Evaluation of the Genotype® MTBDR*sl* Assay for Susceptibility Testing of second-line Anti tuberculosis Drugs

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Abstract

Background: The GenoType® MTBDR*sl* assay (Hains, Lifesciences, Germany) is a new rapid assay for detection of resistance to second-line anti-tuberculosis drugs.

Method: The MTBDR*sl* assay was evaluated on 342 MDR-TB isolates for ofloxacin (OFX), kanamycin (KAN), capreomycin (CAP) and ethambutol (EMB) resistance and results were compared to the agar proportion method. Discrepant results were tested by DNA sequencing.

Result: The sensitivity and specificity of MTBDR*sl* assay was 70.3% and 97.7% for OFX, 25.0% and 98.7% for KAN, 21.2% and 98.7% for CAP and 56.3% and 56.0% for EMB, respectively. DNA sequencing identified mutation that were not detected by MTBDR*sl* assay including: 8/11 phenotypically OFX-resistant isolates had mutation in *gyr*A (2/8 had additional mutation in the *gyr*B gene), 1/11 had mutation only in the *gyr*B gene; 6/21 phenotypically KAN-resistant isolate had mutation in *rrs* gene; 7/26 and 20/26 phenotypically CAP-resistant isolates had mutation in the *rrs* and *tly*A genes, respectively.

Conclusion: The MTBDR*sl* assay showed a lower sensitivity as compared to previous studies. The assay performed favourably for OFX; however the assay was less sensitive for detection of KAN/CAP resistance and demonstrated low sensitivity and specificity for EMB resistance. It is recommended that the MTBDR*sl* assay should include additional genes to achieve a better sensitivity for all the drugs tested.

INTRODUCTION

The increasing problem of multidrug-resistant tuberculosis (MDR-TB) caused by *M. tuberculosis* strains that are resistant to isoniazid (INH) and rifampicin (RIF) and the emergence of extensively drug resistant tuberculosis (XDR-TB), defined as MDR-TB with additional resistance to any fluoroquinolon (FLQ) and to at least one of three injectable second-line drugs, (kanamycin [KAN], amikacin [AK], and/or capreomycin [CAP]) has become a global health problem, threatening the success of TB control programmes (1,2). The WHO developed guidelines for drug susceptibility testing (DST) for first and second-line anti-TB drugs on Lowenstein-Jensen (LJ) medium or Middlebrook agar using the proportion method. However, DST of *M. tuberculosis* to second-line drugs is difficult, expensive, and not well standardised. In

addition results are only available within 3 to 6 weeks. Therefore, there is a major interest in rapid molecular detection methods for resistance to these drugs.

The GenoType® MTBDR*sl* assay (Hain Lifesciences, Germany) was developed for rapid detection of resistance against second-line drugs (3). The assay uses a multiplex PCR and reverse hybridisation to identify *M. tuberculosis* and relevant mutations in genes, *gyr*A, *rrs*, and *emb*B that confer resistance to FLQ, CAP/AK/KAN/viomycin (VIO) and ethambutol (EMB), respectively (3). Previous studies reported that the majority of FLQ-resistant *M. tuberculosis* isolates had mutations in the quinolone resistance determining region (QRDR) in the *gyrA* gene (mutations mostly in codon A90, 91 and D94 and, more rarely, G88 and S91), and less frequently in *gyr*B gene (4,5,6). Resistance to CAP, KAN, and AK has been shown to be associated with mutations at positions 1401 and 1402 and position 1484 in the *rrs* gene (7,8). Mutations 1401 and 1484 were found to cause high-level resistance to all drugs, whereas 1402 causes resistance to only CAP and KAN. In addition, the *tly*A gene, which encodes a putative rRNA methyltransferase, was reported to confer resistance to CAP (9). Ethambutol resistance is reported to be most frequently associated with mutations in the *emb*CAB operon. However, 50% to 70% of EMB resistant *M. tuberculosis* isolates contain missense mutations in the *emb*B gene with the majority (47 to 60%) of the *M. tuberculosis* strains carrying mutations at codon 306 (10,11).

The aim of the study was to evaluate the performance of the MTBDR*sl* assay for the detection of ofloxacin (OFX), KAN, CAP and EMB resistance in a high-TB burden area. The results obtained were compared with those of the standard agar proportion method which is performed in parallel with the MTBDR*sl* assay.

MATERIALS AND METHODS

Study setting and clinical isolates

The study was conducted at Diagnostic Microbiology Laboratory at Tshwane Academic Division located in Pretoria, South Africa. A total of 342 consecutive MDR-TB isolates were collected from the National Health Laboratory Service (NHLS) Diagnostic Microbiology laboratory at the Medical University of Southern Africa. The laboratory receives specimens from the surrounding clinics and hospitals in the referring provinces of Limpopo, North-West and Mpumalanga, a

geographic area with high incidence of TB. All isolates were freshly sub-cultured on Middle-brook agar before being tested by the different methods.

Drug susceptibility testing

Drug susceptibility testing was done for OFX, KAN, CAP and EMB using the agar proportion method. An MDR-TB isolate was classified as resistant when the colonies on the drug-containing quadrant appeared 1% compared to the drug-free control quadrant.

GenoType® MTBDR*sl* assay

The DNA of MDR-TB isolates was extracted according to a method described previously (12). MTBDR*sl* assay was performed as described by the manufacturer. Either the absence of a wild-type band or the presence of a mutant band was an indicative of a resistant isolate.

DNA sequencing of discrepant results

All discrepant isolates were sequenced to evaluate discrepancies between MTBDR*sl* and agar proportion method. The primers used for amplification and sequencing are shown in Table 1.

Statistical analysis

The sensitivity, specificity, positive and negative predictive values was calculated for each drug compared to the gold standard agar proportion method. The agreement between the two methods was determined by the κ statistic. The κ value, a measure of test reliability, was interpreted as follows: < 0.2, poor; 0.21 to 0.4, fair; 0.41 to 0.6, moderate; 0.61 to 0.8, good; ≥ 0.81, excellent (13).

RESULTS

Of the total 342 isolates tested by the MTBDR*sl* assay, 336 (98.2%) gave interpretable results. In 6 isolates, no TUB band was observed and these isolates were excluded from the analysis. No contamination was observed in the negative controls. A total of 21/336 (6.3%) isolates met the

criteria for the classification as XDR-TB by the agar proportion method and 8/336 (2.4%) by the MTBDR*sl* assay. Turnaround times for DST ranged from 6 to 21 days (median, 11 days) for the agar proportion method and from 2 to 3 days (median, 2 days) for the MTBDR*sl* assay. The DST results of the MTBDR*sl* assay as compared to the agar proportion method are shown in Table 2.

Ofloxacin resistance: The sensitivity and specificity of the MTBDR*sl* assay for OFX resistance was 70.3% and 97.7%, respectively (Table 3). Of the total 336 isolates tested, 299 (89.0%) were phenotypically susceptible to OFX. Of these, 292 (97.7%) showed wild-type patterns with the MTBDR*sl* assay and the remaining 7 (2.3%) isolates had mutations. DNA sequencing confirmed the presence of mutations in the *gyr*A gene in all 7 isolates, with one isolate showing additional mutations in the *gyr*B. The phenotypic method identified 37/336 (11.0%) resistant isolates to OFX, of these 26 (70.3%) had mutations with the MTBDR*sl* assay and the remaining 11 (29.7%) showed wild-type pattern. Analysis of the discrepant results by DNA sequencing showed that 8/11 had mutation in the *gyr*A gene, with 2 of the 8 showing additional mutations in the *gyr*B gene. One of the 11 isolates (phenotypically an XDR-TB isolate) had mutation only in the *gyr*B gene (at position 1491 A→G) and was not detected by the MTBDR*sl* assay as the *gyr*B gene is not included in the assay (Table 4).

Among the 33/336 OFX-resistant isolates by MTBDR*sl* assay, the *gyr*A MUT 3C/D94G was the most prevalent mutation occurring in 14/33 (42.4%) isolates followed by the *gyr*A MUT 3D/D94H (10/33; 30.3%), *gyr*A MUT 1/A90V mutation (1/33; 3.0%) and *gyr*A MUT 3B band/D94Y (1/33; 3.0%). In 5 of 33 isolates (15.2%) both *gyr*A MUT 3C/D94G and *gyr*A MUT 1/A90V were present. For 2 isolates (2/33; 6.1%) resistance was indicated by omission of wild-type pattern.

Kanamycin and/or capreomycin resistance: In 4/336 (1.2%) isolates, the *rrs* gene control band was absent. Similar results were obtained after repeating the assay and hence, these isolates were excluded from the analysis. The sensitivity and specificity of the MTBDR*sl* assay was 25.0% and 98.7% for KAN, 21.2% and 98.7% for CAP, respectively (Table 3).

A total of 304/332 (91.6%) were phenotypically susceptible to KAN and of these 300/304 (98.7%) showed wild-type patterns with the MTBDR*sl* assay and the remaining 4 (1.3%) had

mutation in the *rrs* gene. DNA sequencing confirmed the presence of mutations in all 4 isolates. Of the 332 isolates, 28 (8.4%) were phenotypically resistant to KAN and only 7 (25.0%) of these isolates had mutations by the MTBDR*sl* assay, while the remaining 21 (75.0%) had wild-type patterns. Among the 21 discrepant isolates, 6 isolates (including 3 phenotypically XDR-TB isolates) had mutation in the *rrs* gene by DNA sequencing and were not detected by the MTBDR*sl* assay (Table 4). These isolates had various nucleotide changes at different positions in the *rrs* gene (between 87 to 1431bp).

Of the 332 isolates, 299 (90.1%) isolates were phenotypically susceptible to CAP and of these 295/299 (98.7%) showed wild-type patterns with the MTBDR*sl* assay and the remaining 4 (1.3%) had mutation in the *rrs* gene. DNA sequencing of the *rrs* gene showed mutation in all 4 discrepant isolates. Thirty-three of the 332 (9.9%) isolates were phenotypically resistant to CAP, of these only 7 (21.2%) had mutation in the *rrs* gene with the MTBDR*sl* assay and the remaining 26 (78.8%) showed wild-type pattern. Of the 26 discrepant isolates, DNA sequencing identified mutation in 7 isolates (including 3 phenotypically XDR-TB isolate) which were not detected by the MTBDR*sl* assay. These isolates had various nucleotide changes at different positions in the *rrs* gene (between 87 to 1431bp). In addition, the *tly*A gene was sequenced for all the discrepant isolates. DNA sequencing identified nucleotide change at position 33 (A→G) of *tly*A gene in 20/30 isolates phenotypically resistant to CAP and 3/30 CAP-resistant isolates by the MTBDR*sl* assay (Table 4). This mutation was not detected by MTBDR*sl* assay as the *tly*A gene is not included in the assay.

Of the 11 KAN/CAP resistant isolates by MTBDR*sl* assay, 1/11 (9.1%) had *rrs* MUT 1/A1401G and the other 1/11 (9.1%) had *rrs* MUT 2/A1484T mutation. The remaining 9/11 (81.8%) were resistant by absence of wild-type patterns.

Ethambutol resistance: In 20/336 (5.9%) of the isolates tested for EMB, both the wild-type and mutant EMB bands were observed, suggesting heteroresistance and thus were not included in the analysis. The sensitivity and specificity of the MTBDR*sl* assay for detection of EMB resistance was 56.3% and 56.0% respectively (Table 3). Of the 316 isolates, 268 (84.8%) were phenotypically susceptible to EMB and of these 150 (56.0%) showed wild-type pattern and the remaining 118 (44.0%) showed mutations with the MTBDR*sl* assay. Forty-eight of the 316

isolates (15.2%) were phenotypically resistant to EMB. However, only 27 (56.3%) showed mutations with the MTBDR*sl* assay while the remaining 21 (44.0%) showed wild-type patterns. The discrepant isolates were sequenced in the *emb*B306 gene to evaluate discrepancies. Of the 118 isolates resistant by the MTBDR*sl* assay, only 67 (56.8%) isolates had mutation with the DNA sequencing, while the remaining 51 (43.2%) isolates had no mutation (Table 4).

Of the 21 isolates susceptible to EMB by MTBDRsl assay, mutation was detected in 9 (42.9%) isolates by DNA sequencing. The remaining 12 (57.1%) isolates had wild-type patterns. The most prevalent mutation of the 145 EMB resistant isolates with MTBDR*sl* assay was *emb*B MUT 1B/M306V (101/145; 69.7%), followed by *emb*B MUT 1A/M3061 (36/145; 24.8%) and the remaining isolates (8/145; 5.5%) were resistant by absence of wild-type patterns.

DISCUSSION

In this study the largest number of isolates (342 MDR-TB) was used to evaluate MTBDR*sl* assay (3,14,15). A good agreement (Kappa=0.73) was found between the MTBDR*sl* assay and the agar proportion method for OFX, with a sensitivity of 70.3%. Previous studies have reported similar sensitivity values for the MTBDR*sl* assay for detecting FLQ-resistance, ranging from 70% to 87% (3,14,15,16). A significant finding of this study, DNA sequencing identified mutation in 8/11 phenotypically resistant to OFX in the in the *gyr*A gene (2/8 had additional mutation in *gyr*B), while 1/11 (phenotypically XDR-TB isolate) had mutation only in the *gyr*B gene. Similarly, Mokrousov *et al*. (6) found OFX-resistant isolates with only *gyr*B mutations and one of these isolate was an XDR-TB strain. Given these findings, genotypic testing for OFX resistance should not be limited to the analysis of the *gyr*A gene but should also include the *gyr*B gene. Furthermore, inclusion a bigger region of the *gyr*A gene could have picked up 8/11 (72.7%), with a resultant increase sensitivity to 92.0%.

In 3 phenotypically OFX-resistant isolates, no mutation was found in *gyr*A or *gyr*B gene.This finding suggests mutation in another target gene or the use of other mechanisms such as decreased cell-wall permeability to drug, drug efflux pump, or even drug inactivation (17).

Although the specificity of the MTBDR*sl* assay for the detection of KAN/CAP resistance was excellent (98.7% and 98.7%), the sensitivity was low (25.0% and 21.2%). Previous studies

reported higher sensitivity (77% to 100% for KAN and 80.0% to 86.7% for CAP) (3,14,15). However, these studies tested small number of isolates (5-13 isolates) and larger number of isolates need to be tested to assess sensitivity accurately. Recently, Huang *et al*. (16) tested 234 MDR isolates using MTBDR*sl* assay and found low sensitivity (43.2%) for detecting KAN resistance.

The low sensitivity of KAN/CAP in this study could be due to other mutations associated with resistance to these drugs. The MTBDR*sl* assay uses only one gene (*rrs*) for the detection of KAN and CAP resistance. The *tly*A gene is reported to cause CAP resistance (9) and the promoter region of *eis* gene, which encodes an aminoglycoside acetyltransferase, are associated with low-level KAN resistance (18). It has been reported that *tly*A mutations are mainly associated in vitro selected CAP-resistant mutants and are rare in clinical isolates of *M. tuberculosis* (9,19,20). However, in this was not the case in this study, as 20/26 of the discrepant results which were pheonotypically resistant to CAP and 3/4 CAP-resistant by MTBDR*sl* assay had mutation in the *tly*A gene. Therefore, the role of *tly*A gene in CAP resistant clinical isolates should be further investigated.

In 15 phenotypically KAN-resistant isolates and 6 phenotypically CAP-resistant isolates, no mutation was detected in either *rrs* or *tly*A genes. Unidentified mutations associated with CAP and KAN resistance could explain this finding. Further work is needed to determine the mechanism of the unexplained KAN and CAP resistance as resistance to one of these drugs is a marker for XDR-TB.

The sensitivity of MTBDR*sl* assay for detection of EMB resistance in this study was low (56.3%). Similarly, previous studies reported a sensitivity ranging from 56% to 69% (3,14,15,16). EMB is known to be a problematic drug to be tested and often yields less reproducible results (21,22). Of the 118 resistant by MTBDR*sl* assay, DNA sequencing identified mutation in *emb*B306 gene in only 67/118 (56.8%) isolates. The discrepancy could be due to the shorter region of the *emb*B306 gene analysed by sequencing. Thus, some of the mutations could lie outside the region anaysed in this study. The limitation of the study; the discrepant results were not repeated by MTBDR*sl* assay to rule out the non-specific hybridisation, however no contamination was observed in negative controls.

In this study, 20/336 (5.9%) isolates showed heteroresistant for the EMB gene. Heteroresistance of *M. tuberculosis* is considered a precursor to development of full resistance (23). This highlights the important advantage of genotypic over the phenotypic methods, as genotypic methods are able to detect mixed strains.

The low performance of the MTBDR*sl* assay in this study was not unique since molecular methods detect only mutations that are screened for, while phenotypic tests detect resistance independent of the underlying mechanism and in addition not all mutations conferring resistance to second-line anti-TB drugs are known especially for KAN, CAP and EMB. In addition the prevalence of certain strains with specific mutation may vary in different geographical areas and thus will affect the test performance. On the other hand significant challenges exist in phenotypic susceptibility testing to second-line drugs. Although standard protocols exist, phenotypic susceptibility testing to second-line drugs is often unreliable and inaccurate, with poor clinical predictive values (24).

In conclusion, the MTBDR*sl* assay showed a lower sensitivity in this study as compared to previous studies. The sensitivity of the assay was variable among the drugs tested. The MTBDR*sl* assay performed favourably to the agar proportion method for OFX; however the assay was less sensitive for detection of KAN/CAP resistance and demonstrated low sensitivity and specificity for EMB resistance. The resolution of discrepant isolates with DNA sequencing has shown that the inclusion of *gyr*B gene and covering bigger region of *gyr*A may improve the sensitivity of the assay for detection of FLQ resistance. In addition inclusion of other genes such as the *tly*A for detection CAP resistance and other regions of the *emb*B gene as well as other targets associated with EMB resistance could improve the performance of the assay.

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Table 1 Primer sequences for amplification and DNA sequencing of discrepant isolates

Drug	Gene	Sequence (5'-3')	Amplicon size (bp)
OFX	gyrA	F YGGTGGRTCRTTRCCYGGCGA	250
		R CGCCGCGTGCTSTATGCRATG	
	gyrB	F GAGTTGGTGCGGCGTAAGAGC	250
		R CGGCCATCAAGCACGATCTTG	
KAN/CAP	16s (rrs)	F AGAGTTTGATCCTGGCTCAG	1500
		R ACGGCTACCTTGTTACGACTT	
CAP	tlyA	F GGCATCGCACGTCGTCGTCTTTCCGAGG	820
		R GGACGACCAGCAGAACACTGCGATG	
EMB	embB	F CCGACCACGCTGAAACTG	400
		R GTAATACCAGCCGAAGGGATCCT	

OFX=ofloxacin, KAN=kanamycin, CAP=capreomycin, EMB=ethambutol , bp=base pairs

Table 2 Drug susceptibility results of MDR-TB isolates by the GenoType® MTBDR*sl* assay and the agar proportion method

Drug	Total no. of isolates	S by both methods	R by both methods	S by agar R by MTBDRsl	R by agar S by MTBDRsl	Correctly identified as S by MTBDRsl (%)	Correctly identified as R by MTBDRsl (%)	Agreement (%)	Kappa
OFX	336	292	26	7	11	292 (97.7)	26 (70.3)	94.6	0.73
KAN	332	300	7	4	21	300 (98.7)	7 (25.0)	92.5	0.327
CAP	332	295	7	4	26	295 (98.7)	7 (21.2)	91.0	0.283
EMB	316	150	27	118	21	150 (56.0)	27 (56.3)	56.0	0.067

OFX=ofloxacin, KAN=kanamycin, CAP=capreomycin, EMB=ethambutol ,S=susceptible, R=resistant

Table 3 Diagnostic efficiency of the GenoType® MTBDR*sl* compared to the agar proportion method

Drug	No. of isolates	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
OFX	336	70.3	97.7	78.8	96.4
KAN	332	25.0	98.7	63.6	93.5
CAP	332	21.2	98.7	63.6	91.9
EMB	316	56.3	56.0	18.6	87.7

OFX=ofloxacin, KAN=kanamycin, CAP=capreomycin, EMB=ethambutol ,S=susceptible, R=resistant

Table 4 DNA sequencing results for the discrepant isolates between GenoType®MTBDR*sl* assay and agar proportion

Drug	Locus		R by MTBDR*sl* S by agar	R by agar S MTBDR*sl*
	DNA SEQUENCING			
OFX n=18			**7**	**11**
	*gyr*A	Mutation present (n=15)	7	8
		Mutation absent (n=3)	0	3
	*gyr*B	Mutation present (n=4)	1	3
		Mutation absent (n=14)	6	8
KAN n=25			**4**	**21**
	16s (*rrs*)	Mutation present(n=10)	4	6
		Mutation absent (n=15)	0	15
CAP n=30			**4**	**26**
	16s (*rrs*)	Mutation present (n=11)	4	7
		Mutation absent (n=19)	0	19
	*tly*A	Mutation present (n=23)	3	20
		Mutation absent (n=7)	1	6
EMB=139			**118**	**21**
	*emb*B (M306)	Mutation present (n=76)	67	9
		Mutation absent (n=63)	51	12

OFX=ofloxacin, KAN=kanamycin, CAP=capreomycin, EMB=ethambutol ,S=susceptible, R=resistant