

Research Article

Second-line Drug Resistance Characterization in *Mycobacterium tuberculosis* by Genotype MTBDRsl Assay

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ABSTRACT

Tuberculosis (TB) remains a main hurdle for national programs due to increase in drug resistance to antitubercular drugs. World Health Organization (WHO)-endorsed Line Probe Assay, Genotype MTBDRsl Ver 2.0, gives opportunity for rapid diagnosis and molecular characterization of different mutations in drug targets of fluoroquinolone (FQ) and second-line injectable drugs (SLID). We, retrospectively, analyzed the data of Genotype MTBDRsl Ver 2.0 from January 2018 to June 2018. A total of 863 isolates of *Mycobacterium tuberculosis*, 687 rifampicin resistant and 176 isoniazid resistant only, were screened for drug resistance in FQ and SLID. All the isolates were tested for Genotype MTBDRsl Ver 2.0 according to the manufacturer's instructions. The FQ and SLID resistance were detected in 295 (34.2%) and 70 (8.1%) isolates, respectively. Among newly diagnosed and follow-up rifampicin-resistant TB (RR TB) patients, the FQ resistance was 25.8% and 44.5%, respectively. The most common mutation (42.7%) in FQ-resistant isolates was MUT3C in *gyrA* gene. Both SLID and FQ resistance were detected in 59 (6.8%) RR TB isolates. The mono SLID resistance was detected in 12 (1.7%) isolates of RR TB. Genotype MTBDRsl Ver 2.0 assay is a rapid and important tool for the diagnosis and molecular characterization of second-line drug resistance under programmatic conditions.

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1. INTRODUCTION

Drug-resistant tuberculosis (TB) remains a main hurdle for national tuberculosis control programs. As per the estimates of World Health Organization (WHO), in 2016, the multidrug-resistant (MDR) TB/rifampicin-resistant (RR) TB was 19% in previously treated patients and 4.1% in newly diagnosed TB patients [1]. The diagnosis of drug resistance for first-line antitubercular drugs is easy due to availability of WHO-endorsed molecular tests like Xpert MTB/RIF and line probe assays (LPAs). The second-line drug resistance detection is a challenge due to very limited WHO-endorsed tests [2]. The most reliable test for second-line drug resistance is phenotypic drug-susceptibility testing (DST) in liquid culture, which is laborious and time consuming.

The nucleic acid amplification methods have very high sensitivity and specificity and also recommended for detection of drug resistance to first-line drugs, mainly rifampicin and isoniazid. Recently, Genotype MTBDRsl Ver 2.0, LPA for fluoroquinolone (FQ) and second-line injectable drugs (SLID) drug resistance detection based on reverse hybridization technology, was endorsed by WHO for rapid detection of second-line drug resistance [3]. The previous version

of this assay, that is, Genotype MTBDRsl Ver 1.0, includes the detection of FQ, SLID, and ethambutol. According to the meta-analysis by Theron et al. [4], the sensitivity and specificity of this assay for FQ and aminoglycoside resistance detection were 83.1% and 76.9%, respectively. The assay version 1.0 was redesigned and new targets were added for FQ (*gyrB*) and kanamycin (*eis*), and the ethambutol targets were removed [5]. The modified assay detects mutations in quinolone resistance determining region of *gyrA* and *gyrB* for FQ and *rrs* and *eis* for aminoglycosides. The assay includes 27 probes based on reverse hybridization technology for detection of FQ and aminoglycoside drug resistance. Gardee et al. [6] evaluated the assay and found it to have a sensitivity and specificity of 100% and 98.9%, respectively, for FQ drug resistance detection. In our earlier evaluation, we observed a sensitivity and specificity of 97.2% and 99.1%, respectively, for FQ resistance detection by Genotype MTBDRsl Ver 2.0 assay, whereas the sensitivity and specificity for detection of SLID resistance were 89.2–92.5% and 98.5–99.5%, respectively. The detection of extensively drug-resistant TB (XDR TB) was increased compared to the previous version of the assay [7].

Genotype MTBDRsl Ver 2.0 assay is included as a frontline test for second-line resistance detection in MDR TB/RR TB or isoniazid-resistant TB in India. In this study, we retrospectively analyzed the resistance pattern and molecular characterization of FQ and aminoglycoside in MDR TB/RR TB or isoniazid-resistant TB.

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2. MATERIALS AND METHODS

2.1. Study Settings

This retrospective analysis was carried out for two centers of TB diagnosis under Revised National Tuberculosis Control Program (RNTCP), India—the Department of Medical Microbiology, Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, and the Intermediate Reference Laboratory, Patiala, Punjab. Both the laboratories are certified by Central TB Division, India, for the testing of molecular and phenotypic drug sensitivity of *Mycobacterium tuberculosis*.

2.2. Clinical Isolates

A total of 863 consecutive drug-resistant isolates (either MDR TB/RR TB or isoniazid-resistant) received between August 2017 and March 2018 were analyzed. The information related to rifampicin and isoniazid resistance was extracted from RNTCP request form. Under programmatic (RNTCP) conditions in India, all the samples from rifampicin and isoniazid or both resistant patients (tested by the first-line LPA—Genotype MTBDRplus Ver 2.0, Hain Lifescience, or Xpert MTB/RIF) were subjected to second-line LPA (Genotype MTBDRsl Ver 2.0, Hain Lifescience) to check the additional resistance to FQs and aminoglycosides. These isolates were from sputum samples received for diagnosis of second-line drug resistance. The samples were processed by NALC NaOH method, followed by inoculation of 500 µl of processed sample in mycobacteria growth indicator tubes (MGIT) tube containing polymyxin B, amphotericin B, nalidixic acid, trimethoprim, azlocillin (PANTA) and growth supplement [8]. The positive culture tube from BACTEC MGIT 960 instrument was identified as *M. tuberculosis* by using SD Bioline MPT 64 Ag kit (Standard Diagnostics, Inc., Republic of Korea). The cultures were processed for Genotype MTBDRsl Ver 2.0 assay. The drug resistance characterization was done by second-line LPA (Genotype MTBDRsl Ver 2.0) only, and no phenotypic DST was performed.

2.3. Genotype MTBDRsl Ver 2.0 Assay

The Genotype MTBDRsl Ver 2.0 assay was performed as per the manufacturer's instructions [5]. In brief, 1 ml of positive liquid culture was centrifuged and the pellet was taken for DNA extraction by GenoLyse kit Hain Lifescience (Nehren, Germany). Before amplification, the kit components AM-A and AM-B were mixed and then the extracted DNA was added and amplified. The hybridization was performed using TwinCubator/GT-Blot and the results were analyzed. The strip contains 27 probes to check internal controls, identification of *M. tuberculosis* complex, and drug targets *gyrA*, *gyrB* for FQ and *rrs*, *eis* for SLID. The missing of wild probe and presence of mutant probe are considered as resistant (Figure 1).

3. RESULTS

Among 863 drug-resistant isolates, 687 were RR and 176 were isoniazid-resistant but rifampicin-sensitive isolates. The age was ranging from 12 to 94 years and there were 319 (36.9%) females

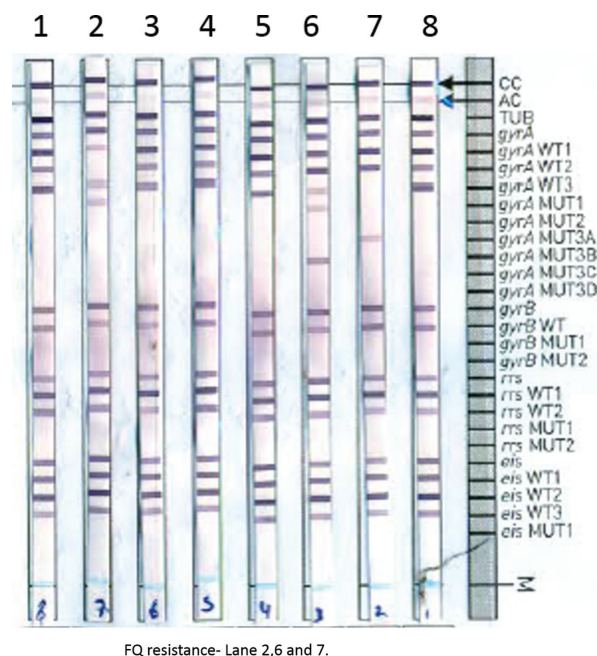


Figure 1 Fluoroquinolones and aminoglycosides drug resistance detection by Genotype MTBDRsl Ver 2.0.

and 544 (63.03%) males. The isolates were obtained by culturing the sputum samples from newly diagnosed and follow-up during the therapy for both RR and/or isoniazid-resistant patients.

3.1. Second-Line Drug Resistance

Among the 863 isolates, 687 were RR including 375 (54.6%) newly diagnosed and 312 (45.4) follow-up isolates. The FQ resistance among RR isolates was detected in 265 (38.6%) isolates, of which FQ resistance among newly diagnosed and follow-up isolates were 97 (25.8%) and 139 (44.5%), respectively. Among 176 isoniazid-resistant isolates, 136 were newly diagnosed and 136 were follow-up isolates. The FQ resistance in isoniazid-resistant but rifampicin-sensitive isolates were 14 (10.3%) and 15 (11%) in newly diagnosed and follow-up isolates, respectively. The SLID resistance was found in 12 (1.7%) in RR isolates, 6 in RR newly diagnosed isolates, and 6 in RR follow-up samples. The SLID resistance was also detected in five isoniazid-resistant isolates, and four newly diagnosed and three follow-up samples were found resistant to SLID. Both FQ and SLID resistance were detected in 59 isolates, 6 in newly diagnosed and 53 in follow-up RR isolates (Table 1).

3.2. Molecular Characterization of Drug Resistance

Among 295 FQ-resistant isolates, 18 different types of banding pattern were observed. The most common banding pattern was MUT3C in 139 cases, including 126 RR samples and 13 isoniazid-resistant samples. The second most common banding pattern was *gyrA* MUT1 in 58 isolates, followed by *gyrA* MUT3A in 30 isolates. The following types of single-defined mutations were detected at *gyrA* codon 94: *gyrA* MUT3C (D94G, 139/324, 42.9%); *gyrA* MUT3A (D94A, 30/324, 9.2%); *gyrA* MUT3D (D94H, 3/324,

0.9%); and *gyrA* MUT3B (D94N/D94Y, 22/324, 6.8%) (Table 2). Other *gyrA*-defined mutations detected at codons 90 and 91 were *gyrA* MUT1 (A90V, 58/324, 17.9%) and *gyrA* MUT2 (S91P, 11/324, 3.4%) (Table 2). Four types of double mutations *gyrA* MUT3A and MUT3C, *gyrA* MUT3B and MUT3D, *gyrA* MUT3B and MUT3C, and *gyrA* MUT1 and MUT3A were found in 8/324 (2.4%) isolates. A total of 26 isolates showed missing of wt probe only, which includes 20, 4, and 2 in WT 3-, WT 2-, and WT 1-2-, respectively. Ten isolates (10/324, 3.1%) missing *gyrB* WT1- showed a low prevalence of *gyrB* mutations in this region. For SLID resistance, 58 defined mutations in *rrs* and *eis* and 18 undefined mutations in *rrs* and *eis* were detected. The most frequently observed mutation (32/73, 43.8%) for KM resistance was *rrs*MUT1 (A1401G); 10 of these isolates indicated the presence of both *rrs* wt and MUT1. The mutation *rrs*MUT2 (G1484T) was observed in 4/73 isolates (Table 2).

4. DISCUSSION

The rapid and accurate diagnosis of second-line drug resistance among RR and isoniazid mono-resistant cases is important for

timely initiation of correct treatment regimen. WHO-endorsed test Genotype MTBDRsl gives rapid and accurate diagnosis for second-line drug resistance detection and has also been started in RNTCP in India. In this study, we demonstrate the use of Genotype MTBDRsl assay for second-line drug resistance detection under programmatic condition in North India. The overall FQ resistance was 38.6% among the RR isolates, which is similar to other studies from India that range from 24 to 59.6%, while the meta-analysis by Ho et al. [13] has shown FQ resistance in MDR patients ranging from 1 to 22%. Among newly diagnosed isolates, the FQ resistance was 25.8% in our study, which was slightly higher than the 22% reported in the recent drug resistance survey carried out in India [14]. Similarly, the FQ resistance in follow-up culture isolates was 44.6% compared to the reported 20.9% in the survey. The overall SLID resistance was 1.7%, which is less than that from other regions of India [15]. Both the FQ and SLID resistance were detected in 8.6% isolates, which is quite higher than reported prevalence of XDR TB worldwide [16]. In our study, we also included the isoniazid mono-resistant isolates and found that the FQ resistance in newly diagnosed and follow-up samples was almost ranging from 10 to 11%. The meta-analysis done by Ho et al. [11] had shown the prevalence of FQ resistance to be

Table 1 | Second line drug resistance profile among rifampicin and isoniazid resistant isolates

Resistance type of isolates	Total	FQ R	FQ S	SLID R	SLID S	FQ and SLID both
Rifampicin resistant newly diagnosed	375	97	272	6	363	6
Rifampicin resistant follow-up	312	139	114	6	139	53
Isoniazid mono-resistant newly diagnosed	136	14	122	2	134	0
Isoniazid mono-resistant follow-up	40	15	25	3	37	

FQ, fluoroquinolones; SLID, second line injectable drug; R, resistant; S, sensitive.

Table 2 | Genotype MTBDRsl Ver 2.0 hybridization pattern observed in resistant isolates

Hybridization band(s) observed	Rifampicin resistant (N)		Isoniazid mono-resistant (N)		Total
	Newly diagnosed	Follow-up	Newly diagnosed	Follow-up	
<i>gyrA</i> gene					
<i>gyrA</i> MUT1	25	27	2	4	58
WT/ <i>gyrA</i> MUT1	1	3	0	0	4
WT1-, WT2-		1	0	1	2
<i>gyrA</i> MUT2	4	7	0	0	11
WT2-	1	2	1	0	4
WT/ <i>gyrA</i> MUT2	1	4	0	0	5
<i>gyrA</i> MUT3A	13	15	2	0	30
<i>gyrA</i> MUT3B	9	12	0	1	22
<i>gyrA</i> MUT3A and MUT3C	0	2	0	0	2
<i>gyrA</i> MUT3B and MUT3D	0	2	0	0	2
<i>gyrA</i> MUT3C	39	87	7	6	139
WT/ <i>gyrA</i> MUT3A	0	1	0	0	1
WT/ <i>gyrA</i> MUT3B	0	1	0	1	2
WT/ <i>gyrA</i> MUT3C	6	6	1	2	15
<i>gyrA</i> MUT3D	0	3	0	0	3
<i>gyrA</i> MUT3B and MUT3C	1	1	0	0	2
<i>gyrA</i> MUT1 and MUT3A	1	1			2
WT3-	2	17	1	0	20
<i>rrs</i> gene					
<i>rrs</i> MUT1	3	27	1	1	32
WT/ <i>rrs</i> MUT1	2	9	0	1	12
WT/ <i>rrs</i> MUT2	2	2	0	0	4
<i>eis</i> gene					
<i>eis</i> MUT1	1	7	0	0	8
<i>eis</i> WT2 absent	4	10	1	0	15
WT/ <i>eis</i> MUT1	0	1	0	1	2

0–4.4% among non-MDR TB patients [11]. The high FQ resistance was also noted in newly diagnosed MDR/RR TB cases, which might be due to the high transmission of the drug-resistant strains. The high rate of FQ resistance in this study is alarming for the national program and indicates injudicious use of FQ. The SLID resistance was detected in 1.7% of RR isolates, which was also quite less than the previously reported 7.7–17% [16,17].

The most common mutation detected by Genotype MTBDRsl in FQ-resistant isolates was a change at codon 94. Among codon 94 mutations, the most prominent mutation was *gyrA* MUT3C in 139 cases, which was comparable to the studies from South Africa, China, and India [6,7,18]. We found the rare *gyrA* MUT3D mutation in three cases that is absent in most of the studies, including the recent study from China [13]. We also found the *gyrB* WT1 missing in 3.1% cases, indicating the presence of *gyrB* mutations in this region. The heteroresistance reported from South Africa was also observed in 27 of our cases [6]. Among the *rrs* mutations, the most common was *rrs*MUT1 found in 32 cases, while *rrs*MUT2 was present in 4 cases. These observations are comparable to the previous studies from India and South Africa [6,7]. We also found the mutations in *eis* region and the most common was *eis*MUT2 absent in 15 cases. The limitation of this analysis lies in the absence of phenotypic DST and sequencing data for confirmation of different drug-resistant related mutations.

In conclusion, the WHO-endorsed rapid method for detection of second-line drug resistance, Genotype MTBDRsl assay, is also able to detect mutations with a short turnaround time, thus enabling early and precise combination therapy decisions.

CONFLICTS OF INTEREST

The authors declare they have no conflicts of interest.

AUTHORS' CONTRIBUTION

SS, RY and PA contributed in conceptualization and writing original draft. RY, JC and NK contributed in data curation. RY, NK and ANA contributed in formal analysis. SS, RK, PA and NK contributed in project administration. NK and RY contributed in methodology. All authors contributed in review draft & editing.

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