Evaluation of the Genotype® MTBDR*sl*® VER 2.0 assay for second-line drug resistance detection of *Mycobacterium tuberculosis* isolates in South Africa

EVALUATION OF MTBDR*sl* VER 2.0: SOUTH AFRICAN STUDY

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ABSTRACT

Early detection of resistance to second-line anti-tuberculosis drugs is important for the management of multidrug-resistant (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) (*gyr*A and *gyr*B genes) and second-line injectable drugs (SLID) (*rrs* and *eis* genes). The study evaluated the diagnostic performance of MTBDR*sl* VER 2.0 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, with rifampicin -mono-resistant (RR) or MDR based on DST were selected. MTBDR*sl* VER 2.0 testing was performed on these isolates and results analysed. The MTBDR*sl* VER 2.0 sensitivity and specificity indices for culture isolates were; FLQ 100% (95% CI, 95.8-100%) ; 98.9% (95% CI, 96.1–99.9%) and SLID 89.2% (95% CI, 79.1-95.6%); 98.5% (95% CI, 95.7–99.7%). The sensitivity and specificity observed for individual SLID were: amikacin 93.8% (95% CI, 79.2–99.2%); 98.5% (95% CI, 95.5–99.7%), kanamycin 89.2% (95% CI, 79.1–95.6%); 98.5% (95% CI, 95.5–99.7%) and capreomycin 86.2% (95% CI, 68.3–96.1%) ; 95.9% (95% CI, 92.2–98.2%). An inter-operator reproducibility of 100% and an overall inter-laboratory performance of 93 % - 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.

KEY WORDS: Genotype® MTBDR*sl* VERSION 2.0; Drug Resistant (DR)-TB; MDR-TB; XDR-TB, line probe assay, LPA, fluoroquinolones, second-line injectable drugs, molecular diagnostic testing, phenotypic drug susceptibility testing

INTRODUCTION

The World Health Organization (WHO) Global Tuberculosis Report 2015 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 rifampicin (RIF). South Africa is listed amongst the 14 countries that appear in all three WHO 2016 -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 fluoroquinolone (FLQ) and at least one second-line injectable drug (SLID) amongst the aminoglycoside drugs (AG) amikacin (AMK), 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 co-epidemic has fuelled 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 labour intensive, time consuming and generally takes between 2 - 3 weeks' 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 hours) for resistance reporting when compared to conventional DST, and in the process alerting clinicians to the emergence of drug resistance in *M. tuberculosis* strains from individual patients. Early detection of drug resistance is crucial to prevent 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* 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% CI, 78.7 - 86.7%) and 97.7% (95% CI 94.3% to 99.1%) for FLQs and 76.9% (95% CI, 61.1 - 87.6%) and 99.5% (95% CI, 97.1 - 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 *gyr*A (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 MTBDR*sl* VER 1.0 and accommodates additional mutations for the molecular detection of resistance to FLQ involving *gyr*A and *gyr*B and SLID resistance covering both *rrs* and *eis* genes (13). The probes target commonly occurring mutations that encode resistance to these agents. The *gyr*A 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 *gyr*B 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 prioritise and facilitate the identification of pre-XDR-TB (resistance to INH, RIF and either resistance 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 inter-operator and inter-laboratory performance of Genotype® MTBDRsl VER 2.0 in the detection of second line drug resistance mutations in *M. tuberculosis* complex culture isolates compared to phenotypic DST.

MATERIALS AND METHODS

Setting and study design. The evaluation of the Genotype® MTBDR*sl* VER 2.0 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 characterised *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 comprised of 92 phenotypically well-characterized *M. tuberculosis* complex isolates using whole genome sequencing (WGS) and 176 anonymized *M. tuberculosis* clinical isolates exhibiting rifampicin mono-resistance (RIF-R) or MDR based on phenotypic DST, tested at CTB or NHLS TB Referral Laboratory, Braamfontein, Johannesburg. Phenotypic testing for higher 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 rifampicin resistance in South Africa. The fully susceptible ATCC® *M. tuberculosis,* H37Rv 27294 reference strain (15) 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 manufacturer's recommendations (16) and considered as 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 (17).

Genotype® MTBDR*sl* VER 2.0. The Genotype® MTBDR*sl* VER 2.0 assay was performed according to manufacturer's instructions (13). Each strip contains 27 reaction zones with probes for all specific targeted regions. Seven probes for *gyr*A (A90V, S91P, D94A, D94N/Y, D94G, and D94H) and 2 probes for *gyr*B (N538D, E540V) are used to detect FLQ resistance. SLID resistance is 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) (18) 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 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, USA). Library preparation was performed using the Illumina Nextera XT® library preparation kit and sequencing reaction using the MiSeq® version 3 cartridge (2 x 300bp). 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 inter-laboratory performance of the assay, a panel of 10 *M. tuberculosis* complex EQA isolates constituting a subset of the isolates evaluated in the present study, comprising 10 isolates present in triplicate was distributed to 5 NHLS laboratories

performing diagnostic culture of *M. tuberculosis* as well as LPA (19). An overall agreement of ≥ 90% was deemed an acceptable score. Inter-operator 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), 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 (20).

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 as detected by the two methods feature in 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, 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® MTBDR*sl* 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® MTBDR*sl* VER 2.0 were determined as 100%, 98.9% and 99.3% respectively, when compared to the gold standard (Table 2). There were 85/120 (70.8%) isolates resistant to FLQ as tested by DST. Genotype® MTBDR*sl* VER 2.0 detected 92 mutations in the *gyr*A and *gyr*B genes among the 85 FLQ (OFX) resistant isolates. The distribution of mutations is summarised in Table 3. The majority of mutations, 52/92 (56.5%) was observed at codon 94. Other *gyr*A 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 *gyr*A codon 94 included the following (see Table 3): *gyr*A MUT3C (D94G) 23/52 (44.2 %), *gyr*A MUT3A (D94A) 7/52 (13.5%), *gyr*A MUT3D (D94H) 6/52 (11.5%) and *gyr*A MUT3B (D94N / D94Y) 5/52 (9.6%). In 3/52 (5.8%) isolates, both *gyr*A mutations MUT3B and MUT3D (D94N / D94Y & D94H) were detected. Other *gyr*A defined mutations detected at codons 90 and 91 were: *gyr*A MUT1 (A90V) 25/29 (86.2%) and *gyr*A MUT2 (S91P) 7/9 (77.8%) (See Table 3).

Table 1: Summary of MTBDRsl VER 2.0 LPA and DST Results

Genotype® MTBDRsl VER 2.0 Assay and Phenotypic DST Results				
DRUG TYPE (n=number of isolates)	**MGIT SL-DST**		**MTBDRsl VER 2.0**	
	RESISTANT	**SUSCEPTIBLE**	**RESISTANT**	**SUSCEPTIBLE**
FLQ (OFX) (n=267)	85(31.8%)	182 (68.2%)	87 (32.6%)	180 (67.4%)
AG (KAN) (n=268)	65 (24.3%)	203 (75.7%)	60 (22.4%)	208(77.6%)
AG (AMK) (n=226)*	32(14.2%)	194(85.8%)	33 (14.6%)	193 (85.4%)
CAP (n=226)*	29 (12.8%)	197 (87.2%)	33(14.6%)	193 (85.4%)

* Excludes isolates from NHLS Braamfontein TB Referral Laboratory

Table 2: Performance of Genotype® MTBDRsl V2 Assay

Pooled Result (n=268)	% SENS	95% CI	% SPEC	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/CP)	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
AMI (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
KANA (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 (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

* Excludes isolates from NHLS Braamfontein TB Referral Laboratory

Table 3: Genotype® MTBDRsl VER 2.0 Assay - Hybridisation patterns observed in LPA resistant strains (FLQ n = 85; SLID n = 65)

PHENOTYPIC RESISTANCE DETECTED	Genotype® MTBDRsl V2 Assay Results: HYBRIDIZATION BAND(S) OBSERVED	MUTATION(S) DETECTED	No. of mutations n*	%
FLQ	*gyr*A MUT1	A90V	25	27.2
	WT/*gyr*A MUT1 & MUT3B	A90V & D94N/D94Y	4	4.3
	*gyr*A MUT2	S91P	6	6.5
	WT/*gyr*A MUT2	S91P	1	1.1
	WT/*gyr*A MUT2 & MUT3B	S91P & D94N/D94Y	2	2.2
	*gyr*A WT2 Absent	None	4	4.3
	*gyr*A WT3 Absent	None	1	1.1
	*gyr*A MUT3A	D94A	6	6.5
	WT/*gyr*A MUT3A	D94A	1	1.1
	*gyr*A MUT3B	D94N / D94Y	3	3.3
	WT/*gyr*A MUT3B	D94N / D94Y	2	2.2
	WT/*gyr*A MUT3B & MUT3C	D94N / D94Y & D94G	2	2.2
	*gyr*A MUT3B & MUT3D	D94N / D94Y & D94H	3	3.3
	*gyr*A MUT3C	D94G	21	22.8
	WT/*gyr*A MUT3C	D94G	2	2.2
	*gyr*A MUT3D	D94H	6	6.5
	*gyr*B MUT1	N538D	0	0.0
	*gyr*B MUT2	E540V	0	0.0
	*gyr*B 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 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

*FLQ mutations (n=92)
*SLID mutations (n=62)

Heteroresistance in *gyr*A was observed in 14/85 (16.5%) OFX resistant isolates. Eight isolates amongst these exhibited two mutation bands involving codons 94, 91 and 90. These were as follows: 4/85 (4.7%) WT/*gyr*A MUT1 and MUT3B (A90V and D94N/D94Y); 2/85 (2.4%) WT/*gyr*A MUT2 and MUT3B (S91P and D94N/D94Y); and 2/85 (2.4%) WT/*gyr*A MUT3B and MUT3C respectively (See 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 amongst these isolates; *gyr*A WT2 4/85 (4.7%), *gyr*A WT3 1/85 (1.1%) and *gyr*B WT1 3/85 (3.3%). WGS performed on 6/8 available isolates confirmed defined mutations at *gyr*A (A90V, S91P and D94Y) missed by the assay, and *gyr*B 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 harboured *gyr*A 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 *gyr*B 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, when compared with phenotypic DST. Individual SLID sensitivity and specificity assessments were respectively: 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 amongst 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 the *rrs* MUT1 (A1401G) with four of these isolates displaying heteroresistance. All 48 isolates were

Table 4. WGS* results for MTBDRsl VER 2.0 Isolates with Undefined Mutations and Discordant DST

MGIT DST (OFX)	MGIT DST (KAN)	MGIT DST (AMK)	MGIT DST (CAP)	MTBDRsl VER 2.0 *gyrA*	WGS *gyrA*	MTBDRsl VER 2.0 *gyrB*	WGS *gyrB*	MTBDRsl VER 2.0 *rrs*	WGS *rrs* (nt)**	MTBDRsl 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

*DNA available for 10/14 isolates with Undefined mutations & 9/20 Discordant isolates

**Nucleotide

confirmed as phenotypically resistant to a SLID (AMK, KAN or CAP). Two isolates had *rrs* WT1 bands absent with no corresponding mutation band observed, 1/2 tested wild type by WGS, both being phenotypically susceptible to SLID. Similarly, there were 4/65 (6.2%) isolates that were interpreted as resistant (absence of *eis* WT2 and no mutation band observed), 3/4 were phenotypically resistant to KAN while 1/4 was phenotypically susceptible to KAN. WGS tested 2/4 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* mutations were 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). Amongst five isolates (5/65; 7,7%) that were phenotypically susceptible to CAP, 2/5 had the *rrs* MUT1 (A1401G) mutation and 3/5 with *eis* missing WT2, and interpreted as resistant to SLID by the assay. WGS performed on these isolates revealed no resistance conferring mutations for *gid*B and *tly*A regions. The mutation, *rrs* MUT2 (G1484T) was not observed amongst any of the isolates tested.

Detection of XDR-TB. Amongst 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%), missing 4/31 (12.9%) isolates, all of which were phenotypically SLID (KAN) resistant but sensitive by the assay. WGS performed for 2/4 missed by the assay, 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. Amongst the 5 laboratories that tested the panel of 30 isolates, the overall performance ranged between 93% – 96 % (Table 6). The inter-operator assessment showed 100% agreement.

Table 5: MGIT DST and Genotype® MTBDRsl VER 2.0 analysis in XDR Isolates identified (n=31)

MGIT DST (FLQ)	MGIT DST (SLID)	MTBDRsl VER 2.0 (FLQ-*gyrA*/*gyrB*)	MTBDRsl VER 2.0 (SLID-*rrs*)	MTBDRsl VER 2.0 (SLID-*eis*)	Frequency n (%)
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[1]	wt[1]	1 (3.2)
R	R	*gyrA* MUT2/S91P	wt[2]	wt[2]	1 (3.2)
R	R	*gyrA* MUT3A/D94A	*rrs* MUT1/A1404G	wt	2 (6.5)
R	R	*gyrA* MUT3B/ D94N/D94Y	wt[2]	wt[2]	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[1]	wt[1]	1 (3.2)
R	R	*gyrA* MUT3D/D94H	*rrs* MUT1/A1404G	wt	4 (12.9)
R	R	*gyrA* MUT1/A90V *gyrB* WTabsent	wt	*eis* MUT1/ C-14T	1 (3.2)

[1] WGS not available
[2] WGS results: wt

Table 6: GenoType® MTBDRsl VER 2.0 - REPRODUCIBILITY ASSESSMENT OF PARTICIPANT LABORATORIES

STRAIN NUMBER (3 Isolates each)	Laboratory A	Laboratory B	Laboratory C	Laboratory D	Laboratory E
Strain 1	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)
Strain 2	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)
Strain 3	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)
Strain 4	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)
Strain 5	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)
Strain 6	No consensus Excluded	No consensus Excluded	No consensus Excluded	No consensus Excluded	No consensus Excluded
Strain 7	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)
Strain 8	2 /3 (67%)	3/3 (100%)	3/3 (100%)	3/3 (100%)	3/3 (100%)
Strain 9	3/3 (100%)	2 /3 (67%)	3/3 (100%)	3/3 (100%)	3/3 (100%)
Strain 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%)

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 the sensitivity and specificity for the determination of molecular resistance to both FLQ (100% (95% CI 95.8-100%); 98.9% (95% CI 96.1–99.9%) and SLID (89.2% (95% CI 79.1-95.6%); 98.5% (95% CI 95.7–99.7%), in comparison with the pooled sensitivity and specificity of the Genotype® MTBDR*sl* VER 1.0 reported by WHO Expert Group (12) and a meta-analysis by Theron and colleagues (5). Agreement between 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 reproducible in terms of inter-laboratory (93 - 96%) and inter-operator (100%) assessment.

FLQ resistance in *M. tuberculosis* is ascribed mainly to *gyr*A mutations, with 57.5% of mutations detected at codon 94 and 31.5% at codon 90 (21). Consistent with published data, the highest frequency of mutations conferring FLQ resistance was observed in *gyr*A codons 94 and followed by codon 90 (Table 3). Furthermore, *gyr*A mutations D94G and D94N were observed in majority of the isolates tested. Previous studies have indicated that isolates harbouring these mutations exhibit high levels of resistance to FLQ (21, 22). The *gyr*A MUT3D/ D94H mutation, reported as a rare *in silico* mutant (13, 23), 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%) with multiple mutation bands observed in some isolates (Table 3) and were confirmed as phenotypically resistant. Cohen et. al., 2011, reported on mixed-strain infections in a hospital setting in KwaZulu-Natal, South Africa (24). 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 *gyr*A wild-type bands were confirmed as phenotypically resistant. WGS results available for four isolates confirmed the presence of *gyr*A 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 *gyr*A MUT2/S91P bands with intensities less than the AC zone, therefore interpreted as negative and reported as resistant due to the absence of *gyr*A wild-type bands. No weak intensity mutation bands were detected in the fourth isolate. An isolate phenotypically susceptible to OFX with *gyr*A MUT 3C/D94G bands and a confirmed Asp94Gly mutation on WGS had *gyr*A WT bands with less intensity than AC control band. The discrepant OFX phenotypic DST is possibly due to a mixed population. The inclusion of selected *gyr*B probes offered limited enhancement to the identification of FLQ mutations as only the absence of *gyr*B WT was observed in 3/268 (1.1%) of isolates. Genotype® MTBDR*sl* VER 2.0 is limited to detecting mutations in selected areas of the QRDR regions of *gyr*A and *gyr*B only; therefore, FLQ resistance mechanisms outside these regions are likely to be missed but does not appear to be a major concern in our setting presently.

Consistent with published data, (23), *rrs* MUT1 A1401G (translating to high level SLID resistance) was the most frequently observed mutation (77.5%) amongst 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. In 2 isolates with *rrs* WT1 bands absent with no corresponding mutation band observed and wild type by WGS, interpreted as resistant to

SLID by the assay, was phenotypically susceptible to SLID. The omission of WT-bands in line probe assay is not a reliable indication of phenotypic resistance and would require confirmatory phenotypic DST (25). 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 (non-standardised) second-line drug preparations available for testing (25). 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., (26) *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 (27).

There were 31 isolates identified as phenotypically XDR and the Genotype® MTBDR*sl* VER 2.0 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 *gyr*A 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 standardised 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 (17, 28).

The reproducibility assessment of selected routine laboratories showed excellent performance consistent with other data on molecular diagnostics (Table 6) (29). 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 turn-around-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 with the gold standard, phenotypic DST. However, there is a need for further studies evaluating the Genotype® MTBDR*sl* VER 2.0 on clinical specimens from patients with MDR-TB, thereby enhancing the turn-around 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 not any 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 (30). Phenotypic DST for higher generation FLQs (moxifloxacin) was introduced in 2015 as per WHO recommendations (17, 28). A general limitation of second-line drug testing is the limited availability of standardized

drug preparations (25). 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 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 is recommended (31, 32). Appropriately trained laboratory staff, quality assurance and availability of laboratory infrastructure are requisite recommendations of the WHO to implement use of this assay (31, 32). Our study provides support and evidence for these recommendations and the implementation of the assay in South Africa.

ACKNOWLEDGEMENTS

The authors thank Hain Lifescience, South Africa for providing Genotype® MTBDRs*l* VER 2.0 reagents and training at participating laboratories, the staff members of CTB NICD/NHLS and the participant NHLS laboratories for their assistance during the study. Funding from the CTB NICD/NHLS supported this study.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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