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Diagnostic Performance of the New Version (v2.0) of GenoType MTBDR*sl* Assay for Detection of Resistance to Fluoroquinolones and Second-Line Injectable Drugs: a Multicenter Study

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Resistance to fluoroquinolones (FLQ) and second-line injectable drugs (SLID) is steadily increasing, especially in eastern European countries, posing a serious threat to effective tuberculosis (TB) infection control and adequate patient management. The availability of rapid molecular tests for the detection of extensively drug-resistant TB (XDR-TB) is critical in areas with high rates of multidrug-resistant TB (MDR-TB) and XDR-TB and limited conventional drug susceptibility testing (DST) capacity. We conducted a multicenter study to evaluate the performance of the new version (v2.0) of the Genotype MTBDR*sl* assay compared to phenotypic DST and sequencing on a panel of 228 *Mycobacterium tuberculosis* isolates and 231 smear-positive clinical specimens. The inclusion of probes for the detection of mutations in the e*is* promoter region in the MTBDR*sl* v2.0 test resulted in a higher sensitivity for detection of kanamycin resistance for both direct and indirect testing (96% and 95.4%, respectively) than that seen with the original version of the assay, whereas the test sensitivities for detection of FLQ resistance remained unchanged (93% and 83.6% for direct and indirect testing, respectively). Moreover, MTBDR*sl* v2.0 showed better performance characteristics than v1.0 for the detection of XDR-TB, with high specificity and sensitivities of 81.8% and 80.4% for direct and indirect testing, respectively. MTBDR*sl* v2.0 thus represents a reliable test for the rapid detection of resistance to second-line drugs and a useful screening tool to guide the initiation of appropriate MDR-TB treatment.

Resistance to fluoroquinolones (FLQ) and second-line injectable drugs (SLID) is becoming more common, especially in eastern European countries, posing a serious threat to effective tuberculosis (TB) infection control. According to the most recent World Health Organization (WHO) report, extensively drug-resistant TB (XDR-TB) had been reported by 100 countries by the end of 2013. On average, an estimated 9.0% (95% confidence interval [CI[, 6.5% to 11.5%) of people with multidrug-resistant TB (MDR-TB) have XDR-TB (1, 2). To date, only 8 countries among the 36 with a high TB and/or MDR-TB burden have established a national surveillance system for resistance to second-line drugs among patients with MDR-TB (1, 2).

Efforts should be made to ensure that all patients diagnosed with MDR-TB undergo testing of susceptibility to FLQ and SLID in order to initiate early effective treatment and appropriate measures of infection control.

Conventional phenotypic methods take weeks to months to fully define the drug resistance profile of *Mycobacterium tuberculosis* isolates due to the low growth rate of the bacterium (3, 4). The development and implementation of rapid molecular assays for the detection of resistance to anti-TB drugs promise more-rapid drug resistance detection, which can be critical in areas with high rates of MDR-TB and XDR-TB and settings with limited conventional DST capacity.

Several molecular tests are available today to detect resistance to first-line drugs. In contrast, the choice of molecular assays for second-line drugs is far more limited. To date, GenoType MTBDR*sl* (Hain Lifescience, Nehren, Germany) is one of the few commercially available molecular tests for detection of resistance to the main second-line anti-TB drugs. It is a qualitative test for the identification of *Mycobacterium tuberculosis* complex (MTBC) and its resistance to FLQ, to aminoglycosides and cyclic peptides (AG/CP), and to ethambutol (EMB) as either clinical isolates or pulmonary smear-positive clinical specimens (5–7). Diagnosis of FLQ resistance is enabled by the detection of mutations in the quinolone resistance-determining regions (QRDR) of the *gyrA* gene (coding for the A-subunit of the DNA gyrase). Resistance to AG/CP is detected by targeting a region of the 16S rRNA gene (encoded by the *rrs* gene), whereas identification of mutations in the *embB* gene enable detection of EMB resistance.

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Several studies have been conducted to evaluate the test performances in different settings and have demonstrated generally good performance characteristics and high confidence for resistance results (2). However, sensitivities varied significantly among studies depending on the genetic background of the strains and were frequently suboptimal for kanamycin (KAN), in particular, with eastern European strains (8–10). In eastern Europe and Russia, where the Beijing subfamily is largely diffused, assays using the *rrs* gene alone failed to detect a large proportion of KAN-resistant isolates (11). In line with this observation, Huang et al. showed that the low sensitivity of the GenoType MTBDR*sl* assay was due to the presence of specific mutations not detected by the test, located in the *eis* gene promoter region (12).

The *eis* gene encodes an aminoglycoside acetyltransferase specific to KAN. Polymorphisms in the *eis* promoter region lead to an increased synthesis of the enzyme and the subsequent inactivation of the drug (13). These mutations are responsible for more than 80% of low-level KAN-resistant cases (13–15), making this genetic region a valuable molecular marker of KAN resistance.

On the basis of published evidence, the WHO decided in 2013 not to endorse the second-line Line Probe assay (LPA) given that, with the test's low pooled sensitivity, negative results cannot be considered to reliably rule out resistance. The WHO Expert Group thus concluded that the assay can be used only as a method to rule in diagnosis of XDR-TB but not as a replacement for conventional phenotypic DST to rule out resistance (16).

To improve the overall performance of GenoType MTBDR*sl*, and in particular its sensitivity for KAN resistance, a new version of the assay (i.e., MTBDR*sl* v2.0) has been recently developed. GenoType MTBDR*sl* v2.0 includes two new target genetic regions: the *eis* promoter region −10 to −14 (in addition to *rrs*) and the *gyrB* quinolone resistance-determining region (QRDR) (in addition to *gyrA*) for the detection of mutations at codons 536 to 541. The target region for EMB (*embB*) has been removed. MTBDR*sl* v2.0 thus includes a total of 27 probes for the detection of resistance exclusively to second-line drugs.

We have conducted an international study to assess the performance of the new version of the GenoType MTBDR*sl* assay for detection of resistance to FLQ and SLID. The performance of the test was evaluated by comparison to DST and sequencing using a panel of 228 clinical isolates and 231 clinical specimens from subjects referred to the different study sites (three TB Supranational Reference Laboratories [SRL] in Europe: SRL Milan, Italy; SRL Solna, Sweden; and SRL Borstel, Germany) from countries with a high MDR-TB prevalence.

MATERIALS AND METHODS

A collection of MTBC clinical isolates and specimens obtained from the National and Supranational Reference Laboratories involved in the FP7 project Pan-European Network for Study and Clinical Management of Drug-Resistant Tuberculosis (TB PAN-NET) (funded by a European Union grant) were considered for the evaluation of the test performances. The collection includes strains isolated in Italy and Sweden and clinical specimens collected in Italy, Germany, and Moldova. Samples were collected from 2009 to 2013. All tests were performed at a TB Supranational Reference Laboratory (SRL) (SRL Milan, Milan, Italy; SRL Solna, Solna, Sweden; or SRL Borstel, Borstel, Germany).

Clinical isolates. The study included 228 mycobacterial strains isolated on liquid (BBL MGIT; BD, Franklin Lakes, NJ, USA) or solid (BBL Löwenstein-Jensen; BD, Franklin Lakes, NJ, USA) media and identified according to standard biochemical test and sequencing, as previously recommended (3). All MTBC strains were phenotypically resistant to rifampin (RIF) and isoniazid (INH), harboring different patterns of mutations in *rpoB* and *katG* or *inhA* genes. The profiles of susceptibility to second-line drugs were determined by conventional phenotypic testing. Two different panels were tested and analyzed independently, one that included 128 samples at SRL Milan, Milan, Italy, and another that included 100 samples at SRL Solna, Solna, Sweden.

Clinical specimens. DNA was obtained from 231 clinical specimens from patients with pulmonary MDR-TB during standard clinical management who were referred to the different study sites (SRL Milan, Milan, Italy; SRL Borstel, Borstel, Germany; and NRL Minsk, Minsk, Belarus) for retesting. All respiratory specimens were classified as acid-fast bacillus (AFB) positive by sputum smear microscopy. Samples were decontaminated according to international guidelines using an N-acetyl-cysteine–NaOH procedure (17). The drug susceptibility profile of the corresponding strains was obtained using a conventional phenotypic assay according to international guidelines.

The development and validation of the MTBDR*sl* v2.0 assay were approved by the Ethical Committee of the San Raffaele Hospital given the role of SRL Milan as the coordinating institution.

DST. Determinations of susceptibility to FLQ (ofloxacin, levofloxacin, and moxifloxacin) and SLID (amikacin, kanamycin, and capreomycin) were performed either on a Bactec MGIT 960 system (MGIT) (BD Bioscience, Erebodegem, Belgium) or on Lowenstein-Jensen (LJ) media using the proportional method according to international guidelines (18). The DST critical concentrations used for MGIT are as follows: for ofloxacin, 2 μg/ml; for levofloxacin, 1.5 μg/ml; for moxifloxacin, 0.5 μg/ml; for amikacin, 1 μg/ml; for kanamycin, 2.5 μg/ml; and for capreomycin, 2.5 μg/ml. The DST critical concentrations used for solid LJ media are as follows: for ofloxacin, 4 μg/ml; for amikacin, 30 μg/ml; for kanamycin, 30 μg/ml; and for capreomycin, 40 μg/ml.

DNA extraction. Genomic DNA was extracted from isolates and decontaminated specimens by thermal lysis and sonication as described elsewhere (19). Alternatively, a commercially available kit, GenoLyse (Hain Lifescience, Nehren, Germany), was used to extract DNA from clinical specimens according to the manufacturer's instructions.

GenoType MTBDR*sl* assay. Amplification and hybridization of DNA extracted from isolates and clinical specimens were performed according to the manufacturer's instructions.

Sequencing. Genomic regions harboring drug resistance mutations were amplified and sequenced (9) by the Sanger method using the designed internal primers for each region. Results were visualized as electropherograms and analyzed by ClustalW application (BioEdit Software; Ibis Biosciences/Abbott Company, Carlsbad, CA), aligning sequences with the corresponding reference strain (*M. tuberculosis* H37Rv; GenBank accession no. AL123456).

Statistical analysis. The performance of the MTBDR*sl* v2.0 assay was evaluated using conventional phenotypic DST and Sanger sequencing as reference standards, following the Standards for Reporting of Diagnostic Accuracy (STARD) recommendations (20). Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), likelihood ratio, and diagnostic accuracy values were calculated according to the Wilson score. All statistical analyses were carried out using Open Source Epidemiologic Statistics for Public Health version 3.03 (21).

RESULTS

Performance characteristics of the GenoType MTBDR*sl* v2.0 assay for detection of FLQ resistance on clinical isolates. The panel included a total of 73 strains phenotypically resistant to at least one FLQ (ofloxacin or moxifloxacin) and 155 susceptible ones (Table 1). The GenoType MTBDR*sl* v2.0 assay correctly identified 61 (83.6%) FLQ-resistant isolates, missing 12 (16.4%) phenotypically resistant strains. All phenotypically susceptible strains were identified as nonmutated by the assay. Eleven phenotypically re-

TABLE 1 Phenotypic drug-susceptibility testing of 228 clinical isolates and 231 specimens used in the study[a]

Drugs	No. of clinical isolates		No. of clinical specimens	
	R	S	R	S
FQs	73	155	57	174
SLIDs	147	81	63	168

[a] FQs, fluoroquinolones; SLIDs, second-line injectable drugs; R, resistant; S, susceptible.

sistant strains showed a mix of wild-type (WT) and mutated bands at the level of the *gyrA* gene, suggesting a mixed infection.

The distribution of gene mutations in the 73 FLQ-resistant isolates is shown in Table 2. The predominant mutations detected by MTBDR*sl* v2.0 as conferring FLQ resistance are the *gyrA* MUT1 A90V and MUT3C D94G mutations, identified in 26 (35.6%) and 15 (20.5%) resistant strains, respectively. Nine additional strains (12%) showed a mix of wild-type sequence and A90V or D94G mutations. *gyrA* mutations MUT3A (D94A) and MUT3B (D94N/D94Y) were detected in 4 (5.5%) and 2 (2.7%) resistant strains, respectively. The *gyrA* mutation MUT2 (S91P) was detected in 3 (4.1%) samples either alone or together with other mutations as a mix of wild-type and mutated bands. In three cases, the lack of the *gyrA* wild-type 3 (WT3) probe was used to infer a mutation at the level of codon 94. By sequencing the *gyrA* gene, we confirmed the presence of mutations D94Y and D94A in two and one resistant strains, respectively.

Mutations in the *gyrB* gene were very rare and were identified in only 1 out of 73 (1.4%) resistant strains. In this unique case, the mutation could be inferred by the lack of the *gyrB* wild-type probe on the GenoType MTBDR*sl* v2.0 strip. DNA sequencing revealed the presence of the N538T mutation, thus confirming the MTBDR*sl* test result.

Overall, the test sensitivity for FLQ resistance measured against culture-based DST was 83.6% (95% confidence interval [CI], 73.4% to 90.3%) whereas the test specificity was 100% (95% CI, 97.6% to 100%). The concordance between phenotypic DST and the MTBDR*sl* v2.0 test was 94.7% (95% CI, 91.0% to 97.0%) (Table 3). We also calculated MTBDR*sl* v2.0 test performance by looking separately at each single FLQ tested, namely, ofloxacin or moxifloxacin. For ofloxacin, we obtained a test sensitivity and specificity of 84.7% (95% CI, 74.7% to 91.2%) and 100% (95% CI, 97.6% to 100%), respectively, whereas the test sensitivity and specificity for moxifloxacin were 90.0% (95% CI, 69.9% to 97.2%) and 97.4% (95% CI, 91.0% to 99.3%), respectively. Results are shown in Table S1 in the supplemental material.

The comparison between the MTBDR*sl* v2.0 test and *gyrA* QRDR sequencing showed an overall concordance of 98.3% (95% CI, 95.6% to 99.3%) (Table 4). Among the 11 phenotypically resistant strains showing a mix of wild-type and mutated bands at the level of the *gyrA* gene, sequencing showed a double-pattern profile in 9 out of 11 cases; it identified only the mutated nucleotide in 1 case, whereas it completely missed the mutation (D94G codon substitutions) in another case. Sequencing also missed one fully mutated sample (i.e., mutation at the level of codon D94 and absence of the corresponding wild-type 3 [WT3] probe) in one case. In contrast, MTBDR*sl* v2.0 missed a D94Y codon substitution and a D89N codon substitution in the *gyrA* gene in two distinct isolates that were instead detected as mixed populations by sequencing (see Table 2). Overall, the test sensitivity for FLQ resistance compared to *gyrA* gene sequencing was 96.7% (95% CI, 88.8% to 99.1%) and the specificity was 98.8% (95% CI, 95.8% to 99.7%) (Table 4).

TABLE 2 Fluoroquinolone resistance and susceptibility results obtained by GenoType MTBDR*sl* v2.0 assay, phenotypic DST, and sequencing on 228 clinical isolates[a]

Phenotypic DST result for *M. tuberculosis* clinical isolates	MTBDR*sl* v2.0 result		Sequencing result			
	gyrA	*gyrB*	*gyrA*	*gyrB*	*n*	%
R	A90V	WT	A90V	NP	25	11.0
R	D94G	WT	D94G	NP	14	6.1
R	D94A	WT	D94A	NP	4	1.8
R	WT/D94G	WT	WT/D94G	NP	3	1.3
R	No WT3	WT	D94Y	NP	2	0.9
R	No WT3	WT	D94G	NP	1	0.4
R	D94N/Y	WT	D94Y	NP	1	0.4
R	S91P	WT	S91P	NP	1	0.4
R	WT/D94N/Y + WT/A90V	WT	WT/D94N + WT/A90V	NP	1	0.4
R	WT/A90V	WT	WT/A90V	NP	2	0.9
R	WT/A90V + WT/D94G	WT	WT/A90V + WT/D94G	NP	1	0.4
R	WT/A90V + WT/S91P + WT/D94G	WT	WT/A90V + S91P + WT/D94G	NP	1	0.4
R	WT/S91P + WT/D94A	WT	WT/S91P + WT/D94A	NP	1	0.4
R	D94G/D94N/Y	WT	WT	NP	1	0.4
R	WT/D94G	WT	D94G	NP	1	0.4
R	WT/D94G	WT	WT	NP	1	0.4
R	WT	WT	WT/D94Y	NP	1	0.4
R	WT	WT	WT/D89N	NP	1	0.4
R	WT	No WT	WT	N538T	1	0.4
R	WT	WT	WT	NP	10	4.4
S	WT	WT	WT	NP	155	68.0

[a] DST, drug susceptibility testing; WT, wild type; R, resistant; S, susceptible; NP, not performed.

TABLE 3 Performance of GenoTypeMTBDR*sl* v2.0 assay compared to phenotypic DST on 228 clinical isolates[a]

Parameter	Phenotypic DST result	
	% or ratio	95% CI
FLQ		
Sensitivity	83.6	73.4–90.3
Specificity	100	97.6–100
PPV	100	94.1–100
NPV	92.8	87.9–95.8
Positive likelihood ratio	Undefined	Undefined
Negative likelihood ratio	0.2	0.1–0.2
Diagnostic accuracy	94.7	91.0–97.0
SLID		
Sensitivity	86.4	79.9–91.0
Specificity	90.1	81.7–94.9
PPV	94.1	88.7–97.0
NPV	78.5	69.1–85.6
Positive likelihood ratio	8.7	6.8–11.2
Negative likelihood ratio	0.15	0.1–0.2
Diagnostic accuracy	87.7	82.8–91.4
Kanamycin		
Sensitivity	95.4	90.4–97.9
Specificity	91.4	83.9–95.6
PPV	94.0	88.6–97.0
NPV	93.4	86.4–96.9
Positive likelihood ratio	11.1	8.7–14.2
Negative likelihood ratio	0.05	0.04–0.07
Diagnostic accuracy	93.8	89.8–96.9

[a] FLQ include ofloxacin and moxifloxacin. SLID include kanamycin, amikacin, and capreomycin. CI, confidence interval; DST, drug susceptibility testing; PPV, positive predictive value; NPV, negative predictive value.

TABLE 4 Performance of GenoType MTBDR*sl* v2.0 assay compared to sequencing on 228 clinical isolates[a]

Parameter	Sequencing result	
	%	95% CI
gyrA		
Sensitivity	96.7	88.8–99.1
Specificity	98.8	95.8–99.7
PPV	96.7	88.8–99.1
NPV	98.8	95.8–99.7
Diagnostic accuracy	98.3	95.6–99.3
rrs		
Sensitivity	100	96.2–100
Specificity	96.2	91.5–98.4
PPV	95.1	89.0–97.9
NPV	100	97.1–100
Diagnostic accuracy	97.8	95.1–99.1
eis		
Sensitivity	94.9	83.1–98.6
Specificity	99.2	95.4–99.9
PPV	97.4	86.5–99.5
NPV	98.4	94.2–99.5
Diagnostic accuracy	98.1	94.6–99.4

[a] CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value.

Performance characteristics of the GenoType MTBDR*sl* v2.0 assay for detection of SLID resistance in clinical isolates. Our panel included 147 isolates resistant to at least one SLID (amikacin, kanamycin, or capreomycin) as determined by phenotypic DST and 81 strains susceptible to all the tested injectable drugs (Table 1). The GenoType MTBDR*sl* v2.0 assay correctly identified 127 out of 147 (86.4%) SLID-resistant isolates and 73 out of 81 (90.1%) fully susceptible ones. The distribution of gene mutations in the 127 SLID-resistant isolates identified by the MTBDR*sl* v2.0 test is shown in Table 5. The great majority of resistant strains carried mutations only at the level of the *rrs* gene, namely, 98 out of 127 (77.2%), including 12 (9.4%) strains with a mixed mutated and wild-type profile. Among these isolates, the most frequent mutation was *rrs* MUT1 A1401G, which was detected in 92 out of 98 (93.9%) cases; three (~3%) strains carried both A1401G and G1484T mutations, two (~2%) isolates carried the C1402T mutation, and one isolate carried the MUT2 G1484T mutation.

Two resistant strains carried mutations in both the *rrs* (MUT A1401G) and *eis* promoter regions, with one sample showing both mutated and wild-type bands. A total of 27 out of 127 (21.3%) SLID-resistant strains carried mutations only at the level of the *eis* promoter region. The most frequent mutation identified was the MUT1 C-14T mutation, which was detected in 12 out of 27 (44.4%) isolates, including one strain with a mixed profile. The absence of the wild-type 2 (WT2) probe in the *eis* region correlated with the presence of mutations G-10A, G-37T, and C-12T in 8, 4, and 3 cases, respectively. All strains with a mixed mutated and wild-type profile, in either the *rrs* or *eis* promoter region, were phenotypically resistant. A total of 20 strains phenotypically resistant to SLID were detected as fully wild-type by the MTBDR*sl* v2.0 assay: the great majority (13 out of 20 [65%]) were resistant to capreomycin but susceptible to both amikacin and kanamycin, 4 isolates (20%) were resistant uniquely to kanamycin, 2 isolates (10%) were resistant to all SLID, and one isolate was resistant uniquely to amikacin.

The GenoType MTBDR*sl* v2.0 test also detected mutations in the *eis* promoter region of 8 phenotypically susceptible strains. Among those, 6 isolates were missing the *eis* WT2 probe corresponding to mutations G-10A and C-12T, as detected by gene sequencing in 5 and 1 cases, respectively, 1 sample showed the *eis* C-14T mutation, and 1 sample showed the G-37T mutation (Table 5).

Overall, the concordance between MTBDR*sl* v2.0 and phenotypic DST for SLID resistance was 87.7% (200/228), whereas the test sensitivity and specificity were 86.4% (95% CI, 79.9% to 91.0%) and 90.1% (95% CI, 81.7% to 94.9%), respectively. As a test for detection of kanamycin resistance measured against culture-based DST, the diagnostic accuracy of GenoType MTBDR*sl* v2.0 was 93.8% (95% CI, 89.9% to 96.3%), whereas the sensitivity and specificity were 95.5% (95% CI, 90.6% to 97.9%) and 91.4% (95% CI, 83.9% to 95.6%), respectively (Table 3).

We then measured the concordance between MTBDR*sl* v2.0 and gene sequencing. The comparison between the assay and *rrs* sequencing showed an overall concordance of 97.8% (95% CI, 95.1% to 99.1%), with the molecular assay correctly identifying all the mutations revealed by gene sequencing (100% sensitivity) (95% CI, 96.2% to 100%). Our panel included 13 strains with a mixed mutated and wild-type profile either in the *rrs* gene or in the *eis* promoter region. Sanger sequencing confirmed the double-pattern profile in 7 out of 13 cases, whereas it missed the mutation in 2 cases and it recognized only the mutated nucleotide in the

TABLE 5 Second-line injectable drug resistance and susceptibility results obtained by GenoType MTBDR*sl* v2.0 assay, phenotypic DST, and sequencing on 228 clinical isolates[a]

Phenotypic DST result for *M. tuberculosis* clinical isolates	MTBDR*sl* v2.0 result		Sequencing result			
	rrs	*eis*	*rrs*	*eis*	*n*	%
R	A1401G	WT	A1401G	WT	82	36.0
R	WT/A1401G	WT	WT/A1401G	WT	4	1.8
R	WT/A1401G	WT	A1401G	WT	2	0.9
R	A1401G + G1484T	WT	A1401G	WT	2	0.9
R	WT/A1401G	WT	WT/A1401G	WT/G-10A	1	0.4
R	A1401G	C-12T/G-10A/C-15G	A1401G	C-12T	1	0.4
R	WT/A1401G	C-12T/G-10A/C-15G	A1401G	WT	1	0.4
R	WT/A1401G	WT	WT/A1401G	WT/G-37T	1	0.4
R	WT/A1401G/G1484T	WT	WT/A1401G	WT	1	0.4
R	G1484T	WT	G1484T	WT	1	0.4
R	C1402T	WT	C1402T	WT	1	0.4
R	WT/A1401G	WT	WT	WT	2	0.9
R	C1402T	WT	WT	WT	1	0.4
R	WT	C-14T	WT	C-14T	11	4.8
R	WT	C-12T/G-10A/C-15G	WT	G-10A	8	3.5
R	WT	G-37T	WT	G-37T	4	1.8
R	WT	C-12T/G-10A/C-15G	WT	C-12T	3	1.3
R	WT	WT/C-14T	WT	C-14T	1	0.4
R	WT	WT	WT	WT	20	8.8
S	WT	C-14T	WT	C-14T	1	0.4
S	WT	C-12T/G-10A/C-15G	WT	C-12T	1	0.4
S	WT	C-12T/G-10A/C-15G	WT	G-10A	5	2.2
S	WT	G-37T	WT	G-37T	1	0.4
S	WT	WT	WT	WT	73	32.0

[a] DST, drug susceptibility testing; WT, wild type; R, resistant; S, susceptible.

remaining 4 cases. We excluded from the analysis one isolate showing the lack of the *rrs* WT1 probe since we were not able to obtain a readable sequence, which was probably due to the low quality or partial degradation of the DNA.

The comparison between MTBDR*sl* v2.0 and sequencing of the *eis* gene promoter region showed an overall concordance of 98.1% (95% CI, 94.6% to 99.4%).

The assay missed two mutations detected by sequencing at the level of codons −10 and −37. In both cases, the sequencing profile showed a double population with the coexistence of both wild-type and mutated nucleotides. In one case, the lack of the *eis* WT2 probe did not correspond to any mutation in the corresponding genetic region of the *eis* promoter by sequencing. Overall, the test sensitivity and specificity measured against *eis* sequencing were 94.9% (95% CI, 83.1% to 98.6%) and 99.2% (95% CI, 95.4% to 99.9%), respectively (Table 4).

Performance characteristics of the GenoType MTBDR*sl* v2.0 assay for detection of resistance to second-line drugs among clinical specimens. Of the 232 respiratory specimens tested with MTBDR*sl* v2.0, 231 showed an interpretable result, with only one sample not providing a valid hybridization profile for any of the 4 genomic regions. The performance for FLQ and SLID resistance detection was evaluated by comparison to DST. Gene sequencing, either direct or using the corresponding isolate, was performed uniquely on samples with a discordant genotypic/phenotypic profile. Results are summarized in Table 6.

Fluoroquinolones. Our panel included a total of 57 clinical specimens phenotypically resistant to at least one FLQ (ofloxacin, levofloxacin, or moxifloxacin) and 174 fully susceptible ones (Table 1). The test correctly identified 53 (92.9%) of the FLQ-resis-

TABLE 6 Performance of GenoTypeMTBDR*sl* v2.0 assay compared to phenotypic DST on 231 clinical specimens[a]

Parameter	Phenotypic DST result	
	% or ratio	95% CI
FLQ		
Sensitivity	93.0	83.3–97.2
Specificity	98.3	95.1–99.4
PPV	94.6	83.4–98.2
NPV	97.7	94.3–99.1
Positive likelihood ratio	53.9	28.0–103.9
Negative likelihood ratio	0.07	0.04–0.117
Diagnostic accuracy	97.0	93.9–98.5
SLID		
Sensitivity	88.9	78.8–94.5
Specificity	91.7	86.5–95.0
PPV	80.0	69.2–87.7
NPV	95.7	91.3–97.9
Positive likelihood ratio	10.7	9.23–12.32
Negative likelihood ratio	0.12	0.09–0.16
Diagnostic accuracy	90.9	86.5–94.0
Kanamycin		
Sensitivity	96.0	86.5,98.9
Specificity	92.2	87.1–95.4
PPV	78.7	66.9–87.1
NPV	98.7	95.5–99.7
Positive likelihood ratio	12.3	10.6–14.4
Negative likelihood ratio	0.04	0.016–0.116
Diagnostic accuracy	93.1	88.9–95.8

[a] FLQ include ofloxacin, levofloxacin, and moxifloxacin. SLID include capreomycin, amikacin, and kanamycin. CI, confidence interval; DST, drug susceptibility testing; PPV, positive predictive value; NPV, negative predictive value.

TABLE 7 Fluoroquinolone resistance and susceptibility results obtained by GenoType MTBDR*sl* v2.0 assay and phenotypic DST on 231 clinical specimens[a]

Phenotypic DST result for *M. tuberculosis* clinical isolates	MTBDR*sl* v2.0 result			
	gyrA	*gyrB*	*n*	%
R	D94G	WT	13	5.6
R	WT/D94G	WT	6	2.6
R	A90V	WT	9	3.9
R	WT/A90V	WT	3	1.3
R	D94A	WT	5	2.2
R	S91P	WT	3	1.3
R	WT/S91P	WT	3	1.3
R	WT/D94N/Y	WT	2	0.9
R	WT/D94G/D94N/Y	WT	2	0.9
R	No WT2	WT	3	1.3
R	WT/D94G/A90V	WT	1	0.4
R	D94G/D94A	WT	1	0.4
R	WT/A90V/S91P/D94N/Y	WT	1	0.4
R	WT	No WT	1	0.4
R	WT	WT	4	1.7
S	WT/D94G	WT	2	0.9
S	WT	No WT	1	0.4
S	WT	WT	171	74.0

[a] DST, drug susceptibility testing; WT, wild type; R, resistant; S, susceptible.

TABLE 8 Second-line injectable drug resistance and susceptibility results obtained by GenoType MTBDR*sl* v2.0 assay and phenotypic DST on 231 clinical specimens[a]

Phenotypic DST result for *M. tuberculosis* clinical isolates	MTBDR*sl* v2.0 result			
	rrs	*eis*	*n*	%
R	A1401G	WT	35	15.2
R	WT/A1401G	WT	10	4.3
R	A1401G	C-12T/G-10A/C-15G	1	0.4
R	C1402T	WT	1	0.4
R	WT	C-12T/G-10A/C-15G	7	3.0
R	WT	C-14T	2	0.9
R	WT	WT	7	3.0
S	WT/A1401G	WT	1	0.4
S	WT/G1484T	WT	2	0.9
S	WT	C-12T/G-10A/C-15G	8	3.5
S	WT	C-14T	3	1.3
S	WT	WT	154	66.7

[a] DST, drug susceptibility testing; WT, wild type; R, resistant; S, susceptible.

tant clinical specimens, missing four resistant cases. In contrast, 3 specimens showed a FLQ-resistant profile by MTBDR*sl* v2.0 while being susceptible by phenotypic DST. Note that 2 out of 3 of these samples showed a double-pattern profile at the level of the *gyrA* gene, suggesting a mixed infection.

The most frequent mutations detected by GenoType MTBDR*sl* among the 53 FLQ-resistant strains were the *gyrA* MUT3C D94G and the MUT1 A90V mutations, each identified in 13 (22,8%) and 9 (15,8%) cases, respectively. In addition, 10 specimens (17.5%) showed a mixed profile (i.e., wild-type sequence and D94G and/or A90V mutations). Other *gyrA* mutations, including MUT2 (S91P) and MUT3A (D94A), were detected either as fully mutant or as mixed populations in 6 and 5 FLQ-resistant specimens, respectively. The full distribution of *gyrA* mutations in our panel is shown in Table 7. Mutations at the level of the *gyrB* gene were very rare (only 2 cases) and could be inferred by the lack of the wild-type probe. DNA sequencing revealed the presence of *gyrB* mutations N538S and T539A, thus confirming the *MTBDRsl* test result. However, only one of the two samples carrying the mutations in *gyrB* was FLQ resistant by phenotypic testing.

Overall, the diagnostic accuracy for FLQ resistance detection in clinical specimens was 97.0% (95% CI, 93.9% to 98.5%), with a sensitivity and specificity of 93.0% (95% CI, 83.3% to 97.2%) and 98.3% (95% CI, 95.1% to 99.4%), respectively. We also calculated MTBDR*sl* v2.0 test performance by looking separately at each single FLQ tested, namely, ofloxacin, levofloxacin, or moxifloxacin. For ofloxacin, we obtained a test sensitivity and specificity of 92.9% (95% CI, 83.0% to 97.2%) and 97.7% (95% CI, 94.3% to 99.1%), respectively; for levofloxacin, the test sensitivity and specificity were 100.0% (95% CI, 67.6% to 100%) and 91.1% (95% CI, 79.3% to 96.5%), respectively, whereas the test sensitivity and specificity for moxifloxacin were 100.0% (95% CI, 78.5% to 100%) and 90.9% (95% CI, 62.3% to 98.4%), respectively. Results are shown in Table S2 in the supplemental material.

Second-line injectable drugs. Our panel included a total of 63 clinical specimens phenotypically resistant to at least one SLID (kanamycin, amikacin, or capreomycin) and 168 fully susceptible ones (Table 1). The test correctly identified 56 (88.9%) of the SLID-resistant samples and 154 out of 168 (91.7%) of the fully susceptible ones. Among the 7 SLID-resistant specimens missed by the assay, 5 were susceptible to kanamycin and resistant to capreomycin or amikacin or both.

Thirty-five out of 56 (62.5%) samples classified as resistant by MTBDR*sl* v2.0 carried the A1401G mutation at the level of the *rrs* gene, with an additional 10 samples (17.5%) showing a mixed wild-type and mutated (MUT A1401G) profile, whereas only one sample carried the *rrs* C1402T substitution. Mutations at the level of the *eis* promoter region were detected in 9 SLID-resistant specimens, with an additional sample showing a double mutation at the levels of both the *rrs* and *eis* regions.

MTBDR*sl* v2.0 test detected mutations within the *eis* promoter region in 11 out of 168 (6.5%) SLID-susceptible samples and mutations in the *rrs* region in 3 out of 168 (1.8%) SLID-susceptible specimens. The lack of an *eis* WT2 probe was the most prevalent mutation, detected in 8 out of 14 susceptible specimens. Direct gene sequencing of these 8 samples confirmed the presence of the C-12T and G-10A mutations in 6 and 2 cases, respectively. All three phenotypically susceptible specimens with mutations in the *rrs* gene showed a double (wild-type and mutation)-pattern profile by MTBDR*sl* v2.0 assay, but sequencing of the corresponding isolates resulted in a wild-type profile. The full distribution of mutations detected by the GenoType MTBDR*sl* assay is summarized in Table 8.

Overall, the diagnostic accuracy of the test for SLID resistance detection in clinical specimens was 90.9% (95% CI, 86.5% to 94.0%), with a sensitivity and specificity of 88.9% (95% CI, 78.8% to 94.5%) and 91.7% (95% CI, 86.5% to 95.0%), respectively. As a test for detection of kanamycin resistance, the diagnostic accuracy of GenoType MTBDR*sl* v2.0 was 93.1% (95% CI, 88.9% to 95.8%), whereas the sensitivity and specificity were 96.0% (95% CI, 86.5% to 98.9%) and 92.2% (95% CI, 87.1% to 95.4%), respectively (Table 6).

Performance characteristics of the GenoType MTBDR*sl* v2.0 assay for XDR-TB identification among clinical isolates and clinical specimens. The GenoType MTBDR*sl* v2.0 assay correctly

TABLE 9 Performance of GenoTypeMTBDR*sl* v2.0 assay for detection of XDR-TB measured against phenotypic DST on 228 clinical isolates and 231 clinical specimens[a]

XDR-TB detection	Phenotypic DST result	
	% or ratio	95% CI
Clinical isolates		
Sensitivity	80.4	66.8–89.4
Specificity	95.6	91.6–97.8
PPV	82.2	68.7–90.7
NPV	95.1	90.9–97.4
Positive likelihood ratio	18.3	14.1–23.7
Negative likelihood ratio	0.2	0.2–0.3
Diagnostic accuracy	92.5	88.4–95.3
Clinical specimens		
Sensitivity	81.8	61.5–92.7
Specificity	98.1	95.2–99.3
PPV	81.8	61.5–92.7
NPV	98.1	95.2–99.2
Positive likelihood ratio	42.8	25.6–71.5
Negative likelihood ratio	0.2	0.1–0.3
Diagnostic accuracy	96.5	93.3–98.2

[a] CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value.

identified 37 out of 46 (80.4%) phenotypically identified XDR-TB isolates, thus missing 9 (19.6%) cases. In particular, the test detected only SLID resistance, missing FLQ resistance, in 7 out of 9 (77.8%) XDR-TB isolates and detected only FLQ resistance, missing SLID resistance, in the remaining two cases (22%). In contrast, the test gave a positive XDR-TB result for 8 isolates resistant uniquely to FLQ by detecting mutations at the level of the *eis* promoter region. Overall, the test accuracy for detection of XDR-TB was 92.5% (95% CI, 88.4% to 95.3%), whereas the test sensitivity and specificity were 80.4% (95% CI, 66.8% to 89.4%) and 95.6% (95% CI, 91.6% to 97.8%), respectively (Table 9).

The GenoType MTBDR*sl* v2.0 assay correctly identified 18 out of 22 (81.8%) phenotypically identified XDR-TB clinical specimens, while it missed FLQ resistance in 2 cases, SLID resistance in 1 case, and both FLQ resistance and SLID resistance in 1 case. However, the test also gave a positive XDR-TB result for 3 specimens that were FLQ resistant only by detecting mutations at the level of the *eis* promoter region and for one sample that was SLID resistant only by failing to detect the *gyrB* WT probe. Overall, MTBDR*sl* v2.0 diagnostic accuracy for detection of XDR-TB was 96.5% (95% CI, 93.3% to 98.2%), with a sensitivity and specificity of 81.8% (95% CI, 61.5% to 92.7%) and 98.1% (95% CI, 95.2% to 99.3%), respectively (Table 9).

DISCUSSION

This was the first multicenter study to assess the diagnostic accuracy of the novel version (v2.0) of the GenoType MTBDR*sl* test for detection of resistance to FLQ (ofloxacin, moxifloxacin, and levofloxacin) and SLID (amikacin, kanamycin, and capreomycin).

Similarly to GenoType MTBDR*sl* v1.0, the concordance between MTBDR*sl* v2.0 and gene sequencing was >97% for all the genomic regions analyzed. Discrepancies were mainly observed in cases of isolates showing a double-pattern profile (15% for *gyrA* and 8.2% for *rrs*), which could be explained by the known lower sensitivity of Sanger sequencing in detecting heteroresistance (22, 23). All heteroresistant strains were phenotypically resistant, thus confirming the presence of the mutated strain.

Second-line injectable drugs. The inclusion of probes for the detection of mutations in the *eis* promoter region increased the overall sensitivity of MTBDR*sl* v2.0 for the detection of SLID resistance to 86.4% (95% CI, 79.9% to 91.0%) and the sensitivity for the detection of kanamycin resistance to 96% for both the isolates and the clinical specimens, which is higher than the pooled sensitivity values for MTBDR*sl* v1.0 (66.9%; 95% CI, 44.1% to 83.8) (2). Notably, the assay primarily missed strains that are resistant uniquely to capreomycin, namely, 13 out of 20 (65%) among isolates and 4 out of 7 (57%) among clinical specimens, which is expected given that the mechanisms of resistance to capreomycin partly differ from those causing resistance to aminoglycosides (13).

If, on the one side, the inclusion of the *eis* promoter region increased the test sensitivity, on the other, it negatively affected its specificity, which decreased from 99.5% (95% CI, 97.1% to 99.9%) in MTBDR*sl* v1.0 (2) to 91.1% (95% CI, 86.43% to 94.9%) and 90.1% (95% CI, 81.7% to 94.9%) in MTBDR*sl* v2.0 for direct and indirect testing, respectively. As expected, the discordance between the results from the SLID-resistant samples as defined by this molecular test and phenotypic DST involved primarily samples carrying mutations in the *eis* promoter region: 8 out of 8 among isolates and 11 out of 14 (78.6%) among clinical specimens. The majority of isolates with a discordant phenotypic/genotypic profile carried the G-10A mutation (62.5%), whereas the most frequent *eis* mutation among the discordant clinical specimens was C-12T (6 of 11; 54.4%), followed by mutations C-14T (27.3%) and G-10A (18.2%). In line with published data, the C-12T *eis* mutation was more often found in sensitive strains and thus appears to be a nonspecific marker of resistance to SLID (24, 25). In contrast, the G-10A nucleotide substitution was reported to correlate well with kanamycin resistance in several studies (13, 24, 25), whereas only Engström et al. found it in 85% of their susceptible strains (26).

More evidence is thus required to univocally define the role of each mutation within the *eis* promoter region in the development of resistance to kanamycin and of cross-resistance to the other classes of injectable drugs. This also implies the necessity to include in the assay specific probes able to discriminate between *eis* mutations at the level of different nucleotides, in particular, in positions −12 and −10. In addition, since resistance to capreomycin and amikacin cannot be automatically inferred on the basis of the presence of *eis* mutations, results from MTBDR*sl* v2.0 testing for SLID resistance should be reported accordingly.

If, on the one hand, our understanding of the role of specific mutations within the *eis* promoter region and SLID resistance is still limited, on the other, phenotypic DST might be a suboptimal gold standard for the assessment of the test performance. Data from a recent publication by the Alland group strongly suggest that *eis* promoter mutants should be considered to have low to moderate kanamycin resistance even if resistance is not detected by LJ-based or even MGIT-based susceptibility tests (27).

Concerning the *rrs* gene, in line with previous observations (9, 24, 28), we found a high correlation between mutations in this genetic region and SLID resistance, with 100 out of 100 mutated isolates and 47 out of 50 (94%) clinical specimens being phenotypically resistant to at least one SLID. Notably, the three specimens giving false SLID resistance results were characterized by a

double-pattern profile compatible with a mixed infection, which could explain the discrepancy with the phenotypic DST result. In such cases, the presence of a predominant susceptible strain might have in fact concealed the resistance (29).

Also, in line with published data (9, 24, 28, 30), we found a high correlation between *rrs* mutations and cross-resistance to amikacin, kanamycin, and capreomycin, with 90% of mutated strains and 98% of mutated clinical specimens being resistant to both aminoglycosides and capreomycin. Notably, the two strains and one clinical specimen carrying the *rrs* mutation C1402T were resistant to all injectable drugs. Overall, the incidence of the *rrs* C1402T and G1484T mutations in our panel was very low, with only 3 strains out of 228 and 3 specimens out of 231 carrying uniquely the G1484T and C1402T substitutions. Given the low prevalence of these mutations (24), *rrs* codons 1484 and 1402 have a low positive predictive value of SLID resistance and are thus unlikely to provide strong clinical significance.

Fluoroquinolones. The inclusion of probes for the detection of mutations in the QRDR of the *gyrB* gene did not significantly improve the overall sensitivity and specificity of the MTBDR*sl* v2.0 test compared to MTBDR*sl* v1.0. Indeed, the incidence of *gyrB* mutations, all inferred by the lack of the *gyrB* WT probe, was very low, with only 1 case among 228 strains tested and 2 cases among 231 clinical specimens. Mutations found in *gyrB*, in the absence of *gyrA* substitutions, correlated with phenotypic FLQ resistance in 2 out of 3 cases. Given the very low prevalence of *gyrB* mutations (31–33) and thus their low positive predictive value for FLQ resistance, the inclusion of *gyrB* probes in the new version of the test is unlikely to be clinically relevant.

Clinical consequences. To date, GenoType MTBDR*sl* has been one of the few rapid molecular assays available on the market for the detection of resistance to second-line drugs. Given its high positive predictive value (PPV) and specificity, a positive result for FLQ resistance, SLID resistance, or XDR-TB can be treated with confidence (2). However, the low sensitivity, especially for detection of kanamycin resistance and its low negative predictive value (NPV), makes it necessary to confirm by conventional DST all cases classified as susceptible.

The inclusion of probes for the detection of mutations at the level of the *eis* promoter region in the new version of the assay, MTBDR*sl* v2.0, leads to a significant increase in test sensitivity for the detection of kanamycin resistance compared to the original version. Importantly, these mutations allow the identification of strains with low or moderate kanamycin resistance which might be erroneously classified as susceptible by conventional phenotypic testing alone. If, on the one hand, this raises the problem of dealing with a suboptimal gold standard when assessing the performance of a new diagnostic test, on the other, it highlights the necessity to understand whether this low-level resistance has a real impact on the treatment efficacy. Therefore, we need to further investigate the correlation between specific mutations and the level of drug responsiveness *in vivo* by routinely collecting clinical data on treatment outcomes. This will be essential for improving our capacity to correctly interpret the mutations identified by genotypic testing.

Summarizing, the MTBDR*sl* v2.0 assay has better performance characteristics for the detection of XDR-TB when performed either directly or indirectly on samples, by correctly identifying more than 80% of XDR-TB cases, than version 1.0, which misses the XDR phenotype in between one in four and one in three cases (2). On clinical specimens, MTBDR*sl* v2.0 showed a PPV and a NPV of 81.8% (95% CI, 61.5% to 92.7%) and 98.1% (95% CI, 95.2 to 99.2), respectively. Notably, the prevalence of XDR-TB cases in our panel of clinical specimens (i.e., a setting with high prevalence of MDR-TB) is around 9.5%, which corresponds to the WHO-estimated prevalence of XDR-TB among MDR-TB cases (2). Therefore, our sample panel provides an evaluation of the diagnostic performance of MTBDR*sl* v2.0 in a real-world setting. Indeed, the use of this rapid assay in countries with a high rate of drug resistance, especially kanamycin resistance, will further increase the test PPV and will reduce the number of XDR-TB cases missed by the test.

Despite some limitations, such as the fact that we tested smear-positive clinical samples only, the fact that FLQ resistance was measured primarily against ofloxacin phenotypic DST, and the lack of data on the MIC for isolates carrying mutations in the *eis* promoter region, this report provides an accurate evaluation of the diagnostic performances of the new version of the GenoType MTBDR*sl* assay based on standardized testing of a large set of samples from different settings. We show that the MTBDR*sl* v2.0 test has better performance characteristics in terms of increased sensitivity for detecting SLID and kanamycin resistance and better accuracy for detecting XDR-TB than the original version of the test. MTBDR*sl* v2.0 thus represents a better screening tool for the rapid detection of resistance to second-line drugs, and it should be recommended in countries with a high burden of MDR/XDR-TB or in settings with high reported rates of resistance to ofloxacin in new and retreatment cases. In such contexts, the test can rapidly identify patients for whom MDR treatment regimens that include earlier-generation fluoroquinolones are likely to fail or who may not be good candidates for the shorter MDR treatment currently being tested in western and central Africa (34).

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