The MeltPro TB/INH assay, recently approved by the Chinese Food and Drug Administration, is a closed-tube, dual-color, melting curve analysis-based, real-time PCR test specially designed to detect 30 isoniazid (INH) resistance mutations in katG position 315 (katG 315), the inhA promoter (positions −17 to −8), inhA position 94, and the ahpC promoter (positions −44 to −30 and −15 to 3) of Mycobacterium tuberculosis. Here we evaluated both the analytical performance and clinical performance of this assay. Analytical studies with corresponding panels demonstrated that the accuracy for detection of different mutation types (10 wild-type samples and 12 mutant type samples), the limit of detection (2 × 10³ to 2 × 10⁴ bacilli/ml), reproducibility (standard deviation [SD], <0.4°C), and the lowest heteroresistance level (40%) all met the parameters preset by the kit. The assay could be run on five types of real-time PCR machines, with the shortest running time (105 min) obtained with the LightCycler 480 II. Clinical studies enrolled 1,096 clinical isolates collected from three geographically different tuberculosis centers, including 437 INH-resistant isolates and 659 INH-susceptible isolates characterized by traditional drug susceptibility testing on Löwenstein-Jensen solid medium. The clinical sensitivity and specificity of the MeltPro TB/INH assay were 90.8% and 90%. Overall, the MeltPro TB/INH assay represents a reliable and rapid tool for the detection of INH resistance in clinical isolates.
curve analysis using dually labeled, self-quenched probes (11). These real-time PCR platform-based assays are easy to use, cover a wide range of drug resistance mutations, and can detect heterogeneous samples, therefore representing a new generation of detection format for drug-resistant TB (12–16). In 2013, two such assays developed by Zeesan Biotech (Xiamen, China)—one for INH resistance and one for RIF resistance—were officially approved by the China Food and Drug Administration (CFDA), marking the legal use of these melting curve analysis-based assays for diagnosis in clinical settings. In the present study, we evaluated the MeltPro TB/INH assay, which is a qualitative in vitro diagnostic method designed to detect INH resistance mutations in katG position 315 (katG 315), the inhA promoter region (positions –17 to –8), inhA position 94 (inhA 94), and the ahpC promoter (positions –44 to –30 and –15 to 3) of M. tuberculosis. The mutation information is retrieved based on the melting temperature (Tm) shift from the wild type. Both analytical performance and clinical performance were studied, and the latter evaluation was conducted by a multicenter validation study that enrolled 1096 clinical isolates collected from three geographically different health care units in China. Bacterial cultivation-based results were used for comparison, and sequencing was used to confirm the mutation types and to clarify those showing inconsistent results between the two methods.

**MATERIALS AND METHODS**

**Clinical isolates.** A total of 1,096 clinical M. tuberculosis isolates collected from three geographically different health care units were used for the clinical validation study. Among them, 357 isolates were from southern China (Shenzhen Center for Chronic Disease Control, Shenzhen, Guangdong), 408 isolates were from central China (Henan Center for Disease Control and Prevention, Zhengzhou, Henan), and 331 isolates were from northern China (309th Hospital of Chinese PLA, Beijing). All isolates were previously characterized by drug susceptibility testing (DST) on Löwenstein-Jensen (L-J) solid medium following a standard proportion protocol (17). INH resistance was defined as the growth of more than 1% of colonies on agar containing the critical concentration of 0.2 μg/ml of INH compared to growth on drug-free control medium.

**DNA extraction.** DNA extraction was carried out by a heating lysis method unless otherwise noted. For M. tuberculosis cultured on solid medium, we collected one ring of bacteria with a 22 standard wire gauge inoculation loop and then suspended them in 250 μl of drug-free drug-free liquid medium, we collected 1 ml of cultured bacteria, centrifuged them at 15,000 rpm for 20 min, and then suspended the precipitate in 250 μl of distilled water. The suspended bacteria were incubated at 99°C for 20 min, followed by centrifugation at 15,000 × g for 10 min. The supernatant was kept at –20°C before being used as a DNA template.

**PCR and melting curve analysis.** The program for amplification and melting curve analysis on a Bio-Rad CFX96 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 mixtures 1 and 2, respectively. Each reaction mixture contained 20 μl of PCR mix and 5 μl of template. The reaction started with a contamination control procedure of 2 min at 50°C to prevent carryover of DNA amplicons using uracil-N-glycosylase. After a denaturation step at 95°C for 5 min, a touchdown program was performed as 10 cycles of 95°C for 10 s and then 71°C for 25 s (–1°C/ cycle) and 75°C for 30 s, followed by 45 cycles of 95°C for 10 s, 61°C for 25 s, and 75°C for 25 s. Melting curve analysis was started with 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 1°C/step with a 5-s stop between each step. Fluorescence was recorded at 6-carboxyfluorescein (FAM) and TET channels. Melting curve was obtained by plotting the negative derivative of fluorescence with respect to temperature versus temperature (–dF/dT), and the Tm values were obtained by identifying the peaks of the melting curves.

**Analytical studies.** The items for analytical studies include the accuracy of mutation detection, reproducibility, limit of detection, heteroresistance level, and cross-platform compatibility. Each item was studied by using the corresponding reference panel, which was prepared using a standard strain, H37Rv (National Tuberculosis Reference Laboratory, National Centre of Diseases Control and Prevention, Beijing, China), and clinical isolates that harbor different mutation types.

**Reference panel for accuracy of mutation detection.** Ten samples of the wild-type strain (104 bacilli/ml) and 12 samples having different mutation types (105–108 bacilli/ml) were included in this reference panel. A blind test was performed to detect all of the samples. The variation of Tm values should be within 1°C for the wild type, and the Tm shift (from at least one detection channel) value should be more than 2°C for the mutant samples.

**Reproducibility evaluation.** Reproducibility assays were carried out using wild-type strains at 2 × 106 and 2 × 103 bacilli/ml, respectively, within a period of 20 days. Each dilution was detected in duplicate and twice within 1 day using identical lots of the kit. The within-run and between-run variations were calculated according to the Guideline for Evaluation of Analytical Performance of In Vitro Diagnostic Kits (18). The largest standard deviation of Tm should be smaller than 0.5°C, and the Tm variation should be <1.0°C.

**Reference panel for the limit of detection.** A wild-type strain and a mutant strain (katG 315 [AGC→ACC]) were included in this reference panel. Both strains were diluted to 2 × 106, 2 × 105, 2 × 104, 2 × 103, and 2 × 102 bacilli/ml. Template DNA was prepared using an AsyPrep bacterial genomic DNA MiniPrep kit (Axygen Scientific, Inc., Union City, CA). Each sample was detected in triplicate at 2 × 103 and 2 × 102 and 2 × 101 and 2 × 100 bacilli/ml. The limit of detection should be more than 2 × 101 bacilli/ml.

**Heteroresistance level evaluation.** A series of mixtures of a wild-type strain and a mutant strain (katG 315 [AGC→ACC]) were used for heteroresistance evaluation. The overall concentrations of the mixtures were 1 × 104 and 1 × 102 bacilli/ml, respectively. The percentages of the mutant strain in the two mixtures were 0, 5, 15, 30, 40, 50, 60, 70, 80, 90, and 100%, respectively. The qualified results are that mutant strains should be correctly detected when their percentage in the mixture is >40%.

**Cross-platform compatibility evaluation.** Five models of real-time PCR machines (i.e., Bio-Rad CFX96, ABI 7500 [Life Technologies, Carlsbad, CA], Roche LightCycler 480 II [Roche, Rotkreuz, Switzerland], Ro-tor-Genie 6000 [Corbett Research, Mortlake, Australia], and Stratagene Mx3005P [Agilent, Santa Clara, CA]) were used to evaluate cross-platform compatibility. The reference panel for accuracy of mutation detection was used for this evaluation. Identical PCR conditions were used in all instruments, except that the melting curve analysis program was adapted for each machine. The variation of Tm values should be within 1°C for the wild type, and the Tm shift (from at least one detection channel) should be more than 2°C for the mutant samples.

**Clinical study.** The clinical validation study was performed at each health care unit. All sample isolates were renumbered by a technician who was solely in charge of the data collection and statistical analysis. The coded samples from each unit were analyzed by a second technician from the corresponding laboratories. The results from each laboratory were coded samples from each unit were analyzed by a second technician from the corresponding laboratories. The results from each laboratory were coded. The within-run and between-run variations were calculated according to the Guideline for Evaluation of Analytical Performance of In Vitro Diagnostic Kits (18). The largest standard deviation of Tm should be smaller than 0.5°C, and the Tm variation should be <1.0°C.

**DNA sequencing.** Four PCRs were carried out for sequencing of the four corresponding regions detected by the MeltPro TB/INH assay. The PCR components for katG 315 were as follows: 25-μl PCR mixture contained 10 mmol/liter Tris–HCl (pH 8.6), 50 mmol/liter KCl, 1.5 mmol/liter MgCl2, 1.0 U Taq DNA polymerase, 200 μmol/liter deoxynucleoside triphosphates (dNTPs), 5% glycerol, 0.2 μmol/liter SEQ-katG-F and

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TABLE 1 Performance characteristics of the MeltPro TB/INH assay with the reference panel for accuracy of mutation detection

<table>
<thead>
<tr>
<th>Name</th>
<th>Mutation type(s)</th>
<th>( T_m ) (°C)ᵃ</th>
<th>katG 315</th>
<th>inhA promoter</th>
<th>ahpC promoter</th>
<th>inhA 94</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type strains</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W1 to W10</td>
<td></td>
<td>68 ± 0</td>
<td>63 ± 0</td>
<td>58 ± 0, 68.3 ± 0.7</td>
<td>66 ± 0</td>
<td></td>
</tr>
<tr>
<td>Mutants</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>katG S315T (AGC→ACC)</td>
<td>63</td>
<td>63</td>
<td>58, 69</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>M2</td>
<td>katG S315N (AGG→AAG)</td>
<td>62</td>
<td>63</td>
<td>58, 69</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>M3</td>
<td>katG S315G (AGG→ACG)</td>
<td>58</td>
<td>63</td>
<td>58, 69</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>M4</td>
<td>katG S315G (AGG→GCG) and inhA promoter −15C→T</td>
<td>61</td>
<td>58</td>
<td>58, 69</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>M5</td>
<td>inhA promoter −15C→T</td>
<td>68</td>
<td>58</td>
<td>58, 69</td>
<td>66</td>
<td></td>
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<tr>
<td>M6</td>
<td>inhA promoter −8T→C</td>
<td>68</td>
<td>57</td>
<td>58, 69</td>
<td>66</td>
<td></td>
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<tr>
<td>M7</td>
<td>ahpC promoter −32G→A</td>
<td>68</td>
<td>63</td>
<td>53, 69</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>M8</td>
<td>ahpC promoter −9G→A</td>
<td>68</td>
<td>63</td>
<td>58, 64</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>M9</td>
<td>katG del and ahpC promoter −15C→T</td>
<td>—ᵇ</td>
<td>—ᵇ</td>
<td>—ᵇ</td>
<td>—ᵇ</td>
<td></td>
</tr>
<tr>
<td>M10</td>
<td>katG del and ahpC promoter −10C→T</td>
<td>—ᵇ</td>
<td>—ᵇ</td>
<td>—ᵇ</td>
<td>—ᵇ</td>
<td></td>
</tr>
<tr>
<td>M11</td>
<td>katG del and ahpC promoter −6G→A</td>
<td>—ᵇ</td>
<td>—ᵇ</td>
<td>59, 59ᵇ</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>M12</td>
<td>inhA promoter −15C→T and inhA S94A (TCG→GCG)</td>
<td>68</td>
<td>58</td>
<td>58, 69</td>
<td>63</td>
<td></td>
</tr>
</tbody>
</table>

ᵃ Values for wild-type strains W1 to W10 are shown as means ± SD. Boldface values represent the \( T_m \) values of mutant types.
b —, no peak was detected because of deletion.

The mutation causes merging of the mutant peak of a high \( T_m \) and the wild-type peak of a low \( T_m \) value.

SEQ-katG-R (see Table S1 in the supplemental material), and 5 µl of template DNA. The thermal conditions were as follows: step 1, denaturation at 95°C for 5 min; step 2, 95°C for 10 s and 70°C with a 1°C decrease per cycle for 20 s and 72°C for 25 s, repeated for 10 cycles; and step 3, 95°C for 10 s, 70°C with a 1°C decrease per cycle for 20 s, and 72°C for 25 s, repeated for 40 cycles. The PCR components for the inhA promoter, ahpC promoter, and inhA 94 were identical: the 25-µl PCR mixture contained 67 mmol/liter Tris-HCl (pH 8.0), 16.6 mmol/liter (NH₄)₂SO₄, 6.7 µmM/liter EDTA, 85 µg/ml bovine serum albumin (BSA), 50 mmol/liter KCl, 5% glycerol, 3 mmol/liter MgCl₂, 2% dimethyl sulfoxide (DMSO), 200 µmM/liter dNTPs, 0.4 µmM/liter (each) primers (see Table S1 in the supplemental material), 1.0 U Taq polymerase, and 5 µl of template DNA. The thermal conditions were as follows: step 1, denaturation at 95°C for 5 min; step 2, 95°C for 10 s and 69°C, with a 1°C decrease per cycle for 30 s, and 72°C for 40 s, repeated for 10 cycles; and step 3, 95°C for 10 s, 60°C for 30 s, and 72°C for 40 s, repeated 40 cycles. PCR products were sequenced using the forward primers for each gene (BGI, Shenzhen, China).

**Statistical analysis.** Results were analyzed by OpenEpi (19) with a 95% confidence interval (95% CI).

**Ethics statement.** All clinical isolates were collected for routine diagnosis by the hospitals. No patient information was used in the present study, informed consent from patients was not required, and Institutional Review Board approval was therefore exempted.

**RESULTS**

**The design of the MeltPro TB/INH assay.** According to the manual for the MeltPro TB/INH assay, it is designed to cover the frequent mutations in *M. tuberculosis* that confer resistance against INH. Mutations in the inhA promoter region (positions −17 to −8), katG position 315 (katG 315), the ahpC promoter (positions −44 to −30 and −15 to 3), and inhA position 94 (inhA 94) are detected in two duplex and dual-color PCRs. Reaction 1 detects mutations in the inhA promoter region by FAM channel and mutations at the katG 315 site by TET channel. Reaction 2 detects mutations in ahpC promoter region by the FAM channel and mutations at the inhA 94 site by the TET channel. The MeltPro TB/INH assay covers 20 mutant sites involving 30 mutation types that can be identified from the \( T_m \) shift from the wild type (see Fig. S1 in the supplemental material). The mutation detected can be located to corresponding genes or regions, while the exact type of the mutation is not identified except for S94A (TCG→GCG) of inhA 94 (as there is only type of mutation detected by one probe). According to the MeltPro TB/INH assay, all samples that have heteroresistant mutations are judged to be mutants regardless of their heteroresistance levels.

**Analytical performance.** We first evaluated the MeltPro TB/INH assay regarding its accuracy of mutation detection. Results from the reference panel showed that all wild-type strains and mutant types gave expected \( T_m \) values. The \( T_m \) values of five peaks from each of the 10 wild type strains were highly reproducible, and the coefficients of variation were between 0 and 0.7°C. The \( T_m \) values of each mutation were highly reproducible, and the coefficients of variation were between 0 and 0.7°C. The \( T_m \) values of the 12 mutant strains each had a \( T_m \) shift from the wild type larger than 3°C, while the deletion led to a complete disappearance of the melting peak (Table 1 and Fig. 1). These results outperformed the requirements for accuracy of mutation detection.

The reproducibility of the assay was evaluated according to the **Guideline for Evaluation of Analytical Performance of In Vitro Diagnostic Kits** (18). The standard deviations (SD) of within-run and between-run variations are listed in Table S2 in the supplemental material. The largest SD value was no more than 0.4°C among all five melting peaks, demonstrating the high reproducibility of this assay.

We then studied the limit of detection using both a wild-type strain and a mutant strain (katG 315 [AGC→ACC]). Both strains gave repeatable positive results at concentrations of \( 2 \times 10^8 \) and \( 2 \times 10^9 \) bacilli/ml in triplicate. The wild-type strain gave 100% positive results at \( 2 \times 10^4 \) and \( 2 \times 10^5 \) bacilli/ml but 70% positive results at \( 2 \times 10^2 \) bacilli/ml. Thus, the limit of detection for the wild type was \( 2 \times 10^5 \) bacilli/ml, corresponding to 5 bacilli per reaction. The mutant strain gave 100% positive results at \( 2 \times 10^4 \) bacilli/ml but 60% positive results at \( 2 \times 10^5 \) bacilli/ml. Thus, the limit of detection for the mutant types was \( 2 \times 10^4 \) bacilli/ml, corresponding to 50 bacilli per reaction. Taken together, we concluded the MeltPro TB/INH assay had a limit of detection of \( 2 \times 10^4 \) bacilli/ml.
$10^3$ bacilli/ml for the wild type and $2 \times 10^4$ bacilli/ml for the katG 315 (AGC→ACC) mutant strain, respectively. This result met with the requirement of the assay.

The heteroresistance level represents the ability of the assay to detect a mutant in the presence of the wild type. The results showed that the two concentrations ($1 \times 10^4$ and $1 \times 10^5$ bacilli/ml) gave identical melting curves in all of the probes. The melting curves obtained with $1 \times 10^4$ bacilli/ml are shown in Fig. 2. A gradual transition was seen from the wild-type to the mutant peaks when the percentage of the mutant increased. At a mutant level of 20 to 30%, the melting curve could be differentiated from the wild type by the increasingly widening peaks. At a 40% mutant level, the melting curve became readily distinguished from the wild type by the appearance of a peak between the mutant and the wild type. These results were concordant with the sensitivity to detect a heteroresistance level (40% mutant level) preset by the assay.

The cross-platform compatibility of the MeltPro TB/INH assay was evaluated on five models of 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, and the Tm shifts of the mutant types were all more than 3°C. These results demonstrated that both the wild-type strains and the mutants could be reliably detected with each of the five real-time PCR machines. We noticed that among the five machines, LightCyler 480 II was the fastest to complete the assay (105 min), followed by Bio-Rad CFX96 (110 min), Qiagen Rotor-Gene 6000 (125 min), Stratagene Mx3005P (130 min), and ABI 7500 (150 min).

**Clinical study.** A total of 1096 M. tuberculosis clinical isolates, including 437 INH-resistant and 659 INH-susceptible isolates, were enrolled from three geographically different health care units to validate the clinical performance of the MeltPro TB/INH assay. The results showed that of the 437 INH-resistant isolates, 397 isolates were detected as mutant and 40 isolates were wild type (Fig. 3). The clinical sensitivity of the assay was calculated to be 90.8% (95% CI, 87.8 to 93.2%). Of the 659 INH-susceptible isolates, 635 isolates were detected as wild type and 24 were mutant. The clinical specificity of the assay was calculated to be 96.4% (95% CI, 94.7 to 97.6%). The positive predictive value, negative predicative value, Youden’s index, and diagnostic accuracy were 94.3%, 94.1%, 0.9, and 94.2%, respectively.

DNA sequencing analysis was carried out for the 437 INH-resistant isolates and 24 INH-susceptible but mutation-containing isolates (Fig. 3). The sequencing results showed that all of the 24 isolates indeed harbored INH-resistant mutations as
found by the MeltPro assay. Of the 437 INH-resistant isolates, all 397 mutation-containing isolates found by the MeltPro assay harbored INH-resistant mutations. Among the 40 INH-resistant isolates that had no mutation detected by the MeltPro assay, 4 isolates had low-level heteroresistant mutations, 5 isolates harbored mutations outside the region of the MeltPro TB/INH assay, and 31 isolates had no mutations in the sequenced regions. Taken together, the sequencing analysis confirmed that, except for the 5 isolates that harbor mutations beyond the range of the MeltPro assay, the MeltPro assay missed 4 heteroresistant isolates among all of the 461 sequenced isolates, yielding an overall concordance rate of 99.1% (457/461) between the two molecular methods.

To gain insight into the performance of the MeltPro assay in the detection of heteroresistant isolates, heteroresistant isolates detected by either the MeltPro assay or sequencing were chosen for further analysis. In total, 17 heteroresistant isolates were detected, representing 4% of all of the 425 mutant isolates detected by both methods (excluding those 5 isolates uncovered by the MeltPro assay). A head-to-head comparison of the results between the two methods was performed for all of the heteroresistant isolates (see Table S3 in the supplemental material). It could be seen that all of the 17 isolates were heteroresistant by the sequencing method, whereas 11 isolates were heteroresistant, 2 isolates were mutant, and 4 isolates were wild type by the MeltPro assay. Of the 2 isolates detected as mutant by MeltPro assay, the melting curve of no. 13 showed the presence of a mutation or mutations in the ahpC promoter within the range of −15 to 3, while the sequencing displayed two heteroresistant mutations: −12C→T (less mutant than wild type) and −10C→T (more mutant than wild type). Likewise, the melting curve of no. 17 revealed the presence of mutation in ahpC promoter within the position range of −15 to 3, while the sequencing showed the existence of two heteroresistant mutations: −9G→A (less mutant than wild type) and −1 insertion (ins) C (less mutant than wild type). As for the 4 heteroresistant isolates (no. 5, 6, 8, and 14) missed by the MeltPro assay, when referred to the sequencing results, all had a low mutation level. The above results demonstrated that the MeltPro assay is capable of detecting most of the heteroresistant mutations. Nevertheless, it is difficult to detect a low level of the mutation in the heteroresistant isolates.

The large number of mutant isolates detected allowed us to reveal information about the mutation frequency and distribution of INH resistance in China. We listed all of the mutation types detected by either the MeltPro INH assay or sequencing (see Table S4 in the supplemental material) and summarized the distribution and frequencies of mutations in Fig. 4. As can be seen, most of the mutations are located in katG, followed by inha and ahpC. Multiple mutations in these regions of M. tuberculosis could be simultaneously detected among a portion of isolates. No mutations were detected at inha 94. Regarding the mutation types, in total, 26 types of mutation were detected. The most frequent mutation was katG S315T (AGC→ACC), followed by inha A→C T, katG S315N (AGC→AAC), and ahpC promoter −10C→T, which together accounted for more than 90% of all of the INH-resistant mutation types, indicating a highly clustering feature of a small number of mutations in INH-resistant M. tuberculosis isolates. Further analysis of the frequencies of the mutation types according to their geographical locations revealed no difference, suggesting that the above mutation frequency would be homogeneous in China. Six outside mutations were found in 5 INH-resistant isolates, in which, katG W328S (TGG→TGC) and katG G279D (GGC→GAC) were previously reported to be associated with INH resistance (20, 21). The other four mutations, i.e., katG D33D (GAC→GAT), katG P325S (CCG→TGC), and ahpC promoter −46 ins AT, had not been reported before. Their significance in INH resistance requires further investigation.

**DISCUSSION**

INH is one of the most effective first-line drugs in anti-TB therapy. The detection of INH resistance is crucial for the treatment and control of MDR-TB. Although RIF resistance was accepted as a surrogate of MDR-TB (22), detection of RIF resistance alone could neglect INH-monoresistant patients as well as RIF-monoresistant patients. The results can be interpreted, on one hand, that INH-monoresistant patients would lose an opportunity to initiate effective treatment and develop acquired resistance to RIF (23); on the other hand, RIF-monoresistant patients (though rare) would exclude INH, a safe and useful drug, from their treatment. Therefore, use of rapid tests to detect both RIF resistance and INH resistance would have better outcomes than tests to detect RIF resistance alone. A recent study reported a high proportion of treatment failure in INH-monoresistant patients, underscoring the importance of early detection and close monitoring of these...
patients with rapid diagnosis (24). In comparison, identical outcomes were achieved between patients with INH-monoresistant TB and those with drug-susceptible TB when early diagnosis of resistance and tailored therapy were implemented (24–26). It is widely accepted that INH resistance and RIF resistance are equally important, and thus their detection is required in various guidelines for drug resistance treatment and prevention. For example, WHO proposes early detection of both INH resistance and RIF resistance for all patients before treatment (23). In this regard, the INH resistance assay is indispensable to the current rapid assay of MDR-TB.

The MeltPro TB/INH assay is the first officially approved assay dedicated solely to detection of INH resistance. This assay is based on melting curve analysis that uses unique dually labeled, self-quenched probes, which have been proved to be accurate, sensitive, and flexible in the detection of multiple mutations in a single reaction (11,27–29). The assay can detect 20 mutant sites that involve 30 mutation types in the inhA promoter region (positions 17 to 8), katG position 315 (katG 315), the ahpC promoter, and inhA position 94 (inhA 94) of M. tuberculosis, constituting the largest number of INH resistance mutations. Such broad coverage of mutations provides the solid base for its high clinical sensitivity. Analytical studies showed the excellent performance of the MeltPro TB/INH assay in many respects. Its accuracy was demonstrated in the correct detection of 12 different mutation types with high reproducibility. The high analytical sensitivity was reflected in the limit of detection of \(2 \times 10^7\) to \(2 \times 10^8\) bacilli/ml, which corresponded to 5 to 50 bacilli per reaction. In the heteroresistance cases, as low as 20 to 30% of the mutants present in the wild-type population can be identified, which is close to the level detected by Sanger sequencing. The cross-platform compatibility study showed that the MeltPro TB/INH assay can be run on all five real-time PCR machines tested, making it compatible with nearly all mainstream real-time PCR instruments on the market. The entire assay procedure ranged from 105 to 150 min, which is much faster than non-real-time PCR assays. In particular, the hands-on time is actually negligible as only a sample addition step is involved for the entire assay. Obviously, the throughput and the simplicity of the TB/INH assay outperform any assays that involve post-PCR manipulations, paving the way for its wide acceptance in clinical settings.

The clinical validation study, which enrolled 1,096 clinical isolates that included 437 INH-resistant and 659 INH-susceptible isolates, represented one of the largest-scale validation studies in terms of sample number. The clinical sensitivity and clinical specificity obtained were 90.8% and 96.4%, respectively. At a similar scale in validation studies, the clinical sensitivity and specificity were 88.7% and 99.2% for GenoType MTBDR assays in a meta-analysis (30) and 80.3% and 95.8% for the Genechip MDR-TB assay in a multicenter evaluation (8), respectively. Thus, the MeltPro assay has a significantly higher clinical sensitivity than the GenoType MTBDR and Genechip MDR-TB assays. We attribute this to the inclusion of the ahpC promoter by the MeltPro assay in addition to the inhA promoter region (positions 17 to 8) and katG 315, the only two regions covered by both GenoType MTBDR and Genechip MDR-TB assays. We attribute this to the inclusion of the ahpC promoter by the MeltPro assay in addition to the inhA promoter region (positions 17 to 8) and katG 315, the only two regions covered by both GenoType MTBDR and Genechip MDR-TB. According to the mutation frequency obtained in this study, mutations in the ahpC promoter account for 13% of the total INH-resistant cases. Obviously, inclusion of the ahpC promoter region allowed an increased number of INH-resistant samples to be detected. This conclusion is further supported by previous observations that the oxyR-ahpC region is associated with 8.9% of INH-resistant strains (31). As for the clinical specificity, the MeltPro TB/INH assay was equivalent to Genechip MDR-TB but less specific than GenoType MTBDR. These results might be due to the fact that both the MeltPro assay and Genechip MDR-TB used the less sensitive solid medium drug susceptibility
test as the comparison method, while GenoType MTBDR used the more sensitive liquid medium drug sensitivity test as the comparison method (8). We noticed that 24 INH-susceptible isolates reported by DST were actually mutants, as confirmed by both the MeltPro TB/INH assay and DNA sequencing analysis. We therefore suspect that these 24 samples might be INH-resistant isolates missed by the less sensitive DST.

One distinct feature of the MeltPro TB/INH assay is that it could detect heteroresistant samples. To date, heteroresistance has been described for INH, RIF, ethambutol, fluoroquinolone, and streptomycin (32, 33). It is detected using conventional DST of several subcultures (34) or by simultaneous detection of the wild type and the mutants using PCR-based techniques, such as restriction fragment length polymorphism (32), DNA sequencing (35), line probe assays (36), pyrosequencing (37), or a sloppy molecular beacon (38, 39). Although no extensive studies have been reported on comparison of the sensitivities to detect heteroresistance levels among different methods, recent studies show that molecular tests are less efficient than conventional culture-based DST in finding resistance in samples with heteroresistant bacteria (40, 41). No molecular methods can detect <1% INH-resistant bacteria in a mixture of susceptible and resistant M. tuberculosis strains; a result could be obtained, however, using phenotypic DST with the Mycobacteria Growth Indicator Tube (MGIT). Accordingly, as a molecular method, the MeltPro TB/INH assay showed no advantage over the culture-based DST methods. Moreover, it seemed even less sensitive than sequencing in detecting low-level heteroresistant mutations. However, we observed that by simply decreasing the temperature ramp rate, a better resolution of the low-level mutant could be detected (data not shown). We thus believe that there should be room for future improvement in detecting low-level heteroresistance by the MeltPro TB/INH assay.

This evaluation study also revealed several limitations of the MeltPro TB/INH assay that deserve improvement. First, as no isolate harboring inhA S94A (TCG→GCC) was found among the 437 INH-resistant isolates, whether it is necessary that the inhA S94A mutation be included in this assay is doubtful, in particular, when considering that this single mutation necessitates a probe and a fluorescence detection channel as well. The inhA S94A mutation, initially identified in INH-resistant laboratory mutants of Mycobacterium smegmatis and Mycobacterium bovis, has recently been confirmed to be associated with low-level INH resistance in M. tuberculosis (3). However, despite the numerous genetic studies carried out on the molecular mechanisms of INH resistance in M. tuberculosis, there have been few reports describing the S94A amino acid substitution from clinical isolates (42). Second, the two regions of the apcC promoter detected by two FAM-labeled probes lead to a complex merged melting curve, which is difficult to analyze when more than one mutation has occurred. This situation became even worse in the case of heteroresistant mutations. It might be advantageous to omit the inhA 94 probe and replace one of the two FAM-labeled probes with a TET-labeled probe for the apcC promoter region. Such modification would allow detection of the two regions of the apcC promoter in separate fluorescence channels, yielding a simpler melting curve for each region. Third, with the availability of multicolor 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.

Finally, we acknowledge that this evaluation study was restricted to clinical isolates of bacteria. The performance of the MeltPro TB/INH assay with real clinical samples, such as sputum, remains to be validated. However, at the present stage, analysis of drug resistance for M. tuberculosis is routinely conducted following the cultivation step to confirm TB infection. Therefore, we can at least recommend that the MeltPro TB/INH assay be used for rapid detection of INH resistance status according to the current protocol.

In conclusion, despite the fact that various MDR assays have been developed and evaluated over a period of years, the MeltPro TB/INH assay is among the first commercially available tests dedicated to detection of INH resistance. Its wide coverage of INH-resistant mutations ensures high clinical sensitivity. The extreme stability of Tm detection by probe-based melting curve analysis guarantees its high reproducibility. Importantly, the real-time-PCR-based detection format features low amplification product contamination, high sample throughput, and simple manipulations that outperform all current reverse hybridization-based platforms. Finally, its compatibility with all mainstream real-time PCR machines allows wider acceptance in clinical settings than other closed-tube systems. We therefore expect that the MeltPro TB/INH assay could provide a valuable molecular tool for rapid and specific detection of INH resistance in M. tuberculosis isolates. Some limitations still exist, but they should be regarded as aspects for future improvement rather than reasons for excluding adoption of this regulatory process-approved product for detection of INH resistance in tuberculosis.

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