Integrated Microfluidic Card with TaqMan Probes and High-Resolution Melt Analysis To Detect Tuberculosis Drug Resistance Mutations across 10 Genes

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ABSTRACT Genotypic methods for drug susceptibility testing of Mycobacterium tuberculosis are desirable to speed the diagnosis and proper therapy of tuberculosis (TB). However, the numbers of genes and polymorphisms implicated in resistance have proliferated, challenging diagnostic design. We developed a microfluidic TaqMan array card (TAC) that utilizes both sequence-specific probes and high-resolution melt analysis (HRM), providing two layers of detection of mutations. Twenty-seven primer pairs and 40 probes were designed to interrogate 3,200 base pairs of critical regions of the gyrA, katG, rpoB, embB, rpsL, rrs, eis, gyrA, gvrB, and pncA genes. The method was evaluated on 230 clinical M. tuberculosis isolates from around the world, and it yielded 96.1% accuracy (2,431/2,530) in comparison to that of Sanger sequencing and 87% accuracy in comparison to that of the slow culture-based susceptibility testing. This TAC-HRM method integrates assays for 10 genes to yield fast, comprehensive, and accurate drug susceptibility results for the 9 major antibiotics used to treat TB and could be deployed to improve treatment outcomes.

IMPORTANCE Multidrug-resistant tuberculosis threatens global tuberculosis control efforts. Optimal therapy utilizes susceptibility test results to guide individualized treatment regimens; however, the susceptibility testing methods in use are technically difficult and slow. We developed an integrated TaqMan array card method with high-resolution melt analysis that interrogates 10 genes to yield a fast, comprehensive, and accurate drug susceptibility result for the 9 major antituberculosis antibiotics.

M ultidrug-resistant tuberculosis (MDR-TB), defined as resistance of the Mycobacterium tuberculosis to isoniazid and rifampin, occurred in an estimated 440,000 individuals worldwide in 2008 (1). Diagnostic assays for drug-resistant tuberculosis are a key element in MDR-TB control, and genotypic drug resistance testing to detect rifampin resistance has become widespread. Once it is detected, however, most countries employ a standardized treatment regimen, despite the fact that better outcomes are associated with individualized drug susceptibility testing (2, 3), because obtaining a complete antibiotic susceptibility profile is resource intensive and requires several culture-based methodologies and significant technical capacity. Furthermore, nonsusceptibility of the pathogen to a number of drugs in the standardized treatment regimens has been associated with the amplification of acquired drug resistance, including the development of extensively drug-resistant (XDR)-TB, while patients are on MDR-TB therapy (4). We sought to develop a single integrated genotypic susceptibility testing method that can provide an accurate result, compared with that of culture, for the most important drugs used to treat MDR-TB, which could significantly improve outcomes and curb the amplification of drug resistance.

There are several drugs used to treat drug-resistant TB, and for each, drug resistance correlates to differing degrees with several mutations in one or more genes. WHO-approved diagnostic assays include the GeneXpert MTB/RIF, INNO-LiPA Rif TB, and GenoType MTBDRplus, which interrogate regions of rpoB to yield information on rifampin resistance, and some assays include inhA and katG for information on isoniazid. The GenoType MTBDRsl adds regions of embB, rrs, and gyrA to yield partial information on ethambutol, aminoglycosides, and quinolones, respectively (5–8). Several important drugs for MDR treatment, such as pyrazinamide and kanamycin, are not currently assayed with these methods, and quinolone coverage is limited because of the lack of gyrB (9). Since additional genes are desirable and new resistance-associated mutations are steadily being identified, the challenges of molecular diagnostic design and interpretation of
results has increased. Sanger sequencing is highly valuable in this regard (10), because it provides precise sequence information that can be compared against resistance databases. However, like most of the other technologies mentioned, it requires multiple steps and postamplification manipulations and has a limited ability to detect mixed populations (11). We therefore sought to create a single, comprehensive, easy-to-perform genotypic method.

The TaqMan array card (TAC) is a customizable 384-well microfluidic real-time PCR system that compartmentalizes each sample into 48 different PCRs simultaneously and has been applied for the detection of multiple respiratory or enteric pathogens (12, 13). In this work, we sourced the largest M. tuberculosis sequence databases to identify the most prominent drug resistance-associated mutations in key regions of 10 genes and design mutation-specific probes. Additionally, to cover rare or unknown mutations in these areas, we also incorporated a high-resolution melt analysis (HRM) to evaluate whether any mutation was present in these regions. This HRM technique is particularly advantageous for genes like pncA, where single resistance-associated mutations are dispersed throughout a large region (14–19), and yet the method has never been attempted in microoliter volumes. Ultimately, we designed 27 amplicons, 40 mutation-specific probes, and HRM to simultaneously and accurately detect in one sample the TB drug resistance-associated mutations in inhA and katG (isoniazid), rpoB (rifampin), embB (ethambutol), rpsL (streptomycin), rrs (streptomycin, amikacin, kanamycin, and capreomycin), eis (kanamycin), gyrA and gyrB (ofloxacin and moxifloxacin), and pncA (pyrazinamide).

RESULTS
Identification of key mutations associated with TB drug resistance and probe design. We first scrutinized the published literature for the key resistance-associated mutations in the above-mentioned genes using the large databases of Campbell et al., Rodwell et al., and the TB Drug Resistance Mutation Database (Table S1 in the supplemental material) (10, 20, 21). We arrived at 38 specific resistance probes that we predicted would detect ~90% of the rpoB mutations that are found in rifampin-resistant tuberculosis, ~90% of the inhA and katG mutations that are found in isoniazid-resistant tuberculosis, ~70% of the embB mutations found in ethambutol-resistant tuberculosis, ~70% of the rpsL and rrs mutations found in streptomycin-resistant tuberculosis, ~80% of the rrs and eis mutations found in amikacin- and kanamycin-resistant tuberculosis, and ~75% of the gyrA and gyrB mutations found in quinolone-resistant tuberculosis. We also included one pncA probe specific to the His57Asp (CAC→GAC) of Mycobacterium bovis and one probe for the common pncA silent mutation Ser65Ser (TCC→TCT). Primer/probe sequences are listed in Table S2. Stringent specificity of probes was required for this application, and many redesigns were required (data not shown). As an illustration of the results, the rrs (amplicon 3) primer/probe set detected a mutant isolate using the rrs-A(1401)G-specific probe (detects the A-to-G change at position 1401 encoded by rrs), while the wild-type, drug-susceptible laboratory strain M. tuberculosis H37Rv was negative (Fig. 1A).

High-resolution melt analysis in the microfluidic card format. To gain additional coverage of the less frequent (e.g., 2 to 10%) resistance-associated mutations, we designed 27 amplicons to adequately cover the necessary gene segments. These included one region for inhA, katG, eis, and gyrA, two for rpoB and rpsL, three for embB and gyrB, four regions for rrs (2 regions for streptomycin resistance and another 2 regions for amikacin, kanamycin, and capreomycin resistance), and nine for pncA (Table S2 in the supplemental material). The HRM assay had not been performed within such a small volume (1 μl), and we constructed an internal control plasmid with one defined mutant in every amplicon region to be included in every card as a positive control, along with M. tuberculosis H37Rv as a negative control. For the melt curve analysis, leftward variation indicates a lower melt temperature (e.g., C→A, C→T, G→A, G→T) and rightward variation a higher melt temperature (e.g., A→C, T→C, A→G, T→G) compared with the melt curves of the M. tuberculosis H37Rv wild-type control. The difference graph uses the melt data but plots the negative first derivative (~dF/dt) on the y axis, with positive curves indicating higher melt temperatures and negative curves indicating lower melt temperatures. Both the mutant isolate and H37Rv were amplified, as indicated by the nonspecific dye SYTO9 (Fig. 1B); however, the HRM result reinforced the probe result for mutant/variant samples (Fig. 1C and D). Similarly, the gyrB (amplicon 2) primer/probe set demonstrated the benefit of combining the use of probes with HRM, since the specific probe (Fig. 1E) detected the important Asp461His (GAC→CAC) transversion, while HRM would not (Fig. 1F, G, and H). However, rare mutations, so long as they were not transversions (Asp461 Ala and Asp461 Asn, for instance), would be categorized as variant by HRM. Other combinations of probe–HRM results for specific mutations are shown for katG, rpoB (amplicon 1), embB (amplicon 3), rpsL (amplicon 1), rrs (amplicon 2), rrs (amplicon 4), and gyrA (Fig. S1). The pncA gene can carry resistance-associated mutations dispersed upstream and throughout its 561-base pair open reading frame. Therefore, resistance probes were not feasible with this platform and, apart from the above-mentioned His57Asp (CAC→GAC) of M. bovis and the Ser65Ser silent mutation probe, we relied on melt curve analysis of 9 overlapping amplicons, whereby a variant in any amplicon is inferred to be a mutation (Fig. S2A and B). HRM examples for all of the other genes, inhA, katG, rpoB, embB, rpsL, rrs, eis, gyrA, and gyrB, are shown in Fig. S3.

Evaluation using clinical isolates. The final TAC card was thus configured (Fig. 2) and was evaluated using 110 Sanger-sequenced clinical TB strains. The layout shows, for example, that rpoB is amplified in 9 wells, using two amplicons. Amplicon 1 is amplified in 4 wells that include distinct resistance probes (511Pro, 513Leu, 513Glu, and 516Val), while amplicon 2 is amplified in 5 wells with 6 distinct probes (526Tyr, 526Asp, 526Leu, 533Pro, and 531Leu/531Trp in multiplex). To evaluate the reliability of the HRM software determinations, the difference values of amplicons versus wild-type control of the 110 isolates were plotted (Fig. 3; Fig. S4 in the supplemental material). Based on receiver operating characteristic (ROC) analysis, difference value cutoffs of between 6 and 10 were optimal to discriminate susceptible and resistant isolates, where lower numbers would be categorized as wild-type and higher numbers would be categorized as variant. A difference value of 9 was optimal for the majority of amplicons (13/26). A benefit of the TAC layout is that most regions are amplified and subjected to HRM in multiple wells, and thus, difference values can be averaged; we observed low standard deviations (Fig. 3). As would be expected, however, transversion mutations were not detected by HRM.

Using these difference value cutoffs, we then prospectively analyzed another 120 clinical isolates (total number analyzed, 230)
and compared the performance of TAC-HRM versus Sanger sequencing of the same regions (Table 1). For this analysis, TAC-HRM was considered variant either if the probe yielded detection or a variant was called by HRM, and a Sanger sequencing gold standard was used that included not only detection of mutations but, also, detection of mutations associated with resistance, as described in Table S1 in the supplemental material. The overall accuracy versus this Sanger-identified resistance-associated mutation standard was 96.1% (2,431/2,530), and the accuracy exceeded 95% for all genes except \textit{pncA} (81%), which was partly explained by transversions (Table 1). Next, we compared the performance of TAC-HRM to the phenotypic drug susceptibility results for these genes.
isolates (Table 2). Different phenotypic methods were used, as indicated in Materials and Methods, and if multiple methods were used, isolates with discordant phenotypic results were excluded. The accuracy of TAC-HRM versus phenotypic drug susceptibility testing averaged 87%, ranging from 72 to 94% for the 7 drugs. Sanger sequencing versus phenotypic results are shown for comparison and revealed similar levels of accuracy (average, 90%, and range, 74 to 95%). We used Sanger sequencing to adjudicate the 175 discrepancies for TAC-HRM versus phenotypic testing and found that most (73%) were supported by the Sanger result.

**DISCUSSION**

This work details the design and development of a comprehensive microfluidic card that targets the most common TB drug resistance-associated mutations with TaqMan probes and, importantly, utilizes high-resolution melt analysis (HRM) to cover gaps missed by the probes. As was predicted by the synthesis of information from TB drug resistance mutation databases, this assay and its choice of mutations yielded a result that was on average 87% accurate in comparison to the culture-based drug susceptibility results, ranging from 72 to 94% for the different drugs. Shortcomings were mostly due to true genotypic-phenotypic discordance and not assay failure, as evidenced by the rigorous comparison against the Sanger sequencing result for the same genes. We remain advocates of Sanger sequencing, since it yields precise genotypic information and can also afford resistance interpretation. However, it does have limited detection of heteroresistance, requires days for amplification and postamplification processing in specialized laboratories, and in contrast to real-time PCR, simply is not readily available in the representative areas of TB endemcity where we work (Thailand, Bangladesh, Tanzania, and Russian Federation) (11). Next-generation-sequencing technologies are being developed, but presently, the number of steps and bioinformatic requirements are cumbersome for field use. Therefore, the use of a closed-system, rapid, comprehensive assay like the TAC-HRM offers a considerable advance. In particular, we have previously demonstrated how well the TAC platform works in laboratories in these countries, since the cards are stable at ambient temperature and all reagents are enclosed (22).

The assay provides two independent layers of resolution for mutations, both through resistance-specific probes and through HRM, which can indicate whether a region is non-wild type. Two layers are useful because neither is perfect (i.e., we encountered instances where probes detected mutations while HRM did not, and vice versa). The strategy of detecting non-wild-type regions is a common one and is, for example, the basis of the rifampin resistance result from the GeneXpert MTB/RIF (Cepheid, United States). However, careful design is required to avoid regions with known silent mutations that would result in false resistance being called; alternatively, nonresistance-specific probes can be included (e.g., as we have done with pncA Ser65Ser) to negate false
variant calls. Another shortcoming of HRM is that transversions may be missed; however, this was overcome with specific probes and was otherwise rarely a problem; for instance, it only occurred in 2 of 14 of the pncA nonvariant isolates.

There were limitations to this work. The TAC platform still requires an expensive real-time PCR platform, and currently, the HRM software requires manual reading of the difference values. Additionally, the HRM requires high-quality DNA from purified...
TABLE 1 Performance of TaqMan array card high-resolution melt compared to sequencing

| Target gene | TAC-HRM result | No. with resistance-associated mutation found or not found by sequencing | % sensitivity | % specificity |
|-------------|----------------|-------------------------------------------------------------------------|--------------|--------------|
| inhA        | Variant        | 28 Found, 1 Not found                                                   | 100          | 100          |
|             | Nonvariant     | 0 Found, 201 Not found                                                  |              |              |
| katG        | Variant        | 157 Found, 2 Not found                                                  | 100          | 97           |
|             | Nonvariant     | 0 Found, 71 Not found                                                   |              |              |
| rpoB        | Variant        | 168 Found, 7 Not found                                                  | 98           | 88           |
|             | Nonvariant     | 3 Found, 52 Not found                                                   |              |              |
| embB        | Variant        | 114 Found, 4 Not found                                                  | 99           | 97           |
|             | Nonvariant     | 1 Found, 111 Not found                                                  |              |              |
| rpsL        | Variant        | 80 Found, 6 Not found                                                   | 99           | 96           |
|             | Nonvariant     | 1 Found, 143 Not found                                                  |              |              |
| rrs-S       | Variant        | 19 Found, 2 Not found                                                   | 100          | 99           |
|             | Nonvariant     | 0 Found, 209 Not found                                                  |              |              |
| rrs-A       | Variant        | 8 Found, 9 Not found                                                   | 89           | 96           |
|             | Nonvariant     | 1 Found, 212 Not found                                                  |              |              |
| eis         | Variant        | 6 Found, 1 Not found                                                   | 100          | 100          |
|             | Nonvariant     | 0 Found, 223 Not found                                                 |              |              |
| gyrA        | Variant        | 28 Found, 8 Not found                                                   | 90           | 96           |
|             | Nonvariant     | 3 Found, 191 Not found                                                 |              |              |
| gyrB        | Variant        | 4 Found, 5 Not found                                                   | 67           | 98           |
|             | Nonvariant     | 2 Found, 219 Not found                                                 |              |              |
| pncA        | Variant        | 81 Found, 29 Not found                                                 | 85           | 79           |
|             | Nonvariant     | 14 Found, 106 Not found                                                |              |              |

a One hundred seventy-seven of 191 showed the transversion AGC→ACC (Ser95Thr), a common polymorphism not associated with drug resistance.
b Four of 81 contained Ser65Ser along with mutations in other positions.
c Two of 14 were transversions.
d Sixteen of 106 consisted of a silent mutation (TCC→TCT; Ser65Ser).

TABLE 2 Correlation of sequencing and TAC-HRM results with phenotypic drug susceptibility test results

| Drug | Gene target (no. of isolates tested)a | Resistance-associated mutation found or not found by sequencing | No. with indicated DST resultb | % accuracy | TAC-HRM result | No. with indicated DST result | % accuracy |
|------|--------------------------------------|---------------------------------------------------------------|-------------------------------|------------|----------------|-------------------------------|------------|
| INH  | inhA or katG (227)                   | Found                                                         | 174                           | 0          | 95             | Variant                       | 171        | 93           |
|      |                                      | Not found                                                     | 12                            | 41         | 91             | Nonvariant                    | 15         | 40           |
| RIF  | rpoB (219)                           | Found                                                         | 153                           | 11         | 91             | Variant                       | 151        | 88           |
|      |                                      | Not found                                                     | 8                             | 47         | 91             | Nonvariant                    | 10         | 42           |
| EMB  | embB (203)                           | Found                                                         | 62                            | 35         | 74             | Variant                       | 60         | 72           |
|      |                                      | Not found                                                     | 18b                           | 88b        | 74             | Nonvariant                    | 20         | 87           |
| STR  | rpsL or rrs-S (190)                  | Found                                                         | 81                            | 10         | 87             | Variant                       | 83         | 88           |
|      |                                      | Not found                                                     | 14                            | 85         | 87             | Nonvariant                    | 12         | 84           |
| AMK  | rrs-A or eis (221)                   | Found                                                         | 8                             | 8          | 95             | Variant                       | 9          | 90           |
|      |                                      | Not found                                                     | 8                             | 8          | 95             | Nonvariant                    | 3          | 199          |
| OFX  | gyrA or gyrB (220)                   | Found                                                         | 31                            | 3          | 95             | Variant                       | 29         | 91           |
|      |                                      | Not found                                                     | 9                             | 177c       | 95             | Nonvariant                    | 11         | 170c         |
| PZA  | pncA (113)                           | Found                                                         | 49                            | 2          | 92             | Variant                       | 45         | 82           |
|      |                                      | Not found                                                     | 7d                           | 55e         | 74             | Nonvariant                    | 11d        | 48e          |

a The number of isolates is not always 230 because not all isolates were tested with phenotypic assays and isolates with discordant phenotypic results were excluded.
b Five of 18 and 22/88 were Glu378Ala, which is a polymorphism not considered associated with drug resistance (10).
c One hundred sixty-three of 177 and 156/170 were gyrA Ser95Thr, which is a transversion polymorphism not associated with drug resistance.
d Two of 7 and 2/11 were a silent mutation (TCC→TCT; Ser65Ser).
e Five of 55 and 3/48 were a silent mutation (Ser65Ser).
f DST, drug susceptibility test; R, resistant; S, susceptible.

M. tuberculosis culture and, thus, would likely not work well in clinical specimens or mixed TB/nontuberculous mycobacterial samples. Additionally, the assay performance appeared suboptimal compared to Sanger sequencing for pncA/pyrazinamide (PZA) (accuracy for phenotypic PZA resistance, 82% versus 92%, respectively). Pyrazinamide resistance is an important consideration in the management of MDR-TB, particularly in the face of new TB drugs where pyrazinamide yields synergy (23). This suboptimal performance was largely a function of the microliter format. We previously developed an HRM assay for PZA, using 7 overlapping amplicons in a 20-μl volume on the Rotorgene platform, that yielded a 94% concordance versus Sanger sequencing results (including on many of these isolates) (15), and even 5 μl in a 384-well plate format improved the sensitivity (data not shown). The performance of TAC-HRM was also poor against the phenotypic results for ethambutol, as was that of Sanger sequencing. The major discrepancies were mutations at codons 306 and 406 that were found in susceptible isolates. Such discordance has been reported previously and has brought into question the association with resistance of codon 306 (24–26); however, allelic exchange experiments do demonstrate that codons 306 and 406 can confer resistance (27–29). Such discordance implicates the phenotypic testing for ethambutol as problematic (30, 31). The concordance between the results of rpoB and rifampin drug susceptibility testing was slightly lower than expected (88%); however, this was similar to that of sequencing and may reflect some comparisons to a phenotypic standard on Lowenstein-Jensen (L-J) medium. Of the 16 TAC-HRM variant samples that were rifampin susceptible, 1 was a silent mutation, as might be expected, 5 were Leu533Pro, which has been associated with discordant susceptibility results previously (32), and 3 had one of the well-known resistance mutations Ser531Leu and Asp516Val.

Taken together, this TAC-HRM assay yields a highly accurate TB drug susceptibility result versus either sequencing or culture-based methodologies for the most common drugs used to treat TB drug resistance.
MDR-TB. It carefully interrogates 3,200 bp of *M. tuberculosis* gene territory and can be performed at a modest cost in laboratories with real-time PCR capabilities without postamplication manipulation or risk. The use of such an assay in the clinical setting could improve MDR-TB treatment outcomes and limit the amplification of resistance by identifying the greatest number of active drugs to include in a regimen (2, 3), instead of the current standard of care in most countries where TB is endemic, which is to use a nonindividualized empirical regimen. To this end, it would be sensible to use this device in a tiered setting (33), for example, on MDR-TB cases diagnosed by GeneXpert, where confirmation of MDR-TB and information on other drug susceptibilities is desirable.

**MATERIALS AND METHODS**

**Mycobacterial strains and culture conditions.** The mycobacterial strains used in this study included *M. tuberculosis* H37Rv (ATCC 27294) and a total of 230 clinical isolates, including 92 from Bangladesh (International Centre for Diarrheal Diseases and Research, Dhaka), 85 from Thailand (Department of Microbiology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok), 28 from the Russian Federation (Scientific Center of Family Health and Reproductive Problems, Siberian Branch, Russian Academy of Medical Sciences, Irkutsk), 2 from the University of Virginia, and 2 from the University of Rwanda (Kilibango Clinical Research Institute, Moshi), and 23 from Tanzania (Centre for Diarrheal Diseases and Research, Dhaka), 85 from Thailand (Kilibango Clinical Research Institute, Moshi), and 2 from the University of Virginia. Most were MDR (72%) based on phenotypic susceptibility testing, and 2 from the University of Moscow (Russian Federation). Isolates from Bangladesh were tested using the L-J proportion methods and to 0.5 McFarland for MGIT susceptibility testing. All work was approved by the University of Virginia Institutional Biosafety Committee and the University of Virginia Institutional Review Board. To confirm MDR-TB cases diagnosed by GeneXpert, where confirmation was assessed by the University of Virginia Institutional Biosafety Committee.

**Prophylactic drug susceptibility testing.** Isolates from Thailand underwent susceptibility testing with the standard agar proportion method for both first-line and 2nd-line drugs. Isolates from Tanzania underwent MGIT for 1st-line and TREK Sensititre MYCOTB for both 1st- and 2nd-line drugs. Isolates from Bangladesh were tested using the L-J proportion method for 1st-line and TREK Sensititre MYCOTB for both 1st- and 2nd-line drugs. Twelve isolates from the Russian Federation were tested using the L-J proportion method and TREK Sensititre MYCOTB for both 1st- and 2nd-line drugs, while 16 isolates were tested only with TREK Sensititre MYCOTB. Pyrazinamide susceptibility tests were performed using the MGIT method. All methodologies have been described previously (15, 34, 35).

**Sanger sequencing.** All 230 TB isolates were sequenced by Sanger sequencing. The amplification and sample preparation for sequencing were described previously (34). The following seven loci were amplified by PCR, using the locus-specific primers of Campbell et al. (10): *inhA* and *kat*G (INH), *rpoB* (RIF), *emB* (EMB), *rrs* (KAN, CAP, and AMK), S-R, and gyrA (OFX and MFX). The rpsL (STR), *rrs* (STR), and gyrB (OFX and MFX) loci were amplified by the following primers designed in this study: rpsL-F, 5′-CCGACACCAAGACAGTTGAAA-3′; rpsL-R, 5′-ACAACTGGCATCGG-TAGAC-3′; rrsS-F, 5′-CTGAGATACGGCCGAGACT-3′; rrsS-R, 5′-TG-CATGCTAACCCGGTTA-3′; gyrB-F, 5′-CGTTAAGGCACGAGGTTT-3′; and gyrB-R, 5′-GTTGTCGGACAAAAACATG-3′.

**TaqMan system.** The TaqMan assay included 27 primer pairs and 40 specific probes, of which 38 specific probes included the most common drug resistance-associated mutations of *inhA*, kat*G*, *rpoB*, *emB*, *rrs*, *rrs*, gyrA, and *gyrB*. Two probes were specific to the *panC* His57Asp (CA/C→GAC) of *M. bovis* and a common silent mutation, Ser665Ser (TCC→TCT). Nine pairs of overlapping primers covered the upstream region and the entire 561-bp *panC* open reading frame. The primers and probes were designed using Primer Express 3 (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA) unless a reference is cited (Table S2 in the supplemental material). Additionally, one primer/probe specific to *M. tuberculosis* 16S rDNA was included in the card as a PCR positive control.

**High-resolution melt plus TaqMan probe assay development.** The optimization of conditions and probe specificity testing were performed in the 384-well format of the ViiA 7 platform (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA). Each primer/probe set provided 0.05 μl each of forward and reverse primer and 0.02 μl of probe from 50 μM stock) was utilized in singleplex amplifications, with 5 μl of PCR mixture containing 2.5 μl of 2X MeltDoctor HRM master mix (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA), 1.38 μl of nuclease-free water, and 1 μl of genomic DNA. The cycling conditions included an initial denaturation step at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 64°C for 1 min. For the high-resolution melt, denaturation occurred at 95°C for 1 min, followed by heteroduplex formation at 50°C for 1 min and then by continuous melting from 70 to 95°C at 0.025°C/s. Microfluidic Card Assay for TB Drug Resistance Mutations

**Evaluation of HRM plus TaqMan probe assay on microfluidic card format.** The optimized primer/probe assay for each gene target allowed singleplex amplification in the microfluidic card format by loading a 100-μl reaction mixture including 50 μl of 2X MeltDoctor HRM master mix, 1 μl each of forward and reverse primer (50 μM), 0.4 μl of probe from a 50 μM stock, 27.6 μl of nuclease-free water, and 20 μl of genomic DNA into an empty microfluidic card that has 8 ports per card, with one sample per port. This was centrifuged at 1,200 rpm for 1 min, and then the card was sealed, the loading ports were excised, and the card was inserted into a ViiA 7 instrument (Life Technologies Corporation, Carlsbad, CA) and run under the same cycling conditions as described above. *M. tuberculosis* H37Rv was included in each run as a wild-type control, and either a mutant sample or a constructed DNA plasmid control was used on every run and served as a positive control for mutations.

**High-resolution melt plus TaqMan probe assay on microfluidic card format.** The optimized primer/probe assay for each gene target allowed singleplex amplification in the microfluidic card format by loading a 100-μl reaction mixture including 50 μl of 2X MeltDoctor HRM master mix, 1 μl each of forward and reverse primer (50 μM), 0.4 μl of probe from a 50 μM stock, 27.6 μl of nuclease-free water, and 20 μl of genomic DNA into an empty microfluidic card that has 8 ports per card, with one sample per port. This was centrifuged at 1,200 rpm for 1 min, and then the card was sealed, the loading ports were excised, and the card was inserted into a ViiA 7 instrument (Life Technologies Corporation, Carlsbad, CA) and run under the same cycling conditions as described above. *M. tuberculosis* H37Rv was included in each run as a wild-type control, and either a mutant sample or a constructed DNA plasmid control was used on every run and served as a positive control for mutations.

**Evaluation of HRM plus TaqMan probe array card.** The primer and probe oligonucleotides were synthesized and spotted onto the microfluidic card by Applied Biosystems (Life Technologies Corporation, Carlsbad, CA). Twenty microliters of input DNA was mixed with 50 μl of 2X MeltDoctor HRM master mix, 2 μl of 2.5 μM ROX reference dye, and 28 μl of water to a 100-μl final volume. This was loaded into each port of the card, whereby each card included six clinical samples, *M. tuberculosis* H37Rv as a wild-type control, and the plasmid containing one mutation of each HRM gene segment as a mutant control for HRM.

**TAC-HRM analysis.** Since our assay combined HRM and a TaqMan probe in the same reaction, HRM was analyzed first. Melt curve data were analyzed and normalized using the HRM software module for the ViiA 7 system on the basis of the differences in the shapes and temperature shifts of the melt curves of the sample and the *M. tuberculosis* H37Rv wild-type control. The HRM curves were analyzed by selecting two normalization regions, one occurring prior to the melt of the amplicon and one following complete separation of the two strands. The results for each sample were compared to the results for the wild-type control and automatically categorized as wild-type or variant by the software. Each well yielded two amplification plots, one on the MeltDoctor channel (SYTO9) and the other on the fluorochrome (VIC/HEX/NED) channel. Then, the parameters were reset for probe-based real-time PCR analysis.

**Statistical analysis.** Means or medians were compared using the t test or Mann-Whitney test. Receiver operating characteristic (ROC) analysis was performed with PASW Statistics software to define a cutoff for categorizing variants by HRM.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.orglookup/suppl/doi:10.1128/mBio.02273-14/-/DCSupplemental.
Figure S1, TIF file, 1.7 MB.
Figure S2, TIF file, 0.8 MB.
Figure S3, TIF file, 1.8 MB.
Figure S4, TIF file, 0.9 MB.
Table S1, DOCX file, 0.03 MB.
Table S2, DOCX file, 0.1 MB.

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