Evaluation of the MeltPro TB/STR assay for rapid detection of streptomycin resistance in *Mycobacterium tuberculosis*

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SUMMARY

Rapid and comprehensive detection of drug-resistance is essential for the control of tuberculosis, which has facilitated the development of molecular assays for the detection of drug-resistant mutations in *Mycobacterium tuberculosis*. We hereby assessed the analytical and clinical performance of an assay for streptomycin-resistant mutations. MeltPro TB/STR is a closed-tube, dual-color, melting curve analysis-based, real-time PCR test designed to detect 15 streptomycin-resistant mutations in *rpsL* 43, *rpsL* 88, *rrs* 513, *rrs* 514, *rrs* 517, and *rrs* 905 e 908 of *M. tuberculosis*. Analytical studies showed that the accuracy was 100%, the limit of detection was 50 e 500 bacilli per reaction, the reproducibility in the form of Tm variation was within 1.0 °C, and we could detect 20% STR resistance in mixed bacterial samples. The cross-platform study demonstrated that the assay could be performed on six models of real-time PCR instruments. A multicenter clinical study was conducted using 1056 clinical isolates, which were collected from three geographically different healthcare units, including 709 STR-susceptible and 347 STR-resistant isolates characterized on Löwenstein–Jensen solid medium by traditional drug susceptibility testing. The results showed that the clinical sensitivity and specificity of the MeltPro TB/STR was 88.8% and 95.8%, respectively. Sequencing analysis confirmed the accuracy of the mutation types. Among all the 8 mutation types detected, *rpsL* K43R (AAG → AGG), *rpsL* K88R (AAG → AGG) and *rrs* 514 A → C accounted for more than 90%. We concluded that MeltPro TB/STR represents a rapid and reliable assay for the detection of STR resistance in clinical isolates.

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1. Introduction

Tuberculosis (TB) remains a burden worldwide, and this burden is increased by drug-resistant TB. Failures to recognize and treat patients who suffer from drug-resistant TB have led to nosocomial outbreaks, increased mortality, and resistance to additional anti-TB drugs [1,2]. Streptomycin (STR), an aminocyclitol glycoside, was the first drug used to treat TB and had created a new era of TB treatment owing to the prominent effect [3,4]. Although STR is no longer regarded as a first-line drug in the US, it is still an alternative first-line anti-TB drug recommended by the World Health Organization (WHO) [5,6]. STR is recommended particularly when primary resistance to other drugs (isoniazid, rifampicin and pyrazinamide) is suspected [7]. However, high rate of STR resistance has accumulated after decades of use. In China, 27.7% of patients with new cases of TB and 37.2% of patients with previously treated TB are...
infected with Mycobacterium tuberculosis resistant to STR [8]. Such circumstances prompt an urgent need for STR resistance detection available to all patients.

Conventional cultivation-based diagnostic methods for the detection of STR resistance are slow, lack sensitivity and are labor-intensive [9,10]. Rapid molecular diagnostic approaches are introduced to detect STR resistance, such as direct DNA sequencing of PCR products [11,12], reverse dot-blot hybridization (RDBH) assay [13,14], single-strand conformation polymorphism (SSCP) analysis [15], restriction fragment length polymorphism (RFLP) method [16], as well as DNA arrays [17]. Such molecular approaches have brought critical improvements to drug-susceptibility testing (DST). However, the technical complexity, high cost, and dependence on dedicated laboratory facilities hinder their application, especially in low-resource, high-burden regions. So far, a diagnostic assay dedicated to the detection of STR resistance in M. tuberculosis remains to be available.

MeltPro TB/STR (Zeesan, Xiamen, China), is a qualitative diagnostic assay developed based on melting curve analysis using dual-labeled, self-quenched probes [18]. This assay was designed to detect 15 mutations of rpsL, rpsL, rrs, rrs, rrs, rrs, and rrs, which covers more than 98% of STR-resistant isolates with point mutations, accounting for 65%–90% STR-resistant isolates [19–21]. The MeltPro assay is a closed-tube format performed in a real-time PCR instrument, from which the mutation information is retrieved based on the melting temperature (Tm) shift from the wild-type. One distinctive feature of this assay is its ease-of-use due to the omission of those complex post PCR manipulations.

In this study, we evaluated MeltPro TB/STR assay systematically with respect to analytical and clinical performance. For the analytical performance evaluation, the accuracy, the limit of detection, the reproducibility, and the detectability of mixed bacterial samples were studied. For the clinical performance evaluation, a multicenter validation was performed that enrolled 1056 cultured isolates collected from three geographically different healthcare units. Bacterial cultivating-based DST results were used for comparison and sequencing was used to clarify those inconsistent results and to confirm the mutation types.

2. Materials and methods

2.1. Clinical isolates

A total of 1056 clinical isolates were collected that met the following eligibility criteria: (a) all were M. tuberculosis, and (b) all isolates were previously characterized by DST on Löwenstein–Jensen (L–J) solid medium in accordance with a standard protocol [22]. STR resistance was defined as the growth of more than 1% colonies on L–J medium containing the critical concentration of 4 μg/mL of STR when compared to the growth on drug-free medium. The clinical isolates were from three healthcare units: 331 isolates were from Southern China (Shenzhen Center for Chronic Disease Control, Shenzhen, Guangdong), 394 isolates were from Central China (Henan Center for Disease Control and Prevention, Zhengzhou, Henan), and 331 isolates were from Northern China (the 309th Hospital of Chinese PLA, Beijing). The clinical isolates were randomly selected from the culture collection centers of the three healthcare units. The collected isolates were confirmed to be M. tuberculosis complex by using p-nitrobenzoic acid (PNB) growth experiment. All the samples were numbered anonymously and no patient information was available.

2.2. DNA extraction

DNA was extracted by a heating lysis method [23] unless otherwise noted. The supernatant was kept at –20 °C before use.

2.3. PCR and melting curve analysis

The program for amplification and melting curve analysis on a Bio-Rad CFX 96 real-time PCR machine (Bio-Rad, Hercules, CA) was as follows: for each sample, 5 μL of extracted M. tuberculosis DNA was added to reaction 1 and 2, respectively, each containing 20 μL of PCR mix. The amplification program started with a contamination control procedure of 2 min at 50 °C to prevent carry-over of DNA amplicon using uracil–N-glycosylase, followed by a denaturation step at 95 °C for 10 min, 10 cycles 95 °C for 10 s, 70 °C for 20 s (–1 °C/cycle) and 75 °C for 20 s, and 30 cycles of 95 °C for 10 s, 60 °C for 20 s and 75 °C for 20 s. Melting curve analysis consisted of a denaturation step of 2 min at 95 °C, a hybridization step of 2 min at 40 °C, and a stepwise temperature increase from 40 °C to 80 °C at 0.5 °C/step with 5 s stop between each step. Fluorescence was recorded in both the FAM and TET channels. The melting curves were obtained by plotting the negative derivative of fluorescence over temperature (−dTm/dT) versus the temperature (T). The Tm values were obtained by identification of the peaks of the melting curves.

2.4. MeltPro TB/STR assay verification

We first tested the validity of MeltPro TB/STR assay by using a company supplied reference panel, which included 10 wild-type samples, eight mutant samples with different mutation types, four samples (two wild-type samples and two mutant samples) for testing the limit of detection, five mixed bacterial samples, and two (one wild-type and one mutant) samples for reproducibility testing (each sample was repeated detected ten times) (see Table S1 in the supporting information). These samples were inactivated bacterial suspensions and stored at –20 °C before use. When used for testing, each sample was mixed with an equal volume of TB DNA extraction buffer. DNA was extracted using the heating lysis method as described. A valid assay should meet the following eligible criteria: The variation of the Tm values should be within 1 °C for the wild-type samples and the Tm shift value (ΔTm, which is calculated by the Tm difference between the wild-type and mutant) should be more than 2 °C for the mutant samples from at least one detection channel. The limit of detection should be as low as 500 bacilli per reaction. For the detection of mixed bacterial samples, the mutant samples should be correctly detected when their percentage in the mixture is no less than 40%.

2.5. Limit of detection study

Wild-type DNA was used for this study. Purified wild-type DNA was isolated from cultured M. tuberculosis using the Axyprep™ Bacterium Genomic DNA Miniprep Kit (Axxygen Biosciences, Union City, CA). The DNA was then quantified using UV spectroscopy and serially diluted in 10-fold with TB DNA extraction buffer, yielding DNA concentrations ranging from 2 × 10² copies/mL to 2 × 10⁴ copies/mL. Negative control reagent was used as no-template control.

2.6. Cross-reactivity with other mycobacterial species

Cross-reactivity was studied by using 37 nontuberculous mycobacteria (NTM) strains obtained from the National Institutes for Food and Drug Control (Beijing, China). The 37 NTM strains were: Mycobacterium avium, Mycobacterium intracellulare, Mycobacterium xenopi, Mycobacterium terrae, Mycobacterium gastri, Mycobacterium nonchromogenic, Mycobacterium shimoidei, Mycobacterium triviale, Mycobacterium malmonense, Mycobacte- rium kansasii, Mycobacterium marinum, Mycobacterium simiae,
Mycobacterium asiaticum, Mycobacterium scrofulaceum, Mycobacterium gordonae, Mycobacterium szulgai, Mycobacterium chelonae, Mycobacterium abscessus, Mycobacterium fortuitum, Mycobacterium smegmatis, Mycobacterium phlei, Mycobacterium aurum, Mycobacterium lentiflavum, Mycobacterium gilvum, Mycobacterium neoaurum, Mycobacterium diesterifer, Mycobacterium vaccae, Mycobacterium farcinogenes, Mycobacterium aichiiense, Mycobacterium duvalii, Mycobacterium gadium, Mycobacterium komossense, Mycobacterium obuense, Mycobacterium parafortuitum, Mycobacterium rhodesiae, Mycobacterium pulveris, and Mycobacterium senegalense. These were inactivated bacterial suspensions with a concentration of 10^6 bacilli per mL and the DNAs were extracted using the heating lysis method as described.

2.7. Detection of STR resistance in mixed bacterial samples

Patients infected with a mixture of STR-susceptible and STR-resistant M. tuberculosis could complicate resistance detection. To evaluate the detection ability of mixed bacterial samples of the assay, we mixed wild-type DNA with a mutant DNA (rpsL 43AGG) to create artificial DNA mixtures with percentages of mutant DNA of 0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100%, respectively at a final concentration of 10^6 copies/mL.

2.8. Cross-platform compatibility evaluation

Six brands of real-time PCR machines, i.e., Bio-Rad CFX 96, ABI 7500 (Life Technologies, Carlsbad, CA), Roche LightCycler 480 II (Roche, Rotkreuz, Switzerland), Rotor-Gene 6000 (Corbett Research, Mortlake, Australia), Stratagene Mx3005P (Agilent, Santa Clara, CA), and SLAN-96P (Zeesan, Xiamen, China) were used to evaluate the cross-platform compatibility program. The reference panel for validity test was used. Identical PCR conditions were used in all instruments except that the melting curve analysis program was adapted to each platform. The variation of the Tm values should be within 1 °C for the wild-type samples and the Tm shift (from at least one detection channel) should be more than 2 °C for the mutant samples.

2.9. Clinical study

The double-blind validation was performed at each healthcare unit as previously described [23]. The operators were blind to the DST and sequencing results. The clinical sensitivity, clinical specificity, positive predictive value, negative predi tive value and diagnostic accuracy were calculated respectively.

2.10. DNA sequencing

Three PCR reactions were carried out for the sequencing of the four corresponding regions detected by the MeltPro TB/STR assay: rpsL 43 and 88; rrs 513, rrs 514, rrs 517; and rrs 905–908, respectively. The PCR components were identical: 25–μL PCR reaction contained 75 mmol/L Tris–HCl (pH 9.0), 20 mmol/L (NH4)2SO4, 0.1% (v/v) Tween 20, 3 mmol/L MgCl2, 2% DMSO, 80 μmol/L dNTP mixture, 0.4 μmol/L of each primer (see Table S2 in the supporting information), 1.0 U Taq polymerase (Takara, Dalian, China), and 5 μL DNA. The amplification program started with a denaturation step at 95 °C for 5 min, followed by a 10 cycles of 95 °C for 10 s, 70 °C for 20 s (−1 °C/cycle) and 72 °C for 20 s, and continued by another 45 cycles of 95 °C for 10 s, 60 °C for 20 s and 72 °C for 20 s. PCR products were sequenced using the forward primers of each gene (BGI, Shenzhen, China). The sequencing results were analyzed with ClustalX software (http://www.clustal.org/).

2.11. Statistical analysis

The sensitivity and specificity of this assay compared to phenotypic DST were calculated. Forest plots displaying sensitivity and specificity estimates and their 95% confidence intervals (CIs) for each specimen were created by using Meta-Disc software, version 1.4 (http://meta-disc.software.informer.com/1.4).

3. Results

3.1. The design of MeltPro TB/STR assay

MeltPro TB/STR assay covers 9 mutant sites involving 15 mutation types in rpsL 43, rpsL 88, rrs 513, rrs 514, rrs 517, and rrs 905–908 in a dual-color, dual-reaction assay (see Figure S1 in the supporting information). Reaction 1 detects two mutations at rpsL 43 in the FAM channel and two mutations at rpsL 88 in the TET channel. Reaction 2 detects four mutations at rrs 513, rrs 514 and rrs 517 in the FAM channel and seven mutations at rrs 905–908 in the TET channel. For a M. tuberculosis strain, the result of this assay will give four Tm values, which could identify the location of the mutation based on the type of channel and reaction. According to the MeltPro TB/STR assay, all mixed bacterial samples are judged to be mutants regardless of their mutant percentage.

3.2. Verification of MeltPro TB/STR assay

Our evaluation study started with the preliminary validation of the assay by using the company supplied reference panel, which contained a set of samples for different aims. The accuracy testing result showed that all 10 wild-type and 8 mutant samples gave qualified Tm values (Figure 1 and Table S3 in the supporting information). The Tm values of the four peaks from each of the 10 wild-type samples had variation between 0 and 1.0 °C. The Tm values of the 8 mutant samples each had one or two Tm shifts from the wild-type strain larger than 3 °C. The four samples for the determination of the limit of detection were correctly detected, which fulfilled the criteria that the limit of detection was as low as 500 bacilli per reaction. Among the five mixed bacterial samples, mutant ratio of 40% and 50% showed double peaks corresponding with the mutant and wild-type samples, whereas mutant ratio of 20%, 30%, and 75% showed one major peak but with a shoulder peak, by which the mixed nature could also be recognized. These results demonstrated the validity of the assay in the detection of mixed bacterial samples. The reproducibility testing results showed that 10 Tm values of the four peaks obtained from the two samples gave Tm value variation between 0 and 1.0 °C, which was within the valid range of ±1.0 °C. Taken together, the above results demonstrated the validity of the assay to be evaluated.

3.3. Limit of detection (LOD)

We tested the LOD of this assay using purified wild-type M. tuberculosis DNA. As shown in Figure 2, except in FAM channel of reaction 2, where DNAs concentration of 50 copies per reaction showed wild-type peak, FAM and TET channel of reaction 1, TET channel of reaction 2 all showed wild-type peaks at 500 copies per
reaction. We thus concluded that limit of detection of the assay was 50–500 genomic copies per reaction.

3.4. Cross-reactivity with other mycobacterial species

We tested the specificity of the assay for *M. tuberculosis* detection by cross-reactivity experiments using 37 NTM strains. The results showed that none of the 37 NTM strains generated peaks in reaction 1 whereas all of them gave peaks corresponding with the wild-type *M. tuberculosis* in reaction 2 (Figure 3). According to MeltPro TB/STR assay, a valid result for *M. tuberculosis* should have all four peaks in this dual-color, dual-reaction assay. We thus concluded that the NTM strains showed no cross-reactivity with *M. tuberculosis* in this assay.

3.5. Detection of STR resistance in mixed bacterial samples

These isolates were detected either as double peaks or as a single peak with alternated shape, depending on the ratio of mutant *M. tuberculosis*. As shown in Figure 4, a gradual transition was seen from the wild-type peak to the mutant peak when the percentage of mutation increased. At a mutant ratio of 20%–40%, the melting curve could be differentiated from the wild-type peak by the increasingly widening peaks. At a mutant ratio of 50%, the melting curve became readily distinguished from the wild-type peak by the appearance of a double-peak. These results were concordant with the sensitivity to detect mixed bacterial samples (40% mutant) preset by the assay.

3.6. Cross-platform compatibility evaluation

The cross-platform compatibility of the MeltPro TB/STR assay was evaluated on six mainstream real-time PCR machines using the reference panel for accuracy of mutation detection. The results showed that, although the absolute *Tm* values varied somewhat among different machines, the variations of *Tm* values from one machine were within 1 °C for the wild-type samples and the *Tm* shifts of the mutant samples were all more than 3 °C. These results demonstrated that both the wild-type and the mutant samples could be reliably detected with each of the six real-time PCR machines.

3.7. Clinical study

A total of 1056 *M. tuberculosis* clinical isolates, including 347 STR-resistant and 709 STR-susceptible isolates collected from three...
healthcare units, were used to validate the clinical performance of MeltPro TB/STR assay. The sensitivity and specificity with reference to DST were 88.8% (308/347) and 95.8% (679/709) (Table 1), respectively. The positive predictive value, negative predictive value, and diagnostic accuracy were 91.1%, 94.6%, and 93.5%, respectively. Among 338 mutation-containing isolates, 8 isolates were wild-type. Taken together, for all the discordant isolates, DNA sequencing results confirmed the correctness of the MeltPro TB/STR assay.

Sequencing analysis results further indicated that MeltPro TB/STR assay could identify the exact mutation type. Of the 338 STR-resistant isolates detected by the MeltPro TB/STR assay, 97 isolates that harbored double mutations. Further analysis of the frequencies of the mutation types according to their geographical locations revealed no difference, suggesting that the above mutations would be homogeneous in China.

The large number of mutant isolates detected allowed us to reveal information about the frequency of STR-resistant mutations. As can be seen, most mutations are located in 

<table>
<thead>
<tr>
<th>Site and total</th>
<th>Phenotypic drug-susceptibility testing</th>
<th>Phenotypic drug-susceptibility testing and discrepant resolution by sequencing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sensitivity</td>
<td>Specificity</td>
</tr>
<tr>
<td></td>
<td>no./total (no. (%))</td>
<td>no./total (no. (%))</td>
</tr>
<tr>
<td>Beijing</td>
<td>116/119 (97.5)</td>
<td>212/212 (100.0)</td>
</tr>
<tr>
<td>Henan</td>
<td>115/130 (88.5)</td>
<td>257/264 (97.3)</td>
</tr>
<tr>
<td>Shenzhen</td>
<td>77/98 (78.6)</td>
<td>210/233 (90.1)</td>
</tr>
<tr>
<td>Total</td>
<td>308/347 (88.8)</td>
<td>679/709 (95.8)</td>
</tr>
</tbody>
</table>

95% CI (%): 85.0—91.9, 94.0—97.1, 98.9—100.0
99.5—100.0
Pie chart depicts the distribution of these mutations. The numbers represent the mutation but not the isolates.

4. Discussion

The correspondence between Table 2 drug resistant TB. In this regard, an assay specifically designed to detect STR-resistant mutations, becomes important in clinical settings [25]. In this study, we demonstrated that the MeltPro TB/STR could rapidly identify the mutations most frequently associated with STR resistance from cultured M. tuberculosis samples. The rapidity lies in its homogenous detection nature, which is based on melting curve analysis using unique dual-labeled, self-quenched probes that have been proved to efficient in the detection of multiple mutations in a single reaction [18,23,26–28]. The high accuracy of mutation detection and the broad coverage of STR-resistant mutations, i.e., 15 mutation types on 9 mutant sites of M. tuberculosis, forms solid base for its high clinical sensitivity.

The analytical study showed the robustness of the MeltPro TB/STR assay in many respects. Its accuracy was demonstrated in the correct identification of 8 different mutant samples and 10 wild-type samples with high reproducibility. The analytical sensitivity was reflected in a limit of detection of 50–500 bacilli per reaction. Moreover, as low as 20% mutant samples present in the wild-type population could be identified, and this is close to the percentage that can be detected by Sanger sequencing. The detectability of mixed bacterial samples would allow close monitoring of the disease status and timely adoption of alternative treatment strategy. Importantly, the MeltPro TB/STR assay showed no cross-reactivity with common NTM strains. This feature not only ensures the specificity of the assay but also may extend its application to varied clinical samples. The cross-platform compatibility study showed that the MeltPro TB/STR assay could be run on all six models of real-time PCR machines, thereby making it an open assay for nearly all mainstream real-time PCR instruments on the market.

The clinical validation involved 1056 clinical isolates including 347 STR-resistant and 709 STR-susceptible isolates. To our best knowledge, this represented the largest scale validation study in term of sample number ever conducted for an STR-resistance assay. The clinical sensitivity and clinical specificity obtained was 88.8% and 95.8%, respectively, which were higher than previously reported [29,30]. We attributed this high clinical sensitivity mainly to the wide coverage of STR-resistant mutations. The reported molecular assays for STR-resistance usually detected mutations of rpsL only. By comparison, MeltPro TB/STR covered additionally seven mutant sites in rrs. Moreover, the high accuracy in mutation detection (100% concordant with sequencing analysis) and the ability in the detection of mixed bacterial samples also made contributions. Notably, all mutations detected in this study could be identified through their unique Tm values. As rpsL mutations were mainly related with high-level drug resistance whereas rrs mutations were mainly related with intermediate-level and low-level drug resistance, identification of mutation types offered a second layer of information in understanding the relationship between the genotypic and phenotypic feature of drug-resistant M. tuberculosis [11,31].

Regarding the 39 (3.69%, 39/1056) samples found to be insensitive to STR by phenotypic DST but wild-type by MeltPro TB/STR assay, additional mechanisms mediating STR resistance might be involved, e.g., cell envelope permeability changes, production of aminoglycoside-modifying enzymes, and alterations in other ribosomal molecules [32]. Identification of these STR-resistant isolates lacking rpsL or rrs substitutions should be valuable in search for alternative resistance mechanisms. The 30 (2.84%, 30/1056) discrepant samples were those susceptible to STR by phenotypic DST but mutation-containing by both MeltPro TB/STR assay and sequencing. After checking the mutation types, we observed they

![Mutation frequency](image)

**Table 2**
The correspondence between Tm value and the type of mutation.

<table>
<thead>
<tr>
<th>ΔTm (°C)*</th>
<th>Tm Prediction</th>
<th>Sequencing data</th>
<th>Concordance rate—no./total no. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5–6.0</td>
<td>rpsL 43 AAG → AGG</td>
<td>rpsL 43 AAG → AGG</td>
<td>57/57 (100.0)</td>
</tr>
<tr>
<td>6.0–6.5</td>
<td>rpsL 88 AAG → AGG</td>
<td>rpsL 88 AAG → AGG</td>
<td>14/14 (100.0)</td>
</tr>
<tr>
<td>4.0–4.5</td>
<td>rrs 514 A → C</td>
<td>rrs 514 A → C</td>
<td>10/10 (100.0)</td>
</tr>
<tr>
<td>7.0</td>
<td>rrs 514 A → T</td>
<td>rrs 514 A → T</td>
<td>1/1 (100.0)</td>
</tr>
<tr>
<td>12.0–13.0</td>
<td>rrs 517C → T</td>
<td>rrs 517C → T</td>
<td>6/6 (100.0)</td>
</tr>
<tr>
<td>5.5</td>
<td>rrs 906 A → G</td>
<td>rrs 906 A → G</td>
<td>3/3 (100.0)</td>
</tr>
<tr>
<td>9.0; 4.5</td>
<td>rpsL 43 AAG → ACG;</td>
<td>rpsL 43 AAG → ACG;</td>
<td>1/1 (100.0)</td>
</tr>
<tr>
<td>5.5; 12.0</td>
<td>rpsL 43 AAG → AGG;</td>
<td>rpsL 43 AAG → AGG;</td>
<td>1/1 (100.0)</td>
</tr>
<tr>
<td>5.0; 12.0</td>
<td>rpsL 88 AAG → CAG;</td>
<td>rpsL 88 AAG → CAG;</td>
<td>1/1 (100.0)</td>
</tr>
<tr>
<td>0</td>
<td>rpsL 43 AAG and AGG</td>
<td>rpsL 43 AAG and AGG</td>
<td>1/1 (100.0)</td>
</tr>
<tr>
<td>0 and 6.5</td>
<td>rpsL 88 AAG and AGG</td>
<td>rpsL 88 AAG and AGG</td>
<td>1/1 (100.0)</td>
</tr>
</tbody>
</table>

* ΔTm = Tm (wild-type) – Tm (mutant).
1 The shape of melting curve changes, and also shows wild-type Tm value.
2 The melting curve results show double-peak.
were all STR resistance associated. This could be explained by the potential technical problems with phenotypic DST, which could give false-negative results due to the low sensitivity according to our experience as well as reported in the literature [33].

One distinct feature of MeltPro TB/STR assay is that it could detect mixed bacterial samples. Among the 1056 clinical isolates, we detected 8 mixed bacterial isolates, which were all resistant to STR, and subsequent sequencing data confirmed our results. Mutation detection is challenging in real-time PCR assays in a background of large amounts of wild-type samples. We postulated that the large \( \Delta T_m \) between wild-type and mutant targets would enable the MeltPro assay to detect mutant targets even when they were present as a minor proportion. Nevertheless, as a molecular method, the MeltPro TB/STR assay showed no advantage over the culture-based DST methods which can usually detect <1% STR-resistant bacteria in a mixture of susceptible and resistant \( M. tuberculosis \).

As a real-time PCR-based assay, MeltPro TB/STR assay featured ease-of-use and high throughput. The entire assay involved only a DNA extraction procedure and a single step of adding DNA into two reaction tubes, and the following procedure could be finished within 3 h. Up to 48 samples could be detected in one assay and even more could be processed if combined with the use of standard thermal cyclers. Such a high throughput enables large-scale screening of STR resistance feasible in a medium-sized laboratory. Moreover, the MeltPro TB/STR assay requires no post-PCR manipulation, thereby minimizing the chances for carryover contamination in the laboratory, facilitating the wide acceptance in clinical settings. Of noted, mutations in rrss genes are also associated with kanamycin resistance despite that the mutation sites are different from those associated with STR. It would be possible to develop similar assays for the detection of resistance to kanamycin.

This evaluation study also revealed some limitations of the MeltPro TB/STR assay that may deserve further improvement. First, with the availability of multi-color real-time PCR machines, it would be more advantageous to combine the two reactions into a single reaction, which would further simplify the manipulation and increase the throughput. Second, this evaluation study was restricted to clinical isolated bacteria. The performance of the MeltPro TB/STR assay in real clinical samples such as sputum remains to be validated. At present stage, drug resistance analysis for \( M. tuberculosis \) is routinely conducted after cultivation. Therefore, MeltPro TB/STR assay could be recommended for rapid detection of the STR-resistance status according to the current protocol. It is noted that none of the NTM showed cross-reactivity with STR-resistance detection, suggesting that the existence of NTM in the sample would not interfere with the detection. This result conferred the potential use for direct detection of STR resistance in clinical samples where a diversity of bacterial species might exist.

In conclusion, the MeltPro TB/STR assay features wide coverage of STR-resistant mutations. The probe-based melting curve analysis ensures its high reproducibility in the detection. Meanwhile, the closed-tube working format is characterized by low risk of amplification products contamination, high sample throughput, and simple manipulations. Lastly, the compatibility with mainstream real-time PCR machines allows easy access to different clinical settings. We therefore expect that the MeltPro TB/STR assay could provide a valuable diagnostic tool for detection of STR resistance in \( M. tuberculosis \).

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Competing interests: Qingge Li is one of the inventors who earn royalties for dual-labeled, self-quenched probes for melting curve analysis.

Ethical approval: Not required.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jtube.2014.12.004.

References


