

Improved rapid molecular diagnosis of multidrug-resistant tuberculosis using a new reverse hybridization assay, REBA MTB-MDR

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Rapid diagnosis of multidrug-resistant tuberculosis (MDR-TB) is essential for the prompt initiation of effective second-line therapy to improve treatment outcome and limit transmission of this obstinate disease. A variety of molecular methods that enable the rapid detection of mutations implicated in MDR-TB have been developed. The sensitivity of the methods is dependent, in principle, on the repertoire of mutations being detected, which is typically limited to mutations in the genes *rpoB*, *katG* and the promoter region of *inhA*. In this study, a new reverse hybridization assay, REBA MTB-MDR (M&D), that probes mutations in the *oxyR-ahpC* intergenic region, in addition to those in *rpoB*, *katG* and the *inhA* promoter region, was evaluated. A set of 240 *Mycobacterium tuberculosis* clinical isolates from patients receiving retreatment regimens was subjected to conventional phenotypic drug-susceptibility testing (DST) and the REBA MTB-MDR assay. The nucleotide sequences of the loci known to be involved in drug resistance were determined for comparison. In brief, the results showed that the REBA MTB-MDR assay efficiently recognized nucleotide changes in the *oxyR-ahpC* intergenic region as well as those in *rpoB*, *katG* and the *inhA* promoter region with higher sensitivity, resulting in an 81.0% detection rate for isoniazid resistance. Inclusion of the *oxyR-ahpC* intergenic region in the REBA MTB-MDR assay improved the overall sensitivity of molecular DST for MDR-TB from 73.1 to 79.9%.

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INTRODUCTION

The emergence of *Mycobacterium tuberculosis* strains resistant to at least isoniazid (INH) and rifampicin (RIF), the two most potent components of front-line anti-tuberculosis (TB) therapy, termed multidrug-resistant TB (MDR-TB), is a serious cause for concern to TB control programmes worldwide. Development of MDR-TB significantly worsens the prognosis for achieving a durable cure, presages treatment failure and prolongs the period of transmission potential. Rapid diagnosis of MDR-TB,

therefore, is essential for the prompt initiation of effective chemotherapy and the prevention of disease spread.

The development of drug resistance is the result of selection of random genetic mutations in genes associated with drug resistance. The mechanism of resistance to RIF involves missense mutations, small deletions or insertions in the *rpoB* gene encoding the β -subunit of RNA polymerase. Studies from diverse countries have shown that 95–96% of all RIF-resistant isolates have mutations within an 81 bp ‘core region’ of *rpoB* (Bártfai *et al.*, 2001; Cavusoglu *et al.*, 2002; Herrera *et al.*, 2003; Mani *et al.*, 2001; Telenti *et al.*, 1993; Yue *et al.*, 2003). In contrast, but perhaps not unexpectedly given its highly complex mechanism of action, the mutations causing INH resistance are located in more than three genes and regions (Barry *et al.*, 1998; Slayden & Barry,

Abbreviations: CI, confidence interval; DST, drug-susceptibility testing; INH, isoniazid; MDR, multidrug resistant; MT, mutated; RIF, rifampicin; TB, tuberculosis; UPS, upstream promoter sequence; WT, wild-type.

2000). Depending on the sample set analysed, it has been reported that 50–95 % of INH-resistant strains contain mutations in codon 315 of the *katG* gene encoding the catalase-peroxidase (Mokrousov *et al.*, 2002; Musser *et al.*, 1996; Piatek *et al.*, 2000; Telenti *et al.*, 1997) and 20–35 % harbour mutations in the *inhA* promoter region (Musser *et al.*, 1996; Piatek *et al.*, 2000; Telenti *et al.*, 1997). Moreover, 10–15 % of INH-resistant strains have mutations in the *oxyR-ahpC* intergenic region, although there is no direct evidence relating mutation at this region and INH resistance (Piatek *et al.*, 2000; Telenti *et al.*, 1997).

There has been an increasing interest in the development of rapid molecular methods for detection of those mutations associated with drug resistance to replace the conventional phenotypic drug-susceptibility testing (DST), which is based on culture and typically requires several months. PCR-based methods with readouts including line probe assays, analysis with DNA microarrays and real-time PCR have been the focus of much recent effort in developing molecular DST methods to determine resistance to RIF and INH (Cirillo *et al.*, 2004; Edwards *et al.*, 2001; El-Hajj *et al.*, 2001; Herrera-León *et al.*, 2005; van Doorn *et al.*, 2003). Among the most successful of these molecular diagnostic methods is the GenoType MTBDR_{plus} assay (Hain Life-Science), which is based on reverse hybridization of amplicons containing *rpoB*, *katG* and *inhA* from test strains to probes carrying specific mutations in these genes. This DNA line probe assay performs well and offers substantial promise as a rapid molecular DST methodology (Hillemann *et al.*, 2007; Mäkinen *et al.*, 2006). This kit was designed to probe mutations only in the *katG* gene and *inhA* promoter region in determining INH resistance; however, approximately 10–15 % of INH-resistant *M. tuberculosis* strains have mutations in the *oxyR-ahpC* intergenic region with additional mutation of *katG* outside codon 315.

The aim of this study was to evaluate the impact of expanding the coverage of polymorphic alleles associated with drug resistance for the detection of MDR-TB using a new reverse hybridization assay, REBA MTB-MDR (M&D), that analyses the *oxyR-ahpC* intergenic region in addition to *rpoB*, *katG* and the *inhA* promoter region.

METHODS

***M. tuberculosis* clinical isolates and DST.** The strains used in this study were obtained from National Masan Hospital or Yonsei University College of Medicine in South Korea. A total of 240 *M. tuberculosis* strains were isolated from TB patients receiving retreatment regimens for TB. DST of these isolates was performed by the absolute concentration method on Löwenstein–Jensen medium containing 0.2 µg INH ml⁻¹ or 40 µg RIF ml⁻¹, as described previously (American Thoracic Society & Centers for Disease Control and Prevention, 2000).

Genomic DNA preparation. A loop of fresh *M. tuberculosis* culture on Löwenstein–Jensen medium was suspended in 400 µl TE buffer [10 mM Tris/HCl (pH 8.0), 1 mM EDTA] in a screw-capped microcentrifuge tube. Following heating at 80 °C for 1 h, the heat-

killed bacteria were centrifuged at 13 400 g for 5 min, and 5 µl of the supernatant was used for PCR amplification.

PCR amplification and sequencing of genes associated with drug resistance. A mixture of oligonucleotide primer sets specific to *rpoB*, *katG*, the *inhA* promoter region and the *oxyR-ahpC* intergenic region was supplied with the REBA MTB-MDR kit. Regions of *rpoB*, *katG*, the *inhA* promoter region and the *oxyR-ahpC* intergenic region were amplified by multiplex PCR using the REBA MTB-MDR primer mixture and the heat-killed bacterial supernatant, prepared as described above, according to the manufacturer's instructions. PCR products verified for the amplification of target DNA by agarose gel electrophoresis were subjected to DNA sequencing of both strands. The same PCR products were used for the reverse blot hybridization assay.

Reverse blot hybridization assay. The REBA MTB-MDR kit contains 13 probes detecting wild-type (WT) sequences and six probes recognizing mutated (MT) sequences for the determination of INH and RIF resistance, as well as one probe specific for *M. tuberculosis* (MTB; Table 1) and a mycobacterial genus-specific probe (Myc; Table 1). Hybridization of the PCR products with the probes immobilized on a membrane was carried out according to the manufacturer's instructions. Briefly, denatured PCR products in 2 × SSPE (150 mM NaCl, 10 mM NaH₂PO₄, 1 mM EDTA)/0.1 % SDS were incubated with REBA MTB-MDR membrane strips at 50 °C on a Mini-Incubation Tray (Bio-Rad). Following washing with 2 × SSPE/0.5 % SDS at 65 °C, the strips were incubated with streptavidin-conjugated alkaline phosphatase in 2 × SSPE/0.5 % SDS at ambient temperature, after which they were washed with 50 mM Tris/HCl (pH 7.5)/150 mM NaCl. The hybridized amplicons carrying biotin incorporated into the PCR primers were detected by incubating the strips with nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate in 100 mM Tris/HCl (pH 9.5)/100 mM NaCl/50 mM MgCl₂. The presence of mutations was indicated either by lack of hybridization to the WT probes and hybridization to the corresponding MT probes, or simply by lack of hybridization to the respective WT probe. All experiments were performed in duplicate. The sensitivity of the REBA MTB-MDR assay was determined as the proportion of drug-resistant isolates identified by the assay compared with those determined to be resistant by phenotypic DST, whilst the specificity was determined as the proportion of drug-susceptible isolates identified by the assay compared with those determined by phenotypic DST. These calculations and the 95 % confidence intervals (CIs) were computed using Prism 5 software (GraphPad).

RESULTS

DST and reverse blot hybridization assay

A total of 240 clinical isolates of *M. tuberculosis* from Korean patients undergoing retreatment for TB were included in this study. Of these isolates, 134 were resistant to both INH and RIF, 40 were resistant only to INH and two were RIF monoresistant, whilst 64 were susceptible to both INH and RIF as determined by phenotypic DST (Table 2). Genotypic analysis of RIF and INH resistance was carried out using the REBA MTB-MDR assay on all the clinical isolates. Fig. 1 shows an example of a typical analysis of six strains. Whilst drug-susceptible *M. tuberculosis* strain H37Rv clearly hybridized to all of the WT probes and none of the MT probes, other drug-resistant isolates gave unique hybridization patterns depending on

Table 1. Probes used in the reverse blot hybridization assay

Probe	Sequence (5'→3')	Description
Myc	GACGTCGTCGCCACCATCGA	Mycobacterial probe
MTB	AAACATGTCGGCGAGCCC	<i>M. tuberculosis</i> probe
<i>rpoB</i> WT1	AGCCAGCTGAGCCAATTC	<i>rpoB</i> codons 509–514 WT
<i>rpoB</i> WT2	ATGGACCAGAACAACCCG	<i>rpoB</i> codons 515–520 WT
<i>rpoB</i> WT3	CCGCTGTCGGGGTTGACC	<i>rpoB</i> codons 521–525 WT
<i>rpoB</i> WT4	TTGACCCACAAGCGCCGA	<i>rpoB</i> codons 524–529 WT
<i>rpoB</i> WT5	CTGTCGGCGCTGGGGC	<i>rpoB</i> codons 530–534 WT
<i>rpoB</i> MT1	CTGTTGGCGCTGGGGC	<i>rpoB</i> codon 531 (TCG→TTG)
<i>rpoB</i> MT2	AAATGTCGGCGCCGGGGCC	<i>rpoB</i> codon 533 (CTG→CCG)
<i>rpoB</i> MT3	TTCATGTACCGAACAACCCG	<i>rpoB</i> codon 516 (GAC→TAC)
<i>katG</i> 315 WT	CACCAGCGGCATCGAG	<i>katG</i> codon 315 AGC WT
<i>katG</i> 315 MT	ATCACCACCGGCATCGAG	<i>katG</i> codon 315 (AGC→ACC)
<i>inhA</i> 15UPS WT	CGCGGCGAGACGATAGG	<i>inhA</i> UPS –15 WT
<i>inhA</i> 15UPS MT	CGCGGCGAGATGATAGG	<i>inhA</i> UPS –15 (C→T)
<i>inhA</i> 8UPS WT	AAAGATAGGTTGTCGGGGTGACT	<i>inhA</i> UPS –8 WT
<i>inhA</i> 8UPS MT	GACGATAGGCTGTCGGGG	<i>inhA</i> UPS –8 (T→C)
<i>ahpC</i> WT1	GCCGATAAATATGGTGTGATATATCA	<i>ahpC</i> –60 to –38 WT
<i>ahpC</i> WT2	CCTTTGCCTGACAGCGACTT	<i>ahpC</i> –38 to –18 WT
<i>ahpC</i> WT3	CACGGCACGATGGAATGTC	<i>ahpC</i> –17 to +2 WT
<i>ahpC</i> WT4	GCAACCAAATGCATTGTCCGC	<i>ahpC</i> +3 to +23 WT
<i>ahpC</i> WT5	TTTGATGATGAGGAGATCATGC	<i>ahpC</i> +24 to +44 WT

the mutations that they were carrying, shown by hybridization to the MT probes and lack of hybridization to the WT probes.

Frequency of mutations associated with RIF resistance

The REBA MTB-MDR assay was able to detect 132/136 RIF-resistant isolates (sensitivity 97%; CI 90.8–98.9) and showed no mutations in the 104 RIF-susceptible isolates. These results were fully confirmed by DNA sequencing. The most frequent point mutation among the 136 RIF-resistant isolates was observed at codon 531 of the *rpoB* gene (66 isolates, 48.5%), with 64 isolates showing a TCG→TTG (Ser→Leu) nucleotide transition. Two isolates lacking hybridization signals from the *rpoB* WT5 probe were determined to have a TCG→TGG (Trp) transversion by DNA sequencing. Other mutations were detected in alternative *rpoB* codons including codon 526 (29 isolates, 21.3%), codon 516 (23 isolates, 16.9%), codons 513 and 533 (six isolates each, 4.4%, respectively), codon 511 (three isolates, 2.2%), codons 517 and 522 (two isolates each, 1.5%, respectively) and codons 510, 512, 515, 523 and 540 (one isolate each, 0.7%, respectively). A CCA deletion overlapping codons 516 and 517 was observed in one isolate (0.7%). Among these isolates with confirmed mutations, double mutations were detected in 11 isolates. Four isolates were found not having any mutation in *rpoB*. No mutation in the *rpoB* gene was detected in any of the 104 RIF-susceptible isolates. These mutations were all identified either by a signal from the specific MT probe and lack of the corresponding WT signal, or by lack of

hybridization to the respective WT probe (Table 2). Overall, 14 different hybridization patterns representing 31 different mutation patterns in the *rpoB* gene were observed.

Frequency of mutations associated with INH resistance

Among the 174 INH-resistant isolates, the REBA MTB-MDR assay detected mutations in at least one of the resistance-associated regions, *katG*, the *inhA* promoter and the *oxyR-ahpC* intergenic region, in a total of 141 strains (81.0% sensitivity, CI 74.4–86.6) and these results were confirmed by DNA sequencing. By far the majority of these were detected in the *katG* gene with a mutation in codon 315, AGC→ACC (Ser→Thr), accounting for 51.1% (89 isolates), and a mutation in codon 315 to AAC (Asn) representing 2.3% (four isolates). The second most common site of mutation was in the upstream promoter sequence (UPS) of *inhA*, accounting for 26.4% (46 isolates). Forty-one isolates (23.6%) carried a C→T transition at the –15 position, and four isolates (2.3%) carried a T→C mutation and one isolate (0.6%) carried a T→A mutation at the –8 position. Seventeen isolates (9.8%) carried mutations in the *oxyR-ahpC* intergenic region, and one of them had a double mutation in this region. Among these isolates, eight (4.6%) had mutations in both *katG* at codon 315 and the *inhA* promoter region. Three isolates (1.7%) had mutations at *katG* codon 315 and in the *oxyR-ahpC* intergenic region, whilst four isolates (2.3%) had mutations in the *inhA* promoter region and *oxyR-ahpC* intergenic regions. Mutations solely in the

Table 2. Hybridization patterns of *M. tuberculosis* clinical isolates by the REBA MTB-MDR assay

+ and – indicate presence and absence of the hybridization signal to the probes, respectively.

Phenotypic DST (no. of isolates)*	<i>rpoB</i>								<i>katG</i> 315		<i>inhA</i> 15UPS		<i>inhA</i> 8UPS		<i>ahpC</i>					No. of isolates	Interpretation (no. of isolates)
	WT1	WT2	WT3	WT4	WT5	MT1	MT2	MT3	WT	MT	WT	MT	WT	MT	WT1	WT2	WT3	WT4	WT5		
RIF ^R INH ^R (134)	+	+	+	+	–	+	–	–	–	+	+	–	+	–	+	+	+	+	+	27	<i>rpoB katG</i> (62)
	+	+	+	+	–	–	+	–	–	+	+	–	+	–	+	+	+	+	+	3	
	+	+	+	+	–	–	+	–	–	–	+	–	+	–	+	+	+	+	+	1	
	+	+	+	+	–	–	–	–	–	+	+	–	+	–	+	+	+	+	+	1	
	+	+	+	–	+	–	–	–	–	+	+	–	+	–	+	+	+	+	+	10	
	+	+	+	–	+	–	–	–	–	–	+	–	+	–	+	+	+	+	+	1	
	+	–	+	+	+	–	–	+	–	+	+	–	+	–	+	+	+	+	+	5	
	+	–	+	+	+	–	–	–	–	+	+	–	+	–	+	+	+	+	+	10	
	+	–	+	+	+	–	–	–	–	–	+	–	+	–	+	+	+	+	+	1	
	–	+	+	+	+	–	–	–	–	+	+	–	+	–	+	+	+	+	+	2	
	+	–	+	+	+	–	+	–	–	+	+	–	+	–	+	+	+	+	+	1	
	+	+	+	+	–	+	–	–	+	–	–	+	+	–	+	+	+	+	+	15	<i>rpoB inhA</i> (25)
	+	+	+	–	+	–	–	–	+	–	–	+	+	–	+	+	+	+	+	5	
	+	–	+	+	+	–	–	+	+	–	–	+	+	–	+	+	+	+	+	1	
	–	+	+	+	+	–	–	–	+	–	–	+	+	–	+	+	+	+	+	2	
	–	+	–	+	+	–	–	–	+	–	–	+	+	–	+	+	+	+	+	1	
	+	+	+	+	–	+	–	–	+	–	+	–	–	+	+	+	+	+	+	1	
	+	+	+	+	–	+	–	–	+	–	+	–	+	–	–	+	+	+	+	1	<i>rpoB ahpC</i> (9)
	+	+	+	+	–	+	–	–	+	–	+	–	+	–	+	+	–	+	+	2	
	+	+	+	–	+	–	–	–	+	–	+	–	+	–	+	–	+	+	+	2	
	+	+	+	–	+	–	–	–	+	–	+	–	+	–	+	–	+	+	+	1	
	+	+	–	+	+	–	–	–	+	–	+	–	+	–	+	+	–	+	+	1	
	+	+	–	+	+	–	–	–	+	–	+	–	+	–	–	+	+	+	+	1	
	–	+	+	–	+	–	–	–	+	–	+	–	+	–	+	+	–	+	+	1	
	+	+	+	+	–	+	–	–	–	+	–	+	+	–	+	+	+	+	+	3	<i>rpoB katG inhA</i> (7)
	+	+	+	–	+	–	–	–	–	+	–	+	+	–	+	+	+	+	+	1	
	+	–	+	+	+	–	–	–	–	+	+	–	–	+	+	+	+	+	+	1	
	–	–	+	+	+	–	–	–	–	+	+	–	–	–	+	+	+	+	+	1	
	+	+	+	+	–	–	+	–	–	–	+	–	–	+	+	+	+	+	+	1	
	+	+	+	+	–	+	–	–	–	+	+	–	+	–	–	+	+	+	+	1	<i>rpoB katG ahpC</i> (2)
	–	+	+	–	+	–	–	–	–	+	+	–	+	–	+	+	–	+	+	1	

Table 2. cont.

Phenotypic DST (no. of isolates)*	<i>rpoB</i>								<i>katG</i> 315		<i>inhA</i> 15UPS		<i>inhA</i> 8UPS		<i>ahpC</i>					No. of isolates	Interpretation (no. of isolates)
	WT1	WT2	WT3	WT4	WT5	MT1	MT2	MT3	WT	MT	WT	MT	WT	MT	WT1	WT2	WT3	WT4	WT5		
INH ^R (40)	+	+	+	+	-	+	-	-	+	-	+	-	-	+	+	+	-	+	+	1	<i>rpoB inhA ahpC</i> (2)
	+	-	+	-	+	-	-	-	+	-	-	+	+	-	+	+	-	+	+	1	<i>rpoB</i> (23)
	+	+	+	+	-	+	-	-	+	-	+	-	+	-	+	+	+	+	+	11	
	+	+	+	+	-	-	-	-	+	-	+	-	+	-	+	+	+	+	+	1	
	+	+	+	-	+	-	-	-	+	-	+	-	+	-	+	+	+	+	+	5	
	+	-	+	+	+	-	-	-	+	-	+	-	+	-	+	+	+	+	+	4	
	-	+	+	+	+	-	-	-	+	-	+	-	+	-	+	+	+	+	+	2	<i>katG</i> (1) <i>inhA</i> (1) <i>katG inhA</i> (1) No mutation (1)
	+	+	+	+	+	-	-	-	-	+	+	-	+	-	+	+	+	+	+	1	
	+	+	+	+	+	-	-	-	-	+	-	+	+	-	+	+	+	+	+	1	
	+	+	+	+	+	-	-	-	+	-	+	-	+	-	+	+	+	+	+	1	
	+	+	+	+	+	-	-	-	-	+	+	-	+	-	+	+	+	+	+	20	
	+	+	+	+	+	-	-	-	+	-	-	+	+	-	+	+	+	+	+	9	<i>inhA</i> (9)
	+	+	+	+	+	-	-	-	+	-	+	-	+	-	+	-	+	+	+	1	<i>ahpC</i> (1)
	+	+	+	+	+	-	-	-	+	-	-	+	+	-	-	+	+	+	+	1	<i>inhA ahpC</i> (1)
	+	+	+	+	+	-	-	-	+	-	+	-	+	-	+	+	+	+	+	9	No mutation (9)
RIF ^R (2)	+	+	+	+	-	+	-	-	+	-	+	-	+	-	+	+	+	+	+	2	<i>rpoB</i> (2)
RIF ^S INH ^S (64)	+	+	+	+	+	-	-	-	+	-	+	-	+	-	+	+	+	+	+	64	No mutation (64)
Total																				240	

*R, Resistant; S, sensitive.

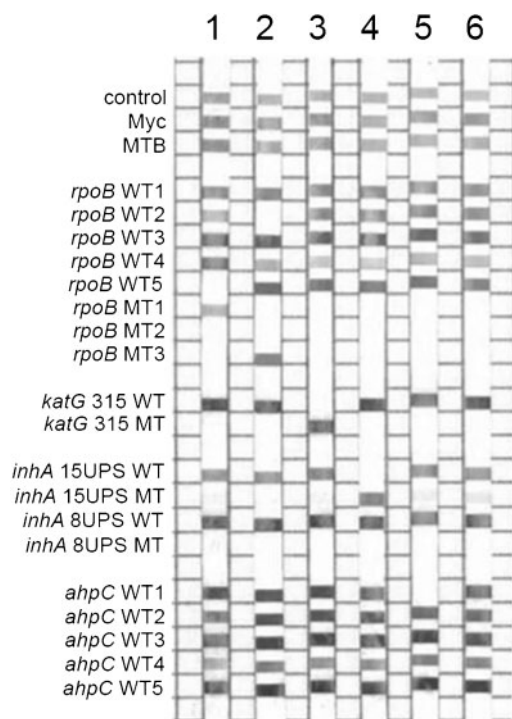


Fig. 1. Hybridization patterns of *M. tuberculosis* strains by the REBA MTB-MDR assay. The probes immobilized on the strip are indicated on the left. Lanes: 1, clinical isolate carrying an *rpoB* mutation at codon 531 (TCG→TTG); 2, clinical isolate carrying an *rpoB* mutation at codon 516 (GAC→TAC); 3, clinical isolate carrying a *katG* mutation at codon 315 (AGC→ACC); 4, clinical isolate carrying a mutation in the upstream promoter sequence (UPS) of *inhA* at the -15 position (C→T); 5, clinical isolate carrying a mutation in the *oxyR-ahpC* intergenic region at the -39 position (C→T); 6, *M. tuberculosis* strain H37Rv.

oxyR-ahpC intergenic region were observed in ten isolates (5.8%). There were 33 INH-resistant isolates (19.0%) without any observed mutations in these loci. The 66 INH-susceptible isolates did not carry any mutations in these three regions.

These results could be visualized directly by the dual signal changes, loss of the WT probe signal and appearance of the MT probe signal, or only by loss of the WT probe signal, in all but two cases. One isolate had a C→T change at the -30 position and both WT (C) and MT (T) sequences at the +2 position in the *oxyR-ahpC* intergenic region, as confirmed by DNA sequencing, suggesting a possible mixed infection. This isolate was determined to be INH-resistant by the REBA MTB-MDR assay because the C→T change at the -30 position was detected by a lack of hybridization signal of the *ahpC* WT2 probe, whilst the *ahpC* WT3 probe still gave the WT pattern. The other isolate also appeared to be a mixed-infection case that had WT (C) and MT (T) sequences simultaneously at the -10 position in the *oxyR-ahpC* intergenic region. Although this

isolate gave a WT pattern by the REBA MTB-MDR assay for the *ahpC* WT3 probe, this isolate was still detected as resistant to INH by the REBA MTB-MDR assay, as it also had mutation in the *inhA* promoter region. Overall, 17 different hybridization patterns representing 22 different mutations at codon 315 of *katG*, in the *inhA* promoter region and in the *oxyR-ahpC* intergenic region were observed.

Sensitivity and specificity of REBA MTB-MDR results

As shown in Table 2, the REBA MTB-MDR assay correctly identified 107/134 MDR isolates, resulting in an overall sensitivity of 79.8% (CI 71.8–86.1). Likewise, no mutations in the four gene regions analysed were detected by the REBA MTB-MDR assay among the 64 RIF- and INH-susceptible isolates, resulting in a specificity of 100%. As already alluded to above, in almost all isolates, the phenotypic DST patterns could be predicted by the REBA MTB-MDR assay. The strains that had double mutations in the same gene, and also showed mutations in other INH resistance-associated regions, were still detected as MDR by the REBA MTB-MDR assay. In total, 44 different hybridization patterns representing 59 different mutations in the *rpoB* hot-spot region, codon 315 of *katG*, the *inhA* promoter region and the *oxyR-ahpC* intergenic region of 107 MDR isolates were detected (Table 2).

DISCUSSION

In this study, we evaluated the utility of incorporating additional putative resistance-related mutations into a REBA MTB-MDR assay using a panel of 240 *M. tuberculosis* clinical isolates, of which 134 were MDR. This assay contains both WT and MT probes in the 81 bp hot-spot region of the *rpoB* gene, probes for codon 315 of the *katG* gene, probes for the -15 or -8 UPS of the *inhA* promoter region and WT probes of the intergenic region of the *oxyR-ahpC* gene immobilized on a membrane. The presence of mutations was indicated either by lack of hybridization to the WT oligonucleotide probes and hybridization to the corresponding mutant oligonucleotide probes, or by lack of hybridization to the respective WT sequence only.

This assay successfully detected 107/134 MDR isolates including nine MDR isolates with mutations in the *rpoB* gene and the *oxyR-ahpC* intergenic region but not in the *katG* or *inhA* genes. These nine MDR isolates would have been missed by use of previous methods that do not assess for the presence of mutations in the *oxyR-ahpC* intergenic region. The additional isolates correctly detected amounted to a 5.7% increase in the INH resistance detection rate from 75.3 to 81.0%, and a 6.8% increase in the MDR-TB detection rate from 73.1 to 79.9%. This increase in the detection rate was achieved by the REBA MTB-MDR assay due to the inclusion of probes spanning

the *oxyR-ahpC* intergenic region. Compared with the data from phenotypic DST for these strains, the sensitivity and specificity of the REBA MTB-MDR assay were determined to be 79.9 and 100 %, respectively. It has been reported previously that 10–15 % of INH-resistant strains have mutations in the *oxyR-ahpC* intergenic region with inactivation of KatG (Piatek *et al.*, 2000; Telenti *et al.*, 1997). The relationship between INH resistance and mutations of the *oxyR-ahpC* intergenic region is not yet clearly understood. Therefore, future studies of the ten INH-resistant strains containing mutations only in the *oxyR-ahpC* intergenic region will be needed to determine their precise MICs and to investigate whether they have additional mutations of the *katG* gene outside codon 315. This future study will give more information about the relationship with INH resistance and mutations in the *oxyR-ahpC* intergenic region.

Signals for both WT and MT probes, suggestive of mixed infection with two distinct strains, one WT and the other a MT strain, were detected in several cases. These isolates had another mutation in the same region and had mutations in other INH resistance-associated regions, and were therefore determined to be MDR by the REBA MTB-MDR assay. Although it is not easy to make a simple recommendation in such cases of mixed infection with resistant and susceptible *M. tuberculosis* strains as observed in this study, or in mixed infections containing *M. tuberculosis* and a non-tuberculous mycobacterium (Hillemann *et al.*, 2007), we favour a conservative approach of reporting such strains as likely to be resistant after exclusion of involvement of a non-tuberculous mycobacterium.

The REBA MTB-MDR assay was able to detect 132/136 RIF-resistant isolates and showed no mutations in the 104 RIF-susceptible isolates, and these results were fully confirmed by conventional DNA sequencing. In general, mutations in the *rpoB* gene account for 95–96 % of RIF-resistant *M. tuberculosis* strains worldwide (Musser, 1995; Raviglione *et al.*, 1995). Similarly, the REBA MTB-MDR assay correctly detected 141 INH-resistant isolates out of 174 isolates (81 %) determined to be drug resistant by phenotypic DST, whilst not finding any polymorphisms in 66 INH-susceptible isolates.

In addition to the mixed-infection cases, reverse line blotting assays will continue to underestimate the true number of resistant strains because of limitations of knowledge regarding the genotypic markers of drug resistance. In this study, we showed that inclusion of a relatively modest number of additional probes to detect additional mutations associated with INH resistance in the *oxyR-ahpC* intergenic region improved the detection rate of drug-resistant TB in this patient population from 73.1 to 79.9 %, which is an improvement over previously implemented line probe assays. This assay is being evaluated on clinical specimens to determine its potential for improving rapid diagnosis of MDR-TB in a prospective clinical trial.

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