JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 2010, p. 1047–1054 Vol. 48, No. 4
0095-1137/10/$12.00 doi:10.1128/JCM.02036-09

Rapid Detection of Rifampicin- and Isoniazid-Resistant *Mycobacterium tuberculosis* by High-Resolution Melting Analysis[▽]

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Received 9 October 2009/Returned for modification 22 December 2009/Accepted 10 February 2010

We have developed a high-resolution melting (HRM) assay to scan for mutations in the *rpoB*, *inhA*, *ahpC*, and *katG* genes and/or promoter regions for the detection of rifampin and isoniazid resistance in *Mycobacterium tuberculosis*. For assay development, 23 drug-resistant isolates of *M. tuberculosis* having 29 different mutations, together with 40 drug-susceptible isolates, were utilized. All 29 mutations were accurately detected by our assay. We further validated the assay with a series of 59 samples tested in a blind manner. All sequence alterations that were within the regions targeted by the HRM assay were correctly identified. Compared against results of DNA sequencing, the sensitivity and specificity of our HRM assay were 100%. For the blinded samples, the specificities and sensitivities were 89.3% and 100%, respectively, for detecting rifampin resistance and 98.1% and 83.3%, respectively, for detecting isoniazid resistance, as isolates with mutations in regions not encompassed by our assay were not detected. A C-to-T sequence alteration at position −15 of the *ahpC* regulatory region, which was previously reported to be associated with isoniazid resistance, may possibly be a polymorphism, as it was detected in an isoniazid-susceptible *M. tuberculosis* isolate. HRM is a rapid, accurate, simple, closed-tube, and low-cost method. It is thus an ideal assay to be used in countries with a high prevalence of drug-resistant *M. tuberculosis* and where cost-effectiveness is essential. As a mutation-scanning assay for detecting drug-resistant *M. tuberculosis*, it can potentially lead to better treatment outcomes resulting from earlier treatment with the appropriate antibiotics.

The emergence of multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant TB (XDR-TB) has hampered the control and treatment of TB (45). MDR-TB is defined as TB that is resistant to at least isoniazid (INH) and rifampin (RIF), two main first-line antitubercular drugs, while XDR-TB is MDR-TB that is additionally resistant to three or more second-line drugs. MDR-TB accounts for an estimated 5% of all TB cases (44); however, patients are often not expeditiously diagnosed, resulting in the delay of appropriate treatment as well as poorer treatment outcomes for patients and the propagation and spread of MDR-TB. Conventional methods for drug susceptibility testing of MDR-TB require an additional culture period, typically between 2 and 5 weeks. An easy-to-implement, cost-effective, and rapid method for drug susceptibility testing is thus of paramount importance to limit the spread of drug-resistant tuberculosis.

Drug resistance in *Mycobacterium tuberculosis* is due to mutations in genes or promoters of genes activating the drug or encoding the drug targets, which are detectable in the majority of drug-resistant isolates (41). Mutations associated with RIF resistance occur mainly in an 81-bp RIF resistance-determining region (RRDR) of the *rpoB* gene (codons 507 to 533; numbering according to the *Escherichia coli rpoB* sequence), with >95% of RIF-resistant isolates containing at least one mutation in this region (12, 13, 22, 28, 31, 36, 43). Mutations associated with INH resistance occur mainly in the *katG* gene (codon 315), the *inhA* gene and regulatory region, and the *ahpC* regulatory region (11, 20, 27, 29, 34, 40, 42).

While several molecular methods have been previously described for drug susceptibility testing of *M. tuberculosis* (2, 6, 7, 9, 26, 38), the cost and technical demands of the assays restrict their usage, especially in countries where funds are scarce. Another problem with the majority of PCR-based molecular methods is the requirement of downstream processing of PCR products, which exposes the PCR products to the environment, increasing the risk of cross-contamination of subsequent assays.

The high-resolution melting (HRM) analysis is a simple, cost-effective, closed-tube method with sensitivity and specificity reported to be higher than those of denaturing high-performance liquid chromatography (dHPLC) (3). HRM does not require the use of costly fluorescent probes and requires no post-PCR handling, making it an attractive alternative method for genotypic drug susceptibility testing of *M. tuberculosis*. The method involves performing a PCR with a saturating double-stranded DNA-binding dye such as Syto9, followed by a high-resolution melt analysis, whereby the amplicons are slowly heated to denaturation with real-time monitoring of the decrease in fluorescence during denaturation. By comparing the melting profile of the sample with a reference, any sequence

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▽ Published ahead of print on 17 February 2010.

TABLE 1. Primer and probe sequences used for PCR

Primer name[a]	Primer/probe sequence	Amplicon size (bp)	Annealing temp (°C)	HRM temp range (°C)	Nucleotide positions[b]
katG_F	5′-CTGGAGCAGATGGGCTTG-3′	195	66	84 to 91	877 to 894
katG_R	5′-GTCCTTGGCGGTGTATTGC-3′				1071 to 1053
mabA-Prom_F	5′-ACACCGACAAACGTCACGAG-3′	143	67	85 to 92	−121 to −102
mabA-Prom_R	5′-CTTCAGTGGCTGTGGCAGTC-3′				22 to 3
inhA-Frag1_F	5′-GCCACTGACACAACACAAGG-3′	153	63	83 to 92	−26 to −7
inhA-Frag1_R	5′-GGTCGAACCCGGTGAGC-3′				127 to 111
inhA-Frag2_F	5′-CTGGTGCTCACCGGGTTC-3′	167	63	88 to 95	106 to 123
inhA-Frag2_R	5′-ACCCCGTCGAGCTTGTTG-3′				272 to 255
inhA-Frag3_F	5′-CGAACTCGACGTGCAAAAC-3′	148	62	85 to 94	183 to 201
inhA-Frag3_R	5′-GTCGAAGAACGGGTTGATG-3′				330 to 312
ahpC-Prom_F	5′-ACTGCTGAACCACTGCTTTG-3′	177	60	81 to 94	−144 to −125
ahpC-Prom_R	5′-CATCATCAAAGCGGACAATG-3′				33 to 14
ahpC_Probe-InvdT	5′-GATGCCGATAAATATGGTGTGATATAT-3′			53 to 65	−66 to −40
rpoB_F	5′-CCGCGATCAAGGAGTTCTTC-3′	129	67	84 to 93	1256 to 1275
rpoB_R	5′-CACGCTCACGTGACAGACC-3′				1384 to 1366

[a] F, forward; R, reverse.
[b] Nucleotide position is relative to the transcriptional start site of each gene.

variance can be detected. Homoduplexes are usually detected by a change in melting temperature (T_m), while heteroduplexes are usually detected by a change in the melt curve shape (24). As it is easier to identify a change in melt curve shape (10), the sample and reference DNA can be mixed together and amplified together by PCR to produce heteroduplexes, as in the method we have developed.

However, as HRM analysis detects all mutations within the PCR amplicon, known polymorphisms that lie within the amplicon can be excluded by the use of an unlabeled oligonucleotide probe as described by Zhou et al. (48). Briefly, a 3′-blocked unlabeled probe, designed to encompass the polymorphism, is included in the PCR.

In this study, we evaluated the efficacy of our assay in comparison with that of standard drug susceptibility testing for the detection of RIF- and/or INH-resistant *M. tuberculosis* strains from clinical specimens.

MATERIALS AND METHODS

***M. tuberculosis* clinical isolates and drug susceptibility testing.** A total of 23 drug-resistant and 40 drug-susceptible clinical isolates of *M. tuberculosis* used for the initial assay development were collected from the Central Tuberculosis Laboratory, Department of Pathology, Singapore General Hospital, and have been described previously (21). Phenotypic drug susceptibility testing was done using the BACTEC 460 system (Becton Dickinson, Towson, MD) with 2 μg/ml of RIF and 0.1 μg/ml of INH. The 59 clinical isolates of *M. tuberculosis* used for the screening in a blind manner were obtained from Hong Kong, and the agar proportion method was used for susceptibility testing for RIF and INH, as described previously (30).

DNA extraction. DNA from the clinical isolates of *M. tuberculosis* used for the initial assay development was extracted from bacterial colonies grown on Löwenstein-Jensen slants as described previously (8). DNA from the clinical isolates of *M. tuberculosis* used for the blinded screening was extracted as described previously (46) and purified using phenol-chloroform-isoamyl alcohol (25:24:1) (Invitrogen). All DNA samples were quantified using the Nanodrop 1000 (Thermo Scientific, Waltham, MA).

Primers and probes. Seven pairs of primers were designed to flank regions that were previously reported to be associated with RIF and INH resistance in *M. tuberculosis*. For *rpoB*, primers were designed to flank the RRDR region. For the *mabA* promoter, primers were designed to include the promoter, starting from 121 bp upstream of *mabA*. For *inhA*, three overlapping sets of primers were designed to detect the majority of mutations, including the 5′ end of the gene (including codon 110). For the *ahpC* promoter, the promoter was included starting from 144 bp upstream from the *ahpC* transcription start site. However, as there is a known polymorphism at position −46 of *ahpC* (11), we designed an additional unlabeled oligonucleotide probe (3′ blocked by an inverted deoxyribosylthymine [dT]) to specifically detect this polymorphism (48). All primer and probe sequences are listed in Table 1. Amplicon lengths were kept below 200 bp, as shorter amplicons produce melt curves of altered shapes that are easier to identify when there is a sequence variant (10).

Real-time PCR and high-resolution melting (HRM). Prior to performing the HRM assay, a preliminary real-time PCR was done to ensure that the DNA concentration of each sample was correctly quantified. Real-time PCR was performed with the *katG* primer set, the assay that has the highest stringency for detecting degraded samples as it has the largest amplicon size (degraded nucleic acids, being shorter in length, may be amplifiable by a PCR assay which amplifies a short region but not by one which amplifies a longer region). No reference DNA was added, and 0.4 ng of sample DNA was used. Samples with threshold cycle (C_T) values varying more than 2 cycles compared to a DNA sample of known high integrity were removed.

For the HRM assay, a PCR was performed in 10-μl reaction mixtures containing 0.2 ng sample DNA, 0.2 ng reference DNA (from *Mycobacterium tuberculosis* H37Rv), and 1× PCR buffer containing 1.5 mM $MgCl_2$, 200 nM deoxynucleoside triphosphates (dNTPs), 200 nM each primer, 1.5 μM Syto9 (Molecular Probes, Eugene, OR), 0.5 U of HotStarTaq polymerase (Qiagen, Hilden, Germany), and 4 μl of mineral oil (Sigma Aldrich, St. Louis, MO). Mineral oil is essential for preventing any evaporation or condensation and to increase the accuracy of the assay.

The PCR for the probe-based assay for *ahpC* (used to detect a known polymorphism) was performed in 10-μl reaction mixtures containing 0.2 ng sample DNA, 0.2 ng reference DNA, and 1× PCR buffer containing 1.5 mM $MgCl_2$, 200 nM dNTPs, 500 nM forward primer, 50 nM reverse primer, 500 nM probe, 300 nM 1.5 μM Syto9, 0.5 U of HotStarTaq polymerase, and 4 μl of mineral oil.

All PCRs were performed in duplicate. The PCR cycling and HRM analysis were performed on the Rotor-Gene 6000 (Corbett Research, Sydney, Australia). PCR cycling parameters were as follows: 95°C for 15 min, 40 cycles at 95°C for 20 s, and at the appropriate annealing temperature (Table 1) for 30 s. The melt curve was generated by heating, using the temperature ranges indicated in Table 1, at increments of 0.1°C/s, except for the *ahpC* probe, which had an additional melt at increments of 0.5°C/s in order to detect polymorphisms within the region encompassed by the *ahpC* probe. These melt curve parameters were optimized experimentally, and the parameters used are the ones that worked best to discriminate the mutants. The HRM curve was analyzed using the Rotor-Gene 1.7.87 software and the HRM algorithm included in the software, and the melt curves were normalized using the software and following the software instructions.

DNA sequencing. The PCR products were sequenced as described previously (16, 21–23) or using the Applied Biosystems 3130XL genetic analyzer (Foster City, CA). The clinical isolates of *M. tuberculosis* used for the blinded screening were sequenced for *katG*, *mabA-inhA*, and *rpoB* genes and promoter regions using an Applied Biosystems 3130 genetic analyzer using the primer sequences listed in Table 2.

TABLE 2. Primer sequences used for sequencing of a blinded series of 59 *M. tuberculosis* clinical isolates

Gene (Rv no.)	GeneID	Primer name	Length[a]	T_m	%GC[b]	Direction[c]	Sequence	Nucleotide positions
katG (Rv1908c)	885638	katG-pcrF	23	58.2	42	F	GATTGTTCGATATCCGACACTTC	−76 to −54
		katG-1F	21	59.78	52	F	AGCAACACCCACCCATTACAG	8 to 28
		katG-2F	18	58.96	67	F	GGCACCTACCGCATCCAC	331 to 348
		katG-3R	19	59.63	53	R	AAAACAATCAGGTCCGCCC	500 to 482
		katG-4F	18	59.74	61	F	CGTCGGCGGTCACACTTT	798 to 815
		katG-5R	18	59.47	61	R	GGTTCCGGTGCCATACGA	924 to 907
		katG-6F	20	59.11	55	F	GTCCCGTTGCGAGATACCTT	1262 to 1281
		katG-7R	22	60.52	50	R	TGTGAGACAGTCAATCCCGATG	1412 to 1391
		katG-8F	18	60.42	61	F	CGCAGGAACAAACCGACG	1724 to 1741
		katG-9R	20	59.02	50	R	TGCCCTTTCCGAGGTAGTTT	1805 to 1785
		katG-10R	18	59.48	56	R	GCCGATCAACCCGAATCA	15 to −2221
mabA-inhA (Rv1483–Rv1484)	886551–886523	inhA-pcrF	21	60.15	52	F	TTCGTAGGGCGTCAATACACC	*mabA*, −200 to −180
		inhA-2F	19	59.87	58	F	TCACGGCGGTAGAAGAGCA	*mabA*, 215 to 233
		inhA-3R	18	59.83	67	R	GCGTTGGACACCAGCACC	*mabA*, 266 to 249
		inhA-4F	18	59.49	56	F	TTTATCCCAGCGAAGCGG	*mabA*, 613 to 630
		inhA-5R	18	60.32	67	R	CCTCGGAAGCCAGGAAGC	*mabA*, 682 to 655
		inhA-6F	17	58.23	65	F	GTTCATGCCGCAGACCG	*inhA*, 288 to 303
		inhA-7R	21	60.25	48	R	AAGCATACGAATACGCCGAGA	*inhA*, 385 to 365
		inhA-pcrR	17	59.91	71	R	GGTCCGCCGAACGACAG	*inhA*, 46 to 30
rpoB (Rv0667)	888164	rpoB-pcrF	22	63.09	55	F	CTTTGTTCGTGGTGAGCGTGAG	−565 to −544
		rpoB-2F	18	60.53	61	F	TGCGGCTCAGCGGTTTAG	−147 to −130
		rpoB-3R	19	61.5	53	R	GCCGCGATAATTTTGTCGG	−70 to −88
		rpoB-4F	22	62.22	55	F	GTCGACGAGTGCAAAGAC AAGG	337 to 358
		rpoB-5R	22	61.45	55	R	GAAGTCACCCATGAACACCGTC	453 to 432
		rpoB-6F	16	60.29	69	F	CGAGCCCCCGACCAAA	834 to 849
		rpoB-7R	18	60.35	61	R	TGCAGCCCGAGCTTCTTG	950 to 933
		rpoB-8F	18	61.06	61	F	CCCGATCGAAACCCCTGA	1434 to 1451
		rpoB-9R	18	62.42	67	R	GCGAGCCGATCAGACCGA	1480 to 1463
		rpoB-10F	19	61.51	58	F	GTGATGCACGACAACGGCA	1960 to 1978
		rpoB-11R	18	59.6	67	R	CGCATCCGGTAGGTACGC	2000 to 1983
		rpoB-12F	19	61.04	68	F	GGTGAGACCGAGCTGACGC	2410 to 2428
		rpoB-13R	19	61.04	68	R	GCGTCAGCTCGGTCTCACC	2428 to 2410
		rpoB-14F	20	64.72	60	F	ACGGCAAGGCCATGCTCTTC	2996 to 3015
		rpoB-15R	19	59.41	63	R	TGTAGGCAGCACCGTAGGC	3232 to 3214
		rpoB-pcrR	22	63.62	50	R	GTTGATCGTCTCCGGCTTTTTG	149 to 128

[a] Length, number of nucleotides.
[b] %GC, number of G's and C's in the primer as a percentage of the total number of nucleotides.
[c] F, forward; R, reverse.

RESULTS

Validation of the HRM assay with reference strains. For the initial assay development, we tested the assay for 29 different mutations within the *mabA* promoter ($n = 3$), *ahpC* promoter ($n = 6$), *katG* ($n = 6$), *inhA* ($n = 2$), and *rpoB* ($n = 12$) from 23 drug-resistant *M. tuberculosis* isolates (Table 3). All 29 mutations were detected by our HRM assay. We also analyzed 40 drug-susceptible isolates to check for false-positive results. All 40 isolates were correctly identified as wild type. The normalized melt curves of PCR products of isolates with different mutations are shown in Fig. 1. Visually, samples with mutations (colored lines) are easily differentiated from the wild type (black lines) by the distinct differences in the shape of the melt curves.

Validation of the HRM assay with a blinded series of strains. To further validate the assay, a series of 59 blinded samples was used to assess the sensitivity and specificity of the assay (Tables 4 and 5). Of the 28 RIF-resistant isolates in our blinded samples, 25 (89.3%) were detected to have mutations in *rpoB*. The three RIF-resistant isolates that were detected as wild type had mutations at I572F (two isolates) and at V169F (one isolate). These two mutations have been shown to associate with rifampin resistance upon transformation into *Mycobacterium smegmatis* (unpublished data). Both of these mutations are not within the RRDR region and were thus not detected by our HRM assay. All 31 RIF-susceptible isolates were correctly typed.

Of the 53 INH-resistant isolates in our blinded samples, 52 (98.1%) were detected to have mutations in *katG*, the *mabA* promoter, *inhA*, and/or *ahpC*. The isolate that was detected as wild type had a Y98C mutation in *katG*, which is not within the region covered by our assay. Five of six (83.3%) INH-susceptible isolates were correctly typed. Sample 48, although phenotypically INH susceptible, was found to contain a C-to-T sequence alteration at position −15 of the *ahpC* regulatory region. Thus, our results suggest that this sequence alteration, which was previously reported to be associated with INH resistance (11, 39), may possibly be a polymorphism.

Three INH-resistant isolates (sample 3, 4, and 46), although containing mutations in *katG* in regions that are not covered by

TABLE 3. Reference strains with known mutations used for assay development

Sample	Gene	Nucleotide change(s)	Amino acid change[a]
IE1	*katG*	TGG→TAG	Trp300Stop
IS28	*katG*	AGC→AGG	Ser302Arg
I86	*katG*	AGC→ACC	Ser315Thr
I92	*katG*	AGC→AAC	Ser315Asn
IR4	*katG*	AGC→ACC, ACG→ACA	Ser315Thr, Thr344Thr
IE5	*katG*	CTG→CGG	Leu336Arg
IRS6	*mabA*	−17 G→T	NA
ISD3	*mabA*	−8 T→A	NA
IRS7	*mabA*	−15 C→T	NA
IR27	*inhA*	GGA→GGC	Gly3Gly
IRS7	*inhA*	ATC→ACC	Ile21Thr
IR27	*ahpC*	−6 G→A	NA
IR5	*ahpC*	−12 C→T	NA
IRS12	*ahpC*	−30 C→T	NA
IS3	*ahpC*	−46 G→A	NA
IRS6	*ahpC*	−55 A→G	NA
I86	*ahpC*	−81 G→A	NA
R3	*rpoB*	CAA→GAA	Gln513Glu
IRE6	*rpoB*	TTC→CTC, GAC→GTC	Phe514Leu, Asp516Val
IR12	*rpoB*	GAC→GTC	Asp516Val
MDR8	*rpoB*	AAC deletion	Asn518 deletion
IR25	*rpoB*	TCG→TTG	Ser522Leu
IR27	*rpoB*	CAC→TAC	His526Tyr
IR7	*rpoB*	CAC→GAC	His526Asp
R1	*rpoB*	CAC→CGC	His526Arg
R2	*rpoB*	CAC→CTC	His526Leu
MDR6	*rpoB*	TCG→TTG	Ser531Leu
IRS12	*rpoB*	TCG→TGG	Ser531Trp
IRE3	*rpoB*	TCG→ATG	Ser531Met

[a] NA, not applicable.

our assay, were still correctly interpreted as INH resistant due to a concurrent mutation in the *ahpC* promoter (Table 4).

DISCUSSION

We have developed and evaluated a HRM-based assay for RIF and INH susceptibility testing of *M. tuberculosis*. This assay allows the detection of mutations that are commonly associated with RIF and INH resistance in five regions: RRDR of *rpoB* for RIF resistance and the *mabA* and *ahpC* promoters, *katG*, and *inhA* for INH resistance.

All 29 (100%) known mutations within the RIF- and INH-resistant strains used for assay development had clearly distinguishable melt curves using the HRM assay described in this study. When our assay was validated with a blinded series of samples, RIF resistance was detected with a sensitivity of 89.3% and a specificity of 100%. However, if the three RIF-resistant isolates with mutations not within the RRDR region were to be excluded, the sensitivity and specificity of our assay would be 100%. Since more than 95% of RIF resistance-associated mutations lie within the RRDR region (19, 35), it may not be necessary to screen outside the RRDR region, as this may increase the number of primer sets required for the assay and also the possibility of detecting polymorphisms, giving rise to false positives.

When evaluated for the detection of INH resistance in a blinded series of isolates, our assay performed with a sensitivity of 98.1% and a specificity of 83.3% (detecting five of six susceptible isolates). Four isolates had mutations in *katG* in regions not encompassed by our assay. The phenotypes of three of these isolates were, however, still interpreted correctly, as another concurrent mutation in the *ahpC* promoter was detected. Since the majority of INH resistance-associated mutations in *katG* are within codon 315 (11, 27, 40), codon 315 has been commonly used as the only target in *katG* for genotypic INH susceptibility assays, in research studies and commercially available kits (2, 15, 18, 33, 47), and thus it may not be necessary to screen other regions of *katG*.

A key advantage of genotypic drug susceptibility assays over phenotypic assays is the shorter time required for the assay, with genotypic assays requiring just hours to complete in contrast to phenotypic tests that can take weeks. Due to the need to grow the organism, phenotypic methods require weeks of culture, during which the patient may be treated with the wrong antibiotics, resulting in poorer treatment outcome or the transmission of resistant strains. As genotypic tests do not require additional culture beyond that required for the initial isolation, there will also be fewer biohazard-related risks. In addition, genotypic tests may also be able to provide information on drug susceptibility in cases in which the phenotypic assay is indeterminate due to primary culture contamination.

In recent years, numerous genotypic assays (2, 6, 7, 9, 26, 38), including several commercial kits (1, 14, 15, 25), have been described for the detection of drug resistance in *M. tuberculosis*. Although rapid, most of them are too costly, labor-intensive, or technically demanding. Another major problem faced with the use of these assays, including the commercial kits, is the risk of cross-contamination with PCR products (44). This is because almost all genotypic drug susceptibility tests involve PCR, followed by the subsequent processing of the PCR products for the detection of mutations, increasing the potential risk of cross-contamination of subsequent reactions. This is often not a problem for a well-set-up molecular biology laboratory, which will have separate rooms or areas for pre-PCR and post-PCR work as well as skilled molecular biologists with stringent work practices. However, most TB labs in high-burden countries are unfortunately not as well equipped in molecular biology and may have space constraints, which may lead to poor-quality results, thus making implementation of these assays problematic.

In comparison to a previously reported HRM RIF susceptibility testing method (17) as well as other genotypic assays which are not closed tube, as they require the reopening of tubes after PCR to mix the samples and reference DNA together prior to HRM, our method has the advantage of being closed tube, thus lowering the risk of cross-contamination. This in itself makes the HRM assay easier to implement, as there will not be a need to make major changes to the lab. Furthermore, the simplicity of the assay, its low cost (estimated at approximately $0.30 per HRM reaction), and its low level of technical requirements makes it even more appealing as a rapid diagnostic test. In comparison to probe-based assays such as the Genotype MTBDRplus assay (Hain Lifescience, Nehren, Germany), our assay is more comprehensive, as it targets the *inhA* gene and *ahpC* promoter in addition to the *katG* gene and *mabA* promoter. This increases the sensitivity of our assay by about 4 to 10% in comparison to results from assays tar-

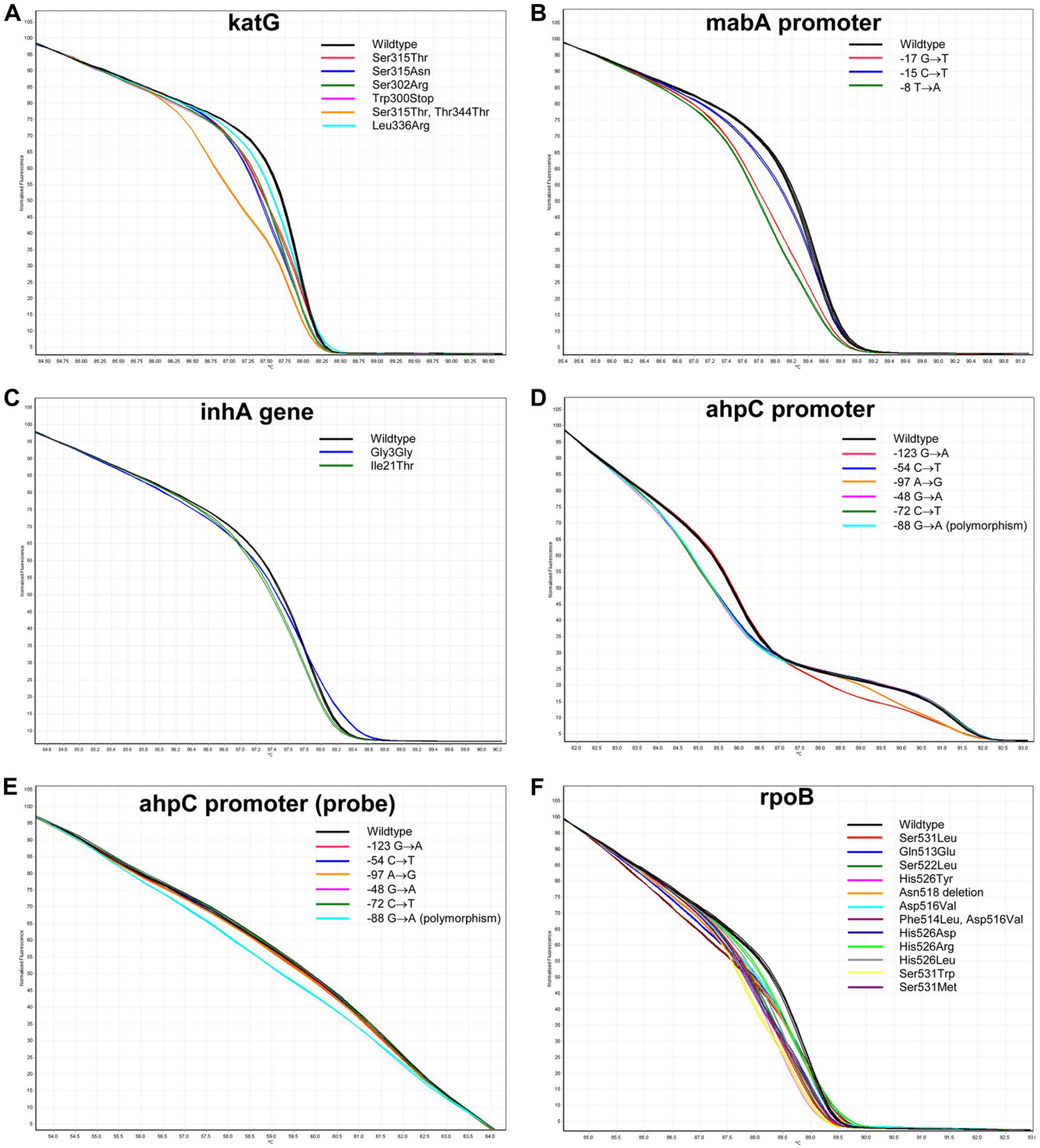

FIG. 1. High-resolution melt curves of *katG* (A), the *mabA* promoter (B), *inhA* (C), the *ahpC* promoter (D), the *ahpC* promoter (probe) (E), and *rpoB* (F), demonstrating the change in melt curve shape caused by mutations. Wild-type samples are shown in black, and samples with mutations are shown in color. Experiments were performed in duplicate.

geting solely the *katG* and the *mabA* promoter (4, 5, 11, 32, 37). HRM is also able to detect mutations over a much broader region (including novel mutations), making it more feasible for detecting drug resistance-associated mutations that are scattered throughout large regions. With increased coverage, the sensitivity of the assay is correspondingly increased. Each HRM reaction is also highly sensitive, requiring only 0.2 ng of sample DNA.

TABLE 4. HRM screening of a blinded series of 59 *Mycobacterium tuberculosis* clinical isolates for rifampin and isoniazid resistance

Sample	Mutation status[a]							HRM interpretation of drug resistance[b]	
	rpoB	*katG*	*mabA* promoter	*inhA* fragment 1	*inhA* fragment 2	*inhA* fragment 3	*ahpC* promoter	Rifampin	Isoniazid
1	NM	NM	M	NM	NM	NM	NM	S	R
2	NM	M	NM	NM	NM	NM	NM	S	R
3	NM	**NM (Q434STOP)**	NM	NM	NM	NM	M	S	R
4	NM	**NM (N655D)**	NM	NM	NM	NM	M	S	R
5	NM	**NM (Y98C)**	NM	NM	NM	NM	NM	S	**S (R)**
6	NM	NM	M	NM	NM	NM	NM	S	R
7	NM	NM	NM	M	NM	NM	M	S	R
8	NM	M	NM	NM	NM	NM	NM	S	R
9	NM	M	NM	NM	NM	NM	NM	S	R
10	NM	NM	M	NM	NM	NM	NM	S	R
11	M	M	NM	NM	NM	NM	NM	R	R
12	M	NM	M	M	NM	NM	NM	R	R
13	M	NM	NM	NM	NM	NM	M	R	R
14	M	NM	M	NM	NM	NM	NM	R	R
15	M	M	NM	NM	NM	NM	NM	R	R
16	M	M	NM	NM	NM	NM	NM	R	R
17	M	NM	M	NM	NM	NM	NM	R	R
18	M	NM	M	NM	NM	NM	NM	R	R
19	M	M	NM	NM	NM	NM	NM	R	R
20	M	M	NM	NM	NM	NM	NM	R	R
21	M	M	NM	NM	NM	NM	NM	R	R
22	**NM (I572F)**	NM	M	NM	NM	NM	NM	**S (R)**	R
23	**NM (V169F)**	M	NM	NM	NM	NM	NM	**S (R)**	R
24	NM	NM	NM	NM	NM	NM	NM	S	S
25	NM	NM	NM	NM	NM	NM	M	S	R
26	NM	M	NM	NM	NM	NM	M	S	R
27	NM	NM	NM	NM	NM	NM	NM	S	S
28	NM	M	NM	NM	NM	NM	NM	S	R
29	NM	NM	M	NM	NM	NM	NM	S	R
30	NM	M	NM	NM	NM	NM	NM	S	R
31	NM	M	NM	NM	NM	NM	NM	S	R
32	NM	NM	M	NM	NM	NM	M	S	R
33	NM	M	NM	NM	NM	NM	NM	S	R
34	NM	NM	M	NM	NM	NM	NM	S	R
35	NM	NM	M	NM	NM	NM	NM	S	R
36	NM	NM	M	NM	NM	NM	NM	S	R
37	NM	NM	M	NM	NM	NM	M	S	R
38	NM	NM	M	NM	NM	NM	NM	S	R
39	M	NM	M	NM	NM	NM	NM	R	R
40	M	NM	M	NM	NM	NM	NM	R	R
41	M	NM	M	NM	NM	NM	NM	R	R
42	M	M	NM	NM	NM	NM	NM	R	R
43	M	M	NM	NM	NM	NM	NM	R	R
44	M	M	NM	NM	NM	NM	M	R	R
45	M	M	NM	NM	NM	NM	NM	R	R
46	M	**NM (A478DEL)**	NM	NM	NM	NM	M	R	R
47	M	NM	M	NM	NM	NM	NM	R	R
48	M	NM	NM	NM	NM	NM	**M (P)**	R	**R (S)**
49	NM	NM	NM	NM	NM	NM	NM	S	S
50	NM	NM	NM	NM	NM	NM	NM	S	S
51	NM	M	NM	NM	NM	NM	NM	S	R
52	NM	NM	M	NM	NM	NM	NM	S	R
53	M	M	NM	NM	NM	NM	NM	R	R
54	M	NM	M	M	NM	NM	NM	R	R
55	NM	NM	NM	M	NM	NM	M	S	R
56	M	M	NM	NM	NM	NM	M	R	R
57	M	M	NM	NM	NM	NM	NM	R	R
58	**NM (I572F)**	NM	M	NM	NM	NM	NM	**S (R)**	R
59	NM	NM	NM	NM	NM	NM	NM	S	S

[a] NM, no mutation; M, mutation; P, polymorphism. Discrepant results between HRM screening and DNA sequencing are shown in bold. The mutations detected by sequencing are in parentheses.

[b] S, susceptible; R, resistant. Discrepant results between HRM screening and phenotypic drug susceptibility testing are shown in bold. The phenotypes detected by drug susceptibility testing are shown in parentheses.

TABLE 5. Sensitivity and specificity of the high-resolution melting (HRM) assay in comparison with drug susceptibility testing

Drug susceptibility	No. of isolates (n = 59): Mutation positive by HRM	No. of isolates (n = 59): Mutation negative by HRM	Sensitivity[a] (95% CI[b])	Specificity[c] (95% CI)
Rifampin			25/28, 89.3% (72.0–97.1)	31/31, 100% (90.4–100)
Resistant	25	3		
Susceptible	0	31		
Isoniazid			52/53, 98.1% (89.1–100)	5/6, 83.3% (41.8–98.9)
Resistant	52	1		
Susceptible	1	5		

[a] [Number of drug-resistant isolates with mutations]/[number of drug-resistant isolates with mutations + number of drug-resistant isolates without mutation].
[b] Statistical calculations were performed with the free software available from http://www.measuringusability.com/wald.htm using the Adjusted Wald method.
[c] [Number of drug-susceptible isolates without mutations]/[number of drug-susceptible isolates with mutations + number of drug-susceptible isolates without mutations].

As all genotypic methods for detecting drug susceptibility rely on the detection of mutations associated with drug resistance, the reliability of genotypic methods ultimately depends on our knowledge of molecular mechanisms for drug resistance. Our current knowledge of molecular mechanisms for drug resistance remains incomplete, especially for second-line antituberculosis drugs, and future research into determining these molecular mechanisms will result in improved genotype-based drug susceptibility assays.

ACKNOWLEDGMENTS

We acknowledge the Central Tuberculosis Laboratory, Department of Pathology, Singapore General Hospital, for providing isolates.

This work was supported by a grant from the Biomedical Research Council (BMRC) of Singapore (BMRC 07/1/31/19/511).

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