Direct Detection of Rifampin and Isoniazid Resistance in Sputum Samples from Tuberculosis Patients by High-Resolution Melt Curve Analysis

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ABSTRACT Drug-resistant tuberculosis (TB) is a major threat to TB control worldwide. Globally, only 40% of the 340,000 notified TB patients estimated to have multidrug-resistant-TB (MDR-TB) were detected in 2015. This study was carried out to evaluate the utility of high-resolution melt curve analysis (HRM) for the rapid and direct detection of MDR-TB in *Mycobacterium tuberculosis* in sputum samples. A reference plasmid library was first generated of the most frequently observed mutations in the resistance-determining regions of *rpoB*, *katG*, and an *inhA* promoter and used as positive controls in HRM. The assay was first validated in 25 MDR *M. tuberculosis* clinical isolates. The assay was evaluated on DNA isolated from 99 *M. tuberculosis* culture-positive sputum samples that included 84 smear-negative sputum samples, using DNA sequencing as gold standard. Mutants were discriminated from the wild type by comparing melting-curve patterns with those of control plasmids using HRM software. Rifampin (RIF) and isoniazid (INH) monoresistance were detected in 11 and 21 specimens, respectively, by HRM. Six samples were classified as MDR-TB by sequencing, one of which was missed by HRM. The HRM-RIF, INH-*katG*, and INH-*inhA* assays had 89% (95% confidence interval [CI], 52, 100%), 85% (95% CI, 62, 97%), and 100% (95% CI, 74, 100%) sensitivity, respectively, in smear-negative samples, while all assays had 100% sensitivity in smear-positive samples. All assays had 100% specificity. Concordance of 97% to 100% (κ value, 0.9 to 1) was noted between sequencing and HRM. Heteroresistance was observed in 5 of 99 samples by sequencing. In conclusion, the HRM assay was a cost-effective (Indian rupee [INR] 400/US\$6), rapid, and closed-tube method for the direct detection of MDR-TB in sputum, especially for direct smear-negative cases.

KEYWORDS DNA sequencing, high-resolution melt curve analysis, tuberculosis, molecular diagnostics, multidrug resistance, sputum

Tuberculosis (TB) is one of the world's deadliest transmittable diseases (1). In 2015, worldwide, 10.4 million people were estimated to be infected with TB, with 1.4 million people dying from the disease. Drug-resistant TB is a menace for TB control worldwide, and 60% of reported TB patients estimated to have multidrug-resistant TB (MDR-TB) were not detected in 2015 (1). India holds the dubious distinction of harboring 27% of the global TB burden, with 2.5% of its new TB cases and 16% of previously treated cases having MDR-TB (1). Conventional *Mycobacterium tuberculosis* culture-based systems for first-line and second-line drug susceptibility testing (DST) are time-consuming, while commercial liquid culture-based DST reduces turnaround times but

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is cost-prohibitive. Microscopic observation drug susceptibility (MODS; Hardy Diagnostics) and thin-layer agar (TLA; NanoLogix, Inc.) assays are other alternatives to conventional culture-based methods for DST of *M. tuberculosis*; however, they are highly operator dependent (2). Line probe assays (LPAs) are World Health Organization (WHO)-endorsed molecular tests for the rapid detection of MDR-TB (INNO-LiPA Rif. TB assay, GenoType MTBDR_{plus} version 1.0 and 2.0, and Nipro NTM+MDR-TB detection kit 2) (3). However, LPAs are endorsed only for sputum smear-positive (SSM-positive) patients and not for SSM-negative specimens (4), and their high sensitivity is marred by their complicated procedural setup and open hybridization format. The recent landscape report suggests their applicability to SSM-negative specimens; however, enough evidence has not been generated for a WHO endorsement (5). The Xpert MTB/RIF assay is a WHO-recommended diagnostic test for TB that enables rapid diagnosis of TB and simultaneous assessment of rifampin (RIF) resistance within 2 h. The test has a higher sensitivity for SSM-positive specimens (98%) than for SSM-negative specimens (67%), indicating the need for improved tests that diagnose resistance in direct smear-negative samples (6). The recent UNITAID document summarizes a host of other new nucleic acid amplification tests (NAATs), including a test made in India by Molbio Diagnostics, which are in development or validation for diagnosing drug resistance; however, none of them have generated enough evidence for a WHO endorsement (5).

Thus, there is an urgent need for a rapid and sensitive diagnostic and resistance screening technique, especially for SSM-negative specimens in resource-limited countries. High-resolution melt curve analysis (HRM) provides quantitative analysis of the melt curve of a DNA fragment after real-time PCR (qPCR) and is an augmentation of amplicon melting analysis. Sequence modifications are demarcated by differences in the melting curve profiles among sample and reference DNA and are dependent on the base composition, length, GC content, and strand complementarity of the amplicon (7). This method is a potentially good substitute for conventional DST in clinical practice and has been evaluated previously for the detection of RIF resistance in *M. tuberculosis* culture isolates from sputum, with an overall sensitivity and specificity of 94% and 99%, respectively (8). HRM has also been used successfully as a molecular DST for other drugs, including isoniazid (INH), fluoroquinolones, streptomycin, and pyrazinamide, in *M. tuberculosis* clinical isolates from sputum, with a sensitivity range of 74 to 100% and specificity range of 83 to 100% (9–16). A microfluidic TaqMan array card that utilizes sequence-specific probes and HRM has also been reported for the detection of mutations involved in MDR and extensively drug-resistant TB (XDR-TB) in clinical isolates, with 96% accuracy (17). To the best of our knowledge, the HRM assay has not been evaluated for the diagnosis and screening of RIF and INH resistance directly from sputum samples. The aims of this study were to (i) develop and validate the HRM assay for MDR-TB diagnosis using sputum specimens and (ii) compare the HRM assay with DNA sequencing and explore the possibility of utilizing it as a standalone test for DST, mainly in SSM-negative samples. Accordingly, we evaluated the utility of HRM for detecting RIF and INH resistance in predominantly SSM-negative sputum samples. The samples were subjected to molecular DST via HRM, and the results were compared to those with DNA sequencing used as the reference standard.

RESULTS

HRM assay optimization with clinical isolates and control plasmids. Twenty-three control plasmids (Table 1) bearing individual mutations determining drug resistance and 25 clinical isolates with a phenotypically confirmed multidrug resistance phenotype were used to develop and evaluate the HRM assay. The clinical isolates, which had mutations at one or more sites, demonstrated variant melting curves for *rpoB* and *katG* and/or the *inhA* promoter region (referred to here onwards as *inhA*) (Fig. 1). For the *rpoB* HRM assay, 16 of the 25 MDR isolates showed a similar pattern and aligned with the melt curve for the 531 plasmid (S531L), and 4 of them showed a profile similar to that of the 516 plasmid control (D516Y) (Fig. 1A and B and 2A). The remaining 5 samples had a mutant genotype at position 526 (H526Y, H526L, and H526N), as

FIG 1 HRM curves for the detection of *M. tuberculosis* drug resistance. (A, C, and E) Normalized melting curves of *rpoB*, *katG*, and *inhA*, respectively. (B, D, and F) Temperature-shifted difference plots of *rpoB*, *katG*, and *inhA* generated by normalizing the melting profiles of all samples (mutant plasmid controls and sample DNA in color) against the WT profile (in black). RFU, relative fluorescence units.

deciphered by HRM, and all 25 isolates were categorized concordantly as mutants by sequencing (Fig. 2A and Table S1 in the supplemental material). For INH resistance, 23 isolates showed a pattern similar to that of the *katG* 315 mutant curve, which was confirmed by sequencing. Two *katG* 315 mutant isolates (determined by sequencing) were incorrectly denoted wild type (WT) by HRM (Fig. 2A and Table S1). Three samples were incorrectly denoted *katG* S315T mutants by HRM but were found to be S315N mutants by sequencing (Fig. 2A and Table S1). All 25 isolates were categorized as WT for the *inhA* region by both HRM and sequencing (Fig. 2A and Table S1).

Direct detection of RIF resistance in sputum. The standardized assays were next applied on 99 sputum sample DNAs. Out of these samples, HRM curves for 83 samples coincided with the WT *rpoB* gene plasmid control, and they were designated WT (Fig. 1A and B and 2B and C). Sixteen samples had melt curves that were clearly distinguishable from the WT pattern and were classified as mutants; 4 samples displayed an S531L pattern, 11 samples revealed a mutant pattern at codon 526 (H526N [$n = 6$], H526Q [$n = 2$], H526L [$n = 2$], and H526G [$n = 1$]), and 1 sample had a D516Y curve (Fig. 2B and C and Table S2 in the supplemental material). When the HRM results were compared with those from sequencing, genotype concordance was found for 96 of 99 samples. All three discrepant samples were in the smear-negative category; 2 samples were assigned as WT by the HRM assay, while they had a H526N mutant genotype by sequencing (Fig. 2C). The third discrepant sample showed a mutation in the *rpoB* region at codon 531 by the HRM assay but actually harbored an H526N codon mutation by DNA sequencing (Fig. 2C). RIF monoresistance (mutation in *rpoB* gene only) was

FIG 2 Categorization of samples in mutant/WT category. (A) *M. tuberculosis* isolates ($n = 25$); 2 *katG* 315 mutants determined by sequencing (S315N and S315T) were incorrectly denoted as WT by HRM. Three samples were categorized as an S315N mutant by sequencing but were incorrectly denoted as an S315T mutant by HRM. (B) Smear-positive sputum samples ($n = 15$). (C) Smear-negative sputum samples ($n = 84$); out of 9 *rpoB* H526N mutant samples (determined by sequencing), three samples showed discrepant results. One sample was incorrectly categorized as an S531L mutant, and 2 samples were categorized as WT by HRM. Out of 4 *katG* S315N mutant samples (determined by sequencing), 3 samples were incorrectly denoted as S315T mutants, 1 was categorized as WT by HRM, and 2 S315T mutants (determined by sequencing) were wrongly denoted as WT by HRM.

detected in 11% of samples by HRM and 12% of samples by DNA sequencing (Fig. 2B and C and Table S2). A mixed genotype was exhibited by 5 sputum samples (Fig. 3 and Table S2). Electropherogram analysis revealed the presence of two distinct peaks at the 526 mutation hot spot, indicating the presence of both wild-type and resistant strains (Fig. 3).

Direct detection of INH resistance in sputum. All 99 sputum DNA samples were evaluated by the HRM assay for mutations in the *katG* gene and *inhA* region. Nineteen samples were determined to be mutants (S315T), and 80 samples had a curve corresponding to the WT in the *katG* assay (Fig. 1C and D, Fig. 2B and C, and Table S2). Sequencing demonstrated 77 samples to be WT and 22 samples to be *katG* 315 mutants, with 18 samples having an S315T mutation and 4 samples exhibiting a *katG* S315N change (Fig. 2B and C and Table S2). Three out of four S315N mutant samples were classified as S315T mutants by HRM and one as WT (Fig. 2C). Two S315T mutants were classified as WT by HRM. All discrepant samples were in the smear-negative category. For the *inhA* region, melt curves for 87 samples were confirmed to be WT, while 12 samples displayed variant curves by both HRM and DNA sequencing, indicating 100% concordance (Fig. 1E and F, Fig. 2B and C, and Table S2). INH monoresistance (mutation in *katG* or *inhA* promoter or both) was detected in 21% of samples by HRM and 20% by DNA sequencing; one discrepant sample (sample 49) was designated MDR by DNA sequencing (Fig. 2B and C and Table S2).

Direct detection of MDR-TB. Out of 99 sputum samples, the HRM assay categorized 5 patient samples as MDR-TB. These samples harbored mutations in both *rpoB* (codon 516, 526, or 531) and *katG* (codon 315) and/or the *inhA* region (−15 and −24 positions) (Table S2). An additional sample (sample 49, SSM negative) was disclosed as MDR by DNA sequencing but was labeled as WT for the *rpoB* and *katG* genes and having a T-8A mutation in the *inhA* region by the HRM assay (Table S2).

FIG 3 Representative electropherograms for heteroresistant samples (at codon 526 of *rpoB*). (A) Wild-type (CAC) and mutant (AAC) sequence (sample 42). (B) Wild-type (CAC) and mutant (CAG) sequence (sample 89).

Sensitivity and specificity of HRM assay. The sensitivities of detection of mutations in genes responsible for RIF and INH resistance were 90.5% (95% confidence interval [CI], 65, 99%) for *rpoB*, 86.4% (95% CI, 65, 97%) for *katG*, and 100% (95% CI, 74, 100%) for *inhA*. All assays had 100% specificity, implying that no WT sample was characterized as a mutant by the HRM assay. The concordance between the HRM assay and DNA sequencing for RIF resistance was 97% ($\kappa = 0.9$), which was identical to that of the *katG* assay. The *inhA* HRM assay demonstrated 100% concordance ($\kappa = 1$). In the SSM-negative category (84/99), mutations in the *rpoB* gene were detected with 89% (95% CI, 52, 100%) sensitivity by HRM, while *katG* mutations were detected at 85% (95% CI, 62, 97%) sensitivity. No smear-negative sample was missed by the *inhA* assay (100% sensitivity; 95% CI, 74, 100%). The MDR-TB sample (sample 49), RIF-monoresistant sample (sample 123), and 2 INH-monoresistant samples (samples 39 and 40) missed by the HRM assay were in the SSM-negative category (Table S2). All assays had 100% sensitivity and specificity in SSM-positive category.

Standard curve and estimation of bacterial load in clinical sample DNA. Standard curves were generated independently for all 3 qPCR assays by serial dilution of *M. tuberculosis* H37Rv WT DNA for 10^2 to 10^7 copies of the *M. tuberculosis* genome to quantitate the bacterial DNA load in each sample (Fig. S2 in the supplemental material). The variation between the various standard curves was negligible and demonstrated the consistency of the assay for single-copy genes (Fig. S2). The DNA load in each sample was estimated by an absolute quantification method from the standard curve constructed for the same group of assayed samples. Average DNA loads (from all 3 assays) ranged from $3.56E + 02$ to $5.09E + 05$ *M. tuberculosis* genome equivalents/ml of sputum.

DISCUSSION

One of the top five priority actions to address the worldwide multidrug resistance crisis as stated in the MDR-TB 2015 update by WHO is to “expand rapid testing and detection of drug-resistant TB cases” by mounting quick testing and detection of all MDR/RIF-resistant TB (MDR/RR-TB) patients (18). In view of this mandate, we have developed and evaluated for the first time, to our knowledge, an HRM-based assay for direct testing of mutations responsible for RIF and INH resistance in sputum samples from a primarily *M. tuberculosis* SSM-negative group (84/99 samples). HRM has been

proposed earlier as an alternative to conventional DST and was employed successfully as a molecular DST method for RIF, INH, and other drugs, including fluoroquinolones, streptomycin, and pyrazinamide, primarily on clinical isolates (8–16, 19).

In the *rpoB* HRM assay, the most common mutation hot spots detected in the RIF resistance-determining region (RRDR) were at codons 526, 531, and 516 (Fig. 2B and C). These mutations have been reported previously at frequency estimates of 47 to 81% (codon 531), 7 to 45% (codon 526), and 5.6 to 22% (codon 516) in various studies in India (20–25) and contribute to 70 to 95% of the total *rpoB* gene mutations leading to RIF resistance (22, 26). We found that H526N was the most frequent mutation, followed by S531L (Fig. 2B and C). Other mutations which were observed were H526Q, H526L, H526G, and D516Y, which have been also reported previously (Fig. 2B and C) (23, 26, 27). Compared with sequencing, the *rpoB* HRM assay had a diagnostic sensitivity of 90.5% (95% CI, 65, 99%), with 100% specificity (95% CI, 96, 100%). The sensitivity was slightly lower than that reported for *M. tuberculosis* culture isolates in a meta-analysis for the detection of RIF resistance (pooled sensitivity and specificity estimates of 94% and 99%, respectively [8]). This might be due to the fact that DNA was isolated directly from sputum samples with a predominantly SSM-negative (84/99 samples) sample set. The 2 samples which were missed were in the SSM-negative category. The *rpoB* HRM assay had 100% diagnostic accuracy in SSM-positive samples. RIF monoresistance was detected in 11 versus 12 samples by HRM and DNA sequencing, respectively, which was consistent with previous findings of ~9% (28) and as high as 15.5 to 16.5% in drug-naïve samples (21, 29).

Mutations responsible for INH resistance have been reported to exist at several genomic loci, including *katG*, *inhA*, *ahpC*, *mabA*, and *ndh* (30). However, the most frequent mutations encoding INH resistance are at codon 315 of the *katG* gene and within the *inhA* promoter region (31). Globally, *katG* codon 315 mutations occur in 64.2% of INH-resistant strains, whereas 19% of INH-resistant strains have *inhA15* promoter mutations, together accounting for 83% INH resistance (31). The *ahpC-oxvR* intergenic region constitutes ~1% of the INH-resistant strains (31) and was therefore not included in this study. In our study, among the INH-resistant cases, ~85% showed mutations at the *katG* 315 codon and the remaining mutations (~15%) were at the *inhA* region (Fig. 2B and C and Table S2). Eight samples had both *katG* and *inhA* promoter mutations. Various Indian studies have reported *katG* mutations at codon 315 in the range of 80 to 89% (22, 24, 32) and *inhA* mutations in the range of 9% to 27% in INH-resistant strains (24, 25, 33). In our study, the INH monoresistance rate was ~21% (mutation in *katG*, *inhA*, or both), which was consistent with previous reports of 11 to 22% in India (29, 34). The 3 samples which were missed by the *katG* HRM assay were in the SSM-negative category. The *katG* HRM assay had 100% diagnostic accuracy in SSM-positive samples. Overall, the *katG* HRM assay had a sensitivity of 86.4% (95% CI, 65, 97%), with 100% specificity (95% CI, 95, 100%), while the *inhA* assay did not miss any samples and had 100% sensitivity (95% CI, 74, 100%) and 100% specificity (95% CI, 96, 100%). Therefore, HRM detected INH resistance at 100% diagnostic accuracy. This was consistent with previously published studies which have reported HRM sensitivity ranging from 80% to 99%, with 83% to 100% specificity in clinical isolates (9–12, 15, 16, 19). A high concordance rate (97 to 100%) between the HRM assay and DNA sequencing (κ value range, 0.9 to 1) indicated an almost-perfect agreement between the two tests for all three assays (35, 36). A total of 6 MDR-TB cases were detected in this study (6/99 samples) by DNA sequencing (Fig. 2B and C and Table S2). HRM missed one case (SSM-negative sample) and could detect 5/99 cases (Fig. 2B and C and Table S2). Even though the WHO estimates 2.5% MDR-TB cases in drug-naïve patients in India, other studies have reported 4 to 12% MDR cases in previously untreated patients (29, 37, 38).

In the present study, DNA sequencing was slightly superior to the developed HRM assay in detecting drug resistance in clinical samples. We tried to speculate on the reason for mutant samples being missed/incorrectly labeled by HRM. In the *katG* assay, some of these discrepant samples (2 samples categorized as WT by HRM assay had a *katG* AGC315ACC mutation confirmed by sequencing) had single nucleotide polymor-

phism (SNP) class 3 mutations. SNP class 3 mutations result in very minute melting temperature (T_m) differences and therefore are more difficult to differentiate from WT (39). Also, the inability of the HRM assay to detect the S315N mutation might be due to the absence of appropriate plasmid control (it being an uncommon mutation). It is unclear why we missed samples with the *rpoB* HRM assay (2 mutants were designated WT, and 1 mutant was incorrectly characterized), as these were class 2 SNPs. Therefore, we believe that the use of a sequencing platform directly from sputum specimens is the way of the future for diagnosing drug resistance. This is especially true for targets, such as *pncA* (a target for pyrazinamide resistance), where mutations are numerous and spread out throughout the gene (40). Whole-genome sequencing has also been previously described as a method for the detection of drug resistance in *M. tuberculosis* (41, 42); however, it is costly and time-prohibitive. Another advantage of targeted sequencing, which was evident in our study, was its ability to detect a minor proportion of a heteroresistant population in our study set. A heteroresistant result is an outcome of the presence of both drug-sensitive and -resistant organisms, which can arise during suboptimal therapy, evolution, or due to mixed infection by strains with different drug susceptibilities, as reported previously (43–45). In our study, 5 heteroresistant cases were identified by DNA sequencing, with both *rpoB* WT and mutant peaks at codon 526 in the electropherogram (Fig. 3). Interestingly, the HRM assay could delegate them only as mutants (single mutant curve), indicating that sequencing is a better tool for detecting the heteroresistant pattern if present. Similar findings have been reported previously in an HRM assay for pyrazinamide resistance detection (46). Heteroresistance has been identified previously in many studies using DNA sequencing (47, 48). Mixed patterns have been reported in India at as high as 29 to 34% of isolates for codons 531, 526, and 516 (25, 44).

Our study has some strengths; for the first time, to our knowledge, sputum DNA was used directly for performing an HRM assay for MDR-TB diagnosis. The performance of molecular methods, like HRM and DNA sequencing, directly from sputum samples mainly depends on bacterial load, DNA extraction method, and purity of the extracted DNA. In this study, the utilization of a concentration-based USP processing method provided pure, good-quality, and inhibitor-free DNA (49), which was a critical determinant for yielding high diagnostic accuracy in SSM-negative samples. The use of reference plasmids for genotypic identification increased the likelihood of mutant identification (along with its genotype) (50). Genetic screening for DST by currently available rapid commercial tests (Xpert MTB/RIF, GenoType MTBDRplus version 1.0 and 2.0, and INNO-LiPA Rif. TB) are limited to previously described mutations and cannot detect resistance-associated mutations encountered outside the mutation hot spots (51). The current version of the HRM assay does not include mutation detection outside the 81-bp RRDR region; however, the assay can be easily modified by including plasmid controls and amplifying specific gene regions to include these novel mutations for resistance screening. However, this possibility was not explored in this study. This risk can be alleviated by the use of techniques, such as DNA sequencing, since it can target any mutation specific to a country/region by not restricting to mutation hot spots *per se*. The cost of the HRM assay per sample for all 3 assays for *rpoB*, *katG*, and *inhA* (INR 400/US\$6) is significantly lower than those of the LPA (INR 1600/~US \$25) and Xpert MTB/RIF (INR 2000/US \$31 in IPAQT laboratories [<http://www.ipaqt.org/about.php>]) assays and is valuable for developing countries, like India (52, 53). Another positive aspect of the study was the use of DNA sequencing as an independent test. DNA sequencing is now economical enough to be amalgamated into routine molecular DST practice undertaken in tertiary-care hospitals and national reference laboratories (NRLs). Our study had some limitations, however: since these assays were performed on archived DNA isolated in 2004 and 2005, phenotypic DST results were not available for this study. At that point in time in India, new patients were not universally put on a DST procedure mandatorily, unless advised specifically by the treating physician or upon first-line treatment failure (54). However, DNA sequencing is being recommended as a gold standard for performing DST, especially for RIF in many studies now (55, 56). In

2017, the WHO will also evaluate the accuracy of genotypic DST (sequencing) and the possibility of it replacing current phenotypic gold standards, at least for drugs, such as rifampin and pyrazinamide (1). Also, since the study was done on previously stored samples, this might have hampered HRM results in few cases. Another limitation is that this technology will not be implementable in a designated microscopy center or a primary health care setting, as performing HRM-based qPCR assays for diagnosing drug resistance in such a setting will not be possible. This test is positioned for a higher-level laboratory (such as a National or Intermediate Reference Laboratory [NRL/IRL]) having access to a real-time PCR machine and DNA isolation facilities. It will be possible to carry out this test (HRM) in laboratories which currently have facilities to perform the Xpert MTB/RIF or LPA test (NRL or IRL).

We believe that high-resolution melt curve analysis has the potential to be used for molecular DST directly from sputum samples. This is a major advantage over previously reported HRM assays, as they have been performed only on DNA isolated from *M. tuberculosis* isolates. This has a potential impact on antitubercular treatment initiation, especially for SSM-negative samples. When first-line treatment is used on an MDR-TB patient, there is a real risk of losing more drugs for future use. The use of an HRM assay as a molecular DST will be better than starting all TB patients on first-line therapy until phenotypic DST results are available. DNA sequencing, on the other hand, has the capacity to revolutionize DST for TB and emerge as a standalone platform for drug resistance determination directly from sputum samples. However, both methods should be analyzed on a larger scale to reliably make conclusions on their applicability to DNA isolated directly from sputum.

MATERIALS AND METHODS

DNA samples. Twenty-five DNA samples from MDR clinical isolates of *M. tuberculosis* from the National JALMA Institute for Leprosy & Other Mycobacterial Diseases, Agra, India, were used for initial assay development (kindly provided by Devendra S. Chauhan). The assay was validated on archived DNA samples isolated directly from 99 *M. tuberculosis* culture-positive sputum specimens, of which 84 were SSM negative. Please note that these 99 archived DNA samples were a subset of 148 specimens analyzed in a previous study (49). The details of sample selection and their characteristics are explained in Fig. S1 in the supplemental material. These sputum samples were collected between 2004 and 2006 after obtaining informed consent from subjects who were attending the DOTS Centre at the National Institute for Tuberculosis and Respiratory Diseases (NITRD) and documented to be treatment naive (49). For this study, the results of *M. tuberculosis* culture and direct-smear microscopy were used for sample selection and assessment (Fig. S1) (49). For direct-smear microscopy, a loopful (5 mm) of sputum sample was taken for smear preparation of 2 by 3 cm, air-dried, and heat-fixed. Ziehl-Neelsen (ZN) staining was done by using carbol fuchsin as a primary stain and methylene blue as a counterstain. *M. tuberculosis* culture was performed by using USP-processed deposits in 7H9 liquid medium containing albumin-dextrose complex and a polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin (PANTA) supplement (Becton Dickinson). The tubes were incubated at 37°C for up to 4 weeks (49). For DNA isolation, a 120- μ l lysis solution containing 10% Chelex 100 (Bio-Rad Laboratories) and 0.3% Tween 20 and 0.03% Triton X-100 (both from Sigma) was added to the processed pellet and heated at 90°C for 40 min (49). The DNA was stored at -20°C until further use. For this study, the stored DNA was purified using phenol-chloroform-isoamyl alcohol (25:24:1 [vol/vol/vol]) extraction (Amresco) prior to HRM and sequence analysis. Since the study involved the generation of various recombinant DNA molecules (reference plasmid DNA library) and use of archived DNA, all protocols were approved by the institutional biosafety committee of the Translational Health Science and Technology Institute (IBSC#28/12).

Preparation of RIF and INH resistance reference plasmid DNA library. The workflow adopted in the present study is shown in Fig. 4. A reference plasmid DNA library of the most frequent mutations encoding RIF and INH resistance was generated for this study (Table 1). The *rpoB*, *katG*, and *inhA* promoter wild-type (WT) resistance-determining regions (including 30- to 150-bp flanking regions) from *M. tuberculosis* H37Rv comprising 261, 196, and 119 bp, respectively, were cloned in the pGEM-T Easy vector system (Promega) (Table 1). Subsequently, site-directed mutagenesis was performed using the WT clones and the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). A pair of mutagenesis primers were designed for each mutation, and the kit protocol was used without any modifications. All 23 control plasmids were cloned in pGEM-T easy vector and were verified by sequencing for the presence of the desired mutation. The plasmid library was used as a positive control in the HRM assays.

HRM primer design and assay optimization. The HRM primers were designed using the NCBI tool for primer design (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi>) flanking the mutation of interest, generating the shortest possible amplicons (<200 bp) with detectable melting temperature variation and to work at an annealing temperature of 60°C. Ten pairs of primers were prepared for each gene, and they were all screened for their ability to discriminate between mutants and WT via qPCR and HRM using plasmid controls. Out of these, the best primer set (i.e., that showing maximum discrimination

FIG 4 Flow chart of the study. *M.tb*, *M. tuberculosis*.

between the WT and various mutant plasmids) was chosen for the final HRM assay of DNA isolated from sputum specimens. The final primers and their characteristics are shown in Table 2. For detecting mutations linked with RIF resistance, a 166-bp fragment spanning codons 507 to 533 of *rpoB* (81-bp RIF resistance-determining region [RRDR]) was selected. To detect mutations linked with INH resistance, (i) a 104-bp region spanning codons 299 to 332, including the most common mutation at codon 315 of the *katG* gene, and (ii) a 119-bp fragment (spanning nucleotides –62 of *inhA* promoter to codon 19 of the *mabA* gene) covering mutations at nucleotides –8, –15, –16, and –24 described for *inhA* promoter were chosen.

TABLE 1 List of plasmids used in assay development

Drug (gene)	Nucleotide change	Amino acid change	
		Position	Amino acids
Rifampin (<i>rpoB</i>)	CTG→CCG	L511P	Leucine→proline
	CAA→CTA	Q513L	Glutamine→leucine
	GAC→GTC	D516V	Aspartic acid→valine
	GAC→TAC	D516Y	Aspartic acid→tyrosine
	CAC→TGC	H526C	Histidine→cysteine
	CAC→TAC	H526Y	Histidine→tyrosine
	CAC→GGC	H526G	Histidine→glycine
	CAC→CTC	H526L	Histidine→leucine
	CAC→AAC	H526N	Histidine→asparagine
	CAC→CGC	H526R	Histidine→arginine
	CAC→GAC	H526D	Histidine→aspartic acid
	CAC→CAG	H526Q	Histidine→glutamine
	TCG→TTG	S531L	Serine→leucine
	CTG→CCG	L533P	Leucine→proline
	WT		
Isoniazid (<i>katG</i>)	AGC→ACC	S315T	Serine →threonine
	WT		
Isoniazid (<i>inhA</i> promoter)	–8 T→A		
	–8 T→G		
	–15 C→T		
	–16 A→G		
	–24 G→T		
	WT		

TABLE 2 Primer sequences used in this study

Primer name	Primer sequence	Amplicon size (bp)	HRM temp range (°C)	Purpose
Rifampin				
<i>rpoB</i> F	ACCGCAGACGTTGATCAACAT	166	86–90	HRM
<i>rpoB</i> R	GGCAGCTCACGTGACAGAC			
<i>rpoB</i> Seq	GGCGAGCTGATCCAAAACC	261		Sequencing
<i>rpoB</i> R	GGCAGCTCACGTGACAGAC			
Isoniazid				
<i>inhA</i> F	CACGTTACGCTCGTGGACAT	119	83–88	HRM and sequencing
<i>inhA</i> R	CAGGACTGAACGGGATACGAA			
<i>katG</i> F	GGAAACTGTTGTCCATTTCG	104	81–86	HRM and sequencing
<i>katG</i> R	GGGCTGGAAGAGCTCGTATG			

Real-time PCR using *rpoB*, *katG*, and *inhA* primers and HRM. DNA from each sputum sample was amplified by qPCR using the final *rpoB*, *katG*, and *inhA* primers. The thermal cycling parameters were 10 min at 95°C and 40 cycles each of 5 s at 95°C and 10 s at 60°C. Plasmids were employed as positive controls (Table 1). In brief, qPCR was performed in 20- μ l reaction mixtures containing 2 to 5 ng of sample DNA, 1 $\times$ Precision melt supermix (Bio-Rad), and 0.5 μ M each primer on the CFX96 real-time PCR detection system (Bio-Rad). The staining method used in the qPCR analysis was based on the EvaGreen dye. This dye was used as a part of the Precision melt supermix (Bio-Rad) and is a 2 $\times$ -concentrated real-time PCR reagent optimized for post-PCR HRM analysis. The use of EvaGreen dye compared to the conventionally used SYBR green was important, as EvaGreen dye is generally less inhibitory toward qPCR, reduces nonspecific amplification, and can be used at a much higher dye concentration than SYBR green, resulting in a more robust PCR signal. It eliminates dye redistribution problems, which can occur with SYBR green during post-PCR DNA melt curve analysis. qPCR amplification was followed by melt curve analysis, which was initiated by a holding step at 65°C for 5 s, followed by a slow temperature increase at a rate of 0.1°C/s to 95°C with continuous fluorescence detection. The HRM analysis of the melt curves was performed using the Precision Melt Analysis software (Bio-Rad). Temperature difference plots were generated by normalizing the melting profiles of all samples (plasmid controls and sample DNA) against the WT profile (Fig. 1). Last, the differences in melting curve patterns were analyzed for all sputum samples to distinguish between WT and mutants and to determine the mutation type. The genotypes of the various patterns were assigned according to the HRM pattern of the control plasmids that were analyzed in parallel.

DNA sequence analysis. Sequencing was performed for the *rpoB*, *katG*, and *inhA* resistance-determining regions using the primers described in Table 2. The amplified products from either routine PCR or qPCR were used for sequencing. Briefly, PCR amplicons produced from *rpoB*, *katG*, and *inhA* were purified using the PCR Cleanup kit (Promega) and subjected to sequencing PCR using BigDye Terminator chemistry version 3.1 (Applied Biosystems). The samples were sequenced on the ABI 3130 genetic analyzer automated sequencer (Applied Biosystems). The results of sequencing were analyzed by ABI Sequence Scanner software version 2 and were further compared with *M. tuberculosis* H37Rv sequence using BLAST analysis (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Test performance and statistical analysis. The various tests employed in the study were compared on the basis of their sensitivity, specificity, and concordance. HRM assay results were assessed using DNA sequencing as a reference standard. HRM sensitivity was defined as (true positives)/(true positives + false negatives) $\times$ 100, wherein true positives are defined as samples identified as mutants by both HRM and DNA sequencing, and false negatives are mutant samples which were missed by HRM but displayed mutations by DNA sequencing. Specificity was defined as (true negatives)/(true negatives + false positives) $\times$ 100, where true negatives are defined as samples without mutations by both HRM and DNA sequencing, and false positives are samples showing mutations by HRM but none by DNA sequencing. Concordance analysis was performed to assess the near-equivalence of the 2 different measuring techniques (HRM and sequencing) used for assessing the presence/absence of mutations (35). Concordance between HRM and sequencing results was calculated as (true positives + true negatives)/(total number of samples) $\times$ 100 (35). The degree of concordance/agreement was measured by Cohen's kappa (κ), which was calculated as described previously (36). Sensitivity, specificity, and concordance were calculated using Stata version 11.1 (StataCorp, College Station, TX). Scatter plots and qPCR standard curves for estimation of bacterial load in sample DNA were plotted using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JCM.02104-16>.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.1 MB.

SUPPLEMENTAL FILE 3, PDF file, 0.4 MB.

SUPPLEMENTAL FILE 4, PDF file, 0.2 MB.

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REFERENCES

1. WHO. 2016. Global tuberculosis report. World Health Organization, Geneva, Switzerland. <http://apps.who.int/iris/bitstream/10665/250441/1/9789241565394-eng.pdf?ua=1>.
2. UNITAID. 2013. Tuberculosis diagnostics technology and market landscape. UNITAID, Geneva, Switzerland. http://tbevidence.org/wp-content/uploads/2013/12/UNITAID-TB_Dx_Landscape-Update_Dec-2013.pdf.
3. Nathavitharana RR, Hillemann D, Schumacher SG, Schlueter B, Ismail N, Omar SV, Sikhondze W, Havumaki J, Valli E, Boehme C, Denking CM. 2016. Multicenter noninferiority evaluation of Hain GenoType MTBDR-plus version 2 and Nipro NTM+MDRTB line probe assays for detection of rifampin and isoniazid. *J Clin Microbiol* 54:1624–1630.
4. WHO. 2008. Molecular line probe assays for rapid screening of patients at risk of multidrug-resistant tuberculosis (MDR-TB): policy statement. World Health Organization, Geneva, Switzerland. http://www.who.int/tb/features_archive/policy_statement.pdf?ua=1.
5. UNITAID. 2015. Tuberculosis diagnostics technology and market landscape, 4th ed. UNITAID, Geneva, Switzerland. https://www.unitaid.eu/assets/Tuberculosis_diagnostics_technology_and_market_landscape_4th_edition_Oct_2015-1.pdf.
6. Steingart KR, Schiller I, Horne DJ, Pai M, Boehme CC, Dendukuri N. 2014. Xpert MTB/RIF assay for pulmonary tuberculosis and rifampicin resistance in adults. *Cochrane Database Syst Rev* (1):CD009593. <https://doi.org/10.1002/14651858.CD009593.pub3>.
7. Garritano S, Gemignani F, Voegelé C, Nguyen-Dumont T, Le Calvez-Kelm F, De Silva D, Lesueur F, Landi S, Tavtigian SV. 2009. Determining the effectiveness of high resolution melting analysis for SNP genotyping and mutation scanning at the TP53 locus. *BMC Genet* 10:5.
8. Yin X, Zheng L, Liu Q, Lin L, Hu X, Hu Y, Wang Q. 2013. High-resolution melting curve analysis for rapid detection of rifampin resistance in *Mycobacterium tuberculosis*: a meta-analysis. *J Clin Microbiol* 51:3294–3299.
9. Yadav RSS, Mewara A, Dhatwalia SK, Gupta D, Sharma M. 2012. Rapid detection of rifampicin, isoniazid and streptomycin resistance in *Mycobacterium tuberculosis* clinical isolates by high-resolution melting curve analysis. *J Appl Microbiol* 113:856–862.
10. Ramirez MV, Cowart KC, Campbell PJ, Morlock GP, Sikes D, Winchell JM, Posey JE. 2010. Rapid detection of multidrug-resistant *Mycobacterium tuberculosis* by use of real-time PCR and high-resolution melt analysis. *J Clin Microbiol* 48:4003–4009.
11. Nagai Y, Iwade Y, Hayakawa E, Nakano M, Sakai T, Mitarai S, Katayama M, Nosaka T, Yamaguchi T. 2013. High resolution melting curve assay for rapid detection of drug-resistant *Mycobacterium tuberculosis*. *J Infect Chemother* 19:1116–1125.
12. Chen X, Kong F, Wang Q, Li C, Zhang J, Gilbert GL. 2011. Rapid detection of isoniazid, rifampin, and ofloxacin resistance in *Mycobacterium tuberculosis* clinical isolates using high-resolution melting analysis. *J Clin Microbiol* 49:3450–3457.
13. Lee AS, Ong DC, Wong JC, Siu GK, Yam WC. 2012. High-resolution melting analysis for the rapid detection of fluoroquinolone and streptomycin resistance in *Mycobacterium tuberculosis*. *PLoS One* 7:e31934. <https://doi.org/10.1371/journal.pone.0031934>.
14. Hong CY, Wang F, Liu XL. 2013. Detection of *pncA* mutation associated with pyrazinamide resistance in *Mycobacterium tuberculosis* by high-resolution melting cure analysis. *Zhonghua Jie He He Hu Xi Za Zhi* 36:198–201. (In Chinese.)
15. Galarza M, Fasabi M, Levano KS, Castillo E, Barreda N, Rodriguez M, Guio H. 2016. High-resolution melting analysis for molecular detection of multidrug resistance tuberculosis in Peruvian isolates. *BMC Infect Dis* 16:260.
16. Ong DC, Yam WC, Siu GK, Lee AS. 2010. Rapid detection of rifampicin- and isoniazid-resistant *Mycobacterium tuberculosis* by high-resolution melting analysis. *J Clin Microbiol* 48:1047–1054.
17. Pholwat S, Liu J, Stroup S, Gratz J, Banu S, Rahman SM, Ferdous SS, Foongladda S, Boonlert D, Ogarkov O, Zhdanova S, Kibiki G, Heysell S, Hout E. 2015. Integrated microfluidic card with TaqMan probes and high-resolution melt analysis to detect tuberculosis drug resistance mutations across 10 genes. *mBio* 6(2):e02273-14. <https://doi.org/10.1128/mBio.02273-14>.
18. WHO. 2015. Update. Multidrug-resistant Tuberculosis (MDR-TB). World Health Organization, Geneva, Switzerland.
19. Choi GE, Lee SM, Yi J, Hwang SH, Kim HH, Lee EY, Cho EH, Kim JH, Kim HJ, Chang CL. 2010. High-resolution melting curve analysis for rapid detection of rifampin and isoniazid resistance in *Mycobacterium tuberculosis* clinical isolates. *J Clin Microbiol* 48:3893–3898.
20. Lingala MAL, Srikantham A, Jain S, Rao KVSM, Rao PVR. 2010. Clinical and geographical profiles of *rpoB* gene mutations in *Mycobacterium tuberculosis* isolates from Hyderabad and Koraput in India. *J Microbiol Antimicrob* 2:13–18.
21. Thirumurugan R, Kathirvel M, Vallayachari K, Surendar K, Samrot AV, Muthaiah M. 2015. Molecular analysis of *rpoB* gene mutations in rifampicin resistant *Mycobacterium tuberculosis* isolates by multiple allele

- specific polymerase chain reaction in Puducherry, South India. *J Infect Public Health* 8:619–625.
22. Gupta A, Prakash P, Singh SK, Anupurba S. 2013. Rapid genotypic detection of *rpoB* and *katG* gene mutations in *Mycobacterium tuberculosis* clinical isolates from Northern India as determined by MAS-PCR. *J Clin Lab Anal* 27:31–37.
 23. Makadia JS, Jain A, Patra SK, Sherwal BL, Khanna A. 2012. Emerging trend of mutation profile of *rpoB* gene in MDR tuberculosis, North India. *Ind J Clin Biochem* 27:370–374. <https://doi.org/10.1007/s12291-012-0228-5>.
 24. Shenai S, Rodrigues C, Mehta A. 2009. Rapid speciation of 15 clinically relevant mycobacteria with simultaneous detection of resistance to rifampin, isoniazid, and streptomycin in *Mycobacterium tuberculosis* complex. *Int J Infect Dis* 13:46–58.
 25. Kumar P, Balooni V, Sharma BK, Kapil V, Sachdeva KS, Singh S. 2014. High degree of multi-drug resistance and hetero-resistance in pulmonary TB patients from Punjab state of India. *Tuberculosis* 94:73–80.
 26. Ramaswamy S, Musser JM. 1998. Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: 1998 update. *Tuber Lung Dis* 79:3–29.
 27. Mani C, Selvakumar N, Narayanan S, Narayanan PR. 2001. Mutations in the *rpoB* gene of multidrug-resistant *Mycobacterium tuberculosis* clinical isolates from India. *J Clin Microbiol* 39:2987–2990.
 28. Coovadia YM, Mahomed S, Pillay M, Werner L, Mlisana K. 2013. Rifampicin mono-resistance in *Mycobacterium tuberculosis* in KwaZulu-Natal, South Africa: a significant phenomenon in a high prevalence TB-HIV region. *PLoS One* 8:e77712. <https://doi.org/10.1371/journal.pone.0077712>.
 29. Negi SS, Gupta S, Lal S. 2003. Drug resistance in tuberculosis in Delhi: a 2 year profile (2001–2002). *J Commun Dis* 35:74–81.
 30. Huyen MNT, Cobelens FGJ, Buu TN, Lan NTN, Dung NH, Kremer K, Tiemersma EW, van Soolingen D. 2013. Epidemiology of isoniazid resistance mutations and their effect on tuberculosis treatment outcomes. *Antimicrob Agents Chemother* 57:3620–3627. <https://doi.org/10.1128/AAC.00077-13>.
 31. Seifert M, Catanzaro D, Catanzaro A, Rodwell TC. 2015. Genetic mutations associated with isoniazid resistance in *Mycobacterium tuberculosis*: a systematic review. *PLoS One* 10:e0119628. <https://doi.org/10.1371/journal.pone.0119628>.
 32. Yadav R, Sethi S, Dhatwalia SK, Gupta D, Mewara A, Sharma M. 2013. Molecular characterisation of drug resistance in *Mycobacterium tuberculosis* isolates from North India. *Int J Tuberc Lung Dis* 17:251–257.
 33. Kumar P, Balooni V, Singh S. 2015. Genetic mutations associated with rifampicin and isoniazid resistance in MDR-TB patients in North-West India. *Int J Tuberc Lung Dis* 19:434–439.
 34. Ramachandran R, Nalini S, Chandrasekar V, Dave PV, Sanghvi AS, Wares F, Paramasivan CN, Narayanan PR, Sahu S, Parmar M, Chadha S, Dewan P, Chauhan LS. 2009. Surveillance of drug-resistant tuberculosis in the state of Gujarat, India. *Int J Tuberc Lung Dis* 13:1154–1160.
 35. Kwiecien R, Kopp-Schneider A, Blettner M. 2011. Concordance analysis: part 16 of a series on evaluation of scientific publications. *Dtsch Arztebl Int* 108:515–521.
 36. Watson PF, Petrie A. 2010. Method agreement analysis: a review of correct methodology. *Theriogenology* 73:1167–1179.
 37. Myneedu VP, Singhal R, Khayyam KU, Sharma PP, Bhalla M, Behera D, Sarin R. 2015. First and second line drug resistance among treatment naive pulmonary tuberculosis patients in a district under Revised National Tuberculosis Control Programme (RNTCP) in New Delhi. *J Epidemiol Glob Health* 5:365–373.
 38. Sethi S, Mewara A, Dhatwalia SK, Singh H, Yadav R, Singh K, Gupta D, Wanchu A, Sharma M. 2013. Prevalence of multidrug resistance in *Mycobacterium tuberculosis* isolates from HIV seropositive and seronegative patients with pulmonary tuberculosis in north India. *BMC Infect Dis* 13:137.
 39. Pietzka AT, Indra A, Stoger A, Zeinzinger J, Konrad M, Hasenberger P, Allerberger F, Ruppmitsch W. 2009. Rapid identification of multidrug-resistant *Mycobacterium tuberculosis* isolates by *rpoB* gene scanning using high-resolution melting curve PCR analysis. *J Antimicrob Chemother* 63:1121–1127.
 40. O'Sullivan DM, McHugh TD, Gillespie SH. 2005. Analysis of *rpoB* and *pncA* mutations in the published literature: an insight into the role of oxidative stress in *Mycobacterium tuberculosis* evolution? *J Antimicrob Chemother* 55:674–679.
 41. Iøerger TR, Koo S, No EG, Chen X, Larsen MH, Jacobs WR, Jr, Pillay M, Sturm AW, Sacchettini JC. 2009. Genome analysis of multi- and extensively-drug-resistant tuberculosis from KwaZulu-Natal, South Africa. *PLoS One* 4:e7778. <https://doi.org/10.1371/journal.pone.0007778>.
 42. Comas I, Borrell S, Roetzer A, Rose G, Malla B, Kato-Maeda M, Galagan J, Niemann S, Gagneux S. 2012. Whole-genome sequencing of rifampicin-resistant *M. tuberculosis* strains identifies compensatory mutations in RNA polymerase. *Nat Genet* 44:106–110. <https://doi.org/10.1038/ng.1038>.
 43. Rinder H, Mieskes KT, Loscher T. 2001. Heteroresistance in *Mycobacterium tuberculosis*. *Int J Tuberc Lung Dis* 5:339–345.
 44. Tolani MP, D'Souza DT, Mistry NF. 2012. Drug resistance mutations and heteroresistance detected using the GenoType MTBDRplus assay and their implication for treatment outcomes in patients from Mumbai, India. *BMC Infect Dis* 12:9.
 45. Hofmann-Thiel S, van Ingen J, Feldmann K, Turaev L, Uzakova GT, Murmusaeva G, van Soolingen D, Hoffmann H. 2009. Mechanisms of heteroresistance to isoniazid and rifampin of *Mycobacterium tuberculosis* in Tashkent, Uzbekistan. *Eur Respir J* 33:368–374.
 46. Pholwat S, Stroup S, Gratz J, Trangan V, Foongladda S, Kumburu H, Juma SP, Kibiki G, Houpt E. 2014. Pyrazinamide susceptibility testing of *Mycobacterium tuberculosis* by high resolution melt analysis. *Tuberculosis (Edinb)* 94:20–25.
 47. Eilertson B, Maruri F, Blackman A, Herrera M, Samuels DC, Sterling TR. 2014. High proportion of heteroresistance in *gyrA* and *gyrB* in fluoroquinolone-resistant *Mycobacterium tuberculosis* clinical isolates. *Antimicrob Agents Chemother* 58:3270–3275.
 48. Engström A, Hoffner S, Jureen P. 2013. Detection of heteroresistant *Mycobacterium tuberculosis* by pyrosequencing. *J Clin Microbiol* 51:4210–4212.
 49. Haldar S, Chakravorty S, Bhalla M, De Majumdar S, Tyagi JS. 2007. Simplified detection of *Mycobacterium tuberculosis* in sputum using smear microscopy and PCR with molecular beacons. *J Med Microbiol* 56:1356–1362.
 50. da Silva JL, Leite GGS, Bastos GM, Lucas BC, Shinohara DK, Takinami JS, Miyata M, Fajardo CM, Luchessi AD, Leite CQ, Cardoso RF, Hirata RD, Hirata MH. 2013. Plasmid-based controls to detect *rpoB* mutations in *Mycobacterium tuberculosis* by quantitative polymerase chain reaction-high-resolution melting. *Mem Inst Oswaldo Cruz* 108:106–109.
 51. Sanchez-Padilla E, Merker M, Beckert P, Jochims F, Dlamini T, Kahn P, Bonnet M, Niemann S. 2015. Detection of drug-resistant tuberculosis by Xpert MTB/RIF in Swaziland. *N Engl J Med* 372:1181–1182.
 52. Puri L, Oghor C, Denkinger CM, Pai M. 2016. Xpert MTB/RIF for tuberculosis testing: access and price in highly privatised health markets. *Lancet Glob Health* 4:e94–95.
 53. Pai M. 2013. Promoting affordable and quality tuberculosis testing in India. *J Lab Physicians* 5:1–4.
 54. RNTCP. 2005. Technical and operational guidelines for tuberculosis control. Revised National Tuberculosis Control Programme, New Delhi, India. <http://health.bih.nic.in/Docs/Guidelines/Guidelines-TB-Control.pdf>.
 55. Van Deun A, Aung KJ, Bola V, Lebeke R, Hossain MA, de Rijk WB, Rigouts L, Gumusboga A, Torrea G, de Jong BC. 2013. Rifampin drug resistance tests for tuberculosis: challenging the gold standard. *J Clin Microbiol* 51:2633–2640.
 56. McAlister AJ, Driscoll J, Metchock B. 2015. DNA sequencing for confirmation of rifampin resistance detected by Cepheid Xpert MTB/RIF assay. *J Clin Microbiol* 53:1752–1753.