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MYCOBACTERIOLOGY AND AEROBIC ACTINOMYCETES

Evaluation of the GenoType MTBDR*sl* Version 2.0 Assay for Second-Line Drug Resistance Detection of *Mycobacterium tuberculosis* Isolates in South Africa

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ABSTRACT Early detection of resistance to second-line antituberculosis drugs is important for the management of multidrug-resistant tuberculosis (MDR-TB). The GenoType MTBDR*sl* version 2.0 (VER 2.0) line probe assay has been redesigned for molecular detection of resistance-conferring mutations of fluoroquinolones (FLQ) (*gyrA* and *gyrB* genes) and second-line injectable drugs (SLID) (*rrs* and *eis* genes). The study evaluated the diagnostic performance of the GenoType MTBDR*sl* VER 2.0 assay for the detection of second-line drug resistance compared with phenotypic drug susceptibility testing (DST), using the Bactec MGIT 960 system on *Mycobacterium tuberculosis* complex isolates from South Africa. A total of 268 repository isolates collected between 2012 and 2014, which were rifampin monoresistant or MDR based on DST, were selected. MTBDR*sl* VER 2.0 testing was performed on these isolates and the results analyzed. The MTBDR*sl* VER 2.0 sensitivity and specificity indices for culture isolates were the following: FLQ, 100% (95% confidence interval [CI] 95.8 to 100%) and 98.9% (95% CI, 96.1 to 99.9%); SLID, 89.2% (95% CI, 79.1 to 95.6%) and 98.5% (95% CI, 95.7 to 99.7%). The sensitivity and specificity observed for individual SLID were the following: amikacin, 93.8% (95% CI, 79.2 to 99.2%) and 98.5% (95% CI, 95.5 to 99.7%); kanamycin, 89.2% (95% CI, 79.1 to 95.6%) and 98.5% (95% CI, 95.5 to 99.7%); and capreomycin, 86.2% (95% CI, 68.3 to 96.1%) and 95.9% (95% CI, 92.2 to 98.2%). An interoperator reproducibility of 100% and an overall interlaboratory performance of 93% to 96% were found. The overall improvement in sensitivity and specificity with excellent reproducibility makes the GenoType MTBDR*sl* VER 2.0 a highly suitable tool for rapid screening of clinical isolates for second-line drug resistance for use in high-burden TB/HIV settings.

KEYWORDS GenoType MTBDR*sl* version 2.0, drug-resistant TB, MDR-TB, XDR-TB, line probe assay, LPA, fluoroquinolones, second-line injectable drugs, molecular diagnostic testing, phenotypic drug susceptibility testing, *Mycobacterium tuberculosis*

The 2015 World Health Organization (WHO) Global Tuberculosis Report notes that approximately 3.3% of new cases of tuberculosis (TB) and 20% of previously treated TB cases in 2014 were multidrug-resistant TB (MDR-TB), defined as resistance to isoniazid (INH) and rifampin (RIF). South Africa is listed among the 14 countries that appear in all three WHO 2016 to 2020 revised high-burden country (HBC) lists (1). South Africa, with a high burden of MDR-TB, also experienced an increase in extensively drug-resistant TB (XDR-TB), accounting for 59% of XDR-TB patients reported globally in 2011 (1, 2). XDR-TB is defined as MDR-TB with additional resistance to any fluoroquin-

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olone (FLQ) and at least 1 second-line injectable drug (SLID) among the aminoglycoside (AG) drugs amikacin (AMK) and kanamycin (KAN) and the cyclic peptide capreomycin (CAP) (2–5). Drug resistance in *Mycobacterium tuberculosis* isolates occurs at a low frequency due to spontaneous chromosomal mutations (6), which are selected through the improper use of anti-TB agents and low patient compliance with treatment, exerting selective pressure for the emergence of drug-resistant mutants (acquired resistance) (7).

In South Africa, despite the emphasis on TB therapy, the HIV epidemic has seriously hampered TB management and severely affected treatment outcomes (8), while the TB-HIV coepidemic has fueled the escalation of both MDR-TB and XDR-TB (9). The introduction of new rapid molecular diagnostic tests in South Africa, notably the Xpert MTB/RIF assay (Cepheid, USA) and line probe assays (LPAs), for the detection of drug-resistant TB has markedly improved patient management with decreased result turnaround times (TATs) for testing. Culture-based phenotypic drug susceptibility testing (DST), considered to be the gold standard for drug resistance determination, is important for MDR-TB confirmation and the assessment of drug resistance to second-line and new drugs in the management of MDR-TB and XDR-TB (10). However, conventional DST is labor intensive, time-consuming, and generally takes between 2 and 3 weeks of incubation to provide meaningful treatment directing results. In addition, second-line DST is fraught with challenges due to variability of methodology, reproducibility in performance, and reliability of results. Currently, there are no rapid genotypic diagnostic tests in the South African TB diagnostic algorithm for the determination of resistance to second-line anti-TB drugs (10). Molecular-based assays designed to detect specific drug resistance-encoding mutations in *M. tuberculosis* have the advantage of achieving faster TATs (within 48 h) for resistance reporting compared to conventional DST, and in the process it alerts clinicians to the emergence of drug resistance in *M. tuberculosis* strains from individual patients. Early detection of drug resistance is crucial to preventing the transmission of drug-resistant TB and averting mortality, as previously described (9, 11).

A current limitation of molecular assays is that they do not accommodate all mutations conferring resistance to anti-TB agents. A WHO Expert Group determined in 2013 that the GenoType MTBDR*sl* version 1.0 (VER 1.0) (Hain LifeScience, Germany) cannot replace phenotypic DST, but it may be used as a rule-in test for XDR-TB (12). In a meta-analysis published in 2014, Theron and colleagues (5) reported respective pooled sensitivity and specificity indices of 83.1% (95% confidence interval [CI], 78.7 to 86.7%) and 97.7% (95% CI, 94.3 to 99.1%) for FLQs and 76.9% (95% CI, 61.1 to 87.6%) and 99.5% (95% CI, 97.1 to 99.9%) for SLID for culture isolates using the GenoType MTBDR*sl* VER 1.0 assay. They concluded that since the assay only targets selected mutations involving *gyrA* (FLQ) and *rrs* (SLID) gene loci, mutations encoding resistance to FLQ and SLID that occur outside these regions would be missed by the assay (5).

GenoType MTBDR*sl* VER 2.0 is redesigned based on GenoType MTBDR*sl* VER 1.0 and accommodates additional mutations for the molecular detection of resistance to FLQ involving *gyrA* and *gyrB* and SLID resistance covering both *rrs* and *eis* genes (13). The probes target commonly occurring mutations that encode resistance to these agents. The *gyrA* probes target codons 85 to 97 of the gene, and *rrs* probes target nucleic acid positions 1401 to 1484. The inclusion of additional targets for selected mutations in the *gyrB* region (codons 536 to 541) and *eis* promoter region (−10 to −14) for low-level KAN resistance are reported to improve the performance of the assay for the detection of FLQ and SLID resistance (13).

In order to prioritize and facilitate the identification of pre-XDR-TB (resistance to INH, RIF, and to any FLQ or SLID but not both) (8) and XDR-TB in South Africa's high-burden setting, the implementation of GenoType MTBDR*sl* VER 2.0 would be a fundamental improvement to case detection and management. An evaluation of this assay was undertaken with the objective of assessing diagnostic performance as well as to determine interoperator and interlaboratory performance of GenoType MTBDRsl VER

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TABLE 1 Summary of GenoType MTBDRsl VER 2.0 LPA and DST results

Drug (total no. of isolates)	Result [no. (%) of isolates] for:			
	MGIT SL-DST		MTBDRsl VER 2.0	
	Resistant	Susceptible	Resistant	Susceptible
FLQ (OFX) (267)	85 (31.8)	182 (68.2)	87 (32.6)	180 (67.4)
AG (KAN) (268)	65 (24.3)	203 (75.7)	60 (22.4)	208 (77.6)
AG[a] (AMK) (226)	32 (14.2)	194 (85.8)	33 (14.6)	193 (85.4)
CAP[a] (226)	29 (12.8)	197 (87.2)	33 (14.6)	193 (85.4)

[a]Excludes isolates from the NHLS Braamfontein TB Referral Laboratory.

2.0 in the detection of second-line drug resistance mutations in *M. tuberculosis* complex culture isolates compared to phenotypic DST.

RESULTS

A total of 268 *M. tuberculosis* complex isolates were included in the study, and the frequency of resistance to OFX, KAN, AMK, and CAP among these isolates was detected by the two methods (Table 1). Of these, 42 isolates from the NHLS Braamfontein Laboratory had phenotypic DST performed for OFX and KAN. As the DNA for the 42 isolates was not available, whole-genome sequencing (WGS) could not be performed. Resistance to one or more of the drugs under evaluation was observed in 120/268 (44.8%) of the *M. tuberculosis* isolates included in the study. All 27 probe bands of GenoType MTBDRsl VER 2.0 strips were interpretable in all samples tested with successful positive and negative quality controls (14). Phenotypically susceptible isolates were correctly classified as susceptible by the assay.

Detection of FLQ resistance. Sensitivity, specificity, and accuracy indices for FLQ (OFX) resistance using the GenoType MTBDRsl VER 2.0 assay were determined as 100%, 98.9%, and 99.3%, respectively, compared to the gold standard (Table 2). There were 85/120 (70.8%) isolates resistant to FLQ as tested by DST. GenoType MTBDRsl VER 2.0 detected 92 mutations in the *gyrA* and *gyrB* genes among the 85 FLQ (OFX)-resistant isolates. The distribution of mutations is summarized in Table 3. The majority of mutations, 52/92 (56.5%), were observed at codon 94. Other *gyrA* mutations detected by the assay were at codon 90 (29/92; 31.5%) and at codon 91 (9/92; 9.8%).

The diversity of single defined mutations at *gyrA* codon 94 included the following (Table 3): *gyrA* MUT3C (D94G), 23/52 (44.2%); *gyrA* MUT3A (D94A), 7/52 (13.5%); *gyrA* MUT3D (D94H), 6/52 (11.5%); and *gyrA* MUT3B (D94N/D94Y), 5/52 (9.6%). In 3/52 (5.8%) isolates, both *gyrA* mutations MUT3B and MUT3D (D94N/D94Y and D94H) were detected. Other *gyrA* defined mutations detected at codons 90 and 91 were the following: *gyrA* MUT1 (A90V), 25/29 (86.2%); and *gyrA* MUT2 (S91P), 7/9 (77.8%) (Table 3).

Heteroresistance in *gyrA* was observed in 14/85 (16.5%) OFX-resistant isolates. Eight isolates among these exhibited two mutation bands involving codons 94, 91, and 90. Results for these isolates were the following: 4/85 (4.7%) wild-type (WT)/*gyrA* MUT1 and MUT3B (A90V and D94N/D94Y), 2/85 (2.4%) WT/*gyrA* MUT2 and MUT3B (S91P and D94N/D94Y), and 2/85 (2.4%) WT/*gyrA* MUT3B and MUT3C, respectively (Table 3).

There were 8/85 (9.4%) isolates with undefined mutations that were interpreted as resistant. The following wild-type bands were not detected among these isolates: *gyrA*

TABLE 2 Performance of GenoType MTBDRsl VER 2.0 assay

Drug ($n = 268$)	% Sensitivity (95% CI)	% Specificity (95% CI)	% PPV (95% CI)	% NPV (95% CI)	Diagnostic efficacy (%)
FQ (OFX) ($n = 267$)	100.0 (95.8–100)	98.9 (96.1–99.9)	97.7 (91.9–99.7)	100.0 (98.0–100.0)	99.3
SLID (AG/CAP)	89.2 (79.1–95.6)	98.5 (95.7–99.7)	95.1 (86.3–99.0)	96.6 (93.2–98.6)	96.3
AMK[a] ($n = 226$)	93.8 (79.2–99.2)	98.5 (95.5–99.7)	90.9 (75.7–98.1)	99.0 (96.3–99.9)	97.8
KAN ($n = 268$)	89.2 (79.1–95.6)	98.5 (95.7–99.7)	95.1 (86.3–99.0)	96.6 (93.2–98.6)	96.3
CAP[a] ($n = 226$)	86.2 (68.3–96.1)	95.9 (92.2–98.2)	75.8 (57.7–88.9)	97.9 (94.8–99.4)	94.7

[a]Excludes isolates from NHLS Braamfontein TB Referral Laboratory.

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TABLE 3 Hybridization patterns observed in strains classified as resistant by LPA using GenoType MTBDR*sl* VER 2.0 assay[a]

Phenotypic resistance type detected and hybridization band(s) observed	Mutation(s) detected	No. (%) of mutations[b]
FLQ		
gyrA MUT1	A90V	25 (27.2)
WT/*gyrA* MUT1 and MUT3B	A90V, D94N/D94Y	4 (4.3)
gyrA MUT2	S91P	6 (6.5)
WT/*gyrA* MUT2	S91P	1 (1.1)
WT/*gyrA* MUT2 and MUT3B	S91P, D94N/D94Y	2 (2.2)
gyrA WT2 absent	None	4 (4.3)
gyrA WT3 absent	None	1 (1.1)
gyrA MUT3A	D94A	6 (6.5)
WT/*gyrA* MUT3A	D94A	1 (1.1)
gyrA MUT3B	D94N/D94Y	3 (3.3)
WT/*gyrA* MUT3B	D94N/D94Y	2 (2.2)
WT/*gyrA* MUT3B and MUT3C	D94N/D94Y, D94G	2 (2.2)
gyrA MUT3B and MUT3D	D94N/D94Y, D94H	3 (3.3)
gyrA MUT3C	D94G	21 (22.8)
WT/*gyrA* MUT3C	D94G	2 (2.2)
gyrA MUT3D	D94H	6 (6.5)
gyrB MUT1	N538D	0 (0.0)
gyrB MUT2	E540V	0 (0.0)
gyrB WT1 absent	None	3 (3.3)
CAP, AMK, KAN		
rrs MUT1	A1401G	44 (71.0)
WT/*rrs* MUT1	A1401G	4 (6.5)
rrs WT1 absent	None	2 (3.2)
CAP, VIO,[c] AMK, KAN		
rrs MUT2	G1484T	0 (0.0)
Low-level KAN		
eis MUT1	C-14T	8 (12.9)
eis WT2 absent	None	4 (6.5)

[a]For FLQ, $n = 85$; for SLID, $n = 65$.
[b]FLQ mutations, $n = 92$; SLID mutations, $n = 62$.
[c]VIO, viomycin.

WT2, 4/85 (4.7%); *gyrA* WT3, 1/85 (1.1%); and *gyrB* WT1, 3/85 (3.3%). WGS performed on 6/8 available isolates confirmed defined mutations at *gyrA* (A90V, S91P, and D94Y) missed by the assay and *gyrB* mutations at codons 274 and 499 that are not covered by the assay (Table 4). All 8 of these isolates were phenotypically resistant to OFX. An isolate that harbored *gyrA* MUT3C (D94G) mutation confirmed by WGS with an A94G mutation was phenotypically susceptible to OFX. In the selection of isolates tested, no defined mutations were detected by the assay in the *gyrB* region (i.e., mutations N538D and E540V).

Detection of SLID resistance. The sensitivity, specificity, and accuracy of GenoType MTBDR*sl* VER 2.0 for SLID resistance was 89.2%, 98.5%, and 96.3%, respectively, compared with phenotypic DST. The respective individual SLID sensitivity and specificity assessments were the following: 93.8% and 98.5% for AMK, 89.2% and 98.5% for KAN, and 86.2% and 95.9% for CAP (Table 2).

GenoType MTBDR*sl* VER 2.0 detected 56 defined mutations and 6 undefined mutations in either the *rrs* or *eis* genes among the 65/120 (54.2%) isolates phenotypically resistant to SLID (Table 3). The most frequently observed mutation (48/56; 85.7%) for SLID resistance was *rrs* MUT1 (A1401G), with four of these isolates displaying heteroresistance. All 48 isolates were confirmed as phenotypically resistant to a SLID (AMK, KAN, or CAP). Two isolates had *rrs* WT1 bands absent with no corresponding mutation band observed and 1/2 tested was wild type by WGS, with both being phenotypically susceptible to SLID. Similarly, there were 4/65 (6.2%) isolates that were interpreted as

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TABLE 4 WGS[a] results for MTBDRsl VER 2.0 isolates with undefined mutations and discordant DST

MGIT DST result for[b]:				MTBDR*sl* VER 2.0		MTBDR*sl*		MTBDR*sl* VER 2.0	WGS	MTBDR*sl*	
OFX	KAN	AMK	CAP	*gyrA*	WGS *gyrA*	VER 2.0 *gyrB*	WGS *gyrB*	*rrs*	*rrs* (nt)	VER 2.0 *eis*	WGS *eis* (nt)
R	S	S	S	Missing WT 3	Asp94Tyr	WT	WT	WT	WT	WT	WT
R	S	S	S	Missing WT 2	Ala90Val Ser91Pro	WT	WT	WT	WT	WT	WT
R	S	S	S	Missing WT 2	Ala90Val Ser91Pro	WT	WT	WT	WT	WT	WT
R	S	S	S	Missing WT 2	Ala90Val Ser91Pro	WT	WT	WT	WT	WT	WT
R	S	S	S	WT	WT	Missing WT 1	Ser274Arg Asn499Ser	WT	WT	WT	WT
R	S	S	S	WT	WT	Missing WT 1	Ser274Arg Asn499Ser	WT	WT	WT	WT
S	R	R	S	WT	Gly247Ser	WT	WT	WT	WT	Missing WT 2	WT
S	R	S	S	WT	Gly247Ser	WT	WT	WT	T517C	Missing WT 2	WT
S	R	R	S	WT	WT	WT	WT	WT	WT	Missing WT 2	C-10T/G-10A
S	S	S	S	WT	WT	WT	WT	Missing WT 1	WT	WT	WT
S	R	R	S	WT	WT	WT	WT	Rrs, MUT1/A1404G	G1401A	WT	WT
S	R	R	S	WT	WT	WT	WT	Rrs, MUT1/A1404G	G1401A	WT	WT
S	R	S	S	*gyrA*, MUT 3C/D94G	Asp94Gly	WT	WT	WT	WT	WT	WT
R	R	S	S	*gyrA*, MUT 3C/D94G	Asp94Gly	WT	Val457Leu	WT	WT	WT	WT
R	R	R	S	*gyrA*, MUT2/S91P	Ser91Pro	WT	Val457Leu	WT	WT	WT	WT

[a]DNA available for 10/14 isolates with undefined mutations and 9/20 discordant isolates.
[b]R, resistant; S, susceptible.

TABLE 5 MGIT DST and GenoType MTBDR*sl* VER 2.0 analysis of XDR isolates identified ($n = 31$)

MGIT DST result for:		MTBDR*sl* VER 2.0 result for:			Frequency
FLQ	SLID	FLQ-*gyrA/gyrB*	SLID-*rrs*	SLID-*eis*	[no. (%)]
R	R	*gyrA* MUT1/A90V	*rrs* MUT1/A1404G	WT	5 (16.1)
R	R	*gyrA* MUT1/A90V	*rrs* MUT1/A1404G	*eis* MUT1/C-14T	1 (3.2)
R	R	*gyrA* MUT1/A90V	WT	*eis* MUT1/C-14T	5 (16.1)
R	R	*gyrA* MUT1/A90V	WT[a]	WT[a]	1 (3.2)
R	R	*gyrA* MUT2/S91P	WT[b]	WT[b]	1 (3.2)
R	R	*gyrA* MUT3A/D94A	*rrs* MUT1/A1404G	WT	2 (6.5)
R	R	*gyrA* MUT3B/D94N/D94Y	WT[b]	WT[b]	1 (3.2)
R	R	*gyrA* MUT3B/D94N/D94Y	WT	*eis* MUT1/C-14T	1 (3.2)
R	R	*gyrA* MUT 3C/D94G	*rrs* MUT1/A1404G	WT	8 (25.8)
R	R	*gyrA* MUT 3C/D94G	WT[a]	WT[a]	1 (3.2)
R	R	*gyrA* MUT3D/D94H	*rrs* MUT1/A1404G	WT	4 (12.9)
R	R	*gyrA* MUT1/A90V *gyr*B WT absent	WT	*eis* MUT1/C-14T	1 (3.2)

[a]WGS not available.
[b]WGS result: WT.

resistant (absence of *eis* WT2 and no mutation band observed), 3/4 were phenotypically resistant to KAN, and 1/4 was phenotypically susceptible to KAN. WGS tested 2/4 isolates as wild type, with a C-10T/G-10A mutation observed in 1/4 isolates. The *eis* MUT1 (C-14T) mutation was observed in 8/65 (12.3%) of the SLID-resistant isolates and confirmed phenotypically as KAN resistant. In 6/65 (9.2%) isolates phenotypically resistant to KAN, no *rrs* or *eis* mutation was observed. These were interpreted as susceptible to SLID by the assay. DNA available for 3/6 of these isolates tested wild type by WGS (Table 4). Among five isolates (5/65; 7,7%) that were phenotypically susceptible to CAP, 2/5 had the *rrs* MUT1 (A1401G) mutation and 3/5 had *eis* missing WT2, and they were interpreted as resistant to SLID by the assay. WGS performed on these isolates revealed no resistance-conferring mutations for *gidB* and *tlyA* regions. The mutation *rrs* MUT2 (G1484T) was not observed among any of the isolates tested.

Detection of XDR-TB. Among the isolates tested, 31/120 (25.8%) were XDR by phenotypic DST (Table 5). Agreement between GenoType MTBDR*sl* VER 2.0 and phenotypic DST was calculated as 97.0% for the detection of XDR-TB. GenoType MTBDR*sl* VER 2.0 correctly identified 27/31 (87.1%) isolates, missing 4/31 (12.9%) isolates, all of which were phenotypically SLID (KAN) resistant but sensitive by the assay. WGS was performed for 2/4 isolates missed by the assay, and they were wild type for *rrs* and *eis*; however, both isolates were phenotypically resistant to KAN (SLID). All isolates with a defined mutation showed phenotypic resistance.

Reproducibility. Among the 5 laboratories that tested the panel of 30 isolates, the overall performance ranged between 93% and 96% (Table 6). The interoperator assessment showed 100% agreement.

DISCUSSION

To our knowledge, the evaluation of GenoType MTBDR*sl* VER 2.0 in conjunction with a reproducibility assessment is the first such study conducted in a high TB-HIV setting in Africa. GenoType MTBDR*sl* VER 2.0 has shown an improvement in sensitivity and specificity for the determination of molecular resistance to both FLQ (100% [95% CI, 95.8 to 100%] and 98.9% [95% CI, 96.1 to 99.9%]) and SLID (89.2% [95% CI 79.1 to 95.6%] and 98.5% [95% CI 95.7–99.7%]) compared with the pooled sensitivity and specificity of GenoType MTBDR*sl* VER 1.0 reported by the WHO Expert Group (12) and a meta-analysis by Theron and colleagues (5). Agreement between the phenotypic gold standard and GenoType MTBDR*sl* VER 2.0 for FLQ (OFX) and SLID was 99.3% and 96.3%, respectively, across a wide distribution of mutations. The assay was found to be highly

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TABLE 6 Reproducibility assessment of participant laboratories for GenoType MTBDR*sl* VER 2.0

Strain no. (3 isolates each)	Result [no. positive/total no. (%)] for laboratory:				
	A	B	C	D	E
1	3/3 (100)	3/3 (100)	3/3 (100)	3/3 (100)	3/3 (100)
2	3/3 (100)	3/3 (100)	3/3 (100)	3/3 (100)	3/3 (100)
3	3/3 (100)	3/3 (100)	3/3 (100)	3/3 (100)	3/3 (100)
4	3/3 (100)	3/3 (100)	3/3 (100)	3/3 (100)	3/3 (100)
5	3/3 (100)	3/3 (100)	3/3 (100)	3/3 (100)	3/3 (100)
6	No consensus; excluded	No consensus; excluded	No consensus; excluded	No consensus; excluded	No consensus; excluded
7	3/3 (100)	3/3 (100)	3/3 (100)	3/3 (100)	3/3 (100)
8	2/3 (67)	3/3 (100)	3/3 (100)	3/3 (100)	3/3 (100)
9	3/3 (100)	2/3 (67)	3/3 (100)	3/3 (100)	3/3 (100)
10	3/3 (100)	2/3 (67)	1/3 (33)	2/3 (67)	2/3 (67)
Overall score	26/27 (96)	25/27 (93)	25/27 (93)	26/27 (96)	26/27 (96)

reproducible in terms of interlaboratory (93 to 96%) and interoperator (100%) assessment.

FLQ resistance in *M. tuberculosis* is ascribed mainly to *gyrA* mutations, with 57.5% of mutations detected at codon 94 and 31.5% at codon 90 (15). Consistent with published data, the highest frequency of mutations conferring FLQ resistance was observed in *gyrA* codon 94, followed by codon 90 (Table 3). Furthermore, *gyrA* mutations D94G and D94N were observed in the majority of the isolates tested. Previous studies have indicated that isolates harboring these mutations exhibit high levels of resistance to FLQ (15, 16). The *gyrA* MUT3D/D94H mutation, reported as a rare *in silico* mutant (13, 17), was observed in 9/85 (10.6%) of mutations identified from these clinical isolates. Heteroresistance involving either FLQ or SLID was detected in 18/85 (21.2%) of isolates, with multiple mutation bands observed in some isolates (Table 3), and were confirmed as phenotypically resistant. Cohen et al. reported on mixed-strain infections in a hospital setting in KwaZulu-Natal, South Africa (18). Further investigation is required to elucidate the frequency and clinical relevance of mixed infections across a more widespread South African setting. Based on our findings, interpretation of these strains as resistant was consistent with the phenotypic DST results.

Undefined mutations with the absence of *gyrA* wild-type bands were confirmed as phenotypically resistant. WGS results available for four isolates confirmed the presence of *gyrA* mutations that the assay missed. The GenoType MTBDR*sl* VER 2.0 guideline for interpretation of results states that "only bands with intensities as strong as or stronger than the amplification control zone (AC) are to be considered" (13). Three out of four of these discordant isolates had *gyrA* MUT2/S91P bands with intensities less than that of the AC zone and therefore were interpreted as negative and reported as resistant due to the absence of *gyrA* wild-type bands. No weak-intensity mutation bands were detected in the fourth isolate. An isolate phenotypically susceptible to OFX with *gyrA* MUT 3C/D94G bands and a confirmed Asp94Gly mutation on WGS had *gyrA* WT bands with less intensity than the AC control band. The discrepant OFX phenotypic DST is possibly due to a mixed population. The inclusion of selected *gyrB* probes offered limited enhancement to the identification of FLQ mutations, as only the absence of *gyrB* WT was observed in 3/268 (1.1%) isolates. GenoType MTBDR*sl* VER 2.0 is limited to detecting mutations in selected areas of the quinolone resistance-determining regions (QRDR) of *gyrA* and *gyrB* only; therefore, FLQ resistance mechanisms outside these regions are likely to be missed but do not appear to be a major concern in our setting presently.

Consistent with published data (17), *rrs* MUT1 A1401G (translating to high-level SLID resistance) was the most frequently observed mutation (77.5%) among tested isolates. The overall SLID sensitivity has improved in the new assay to 89.2% compared with previously published data (5, 12). The inclusion of *eis* promoter region probes improved the detection of SLID resistance, as observed in 8/62 (12.9%) low-level KAN-resistant isolates, all phenotypically KAN resistant and not associated with *rrs* mutations. Two

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isolates, with *rrs* WT1 bands absent, no corresponding mutation band observed, wild type by WGS, and interpreted as resistant to SLID by the assay, were phenotypically susceptible to SLID. The omission of WT bands in the line probe assay is not a reliable indication of phenotypic resistance and would require confirmatory phenotypic DST (19). In 6 phenotypically KAN-resistant isolates that were AMK susceptible, no mutations were detected by the assay or WGS. Although other factors related to phenotypic resistance cannot be excluded, the discrepant results may be due to the inherent challenges of in-house (nonstandardized) second-line drug preparations available for testing (19). Five isolates that were phenotypically susceptible to CAP, interpreted as SLID resistant by the assay and wild type by WGS, displayed resistance only to KAN and AMK phenotypically. As indicated by Georghiou et al. (20), *rrs* MUT1 A1401G mutation is a moderate predictor of CAP resistance. Therefore, CAP resistance should be confirmed phenotypically prior to exclusion of the drug from a treatment regimen (21).

There were 31 isolates identified as phenotypically XDR, and the GenoType MTBDR*sl* VER 2.0 assay achieved 97.0% agreement between the index and gold standard tests. Of the four XDR cases misclassified by the assay, all showed FLQ resistance but missed SLID resistance. All were phenotypically KAN resistant. One isolate was classified as XDR by LPA with *gyrA* MUT1 (A90V) and a missing *eis* WT2 only (C-12T/G-10A) but was phenotypically susceptible to KAN. We used strict criteria, and resistance to any one SLID was accepted as a criterion to classify a strain as XDR. Due to limited availability of standardized drug preparations for second-line DST, in routine practice, laboratories would normally only test one drug out of this class for resistance determination. This is a limitation of the data available for this sample set, as mutations in the assay are known to correlate well with OFX resistance and is evident in our observations. As reported, correlation of these mutations with higher FLQs is uncertain and may still show phenotypic susceptibility despite the presence of these mutations; thus, phenotypic testing remains important for these drugs (22, 23).

The reproducibility assessment of selected routine laboratories showed excellent performance consistent with other data on molecular diagnostics (Table 6) (24). GenoType MTBDR*sl* VER 2.0 LPA performed well with excellent reproducibility and high sensitivity and specificity for FLQ and SLID resistance determination. The average reporting turnaround times for GenoType MTBDR*sl* VER 2.0 LPA varied from 2 to 4 days subsequent to the identification of a positive TB culture, whereas second-line phenotypic DST results were only available in 14 to 21 days. The longer TAT for DST results is largely dependent on the viability/fitness of the isolate, especially for XDR isolates. It also highlights the importance of direct testing in providing clinically relevant and reliable results.

Furthermore, adoption of the assay in laboratories already performing the GenoType assays was relatively easy and quick to implement. GenoType MTBDR*sl* VER 2.0 LPA is therefore suitable for testing clinical isolates as a rapid screening tool for detection of second-line drug resistance in countries with a high burden of MDR-TB.

A limitation of the study was that only culture isolates were used and not clinical specimens, as this study was performed to compare directly the GenoType MTBDR*sl* VER 2.0 assay with the gold standard, phenotypic DST. However, there is a need for further studies evaluating the GenoType MTBDR*sl* VER 2.0 assay on clinical specimens from patients with MDR-TB, thereby enhancing the turnaround time for resistance reporting and pre-XDR/XDR case detection in high-risk settings. Another limitation was that of the FLQs, only OFX was tested and none of the other later-generation FLQs (isolates collected between 2012 and 2014). This probably explains the higher sensitivity found in our study compared with other studies (25). Phenotypic DST for later-generation FLQs (moxifloxacin) was introduced in 2015 per WHO recommendations (22, 23). A general limitation of second-line drug testing is the limited availability of standardized drug preparations (19). A further limitation was that samples from the Braamfontein TB Referral Laboratory could not be included for WGS resolution testing, as the DNA was not available.

In a press release by the WHO in May 2016, the use of the GenoType MTBDR*sl* VER

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2.0 assay as "an initial test, instead of phenotypic culture-based DST" to detect FLQ and SLID resistance in confirmed RIF-R and MDR patients was recommended (26, 27). Appropriately trained laboratory staff, quality assurance, and availability of laboratory infrastructure are requisite recommendations of the WHO to implement use of this assay (26, 27). Our study provides support and evidence for these recommendations and the implementation of the assay in South Africa.

MATERIALS AND METHODS

Setting and study design. The evaluation of the GenoType MTBDR*sl* VER 2.0 assay was conducted at the Centre for Tuberculosis, National Institute for Communicable Diseases (CTB, NICD), Johannesburg, South Africa. The laboratory is a designated WHO Supranational Laboratory (SRL) accredited to ISO 15189:2012 (14). The Human Research Ethics Committee of the University of Witwatersrand, Johannesburg, South Africa, approved the study (M150752). The study was structured with two interrelated components involving the validation of characterized *M. tuberculosis* isolates and a reproducibility assessment using a subset of these isolates tested by selected National Health Laboratory Service (NHLS) laboratories in South Africa, performing diagnostic tests involving culture of *M. tuberculosis*.

Mycobacterial isolates. A total of 268 repository isolates collected between 2012 and 2014 were tested. These were comprised of 92 phenotypically well-characterized *M. tuberculosis* complex isolates using whole-genome sequencing (WGS) and 176 anonymized *M. tuberculosis* clinical isolates exhibiting rifampin monoresistance (RIF-R) or MDR based on phenotypic DST, tested at the CTB or NHLS TB Referral Laboratory, Braamfontein, Johannesburg, South Africa. Phenotypic testing for later generations of FLQ and SLID was introduced in 2015 (2). These isolates originated from Gauteng, Limpopo, Northern Cape, North West, KwaZulu-Natal, and Mpumalanga provinces, collected during surveillance activities for rifampin resistance in South Africa. The fully susceptible ATCC *M. tuberculosis* H37Rv 27294 reference strain (http://www.atcc.org/Products/All/27294.aspx) was used as the DST and LPA positive quality control culture.

Phenotypic DST. Bactec MGIT 960 DST using EpiCenter software (Becton, Dickinson and Company Diagnostic Systems, Sparks, MD, USA) for interpretation of results was performed according to the manufacturer's recommendations (28) and considered the gold standard for resistance determination. The following critical concentrations of drugs recommended by WHO for testing of drug-resistant TB using Bactec MGIT 960 DST were used: ofloxacin (OFX), 2.0 μg/ml; AMK, 1.0 μg/ml; KAN, 2.5 μg/ml; and CAP, 2.5 μg/ml (22).

GenoType MTBDR*sl* VER 2.0. The GenoType MTBDR*sl* VER 2.0 assay was performed according to the manufacturer's instructions (13). Each strip contains 27 reaction zones with probes for all specific targeted regions. Seven probes for *gyrA* (A90V, S91P, D94A, D94N/Y, D94G, and D94H) and 2 probes for *gyrB* (N538D and E540V) were used to detect FLQ resistance. SLID resistance was detected by selected *rrs* (A1401G, C1402T, and G1484T) and *eis* (C-14T and C-12T) probes. The presence of all wild-type bands and absence of mutation bands indicated susceptibility. The development of specific mutation bands (defined mutation) or the absence of wild-type bands (undefined mutation) (29) related to a specific gene on the hybridization strip was interpreted as resistance to the respective drug (13). Heteroresistance was demonstrated when both wild-type and/or mutation band(s) were present and was recorded as resistant for interpretation purposes. SLID resistance referred to resistance to at least one of the 3 injectable drugs (AMK, KAN, and CAP) (5).

Discordance resolution by whole-genome sequencing. WGS was used to resolve discordance between phenotypic and genotypic methods. WGS was performed using the MiSeq platform (Illumina, San Diego, CA). Library preparation was performed using the Illumina Nextera XT library preparation kit and sequencing reaction using the MiSeq version 3 cartridge (2 $\times$ 300 bp). Variant detection was performed using the resequencing module with the reference strain *M. tuberculosis* H37Rv (NC_000962) on CLC Genomics Workbench (v7.5.1).

Reproducibility. In order to evaluate the interlaboratory performance of the assay, a panel of 10 *M. tuberculosis* complex external quality assurance (EQA) isolates, constituting a subset of the isolates evaluated in the present study comprised of 10 isolates present in triplicate, was distributed to 5 NHLS laboratories performing diagnostic culture of *M. tuberculosis* as well as LPA (30). An overall agreement of $\geq$90% was deemed an acceptable score. Interoperator reproducibility was performed by comparing findings on 45 EQA isolates tested by two operators at CTB, working independently.

Statistical analysis. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV), based on agreement between the DST gold standard and the index assay and 95% confidence intervals (95% CIs), were calculated for both the antibiotic class and individual drugs. Agreement between the gold standard and index assay was calculated using the McNemar test. Statistical analyses were performed using Stata release 13 (31).

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