Original Article

Genotype MTBDRsl Version 2 and Phenotypic Drug Resistance Detection of *Mycobacterium tuberculosis* for Fluoroquinolones and Aminoglycosides

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Abstract

Background: Information on genotypic with comparison of phenotypic drug sensitivity test of anti-tuberculosis (TB) has been reported in several studies, which have variable results. The present study aimed to assess the Genotype MTBDRsl version 2.0/Line probe assay (LPA) for the detection of fluoroquinolones (FQ) and aminoglycosides (AMGs) resistance mutations among drug-resistant *Mycobacterium* TB (MTB) strains and also to compare the patterns of genotypic mutations of gyrA/B, *rrs,* and *eis* with mycobacteria growth indicator tube (MGIT 960). **Methods:** A total of 1416 samples were subjected to Genotype MTBDRsl version 2.0 assay. One hundred and twenty sputum smear positive MTB isolates and 37 sputum smear negative MTB isolates confirmed multiple drug resistance resistant to FQ and AMG by the Genotype MTBDRsl version 2.0 were subjected to phenotypic drug susceptibility testing (DST) were analyzed. **Results:** The association sensitivity, specificity, positive predictive value, negative predictive value, and accuracy for the resistance detection between MGIT (DST) and the Genotype MTBDRsl version 2.0 assay was significant ($P < 0.01$) of moxifloxacin (MFX) concentration. Sensitivity and specificity value for kanamycin (KAN) resistance was 76% and 89%; 47% and 94% for capreomycin (CAP); and 60% and 76% for low-level KAN, respectively. **Conclusion:** Our results indicate that MFX (0.25and 1 µg/mL), KAN (2.5 µg/mL), and CAP (2.5 µg/mL) significantly ($P < 0.01$) and support the World Health Organization guidance to test FQ and AMG by genotypic test.

Keywords: Aminoglycosides, fluoroquinolones, line probe assay

Submitted: 01-May-2023 **Revised:** 01-Jul-2023 **Accepted:** 31-Jul-2023 **Published:** 15-Sep-2023

Introduction

In 2022, eight countries accounted for two-thirds of the global total tuberculosis (TB) cases, where India had the highest burden (28%) followed by Indonesia (9.2%), China (7.4%), the Philippines (7.0%), Pakistan (5.8%), Nigeria (4.4%), Bangladesh (3.6%), and Democratic Republic of Congo (2.9%). Globally in 2021, 71% of people (2.4/3.4 million) diagnosed with bacteriologically confirmed pulmonary TB were tested for rifampicin resistance, the same level of coverage as in 2020 (2.1/3.0 million) and up from 61% (2.2/3.6 million) in 2019. Among those tested, 141,953 cases of multiple drug resistance (MDR) rifampicin-resistant (RR)-TB and 25 038 cases were extensive drug-resistant TB (XDR-TB) or Pre-XDR-TB.[1]

The effective management of MDR/XDR TB relies on the rapid diagnosis and treatment of resistant infections.[2] Rapid identification of DR TB is achieved using a combination of nucleic acid amplification technology (NAAT) either by cartridge-based GeneXpert or chip-based NAAT by TrueNAT, first-and second-line line probe assay (LPA)[3] and liquid culture drug susceptibility testing (LC-DST) for specific drugs including newer and repurposed drugs.[4] In

Access this article online

Quick Response Code:

Website: https://journals.lww.com/IJMY

DOI: 10.4103/ijmy.ijmy_120_23

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How to cite this article: Radhakrishnan R, Prabuseenivasan S, Hannah S, Vaishanavee V, Senthildevi V, Kannadhasan T, *et al*. Genotype MTBDRsl version 2 and phenotypic drug resistance detection of *Mycobacterium tuberculosis* for fluoroquinolones and aminoglycosides. Int J Mycobacteriol 2023;12:299-304.

2016, the World Health Organization (WHO) issued a new policy recommendation on use of Genotype MTBDRsl version 2.0 assay (LPA) for the drug resistance detection to fluoroquinolones (FQ) and aminoglycosides (AMGs) that includes kanamycin (KAN), capreomycin (CAP), and amikacin (AMK). Since LC DST is the gold standard for *Mycobacterium* TB (MTB) diagnosis and detection of drug resistance, the WHO recommends that the genotypic FQ resistance reported by LPA need to be correlated with phenotypic results obtained for the drugs.[5]

As per guidelines issued by the WHO in 2018, the critical concentration (CC) for moxifloxacin (MFX) by mycobacteria growth indicator tube (MGIT) DST was reduced from 0.5 µg/mL to 0.25 µg/mL. However, there was some overlay between the minimum inhibitory concentration (MIC) values of low-level *gyrA* mutants and pWT isolates, leading to false-susceptible results. Hence, the clinical breakpoint (CB) was also reduced from 2 to 1 µg/mL, as this detected most of the low-level resistant mutants (e.g. gyrA A90V and D94A). Based on this, it was recommended that the strains with MICs above the CC but below or equal to the CB may be treated with higher dose of MFX (800 mg daily) whereas strains below CC may be treated with lower dose of MFX (400 mg),[4] While phenotypic tests provide CC and CB for determining drug resistance, the detection of target mutations by genotypic tests serves as an important tool for understanding drug resistance patterns.[6] Widespread use of fluoroquinolones-resistant TB has led to unfavorable treatment outcomes, failure, relapse, lost to follow-up, and death.[7,8] To overcome this, we need a combination of genotypic and phenotypic tests to provide an upfront accurate diagnosis of FQ and AMG resistance, also phenotypic drug susceptibility testing has CC and CB which can help to decide drug dosing.

In this study, we aimed to correlate the Genotype MTBDRsl version 2.0 assay (LPA) in the detection of FQ and AMG resistance mutation in *MTB* culture isolates with phenotypic DST with CC and CB concentrations of MFX and CC of KAN and CAP. In addition, we analyzed the frequency of mutations associated with FQ and AMG.

Materials

Ethical clearance

The study was approved by the Institutional Eethical C of the National Institute for Research in Tuberculosis (NIRT) (IEC number: 013/2021).

Study settings

This study was carried out at ICMR-NIRT as part of the National Tuberculosis Elimination Program activity.

Study sample and procedure

A retrospective analysis of test results of LPA and phenotypic DST (MGIT) obtained from 157 patients from August 2019 to January 2021 was carried out. A total of 1416 (1117 direct smear positive and 299 MTB isolates from positive cultures of AFB smear-negative sputum samples) samples confirmed MDR or RR or isoniazid resistant by first line (LPA) were subjected to Genotype MTBDR*sl* version 2.0 assay.[5] Around 157 *MTB* isolates that were either resistant to FQ or AMG or both were subjected to MGIT 960 for the following drugs: MXF, KAN, CAP.[4]

Specimen processing

The sputum specimens were decontaminated by the NALC-NaOH method as described previously.[9] The deposit of the smear-positive samples was processed for DNA extraction by the Genolyse kit as recommended by the manufacturer.[10] Smear-negative samples were cultured by MGIT and positive cultures were further processed for LPA assay after Genolyse DNA extraction.

Genotype MTBDRsl version 2.0 (line probe assay)

The assay was performed as per the manufacturer's instruction. After sputum decontamination and processing, the test was done by direct or indirect methods as mentioned in the Kit manual.[11]

Mycobacteria growth indicator tube culture and drug susceptibility testing

A total of 500 µL of the processed sample was inoculated into the respective MGIT 960 TB culture tube as per the manufacturer's instruction.[12] The positive culture from MGIT 960 instrument was further confirmed as MTB using SD Bioline MPT 64 Ag kit and brain heart infusion agar plating and by identification of acid-fast bacilli by AFB smear and staining.[10,12] A total of 157 strains were subjected to drug susceptibility testing for MFX (0.25 µg/mL^{-2} µg/mL); KAN (2.5 µg/mL); and CAP (2.5 µg/mL) by MGIT.[13]

MTB H37Rv was used as quality control (QC) for all methods. The QC strains received as external quality of panel culture for proficiency testing of mycobacterial culture and DST from ITM, Antwerp, Belgium, was used as a resistance control.

Statistical analysis

The association, sensitivity, specificity, positive (positive predictive value [PPV]), and negative predictive values (NPVs) of LPA was compared with MGIT DST of MFX (four concentration), KAN, and CAP. The statistical significance of the observed difference and agreement between LPA and MGIT DST was assessed using the Fisher's exact test and Chi-square test.

Results

FQ and aminoglycoside resistance by line probe assay

A total of 157/1416 MTB positive samples confirmed MDR or RR or isoniazid resistant were subjected to LPA, 37 were from culture of AFB smear-negative sputum samples, and 120 were directly from AFB smear-positive sputum samples. FQ resistance with mutations in *gyr*A and *gyr*B genes were seen in 122/1416 (8.6%) samples and AMG mutations in the *rrs* and *eis* genes were seen in 35/1416 (2.47%) samples [Table 1].

The *gyr* A mutations of MUT1 was seen in 43.3% isolates at codon 94 and 19.1% isolates at codon 90 and *gyr*A mutations of MUT2 was seen in 5.7% of isolates at codon 91 (S91P). The *gyr*B mutations of MUT1 and MUT2 is seen in 2.5% and 0.6 isolates at codon N538D and E540V, respectively [Table 1].

Heteroresistance (coexistence of populations with wild type (WT) and mutation in drug resistance locus). Ten isolates involving the combination of 3A, 3B, and 3C at *gyr*A were observed in FQ resistance, and eight isolates with WT of *rrs* MUT1 (5.0%) were observed in AMG resistance [Table 1].

The most frequently observed mutation for AMG resistance was *rrs* (A1401G) (15.9%) and one mutation with type 2 band was detected among these isolates at *rrs* MUT2 (G1484T) and *eis* MUT1 (0.6%) (C-14T) [Table 1].

Association between mycobacteria growth indicator tube DST and line probe assay

The sensitivity, specificity, PPV, NPV, and accuracy for the MGIT DST and Genotype LPA assay was observed significant for MFX (0.25 µg/mL) 95%; 31%; 69%; 78% and 71% and 95%; 18%; 28%; 91%; and 38% (1 µg/mL), respectively [$P < 0.01$, Figure 1]. The resistance pattern for FQ between MGIT DST and Genotype LPA assay had significant correlation for 0.25 µg/mL (94.9%) and 0.5 µg/mL (93.6%) of MFX concentration respectively ($P < 0.0$). Similarly for AMG resistance the correlation between both methods was 86.6% in KAN resistance and 46.8% in CAP resistance [$P < 0.01$, Table 2]. The correlation and distribution of isolates with mutation at codon D94G (0.25, 0.5, and 1); D94N/D94Y (0.5 and1); D94N/D94Y and D94G (0.5 and 1) was predominant at CC and CB ($P > 0.001$) whereas *gyr* MUT 1; MUT 3A; 3B; 3C; 3A and 3C was distributed significantly [Table 3, $P > 0.001$] among resistant and susceptible isolates of MFX (0.25–1 µg/mL). The *rrs* WT1 and MUT1 (A1401G) was found resistant to 33/35 for KAN and 15/35 CAP ($P < 0.01$) in MGIT.

Identification of multiple drug resistance/preextensive drug resistant

Around 4 isolates out of 157 (2.54%), isolates were resistant to fluoroquinolones along with second-line injectable by MGIT.

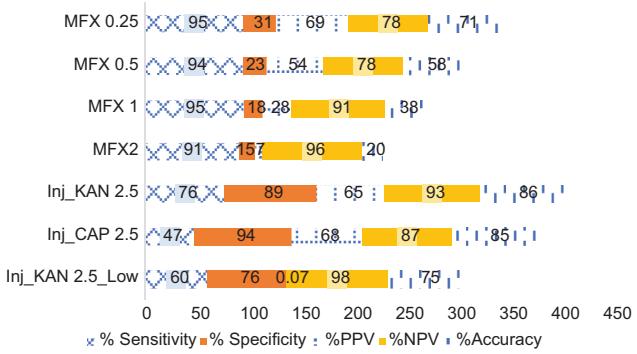

Figure 1: Comparison of moxifloxacin (FQ) and aminoglycosides by mycobacteria growth indicator tube and Genotype MTBDR

Genotype LPA correctly identified *gyr*A MUT1 in 3/157 (1.9%) isolates and missed *rrs* MUT1 but was phenotypically identified as AMG and FQ (MFX, KAN, and CAP) resistant by MGIT DST.

Table 1: Frequency of mutations in *gyr*A and *gyr*B and *rrs* and *eis* of *mycobacterium tuberculosis* strains determined by Genotype MTBDRsl version 2.0 assay

Genotypic resistance type detected, and hybridization bands observed	Mutation (s) detected	Number of mutations (%)
FQ		
*gyr*A, MUT 1	A90V	30 (19.1)
*gyr*A MUT 2	S91P	9 (5.7)
*gyr*A, MUT 3A	D94A	10 (6.3)
*gyr*A MUT 3B (high)	D94N/D94Y	8 (5.0)
*gyr*A MUT 3C (high)	D94G	48 (30)
*gyr*A MUT 3D (high)	D94H	2 (1.2)
*gyr*A MUT 1 and 3C	A90V; D94G	4 (2.5)
*gyr*A MUT 2 and 3C	S91P; D94G	3 (1.9)
*gyr*A MUT 3A and 3C	D94A; D94G	1 (0.6)
*gyr*A MUT 3B and 3C	D94N/D94Y; D94G	2 (1.9)
*gyr*B MUT 1	N538D	4 (2.5)
*gyr*B MUT 2	E540V	1 (0.6)
CAP, Amk, KAN		
rrs MUT1	A1401G	25 (15.9)
WT/*rrs* MUT1	A1401G	8 (5.0)
CAP vio, Amk, KAN		
rrs MUT2	G1484T	1/157 (0.6)
Low-level KAN		
eis MUT1	C-14T	1/157 (0.6)
eis WT2 absent	None	

FQ mutation (n=122), KAN, CAP, Amk mutation (n=35). KAN: Kanamycin, CAP: Capreomycin, Amk: Amikacin, WT: Wild type

Table 2: Association of moxifloxacin (fluroquinolone) and aminoglycosides by mycobacteria growth indicator tube and genotype MTBDRsl version 2.0 assay

Drugs (µg/mL)	MGIT	LPA, number of strains (%)		Total number of strains	*P*
		Resistant	Susceptible		
Moxi 0.25	R	93 (94.9)	5 (5.1)	98	<0.0
	S	41 (69.5)	18 (30.5)	59	
MFX 0.5	R	73 (93.6)	5 (6.4)	78	
	S	61 (77.2)	18 (22.7)	79	
MFX 1	R	38 (95)	2 (5)	40	
	S	96 (82.1)	21 (17.9)	117	
MFX 2	R	10 (90.9)	1 (11.1)	11	1
	S	124 (84.9)	22 (15.1)	146	
KAN 2.5	R	26 (86.6)	8 (23.4)	34	<0.0
	S	14 (11.3)	109 (88.6)	123	
CAP 2.5	R	15 (45.4)	18 (54.5)	33	
	S	7 (5.6)	118 (94.4)	125	

MGIT: Mycobacteria growth indicator tube, MFX: Moxifloxacin, KAN: Kanamycin, CAP: Capreomycin, LPA: Line probe assay

Table 3: Correlation of different mutations in *gyrA* and *gyrB* genes with phenotypic susceptibility profile by proportion method minimum inhibitory concentration of moxifloxacin

Gene	Mutation/ WT probe	Probe band	Mutated codon region	MFX 0.25 (μg/mL)		MFX 0.5 (μg/mL)		MFX 1 (μg/mL)		MFX 2 (μg/mL)	
				Susceptible (*n*=27)	Resistant (*n*=96)	Susceptible (*n*=39)	Resistant (*n*=84)	Susceptible (*n*=80)	Resistant (*n*=43)	Susceptible (*n*=114)	Resistant (*n*=9)
gyrA	MUT 1	A90V		10	20	12	18	26	4*	28	2
	MUT 2	S91P		7	2	6	3	8	1	9	0
	MUT 3A	D94A		0	10*	5	5	9	1	10	0
	MUT 3B (high)	D94N/D94Y		1	7	1	7*	2	6*	5	3
	MUT 3C (high)	D94G		3	45*	7	41*	21	27*	44	4
	MUT 3D (high)	D94H		0	2	0	2	2	0	2	0
	MUT 1 and 3C	A90V; D94G		0	4	1	3	3	1	4	0
	MUT 2 and 3C	S91P; D94G		2	1	2	1	3	0	3	0
	MUT 3A and 3C	D94A; D94G		0	1*	0	1	0	1	1	0
	MUT 3B and 3C	D94N/D94Y; D94G		1	2	1	2	2	1	3	0*
gyrB	MUT1	N538D		3	1	3	1	3	1	4	0
	MUT2	E540V		0	1	1	0	1	0	1	0

*<0.05. MFX: Moxifloxacin, WT: Wild type

Discussion

In India, MDR-TB with additional FQ resistance ranges about 18.6%–25.1% from the year 2020 to 2021 and decreased to 17.8% in the year to 2022.[14-16] Study showed that increase in fluoroquinolones resistance 56% among MDR-TB and RR patients in Lucknow.[17] The mismanagement of empirical TB treatment in India have demonstrated that all nonspecialist private practitioners began antibiotic treatment, especially quinolones, for persistent cough before prescribing a test.[18] In Mumbai, unregulated private health sector that operates in parallel with the public DOTS programme, in which suboptimal TB treatment regimens are commonly being prescribed.[19] Mutations occurring in the quinolone resistance determining region of *gyr*A are mainly responsible for development of FQ resistance. Globally, the *gyr*A mutations were reported in codons 88–94 accounted for roughly 60.0%–90.0%.[20] The frequency of mutation for gyrA/B 123/157 (78.3%) in the present study which is similar to Delhi (78%).[21] In addition, 27 out of 48 (62.7%) with D94G *gyr*A was MFX resistant and 21 out of 48 (26.2%) were MFX sensitive (1.0 μg/mL) by MGIT this is statistically significant. This indicates that additional phenotypic testing at CB is required for FQ testing where 26.2% of D94G strains misclassified as high level MFX resistant by LPA assay and which is considered to the findings of recent study conducted in India.[22]

MTB isolates 26 out of 30 (86.6%) with A90V mutation with low-level resistance was phenotypically susceptible at CB [Table 3] which is significant ($P < 0.01$). Others have previously reported that similar mutations A90V are associated with a low level of resistance of MFX having the MICs of <2 μg/mL [Table 3]. Studies also reported that the occurrence of *gyrA* targeted mutation D94G was the frequently observed mutation followed by A90V in MDR-TB strains is constantly high among Indian patients.[23]

LPA detected 35/157 (22.2%) mutation probes where 33 was resistant by *rrs* MUT1 at position A1401G and 1 *eis* MUT1 at position C-14T and 1 was sensitive for *rrs* MUT 2 which is less than the study conducted in India 15/27 (55.6%).[24] Out of 33 high level KAN resistant isolates detected by LPA (*rrs*) 18 (54.5%) were phenotypically susceptible and 15 (45.4%) were resistant to CAP [Table 2]. This indicates that *rrs* MUT1 A1401G mutation could be of moderate level of CAP resistance which is also reported by a South African study;[25] hence, additional DST by phenotypic method is essential before excluding the drug from treatment regimen.[26]

There were 4/157 (2.54%) isolates that showed resistance to FLQ along with second-line injectables by MGIT. LPA correctly identified *gyr*A MUT1 in 3/157 (1.9%) isolates but missed one isolate *rrs* MUT1 which was resistant to MFX, KAN, and CAP by the BACTEC MGIT phenotypic method.

LPA assay has shown a significant improvement in association sensitivity and specificity for the determination of molecular resistance compared to DST of FQ for lower concentration

to the higher concentration 91%–95% (95% confidence interval [CI], 91%–95%) and 15% to 31% (95% CI, 15%–31%) and AMG [$P < 0.0$, Table 2 and Figure 1]. Agreement between the phenotypic gold standard and LPA assay for MFX (0.25 µg/mL) and MFX (1 µg/mL) was 95% and 31%; 95% and 18%; 71 and 38 ($P < 0.001$) which is less similar across a wide distribution of mutations and KAN was 76%; 89%; and 86% with a recent study (89.2% and 98.5%) especially for *gyr*A and *rrs* associated with resistance compared to mutation catalog and observed high sensitivity.[27] The performance of LPA assay was excellent for 120 directly sputum positive specimens which has higher sensitivity of FQ range 91%–95% when compared to Bangladesh study.[28]

Our study has some additional information that the susceptibility testing was performed from (0.25 µg/mL^{-2} µg/mL) concentrations for MFX in MGIT DST and compared with FQ bands of LPA assay. The association between LPA assay and CC MFX (0.25 µg/mL) was 94.9% as well as CB of drug MFX (1 µg/mL) (90%). A similar association was noted for KAN 86.6% and CAP 46.8% indicating significant association ($P > 0.001$) for CB and CC of MFX and KAN and CAP as recommended by the WHO. The isolates found to be less resistant with phenotypic susceptibility profile at different CC and CB (1 µg/mL) MFX [Tables 2 and 3] which was observed in earlier reports.[29]

Conclusions

On comparison of a limited number of strains on MFX (0.25 µg/mL) and MFX (1 µg/mL) by phenotypic culture DST (MGIT 960) and LPA assay showed good concordance between the two tests with significant association ($P < 0.01$) indicating tests that can predict fluoroquinolone and AMG resistance. This study provides evidence to reflect the current WHO recommendation to use LPA assay.

Limitations of the study

1. Sequencing the isolates for detecting additional resistance and the isolates that had discordance resolution between Genotype MTBDRsl version 2.0 assay and MGIT DST for FQ, KAN and CAP resistance could not be done
2. MIC testing was carried out only for MFX
3. The CC for KAN and CAP between new and previously treated TB cases was not differentiated or categorized
4. Sputum bacillary load assay could not be performed for the conclusive and inconclusive results of Genotype MTBDRsl version 2.0 and MGIT DST as reported in previous study.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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