resistant (XDR) TB, which is defined as MDR-TB with additional resistance to all fluoroquinolones and any of the second-line injectable agents.³ ⁴ Furthermore, the transmission of these resistant isolates could create an unmanageable TB epidemic. In China, which has the highest burden of MDR-TB in the world, ~8% of MDR-TB cases are XDR-TB, most of which results from primary transmission.⁵ ⁶ Therefore, rapid diagnosis of MDR-TB and second-line drug resistance is urgently needed to both initiate effective treatment regimens and prevent secondary transmission.

Similar to resistance to first-line drugs, *Mycobacterium tuberculosis* develops resistance to second-line drugs mainly through chromosome mutations. Resistance to fluoroquinolones is mainly associated with mutations in the fluoroquinolone resistance-determining region (QRDR) of DNA gyrase-coding genes *gyrA* and *gyrB*.⁷ The mechanism of second-line injectable drug resistance is more complicated and associated with several
genes. The rrs A1401G mutation is associated with cross-resistance to capreomycin, amikacin and kanamycin. Mutations in tlyA are responsible for capreomycin resistance and mutations in the promoter of eis are associated with low-level resistance to kanamycin.

Due to the extremely slow growth of M. tuberculosis, the traditional phenotypic drug susceptibility tests pose serious delays to the detection of resistance. Several molecular methods, including both commercial assays such as GeneXpert MTB/RIF, Genotype MTBDRplus/MTBDRsl and in-house assays such as high-resolution melting curve analysis and pyrosequencing, provide rapid diagnosis of both first- and second-line drug resistance based on the detection of resistance mutations. However, most of these methods are of high cost and some of them include complicated experimental procedures, restricting their application in resource-limited settings. Thus, there is still an urgent need for simple, cheap and rapid diagnostic tools to detect drug resistance mutations. We have recently developed a widely applicable assay based on probe melting curve analysis for detecting first-line drug resistance mutations. In the present study, we further developed a triplex real-time PCR melting curve assay to detect mutations conferring resistance to fluoroquinolones, aminoglycosides and capreomycin in M. tuberculosis.

Materials and methods

Clinical isolates, drug susceptibility tests and sequencing

Phenotypic drug susceptibility testing was routinely performed for four first-line drugs (isoniazid, rifampicin, streptomycin and ethambutol) using the indirect agar proportion method at the Tuberculosis Reference Laboratory (TRL) in the Shanghai Center for Disease Control and Prevention (CDC). In 2009, a total of 420 isolates were found to be resistant to at least one of these four drugs. These isolates were further tested for susceptibility to four second-line drugs using the indirect agar proportion method with the following concentrations: amikacin, 20 mg/L; capreomycin, 40 mg/L; kanamycin, 30 mg/L; and ofloxacin, 2 mg/L. The laboratory reference strain M. tuberculosis H37Rv, containing no mutations and susceptible to all drugs, was used as a negative control. A total of 62 ofloxacin-resistant isolates and 47 isolates resistant to any of capreomycin, amikacin and kanamycin were obtained. Among the 47 isolates that were resistant to any of capreomycin, amikacin and kanamycin, 14 were cross-resistant to all three drugs, 1 was resistant to both amikacin and kanamycin, 1 was resistant to both capreomycin and kanamycin, 5 were monoresistant to amikacin, 9 were monoresistant to capreomycin and 17 were monoresistant to kanamycin. DNA samples of all 109 clinical isolates and H37Rv were obtained by a rapid boiling method. The QRDR of gyrA was amplified and sequenced for the 62 ofloxacin-resistant isolates. Fragments of rrs 1275–1574 and the promoter regions of eis were sequenced for 47 capreomycin-, amikacin- or kanamycin-resistant isolates. The primers for amplification and sequencing are listed in Table 1.

Real-time PCR

The principle of this real-time PCR-based method was described in our previous study. Three dually labelled probes and three pairs of primers were designed to detect mutations in gyrA, rrs and the promoter of eis. Each of the three probes was labelled with a different fluorophore at the 5’ end to enable multichannel detection. A quencher was added to the 3’ end of each probe (Table 1). For each target, asymmetrical PCR was performed to accumulate the strand that is complementary to the corresponding probe. To achieve asymmetrical amplification, the final concentrations of the two primers (from one primer pair) were 20-fold different and the probe was designed to complement with the amplicons extended from the more abundant primer. All primers and probes were mixed together into a single triplex reaction. The real-time PCRs were performed on a Bio-Rad CFX96 with a final volume of 20 μL. The optimal concentrations and sequences of the primers and probes are shown in Table 1. The dTTP DNA polymerase (Promega) without 5’ to 3’ exonuclease activity was used so as to avoid hydrolysis of the probes during amplification. Betaine was introduced into the mixture at a final concentration of 1.0 M as described previously. Extracted DNA (1 μL) was added to each reaction. The cycling conditions were denaturation at 95°C for 1 min followed by 40 cycles of amplification at 95°C for 5 s, 55°C for 30 s (with a single acquisition of fluorescence) and 72°C for 20 s. The melting programme was 30 s at 95°C followed by continuous detection of fluorescence from 45°C to 85°C with a temperature increase rate of 0.5°C/step. A negative control with double-distilled water and a standard wild-type control with DNA of H37Rv were performed for each real-time PCR experiment.

Method validation and blind analysis

A selection of 13 isolates with different mutations in each target was first used to test the Tm deviations caused by the mutations and the reliability of the probes. Three repeats of real-time PCRs were performed for each isolate. The assay was then performed with all 109 clinical strains resistant to any of ofloxacin, amikacin, capreomycin and kanamycin in a blinded manner. Validity was assessed by comparison between the results of our assay and the sequencing data.

Sensitivity analysis

To test the analytical sensitivity of our method, we performed the assay with serially diluted genomic DNA (gDNA) samples of the wild-type strain H37Rv and three clinical isolates that carried the mutations rrs A1401G, eis C→T and gyrA D94G, respectively. The gDNAs of these isolates were extracted using the cetyltrimethylammonium bromide (CTAB) method.

The concentration of gDNA was determined by an ND-2000 UV-visible spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Serially diluted gDNA with concentrations ranging from 1×10^5 to 1×10^1 copies/μL was used to test the analytical sensitivity of our method. For each concentration, three technical experimental repeats were performed.

Results

Design of the probes

Three probes labelled with different fluorophores were designed to detect mutations associated with resistance to fluoroquinolones, aminoglycosides and capreomycin. For cross-resistance to capreomycin, amikacin and kanamycin, probe rrsP was designed to cover a fragment of rrs from position 1396 to position 1421. As the mutations responsible for low-level resistance to kanamycin span a long frame in the eis promoter (from position −10 to −14 and −37), we designed a special probe, eisP, to cover both regions, which allows the strand to hybridize with the probe by forming a loop structure (Figure 1). For detecting resistance to fluoroquinolones, probe gyrAP was designed to cover the QRDR of gyrA from codon 89 to codon 94. This probe did not cover the S/T (AGC/ACC) polymorphism in gyrA codon 95 as it is not associated with resistance to fluoroquinolones.
To test the ability of our design to detect drug-resistance mutations, 13 clinical isolates carrying different mutations in three probe-targeted regions of gyrA, rrs and the promoter of eis were selected for the triplex assay. All the isolates with no mutations in the targeted regions displayed highly reproducible Tm with deviations of 0.8°C (data not shown) compared with the wild-type control. By contrast, isolates carrying mutations displayed significantly lower Tms for the corresponding probes. For probe rrsP, covering 1396–1421 of rrs, mutations A1401G and C1402T led to deviations of 2.5°C and 2.8°C, respectively, from the wild-type control (Table 2; Figure 2b). For probe eispP, eis promoter mutations −8G deletion, C→T and C→A led to Tm deviations of −6°C, −7°C and −6.5°C, respectively (Table 2; Figure 2a). With regard to probe gyraP, although it was designed not to cover the polymorphisms in gyrA codon 95, a stable Tm difference of 1°C was detected between gyrA 95S (wild-type control, H37Rv) and gyrA 95T. Since this Tm deviation of 1°C is significantly larger than the standard deviations (<0.5°C) of the wild-type control, the polymorphism in gyrA codon 95 could be clearly differentiated. This deviation may be explained by the difference in the interactions between the fluorophores/quenchers and the DNA templates.22 The ROX attached

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<th>Table 1. Optimal concentrations and sequences of the primers and probes</th>
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<td><strong>Primers/probes</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
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<td><strong>Primers for sequencing and real-time PCR</strong></td>
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<td><strong>Dually labelled probes for real-time PCR</strong></td>
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<sup>a</sup>F1, forward primer for sequencing; R1, reverse primer for sequencing; F2, forward primer for real-time PCR; R2, reverse primer for real-time PCR; P, dually labelled probe.

<sup>b</sup>Nucleotides are numbered from the first position of the start codon. Negative numbers indicate positions before the start codon.

Figure 1. Illustration of the probe eispP. (a) Sequences of the eis promoter and probe eispP. The two regions associated with kanamycin resistance are marked. The broken line indicates gaps in the probe sequence. (b) Schematic representation of hybridized structure of probe eispP and the DNA template of the eis promoter. Hybridization was achieved by forming a loop in the DNA template.

**Tm deviations for mutations in rrs, gyrA and promoter of eis**

To test the ability of our design to detect drug-resistance mutations, 13 clinical isolates carrying different mutations in three probe-targeted regions of gyrA, rrs and the promoter of eis were selected for the triplex assay. All the isolates with no mutations in the targeted regions displayed highly reproducible Tm with deviations of <0.5°C (data not shown) compared with the wild-type control. By contrast, isolates carrying mutations displayed significantly lower Tm for the corresponding probes. For probe rrsP, covering 1396–1421 of rrs, mutations A1401G and C1402T led to deviations of −2.5°C and −5°C, respectively, from the wild-type control (Table 2; Figure 2b). For probe eispP, eis promoter mutations −8G deletion, C→T and C→A led to Tm deviations of −6°C, −7°C and −6.5°C, respectively (Table 2; Figure 2a). With regard to probe gyraP, although it was designed not to cover the polymorphisms in gyrA codon 95, a stable Tm difference of 1°C was detected between gyrA 95S (wild-type control, H37Rv) and gyrA 95T. Since this Tm deviation of 1°C is significantly larger than the standard deviations (<0.5°C) of the wild-type control, the polymorphism in gyrA codon 95 could be clearly differentiated. This deviation may be explained by the difference in the interactions between the fluorophores/quenchers and the DNA templates.22 The ROX attached
at the 5’-end of the probe gyraP may exhibit a stronger interaction with templates that carry the AGC in codon 95 than those that carry ACC, which led to a \( T_m \) difference of 1°C between them. The \( T_m \) deviations caused by mutations in gyrA codons 90, 91 and 94 ranged from 4.0 to 7.5°C, which were much larger than the deviation (1°C) caused by the polymorphism in gyrA codon 95 (Table 2; Figure 2c). Thus, the resistance mutations could be distinguished unambiguously from the single gyrA 95T polymorphism.

**Analytical sensitivity of the triplex assay**

To assess the analytical sensitivity of our assay, we performed the triplex reaction on serially diluted gDNAs of H37Rv and three resistant isolates with concentrations ranging from \( 1 \times 10^5 \) to \( 1 \times 10^7 \) copies/reaction. The results indicated that the triplex reaction could produce reliable \( T_m \) values for all three probes when the concentration was as low as \( 1 \times 10^5 \) copies/reaction (Figure S1, available as Supplementary data at JAC Online). As the concentrations of gDNA extracted from cultured samples are \( \sim 10^5 \) to \( 10^7 \) copies/μL, the analytical sensitivity of our method is high enough to detect second-line drug-resistance mutations from cultured isolates.

**Discussion**

Drug-resistant TB, especially MDR- and XDR-TB, is now a major threat to global TB control.\(^6\) The treatment course for drug-resistant TB is substantially more costly and laborious, with higher rates of treatment failure and mortality.\(^5,6\) Rapid diagnosis of drug resistance is essential for both initiating effective treatment and preventing the spread of drug-resistant strains.\(^3,4\) In a previous study we developed a widely applicable assay based on the melting curve analysis of dual labelled probes to detect
first-line drug-resistance mutations. Based on the same principles, we further developed a triplex assay to detect second-line drug resistance-associated mutations in a single real-time PCR.

Our assay successfully predicted 51 of the 62 (82.3%) ofloxacin-resistant isolates by detecting mutations in the QRDR of gyrA. According to previous studies, mutations in gyrA and gyrB are both responsible for resistance to fluoroquinolones. However, the frequency of gyrB mutations is very low and most fluoroquinolone resistance in clinical isolates is due to mutations in the QRDR of gyrA. Therefore, most assays for detecting fluoroquinolone resistance only include gyrA mutations. However, because there were 11 ofloxacin-resistant isolates that carried no mutations in the QRDR of gyrA in this study, we speculate that the resistances of these isolates were caused by mutations in gyrB or other mechanisms, such as active efflux pumps. Due to the limited number of isolates, the gyrA mutations tested in this study mainly belonged to the most common ones in codons 90, 91 and 94 for clinical resistance. Although our assay was supposed to be able to differentiate single-base variations, further study is needed to perform tests with other, less frequent mutations in the probe-covered region. In addition to detecting mutations accurately, our assay also shows the potential to detect fluoroquinolone heteroresistance, which is common in clinical settings and accounts for 9%–20% of fluoroquinolone-resistant cases. Among the 62 ofloxacin-resistant cases included in this study, 12 (19.4%) were correctly identified as containing both mutant and wild-type isolates or additional resistance mutations.

The mutations in rrs and the promoter of eis were successfully detected by our assay to predict cross-resistance to capreomycin, amikacin and kanamycin and low-level resistance to kanamycin, respectively. According to our results, the most common mutation, rrs A1401G, was detected in 11 of 14 (78.6%) isolates that showed cross-resistance to capreomycin, amikacin and kanamycin. According to previous studies, mutation rrs C1402T is also associated with resistance to aminoglycosides, but with a very low frequency in clinical isolates. Considering the balance between the cost and sensitivity of detection, we chose not to include this mutation in our assay. Furthermore, the three cross-resistant isolates carrying no mutations in rrs1401 also exhibited no mutation in rrs 1484 in this study. This result suggests that there are other mechanisms or unknown mutations associated with cross-resistance to aminoglycosides and capreomycin. Monoresistance to capreomycin is
associated with mutations in \textit{rrs} and \textit{tlyA}. \cite{30} In our study, one of the nine isolates monoresistant to capreomycin was found to carry the \textit{rrs} C1402T mutation. Since mutations in \textit{tlyA} occur at low frequency in clinical capreomycin-resistant isolates and are scattered across the whole gene, we chose not to detect these mutations in our assay. Furthermore, some of them have been proved to be genetic polymorphisms and have no relationship with resistance. \cite{31} Therefore, we speculate that the capreomycin resistance of the other eight isolates may be caused by mutations in \textit{tlyA} or other mechanisms. Few assays have been developed to detect mutations in the \textit{eis} promoter for prediction of low-level resistance to kanamycin.\cite{12,33} However, several recent studies indicate the importance of including these mutations to enhance sensitivity for detecting kanamycin monoresistance.\cite{11,33} In our study, 7 of the 17 isolates monoresistant to kanamycin carried mutations in the \textit{eis} promoter and all these isolates were MDR-TB. Therefore, the detection of these mutations provides valuable information to support the initiation of effective treatment regimens for MDR-TB cases. The remaining 10 isolates, which had no mutation in the promoter of \textit{eis}, may gain their resistance through mutations in \textit{rrs} that were not detected in the current study or through other mechanisms.\cite{30}

Compared with the other available assays for the rapid diagnosis of second-line drug resistance, such as GenoType MTBDR\textregistered S, high-resolution melting curve analysis and pyrosequencing, our method shows several advantages. First, our assay is widely applicable for most real-time PCR platforms, which are available in most laboratories and hospitals. Second, our assay can detect mutations in three target genes in a single reaction. Considering that most real-time PCR instruments are multichannel, the three probes were labelled with different fluorophores to avoid the overlapping of melting peaks and to enable more simplified and reliable data analysis compared with our previous assay.\cite{18} Finally, although triplex asymmetrical amplification was applied, our assay still displayed relatively high sensitivity and enabled the production of reliable 

\textit{Tm} values for concentrations as low as \( \times 10^2 \) copies/\( \mu L \) of gDNA.

In conclusion, we have developed a simple and widely applicable real-time PCR assay to detect second-line drug resistance of \textit{M. tuberculosis}. This assay, combined with the method we developed previously or other commercial assays for detecting \textit{M. tuberculosis} and first-line drug-resistance mutations,\cite{34} provides an efficient and reliable method for the rapid diagnosis of MDR- and XDR-TB.

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**Transparency declarations**

None to declare.

**Supplementary data**

Figure S1 is available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

**References**


