GenoType MTBDR_{plus} Assay for Molecular Detection of Rifampin and Isoniazid Resistance in *Mycobacterium tuberculosis* Strains and Clinical Samples[▽]

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The purpose of this study was to evaluate the GenoType MTBDR_{plus} assay (Hain Lifescience GmbH, Nehren, Germany) for its ability to detect resistance to rifampin (RIF) and isoniazid (INH) in *Mycobacterium tuberculosis* clinical strains and directly in clinical samples. A total of 62 clinical strains characterized with the Bactec 460TB system were included. For the INH-resistant strains, the MIC was measured and sequencing was performed. Sixty-five clinical samples from 28 patients (39 smear-positive samples and 26 smear-negative samples) were also tested directly. The corresponding isolates of the clinical specimens were studied with the Bactec 460TB system. The overall rates of concordance of the MTBDR_{plus} assay and the Bactec 460TB system for the detection of RIF and INH susceptibility in clinical strains were 98.3% (61/62) and 79% (49/62), respectively. The rate of concordance between the Bactec 460TB system and the MTBDR_{plus} test for the detection of INH resistance in the group of 27 strains with low-level resistance was 62.9% (17/27), and that for the detection of INH resistance in the group of 21 strains with high-level resistance was 85.71% (18/21). Valid test results were obtained for 78.45% (51/65) of the clinical samples tested. The rates of concordance between both assays for the detection of drug resistance in these samples were 98% (50/51) for RIF and 96.2% (49/51) for INH. Taking into account only one sample per patient, the overall rate of concordance between both tests was 92.85% (26/28). The GenoType MTBDR_{plus} assay is easy to perform and is a useful tool for the management of tuberculosis, as it allows the detection of resistance to RIF and INH in *M. tuberculosis* strains and also in clinical samples.

Tuberculosis (TB) is an important public health problem and remains one of the most threatening curable infectious diseases, despite improvements in diagnostic and drug susceptibility tests. The effective control of TB is based on the immediate detection of *Mycobacterium tuberculosis*, followed by the prompt implementation of adequate antituberculous therapy (29).

The emergence of strains resistant to the major anti-TB drugs speeds up the need for rapid methods for the identification of resistant *M. tuberculosis* strains in order to treat the disease effectively and, at the same time, prevent the spread of resistant strains (6, 8, 9, 16). Multidrug-resistant (MDR) *M. tuberculosis* strains, which are resistant at least to rifampin (RIF) and isoniazid (INH), have emerged worldwide and seriously threaten TB control and prevention programs (30).

The main mutations that confer RIF resistance are located in the *rpoB* gene, specifically, in the well-defined 81-bp core region (22, 24). About 95% of RIF-resistant strains have a mutation in this region, which facilitates the rapid development of approaches for the detection of resistance to this drug (23, 24, 26). However, the molecular basis of resistance to

INH is more complex because it involves mutations in more than one gene or gene complex (22, 25), such as the *katG*, *inhA*, and *kasA* genes and the intergenic region of the *oxyR-ahpC* complex.

In recent years, the development of new molecular methods based on PCR and sequencing has allowed the rapid identification and detection of the genetic mutations and single-nucleotide polymorphisms related to resistance, specifically, resistance to RIF and INH (1, 5, 7, 23). However, the technology is very laborious, not fully standardized, and unsuitable for use in daily clinical practice. Easy-to-use commercial kits have been developed to address this issue. These assays, based on multiplex PCR combined with reverse hybridization on nitrocellulose strips, target common mutations in *rpoB* (INNO LiPA Rif.TB; Innogenetics, Ghent, Belgium) or *rpoB* and *katG* (GenoType MTBDR; Hain Lifescience GmbH, Nehren, Germany) in both clinical strains and clinical samples (2, 10, 14, 27). In order to increase the ability to detect RIF and INH resistance, a new version of the GenoType MTBDR assay, the GenoType MTBDR_{plus} assay, that covers more mutations has recently been developed. The GenoType MTBDR_{plus} assay targets the mutations included in the previous test and includes a broader selection of probes for the wild-type *rpoB* gene and mutations in the promoter region of *inhA* gene.

The first objective of the present study was to evaluate the ability of the GenoType MTBDR_{plus} assay to detect muta-

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tions in *rpoB*, *katG*, and *inhA* in clinical strains of *M. tuberculosis* previously characterized by Bactec 460TB system, MIC, and DNA sequencing analysis. The second goal was to determine its accuracy for the direct detection of INH and RIF resistance in smear-negative and smear-positive clinical specimens and to compare the results with those obtained by the reference Bactec 460TB system.

MATERIALS AND METHODS

Clinical strains. A total of 62 previously studied and well-characterized *M. tuberculosis* strains were included in the study. All of them were phenotypically studied by the radiometric method with the Bactec 460TB system, and for the resistant strains, the MIC was measured. The latter were also genotypically characterized by sequencing.

Clinical specimens. Sixty-five clinical samples (53 sputum samples, 3 bronchoalveolar lavage samples, 5 bronchial aspirate samples, 3 pleural effusion samples, and 1 lymph node sample) from 28 patients were retrospectively selected from the samples collected between September 2006 and October 2007 during the clinical routine. Before they were tested, all the samples were processed as follows: they were first digested and decontaminated by the Kubica *N*-acetyl-L-cysteine NaOH method (15, 17). After decontamination, the concentrated sediment was suspended in 2 ml sterile phosphate buffer (pH 7.0) and auramine-rhodamine acid-fast staining was performed. The results for specimens positive by fluorochrome staining were confirmed by Ziehl-Neelsen staining. Thirty-nine samples were smear negative and 26 were smear positive. An aliquot of the decontaminated specimens was cultured on Löwenstein-Jensen solid medium and MB/BacT liquid medium (BioMérieux, Marcy l'Etoile, France). After inoculation for growth detection, the remaining decontaminated specimen was stored at -20°C . Isolates of the *M. tuberculosis* complex were detected in all samples included in the evaluation. The identification of *M. tuberculosis* in cultures with growth was confirmed by the Inno-Lipa Mycobacteria (version 2) assay (InnoGenetics, N.V.).

Drug susceptibility. Testing for susceptibility to INH and RIF was performed by the radiometric method with the Bactec 460TB system by using critical concentrations of 0.1 $\mu\text{g/ml}$ for INH and 2 $\mu\text{g/ml}$ for RIF (12). Among the 62 previously characterized strains, 14 were susceptible and 48 were resistant (36 were RIF^r and INH^r and 12 were RIF^r and INH^s). For the strains resistant to INH, the MICs were determined on Middlebrook 7H10 medium by the serial double-concentration method. The strains were incubated for 21 days with INH concentrations of 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, and 32 $\mu\text{g/ml}$. According to the MIC, 27 of the strains had low-level resistance (MICs $\leq 1 \mu\text{g/ml}$) and 21 had high-level resistance (MICs $> 1 \mu\text{g/ml}$). The results obtained with the Bactec 460TB system and the MICs for the INH^r and MDR strains are shown in Table 1.

Genotypic characterization. The 48 resistant strains were genotypically characterized in a previous study (5) by sequencing four genes involved in RIF and INH resistance: *rpoB*, *katG*, *inhA*, and *oxyR-ahpC*. After DNA extraction, 165 bp of *rpoB*, 2,405 bp (six fragments) of *katG*, 248 bp of the *mabA-inhA* regulatory region, and specific regions (105 bp) located upstream of *oxyR-ahpC* were amplified. All of the primers used for the amplification of the different genes except the ones used for the amplification of *rpoB* were derived from published sequences. The primers used for the amplification of *rpoB* were designed with the Primer Express program (Applied Biosystems, Foster City, CA). DNA sequencing was performed with the *fmol* DNA cycle sequencing system (Promega Corporation, Madison, WI) with the ALF Express II system (Amersham Pharmacia Biotech). The sequencing results for these sets of strains are shown in Table 1.

GenoType MTBDRplus assay. The GenoType MTBDRplus line probe assay was carried out according to the manufacturer's instructions. For the testing of the clinical strains, a few colonies from solid media were resuspended in 300 μl of molecular biology-grade water. The specimen was then killed with heat at 95°C for 20 min and sonicated for 15 min. The samples were then centrifuged at $22,000 \times g$ for 5 min, and the supernatant was collected. For the testing of the clinical specimens, 1 ml of the decontaminated and concentrated specimen was centrifuged at $10,000 \times g$ for 15 min. The supernatant was then discarded and the pellet was resuspended in 100 μl of molecular biology-grade water. The remaining steps of the DNA extraction were performed as described above for the cultured strains. Five microliters of the supernatant was used for amplification, which was performed in an automated thermocycler (GeneAmp PCR system 9700) according to the following protocol: 15 min of denaturation at 45°C ; 10 cycles of denaturation at 95°C for 30 s and elongation at 58°C for 120 s; 20 cycles

of denaturation at 65°C for 25 s, annealing at 53°C for 40 s, and elongation at 70°C for 40 s; and a final extension step at 70°C for 8 min. The PCR protocol was modified by increasing the number of cycles from 20 to 30 for the smear-positive samples and to 45 for the smear-negative ones.

After denaturation, the biotin-labeled amplicons were hybridized to the single-stranded membrane-bound probes. After a stringent washing, a streptavidin-alkaline phosphatase conjugate was added to the strips and an alkaline phosphatase-mediated staining reaction was observed in the bands where the amplicon and the probe had hybridized.

The MTBDRplus assay strip contains 27 reaction zones; 21 of them are probes for mutations and 6 are control probes for verification of the test procedures. The six control probes include a conjugate control, an amplification control, an *M. tuberculosis* complex-specific control (TUB), an *rpoB* amplification control, a *katG* amplification control, and an *inhA* amplification control. For the detection of RIF resistance, the probes cover the *rpoB* gene, while the INH resistance-specific probes cover positions in *katG* and *inhA*, as shown in Fig. 1. The absence of at least one of the wild-type bands or the presence of bands indicating a mutation in each drug resistance-related gene implies that the sample tested is resistant to the respective antibiotic. When all the wild-type probes of a gene stain positive and there is no detectable mutation within the region examined, the sample tested is susceptible to the respective antibiotic. In order to give a valid result, all six expected control bands should appear correctly. Otherwise, the result is considered invalid. Examples of valid and invalid results are shown in Fig. 1. The person who read and recorded the band results obtained by the MTBDRplus assay was blind to the susceptibility determined with the Bactec 460TB system and the sequencing results.

RESULTS

MTBDRplus assay results for the clinical strains. Sixty-two *M. tuberculosis* clinical strains were processed by the MTBDRplus assay, and valid results were obtained for all of them. The antibiotic susceptibility patterns obtained with the MTBDRplus assay were compared with those obtained with the Bactec 460TB system and are shown in Table 2. The specificity of the assay for the detection of RIF and INH resistance was 100% for both drugs. The sensitivities for the detection of resistance to RIF and INH were 91.7% (11/12) and 73% (35/48), respectively. Figure 2 shows the distribution of the MTBDRplus assay results according to the results obtained with the Bactec 460TB system. The levels of agreement between the results obtained by the MTBDRplus assay and with the Bactec 460TB system were 98.3% (61/62) ($\kappa = 0.947$; 95% confidence interval [CI] = 0.859) for RIF and 79% (49/62) ($\kappa = 0.549$; 95% CI = 0.382) for INH.

All 50 strains susceptible to RIF were correctly identified as being sensitive by the MTBDRplus assay, and the results of the MTBDRplus assay were 100% concordant with those obtained with the Bactec 460TB system. Among the 12 strains resistant to RIF according to the results obtained with the Bactec 460TB system, 11 strains showed a resistance pattern according to the results obtained with the MTBDRplus system. The mutations in *rpoB* identified by the assay were as follows: S531L in six strains, D526Y in one strain, H526D in one strain, D516V in two strains, and S531W in the last strain. The last mutation matched the lack of a band for the WT8 probe. One strain was identified as susceptible by use of the MTBDRplus system, although sequencing detected a D516Y mutation (Table 1).

Among the 48 INH^r strains, 27 had low-level resistance and 21 had high-level resistance, according to their MICs. Table 3 shows the distribution of the MTBDRplus assay results according to the sequencing results for *katG*, *inhA*, and *oxyR-ahpC* for both groups of strains.

TABLE 1. MTBDR_{plus} assay, MIC, Bactec 460TB system, and sequencing results for the 48 clinical resistant strains included in the study^a

Clinical strain	INH								RIF			
	MIC (μg/ml)	Bactec system result	MTBDR _{plus} assay results		Sequencing results			Bactec system result	MTBDR _{plus} assay results		Sequencing results for <i>rpoB</i> mutation	
			Result	Mutation detected	<i>katG</i> mutation(s)	<i>inhA</i> mutation	<i>oxyR-ahpC</i> mutation		Result	Mutation detected		
058R	0.125	R	S	—	wt	wt	wt	S	S	—	ND	
009R	0.25	R	S	—	W728Y	wt	—12 G→A	S	S	—	ND	
034R	0.25	R	R	<i>inhA</i> -8 T→C	wt	-8 T→C	wt	S	S	—	ND	
037R	0.25	R	S	—	wt	wt	wt	S	S	—	ND	
038R	0.25	R	R	<i>inhA</i> -15 C→T	wt	-15 C→T	wt	S	S	—	ND	
039R	0.25	R	S	—	wt	wt	wt	R	R	S531L	S531L	
040R	0.25	R	S	—	D94N	wt	wt	S	S	—	ND	
041R	0.25	R	S	—	wt	wt	wt	S	S	—	ND	
042R	0.25	R	R	<i>inhA</i> -8 T→C	wt	-8 T→C	wt	S	S	—	ND	
046R	0.25	R	R	<i>inhA</i> -15 C→T	R463L	-15 C→T	wt	S	S	—	ND	
052R	0.25	R	R	<i>inhA</i> -15 C→T	wt	-15 C→T	wt	R	R	S531L	S531L	
054R	0.25	R	S	—	640 deletion	wt	wt	S	S	—	ND	
055R	0.25	R	R	<i>inhA</i> -15 C→T	wt	-15 C→T	wt	S	S	—	ND	
061R	0.25	R	R	<i>inhA</i> -15 C→T	wt	-15 C→T	wt	S	S	—	ND	
067R	0.25	R	R	<i>inhA</i> -15 C→T	wt	-15 C→T	wt	S	S	—	ND	
001R	0.5	R	R	<i>inhA</i> -15 C→T	wt	-15 C→T	wt	S	S	—	ND	
011R	0.5	R	R	<i>inhA</i> -15 C→T	wt	-15 C→T	Insertion at -38/-39	S	S	—	ND	
013R	0.5	R	R	<i>inhA</i> -15 C→T	wt	-15 C→T	wt	S	S	—	ND	
014R	0.5	R	R	<i>inhA</i> -15 C→T	wt	-15 C→T	wt	S	S	—	ND	
033R	0.5	R	S	—	wt	wt	wt	S	S	—	ND	
047R	0.5	R	R	<i>inhA</i> -15 C→T	wt	-15 C→T	wt	R	R	H526Y	H526Y	
050R	0.5	R	S	—	wt	wt	wt	S	S	—	ND	
003R	1	R	R	<i>inhA</i> -15 C→T	wt	-15 C→T	wt	R	R	H526D	H526D	
036R	1	R	R	<i>inhA</i> -15 C→T	Y678C	-15 C→T	wt	S	S	—	ND	
053R	1	R	S	—	wt	-15 C→T	wt	S	S	—	ND	
059R	1	R	R	<i>katG</i> S315T	wt	wt	wt	S	S	—	S512T	
060R	1	R	R	<i>inhA</i> -15 C→T	wt	-15 C→T	wt	S	S	—	ND	
007R	2	R	S	—	204W→stop	wt	wt	R	R	Absence of wt8 probe	S531W	
057R	2	R	R	<i>inhA</i> -15 C→T	D189H	-15 C→T	wt	S	S	—	ND	
004R	4	R	R	<i>katG</i> S315T	S315T	wt	wt	S	S	—	ND	
002R	8	R	R	<i>katG</i> S315T	S315T	wt	wt	S	S	—	ND	
006R	8	R	R	<i>katG</i> S315T	S315T	wt	wt	S	S	—	ND	
010R	8	R	R	<i>katG</i> S315T	S315T	wt	wt	S	S	—	ND	
012R	8	R	R	<i>katG</i> S315T	S315T, R463L, A234G	wt	Insertion at -45/-46	S	S	—	ND	
015R	8	R	R	<i>katG</i> S315T	S315T	wt	wt	S	S	—	ND	
016R	8	R	R	<i>katG</i> S315T	S315R	wt	wt	R	R	S531L	S531L	
035R	8	R	R	<i>katG</i> S315T	S315T	wt	wt	S	S	—	ND	
044R	8	R	R	<i>katG</i> S315T	S315T	wt	wt	R	R	S531L	S531L	
045R	8	R	R	<i>katG</i> S315T	S315T	wt	wt	S	S	—	ND	
048R	8	R	R	<i>katG</i> S315T	S315T	wt	wt	R	R	S531L	S531L	
049R	8	R	R	<i>katG</i> S315T	S315T	wt	wt	S	S	—	ND	
056R	8	R	R	<i>katG</i> S315T	S315T	wt	Insertion at -45 -44	R	R	S531L	ND	
062R	8	R	R	<i>katG</i> S315T	S315T	wt	wt	S	S	—	ND	
063R	8	R	R	<i>katG</i> S315T	S315T	wt	wt	R	R	D516V	D516V	
064R	8	R	R	<i>katG</i> S315T	S315T	wt	wt	R	R	D516V	ND	
066R	8	R	R	<i>katG</i> S315T	S315T	wt	wt	S	S	—	ND	
065R	16	R	S	—	S315T, R463L	wt	wt	S	S	—	ND	
005R	32	R	S	—	234 deletion, 155-158 deletion	wt	wt	R	S	—	D516Y	

^a Abbreviations and symbols: ND, not done; wt, wild type; R, resistant; S, susceptible; —, no mutation found by the MTBDR_{plus} assay.

The rate of concordance between the results obtained with the Bactec 460TB system and those obtained with the MTBDR_{plus} test for the detection of INH resistance in the group of 27 strains with low level resistance was 62.9% (17/27). In 10 cases, the MTBDR_{plus} test results revealed INH susceptibility. Six of them were wild type for the three genes sequenced and two

had a single mutation in the *katG* gene (Asp94Asn, deletion at codon 640) that could not be identified by the MTBDR_{plus} test because the test probes do not cover codon mutations at these positions. One strain had two mutations that could not be identified: Trp728Tyr in *katG* and a G→A mutation at position -12 in *aphC-oxyR*. In the last strain, the assay was not able to

FIG. 1. Representative DNA patterns obtained by the MTBDR_{plus} assay. Examples of invalid results are also included. Lane 1, example of a pattern of RIF^s and INH^s; lane 2, example of a pattern of RIF^r and INH^r; lane 3, example of a pattern of RIF^s and INH^r; lanes 4 to 9, examples of invalid results. The positions of the oligonucleotides and the control probes are given on the left. The targeted genes and the specific probes lines are shown from top to bottom, as follows: conjugate control (CC); amplification control (AC); *M. tuberculosis* complex-specific control (TUB); *rpoB* amplification control; *rpoB* wild-type probes WT1 to WT8 (505 to 533); four *rpoB* mutant probes (probes MUT1, MUT2A, MUT2B, and MUT3) in codons D516V, D526Y, H526D, and S531L, respectively; *katG* amplification control; *katG* codon 315 wild-type probe; two *katG* codon 315 mutant probes (probes MUT1 and MUT2) with AGC-ACC (S315T1) and AGC-ACA (S315T2) mutations, respectively; *inhA* amplification control; *inhA* wild-type probes WT1 and WT2 covering positions -15 and -16 of the gene regulatory region; four *inhA* mutant probes (probes MUT1, MUT2, MUT3A, and MUT3B) with mutations C→T at position -15, A→G at position -16, T→C at position -8, and T→A at position -8, respectively. M, colored marker.

detect a C→T *inhA* mutation at position -15, whereas it was identified by sequencing. For the 17 correctly identified INH-resistant strains, the mutations detected in *inhA* were as follows: 14 had a C→T mutation at position -15 and 2 had a T→C mutation at position -8, while the remaining strain had a S315T mutation in *katG* (Table 1).

The concordance between the results obtained with the Bactec 460TB system and by the MTBDR_{plus} assay for the detection of INH resistance in the group of 21 strains with high-level resistance was 85.71% (18/21). In two strains, sequencing detected mutations in *katG* different from the ones covered by the assay: one strain had two deletions in codons 234 and 155 to 158, and the other one had a stop mutation in codon 204. The remaining strain had two mutations in *katG*: a S315T mutation that was detected by sequencing but not by the MTBDR_{plus} test and a G→T mutation at codon 463. For the 18 strains identified to be INH^r, 17 had an S315T mutation in *katG*, while the remaining one had a C→T mutation at position -15 in *inhA*.

Overall, by consideration of the group of 48 INH^r strains, in 14 cases the pattern of susceptibility revealed by the MTBDR_{plus} assay did not match the one obtained with the Bactec 460TB system. In 3 cases, the responsible mutations should have been identified by the assay, whereas the 11 remaining disagreements could be explained: in 6 cases DNA sequencing

indicated wild-type sequences, and in the other 5 cases, the mutations identified were outside the regions covered by the assay.

MTBDR_{plus} assay results for the clinical specimens.

Among the 65 clinical specimens tested directly, 31 strains were fully susceptible and 34 were resistant (29 were RIF^r and INH^r and 5 were RIF^s and INH^r). Among the 26 smear-negative samples, the strains isolated were fully susceptible in 19 cases, had the INH^r and RIF^s pattern in 4 cases, and had the INH^r and RIF^r pattern in 3 cases. In the 39 smear-positive samples, 12 strains were fully susceptible, 1 had the INH^r and RIF^s pattern, and 26 had the INH^r and RIF^r pattern. Figure 3 shows the distribution of the MTBDR_{plus} assay results according to the results obtained with Bactec 460TB system for the 65 samples. We obtained valid test results for 51/65 (78.4%) samples (Table 4). For the 14 remaining samples, all of which were smear negative, the results were invalid. However, the *M. tuberculosis* complex-specific (TUB) control band was reported in nine of these samples. The overall rate of concordance between the results of the MTBDR_{plus} assay and those of the Bactec 460TB system for the assessment of RIF resistance was 98% (50/51) ($\kappa = 0.960$; 95% CI = 0.859). The level of agreement for RIF resistance for the smear-positive samples was 100% (39/39; $\kappa = 1$; 95% CI = 0), and that for smear-negative specimens was 91.6% (25/26; $\kappa = 0.8$; 95% CI = 0.625). The discordant sample was RIF susceptible according to the Bactec 460TB system, but the pattern obtained by the MTBDR_{plus} assay indicated that the sample tested was resistant because the band for the WT8 *rpoB* probe was missing. The overall rate of concordance between both tests for the assessment of INH susceptibility was 96.2% (49/51; $\kappa = 0.920$; 95% CI = 0.797). The levels of agreement for INH susceptibility were 94.8% (37/39; $\kappa = 0.885$; 95% CI = 0.740) for the smear-positive samples and 100% (26/26; $\kappa = 1$; 95% CI = 0) for the smear-negative samples. The two discordant samples were INH resistant according to the Bactec 460TB system but INH susceptible according to the MTBDR_{plus} assay. Both samples were from the same patient.

Given that more than one specimen was collected from some patients, the rate of concordance was also calculated by taking into account only one sample per patient ($n = 28$). The overall rate of concordance between the MTBDR_{plus} assay and the Bactec 460TB system was 92.85% (26/28). Therefore, the rate of concordance between the MTBDR_{plus} assay and the Bactec 460TB system for the assessment of RIF resistance was 96.42% (27/28; $\kappa = 0.909$; 95% CI = 0.089). The levels of agreement for RIF resistance were 100% (20/20; $\kappa = 1$; 95% CI = 0) for smear-positive samples

TABLE 2. MTBDR_{plus} assay results according to Bactec 460TB system results for the 62 clinical strains

MTBDR _{plus} test result	Bactec 460TB system result (no. [%] of strains)			
	INH		RIF	
	Susceptible ($n = 14$)	Resistant ($n = 48$)	Susceptible ($n = 50$)	Resistant ($n = 12$)
Susceptible	14 (100)	13 (27)	50 (100)	1 (8.3)
Resistant	0	35 (73)	0	11 (91.7)

FIG. 2. Distribution of MTBDR_{plus} assay results according to the results obtained with the Bactec 460TB system for the 62 clinical strains.

and 87.5% (7/8; $\kappa = 0.600$; 95% CI = 0.343) for the smear-negative ones. For the 7/28 RIF-resistant samples, the mutations in *rpoB* identified by the MTBDR_{plus} assay were D516V in 3 specimens, D526Y in 2 specimens, and S531L in 2 specimens. The rate of concordance between both tests for the assessment of INH resistance was 96.42% (27/28; $\kappa = 0.909$; 95% CI = 0.089). The levels of agreement were 95% (19/20; $\kappa = 0.894$; 95% CI = 0.113) for the smear-positive samples and 100% (8/8; $\kappa = 1$; 95% CI = 0) for the smear-negative ones. The mutations identified by the MTBDR_{plus} assay for the 7/28 INH^r samples were as follows: in 5 specimens a *katG* G→C mutation at position 315 and in 2 specimens both a G→C mutation at position 315 in *katG* and a C→T mutation at position -15 in *inhA*.

DISCUSSION

The GenoType MTBDR assay, the previous version of the GenoType MTBDR_{plus} assay, has been widely evaluated with clinical samples and strains (4, 14, 18, 20). In earlier studies, the sensitivity of the MTBDR assay for the detection of RIF resistance was found to vary from 95% to 99%, depending on the study. This high rate of detection can be explained by the fact that the mutations responsible for RIF resistance are mainly located in the 81-bp hot-spot region and that mutations outside this location are rare and are associated with low-level resistance (11, 14).

The main limitation of the MTBDR assay for the detection of INH resistance is that it has a low sensitivity because the test detects only one mutation, the S315T mutation, in *katG*. Brossier et al. (3) reported that the assay had a sensitivity of

67.3% (64/95) for the detection of INH resistance, although when only the strains with high-level resistance were considered, this value increased to 89.4% (59/66). A similar trend was found by Cavusoglu et al. (4), whose values of sensitivity rose from 72.9% to 87.1% when only the strains with high-level resistance were considered. Mutations in the upstream region of *inhA* have also been described to be responsible for resistance in INH^r strains, especially those with low-level resistance (3, 23). To that effect, the new version includes six more probes aimed at the promoter region of *inhA*: two are for the wild type and four cover mutations at different positions (nucleotides -8 [two probes], -15, and -16). In our experience, the inclusion of new probes for *rpoB* and *inhA* has increased the sensitivity of detection of RIF resistance by 8.4% and that of detection of INH resistance by 31.4% in comparison to the sensitivities of the old version.

In our study, the sensitivities of the GenoType MTBDR_{plus} assay for the detection of RIF and INH resistance in clinical strains were 91.7% and 73%, respectively. Hillemann et al. (13) reported considerably higher values: 98.7% for RIF and 92% for INH. The higher value of sensitivity for the detection of INH resistance may be due to the fact that in 71 of the 75 strains evaluated by Hillemann and coworkers, the mutation causing resistance was located in *katG* codon 315. In contrast, in the study of Miotto et al. (19), only 117 of the 173 strains studied had the mutation in *katG* codon 315, resulting in a sensitivity for the detection of INH resistance closer to ours: 79.1%.

For the detection of RIF resistance, the new version of the MTBDR assay includes three more probes covering

TABLE 3. Distribution of MTBDR_{plus} assay results according to the sequencing results for *katG*, *inhA*, and *oxyR-ahpC* for the 48 INH^r strains

MTBDR <i>plus</i> test result	No. of the following INH ^r strains with the indicated sequencing results:								
	Low-level INH ^r (MICs ≤ 1 μg/ml)					High-level INH ^r (MICs > 1 μg/ml) ^a			
	<i>katG</i> mutation	<i>inhA</i> mutation	<i>oxyR-ahpC</i> mutation	Wild type	Total	<i>katG</i> mutation	<i>inhA</i> mutation	Wild type	Total
INH ^r	1	16	0		17	17	1	0	18
INH ^s	2 ^b	1 ^c	1 ^d	6	10	3 ^e	0	0	3
Total	3	17	1	6	27	20	1	0	21

^a None of the strains had the wild-type sequence.

^b Both mutations were outside the *katG* hot-spot region studied by the MTBDR_{plus} assay.

^c The MTBDR_{plus} assay did not identify a C→T *inhA* mutation at position -15 found by sequencing.

^d This strain also had a Trp728Tyr change in *katG*.

^e Two of the three strains had mutations outside the *katG* hot-spot region studied by the MTBDR_{plus} assay. The other strain had a S315T mutation that was not detected by the MTBDR_{plus} assay.

FIG. 3. Distribution of MTBDR_{plus} assay results according to the results obtained with the Bactec 460TB system for the 51 clinical specimens with a valid result.

wild-type *rpoB* sequences in order to improve the sensitivity of the test. Interestingly, we found a sample that was RIF resistant according to the results obtained with the Bactec 460TB system, and the pattern obtained by the MTBDR_{plus} assay also indicated resistance due to the absence of one of these new probes, the WT8 probe, which matches the S531W sequencing result.

Regarding the level of resistance to INH, our sensitivity value increased from 62.9% when only the group of strains with low-level resistance was considered to a value of 85.71% when the set of strains with high-level resistance was considered. This difference can partially be explained by the distribution of resistance-associated mutations according to the level of resistance to INH. As has previously been described by several authors, the most common mutation involved in INH resistance is the S315T substitution in *katG*, which has also been related to high levels of INH resistance (MICs > 1 µg/ml) (22, 23, 28). In contrast, mutations causing low levels of INH resistance are not as clearly elucidated, as they are much more complex and involve different genes; however, a firm relationship has been found between mutations in the *inhA* regulatory region and low or intermediate levels of resistance (3, 23). In our experience, we have confirmed this distribution of mutations according to the MIC. In the case of strains with low-level resistance, among the mutations detected by the MTBDR_{plus} assay, 59% were in the *inhA* regulatory region (the C→T mutation at position -15 in 14 cases and the T→C mutation at position -8 in 2 cases) and only 3.7% were in *katG* (S315T in 1 case). In strains with high-level resistance, we noticed the opposite distribution: 80.9% of the mutations corresponded to the S315T mutation in *katG* and only 4.7% were located in *inhA*.

The MTBDR_{plus} assay can also be applied directly to clinical specimens (13, 19). Hillemann et al. (13) obtained 98.6% valid test results for a set of smear-positive samples. Miotto et al. (19) obtained 100% (47/47) valid test results for smear-positive specimens and 70.9% (22/31) valid test results for smear-negative specimens. Our results with clinical samples also revealed 100% valid test results for smear-positive specimens and 46.1% (12/26) valid test results for smear-negative specimens. If the test is used only once, the smear result is positive, and the presence of bacilli in the clinical sample has been confirmed, as recommended by the manufacturer, the test performs well.

Interestingly, for the 14 Ziehl-Neelsen staining-negative samples, we found two kinds of invalid result. For nine samples, there was an absence of gene-specific hybridization bands along the strip, despite the presence of amplification and the *M. tuberculosis* complex-specific control (TUB) band (shown in Fig. 1, lane 9). In these cases at least, the presence of *M. tuberculosis* in clinical samples with a negative Ziehl-Neelsen staining result was detected. The other kind of invalid result (Fig. 1, lanes 4 to 8), present in five cases, is more confusing. The pattern of resistance was incomplete because of the absence of control, wild-type, and mutation bands for one or two of the genes studied by use of the assay.

The test has a short turnaround time and simultaneously provides the RIF and INH susceptibility pattern in 1 working day (approximately 2 h of hands-on time and 6 h of incubation). These elements are of importance in order to avoid the transmission of resistant *M. tuberculosis* strains and at the same time start antimicrobial treatment early while awaiting conventional drug susceptibility testing results. The results are easy to interpret, although for some samples the intensities of the hybridization bands varied and some training in the reading of the strip was necessary. The equipment required is the basic equipment available in a molecular biology laboratory (a thermocycler and a hybridizer). The MTBDR_{plus} assay is approved for sale only outside the United States, as it has not received final approval for use from the U.S. Food and Drug Administration.

Molecular methods designed to detect resistance in *M. tuberculosis* have some limitations. Since only the more frequent mutations related to RIF and INH resistance are detected by the assay, the results must be confirmed by phenotypic meth-

TABLE 4. Valid and invalid MTBDR_{plus} results according to the Ziehl-Neelsen microscopy staining for the 65 clinical samples

MTBDR _{plus} test result ^a	No. (%) of specimens		
	Smear-positive	Smear-negative	Total
MTBDR _{plus} +	39 (100)	12 (46.1)	51 (78.4)
MTBDR _{plus} -	0	14 (53.9)	14 (21.6)
Total	39	26	65

^a MTBDR_{plus} +, valid test result; MTBDR_{plus} -, invalid test result.

ods. Although the common mutations predictive of resistance are well known for some drugs, in some cases the mutations identified are silent and are not always related to the acquisition of resistance. In addition, the exact ratio of resistant to susceptible bacilli that results in phenotypic resistance is unclear. This means that in practice, as we have noticed, a molecular assay result can differ from the one obtained by a susceptibility proportion method, such as the method conducted with the Bactec 460TB system. The identification of a resistance mutation by a molecular test in clinical samples is clinically informative and useful, whereas the absence of a mutation in the target sequence analyzed must be interpreted cautiously.

A combination of a susceptible result by the MTBDR_{plus} assay but a pattern of resistance with the Bactec 460TB system for INH could reflect an infrequent mutation and could be related to low-level resistance. However, this misclassification probably would not affect a patient's treatment, especially if treatment with the four first-line drugs is prescribed. In addition, by consideration of RIF resistance alone as an accurate marker for MDR (21, 25), the sensitivity of the test for the detection of MDR increases, even though the INH pattern can be misclassified as susceptible. In our experience, the GenoType MTBDR_{plus} assay could be implemented in particular for those patients with risk factors for MDR, such as immigrants from countries with a high prevalence of MDR and patients with relapses or undergoing retreatment.

In conclusion, the MTBDR_{plus} assay may be a very useful tool for the management of TB because it allows the identification of RIF- and INH-resistant *M. tuberculosis* in both clinical samples and strains.

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