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GENOTYPIC CHARACTERIZATION OF FLUROQUINOLONE DRUG RESISTANCE IN MYCOBACTERIUM TUBERCULOSIS BY LINE PROBE ASSAY AT A TERTIARY CARE HOSPITAL IN MUMBAI

Clinical Microbiology

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ABSTRACT

Background: Multidrug-resistant tuberculosis (MDR-TB) remains a significant challenge due to the increasing burden of drug-resistant TB strains. Rapid detection of resistance to second-line drugs is critical for effective patient management. The World Health Organization (WHO) endorsed line probe assay (LPA), GenoType MTBDRsl version 2.0 which is also approved by National Tuberculosis Elimination Program (NTEP) and enables rapid identification of mutations associated with resistance to fluoroquinolone (FQ) and second-line injectable drugs (SLID).**Methods:** This was a prospective study from January 2023 to December 2023. A total of 763 Mycobacterium tuberculosis (MTB) samples from clinically suspected MDR-TB cases, including 178 MDR- TB, 10 mono rifampicin resistant and 43 isoniazid mono-resistant isolates, were tested for resistance to FQ and SLIDs by second line-line probe assay (SL-LPA) using Genotype MTBDRsl Ver 2.0 according to the manufacturer's instructions. **Results:** Resistance to FQ and SLID was detected in 135 (58%.4) and 15 (6.5%) isolates, respectively. The most common mutation among FQ- resistant isolates was MUT3C in the gyrA gene (57%) which is associated with high level resistance to FQ. Concurrent resistance to both FQ and SLIDs was identified in 13 (5.6%) isolates, while mono- resistance to SLIDs was detected in 2 (0.8%) isolates. Conclusion: LPA is a rapid and reliable tool for the diagnosis and molecular characterisation of second-line drug resistance in MTB and is valuable for use under programmatic conditions.

KEYWORDS

Multidrug-resistant tuberculosis (MDR-TB), line probe assay (LPA), Mycobacterium tuberculosis, fluoroquinolone.

INTRODUCTION

Drug-resistant tuberculosis (TB) continues to pose a major challenge even to today's times. According to World Health Organization (WHO) estimates from 2023, multidrug-resistant (MDR) or rifampicin-resistant (RR) TB accounted for 16% of previously treated TB cases and 3.2% of newly diagnosed cases [1]. Detection of resistance to first-line antitubercular drugs is relatively straightforward due to the availability of WHO-endorsed molecular diagnostics, such as Xpert MTB/RIF and line probe assays (LPAs). In contrast, identifying resistance to second-line drugs remains difficult because of the limited number of WHO-endorsed diagnostic tests [2]. Phenotypic drug-susceptibility testing (DST) using liquid culture is considered the gold standard for detecting second-line drug resistance; however, it is labor-intensive and time-consuming.

Nucleic acid amplification techniques demonstrate high sensitivity and specificity and are recommended for detecting resistance to first-line drugs, particularly rifampicin and isoniazid. The WHO endorsed the Genotype MTBDRsl version 2.0 assay, a SL-LPA based on reverse hybridization technology, for the rapid detection of resistance to fluoroquinolones (FQs) and second-line injectable drugs (SLIDs) [3]. The earlier version of this assay, Genotype MTBDRsl version 1.0, targeted resistance to FQs, SLIDs, and ethambutol. A meta-analysis by Theron et al. [4] reported sensitivities of 83.1% for FQ resistance and 76.9% for aminoglycoside resistance detection using version 1.0. Subsequently, the assay was redesigned to include additional targets for FQs (gyrB) and kanamycin (eis), while ethambutol targets were removed [5]. The updated assay detects mutations in the quinolone resistance– determining regions of gyrA and gyrB for FQ resistance, and in rrs and eis for aminoglycoside resistance, utilizing 27 probes through reverse hybridization technology.

Evaluation studies have demonstrated improved performance of the revised assay. Gardee et al [6] reported sensitivity and specificity of 100% and 98.9%, respectively, for FQ resistance detection. Yadav R et al [7] reported that Genotype MTBDRsl version 2.0 assay showed sensitivity and specificity of 97.2% and 99.1% for FQ resistance, while sensitivity and specificity for SLID resistance ranged from 89.2–92.5% and 98.5–99.5%, respectively. Additionally, detection of extensively drug- resistant TB (XDR-TB) was higher with version 2.0 compared to the earlier version.

In India, the Genotype MTBDRsl version 2.0 assay has been implemented as a frontline diagnostic tool for detecting second-line drug resistance in MDR-TB/RR-TB and isoniazid-resistant TB cases under the NTEP. In the present study, we analysed resistance patterns and conducted molecular characterization of FQ resistance among MDR- TB/RR-TB and isoniazid-resistant TB patients.

Materials and Methods

Ethics statement: This study was conducted after obtaining ethical clearance from the Institutional Ethics Committee, Grant Government Medical college & Sir JJ Group of Hospitals, Mumbai vide letter number IEC/PG/969/NOV/2022 dated November 17, 2022.

Study setting: This prospective observational study was conducted at TB culture & drug-susceptibility testing (DST) laboratory operating under the NTEP, India at the Department of Medical Microbiology, Grant Government Medical college & Sir JJ Group of Hospitals, Mumbai. The laboratory is certified by the Central TB Division, Government of India, for performing molecular and phenotypic DST of MTB.

Inclusion criteria: All CBNAAT MTB detected pulmonary & extra-pulmonary samples from clinically suspected MDR-TB cases.

Sample processing:

From a total of 763 MTB detected samples on CBNAAT, 231 samples were drug-resistant *Mycobacterium tuberculosis,* including multidrug- resistant (MDR-TB), mono rifampicin-resistant (RR-TB) and mono isoniazid-resistant TB (Hr-TB), were included in the analysis. Under programmatic conditions in India, samples from patients identified as rifampicin-resistant, isoniazid-resistant, or resistant to both drugs— based on first-line testing using Genotype MTBDRplus version 2.0 (Hain Lifescience) or Xpert MTB/RIF—are routinely subjected to second-line line probe assay (Genotype MTBDRsl version 2.0, Hain Lifescience) to assess additional resistance to fluoroquinolones (FQs) and aminoglycosides.

All samples were initially subjected to decontamination using the N-acetyl-L-cysteine–sodium hydroxide (NALC–NaOH) method followed by acid-fast bacilli (AFB) smear microscopy and cartridge-based nucleic acid amplification testing (CBNAAT). Of the 763 samples included in the study, 534(70%) were smear-positive and were therefore processed directly by First Line LPA(FL-LPA) followed by SL-LPA based on resistance pattern. The remaining 229 samples (30%), which were smear-negative, were subjected to indirect FL-LPA following culture positivity using the Mycobacterial Growth Indicator Tube (MGIT) 960 system followed by SL-LPA based on resistance pattern.

Genotype MTBDRsl Version 2.0 Assay

The Genotype MTBDRsl version 2.0 assay was performed in accordance with the manufacturer's instructions [5]. Briefly, 1 mL of positive liquid culture was centrifuged, and the resulting pellet was used for DNA extraction using the GenoLyse kit (Hain Lifescience, Nehren, Germany). Prior to amplification, the amplification mixes AM-A and AM-B were combined, after which the extracted DNA was added and subjected to polymerase chain reaction.

Following PCR amplification, the amplicons were subjected to reverse hybridization using TwinCubator/GT-Blot system with specific oligonucleotide probes immobilized on nitrocellulose strips. Each strip contained 27 reaction zones. The developed strips were interpreted by alignment with the respective internal control and probes for identification of the *Mycobacterium tuberculosis* complex. The conjugate control (CC) band confirmed proper conjugate function, while the amplification control (AC) band validated successful amplification. A test result was considered positive when specific mutation or wild-type bands corresponding to the target genes (e.g., *gyrA* and *gyrB* for fluoroquinolone resistance, and *rrs* and *eis* for resistance to second-line injectable drugs) were present along with the CC and AC bands.

Resistance was inferred by the absence of a corresponding wild-type probe and the presence of a mutation-specific probe, as illustrated in figures:1

Figure: 1

RESULTS

A total of 763 samples were subjected to First Line LPA(FL-LPA), among them 532 were Drug sensitive TB, 178 were MDR-TB, 10 were mono rifampicin-resistant (RR-TB), while 43 were mono isoniazid-resistant (Hr- TB). (Table 1)

Table 1: showing FL-LPA results analysis (Total = 763)

FL-LPA	NO. OF SAMPLES(Total=763)
Sensitive to both Rif & Inh	532
Resistant to both Rif & Inh	178
Mono Rif resistant	10
Mono Inh resistant	43

Among 231 DR-TB isolates which were subjected to SL-LPA , Fluoroquinolone (FQ) resistance detected in 135 (58.6%) cases. Resistance to second-line injectable drugs (SLIDs) was identified in 15 (6.49%) isolates. Concurrent resistance to both FQs and SLIDs was observed in 13(5.62%) DR-TB isolates. (Table 2)

Table 2: showing SL-LPA results analysis (Total = 231)

SL-LPA	No. of samples(Total= 231)
FQ Sensitive	86(37.2%)
SLID Sensitive	216(93.5%)
FQ & SLID Sensitive	71(30.7%)
FQ Resistant	135 (58.6%)
Inferred FQ Resistant	10(4.3%)
SLID Resistant	15(6.49%)
Concurrent resistance to FQ & SLID	13(5.62%)

Molecular Characterization of Fluroquinolone Drug Resistance

Among the 135 fluoroquinolone (FQ)-resistant isolates, 09 distinct banding patterns were identified. The most frequently observed pattern was gyrA MUT3C, detected in 77 isolates which is associated with high level resistance to FQ. The second most common pattern was gyrA MUT1, identified in 39 isolates which is associated with low level resistance to FQ. Other low level resistance associated mutations were gyrA MUT3A in 10 isolates and gyrA MUT2 in 03 isolates. High level resistance associated mutations were gyrA MUT3B in 05 isolates, and gyrA MUT3B-3C in 01 specimen.

Single, well-defined mutations at gyrA codon 94 were the most prevalent and included gyrA MUT3C (D94G; 77/135, 57%), gyrA MUT3A (D94A; 10/135, 7.4%), and gyrA MUT3B (D94N/D94Y; 05/135, 3.7%).

Additional gyrA mutations were detected at codons 90 and 91, comprising gyrA MUT1 (A90V; 39/135, 28.9%) and gyrA MUT2 (S91P; 03/135, 2.2%). A double mutation gyrA MUT3B and MUT3C—were observed in 01/135 isolates(0.7%). A total of 10(4.3%) isolates exhibited loss of wild-type (WT) probe bands only, including 06 isolates with missing WT3, 02 with missing WT2 bands. Mutations involving gyrB were uncommon; 02 isolates showed absence of the gyrB WT1 probe, indicating a low prevalence of mutations in this region. (Table 3)

Table 3: Genotype MTBDRsl Ver 2.0 hybridization pattern observed in FQ resistant isolates (Total= 145)

Hybridization bands observed	No. of samples	Interpretation for FLQ
MUT1 (gyrA gene)	39	Low level resistance
MUT2 (gyrA gene)	03	Low level resistance
MUT3A (gyrA gene)	10	Low level resistance
MUT3B (gyrA gene)	05	High level resistance
MUT3C (gyrA gene)	77	High level resistance
MUT3B-3C (gyrA gene)	01	High level resistance
WT3 (gyrA gene)	06	Resistance inferred
WT2(gyrA gene)	02	Resistance inferred
WT1(gyrB gene)	02	Resistance inferred

DISCUSSION

Rapid and accurate identification of second-line drug resistance among first line drug resistant TB cases is critical for the timely initiation of appropriate treatment regimens. The WHO-endorsed Genotype MTBDRsl assay enables rapid and reliable detection of resistance to second-line anti-tubercular drugs and has been implemented under the NTEP in India. In the present study, we assessed second-line drug resistance under routine programmatic conditions and characterized molecular gene patterns associated with FQ resistance. The overall prevalence of FQ resistance among MDR-TB isolates in this study was 58.6%, which is comparable to findings from other regions of India, where reported rates range from 24% to 59.6%. This observation was also consistent with the study done by Bhanushali et al in Mumbai, India [13]. In contrast, a meta-analysis by Ho et al [14] documented considerably lower FQ resistance rates among MDR-TB patients globally, ranging from 1% to 22%, suggesting possible amplification of resistance during treatment.

Resistance to second-line injectable drugs (SLIDs) was detected in 6.49% of MDR TB isolates, a proportion at par with that reported from other regions of India [15]. However, concurrent resistance to both FQs and SLIDs was identified in 5.6% of isolates, which is lower than the globally reported prevalence of extensively drug-resistant tuberculosis (XDR-TB) but comparable with the Indian data [16]. These findings highlight a concerning burden of advanced drug resistance within the study population.

Molecular analysis revealed that the most common mutation associated with FQ resistance occurred at gyrA codon 94. Among these, the gyrA MUT3C mutation was predominant, detected in 77 isolates, consistent with reports from studies conducted in South Africa, China, and India [6,7,17]. This is similar to the results of a study done in India by Yadav Narayan et al [18]. In another study in India by Singhal et al [19] out of 25/152 isolates had mutation in the gyrA gene with most common mutation at D94G. Bhanushali et al conducted a study in Mumbai in the year 2022 where 70 samples were resistant to FQ. Additionally, loss of the gyrBWT1 probe was observed in 2 of isolates, indicating the presence of gyrB mutations in this region.Furthermore, phenotypic drug susceptibility testing and whole genome sequencing need to be carried out which will provide confirmatory evidence for the detected resistance-associated mutations.

CONCLUSION

In this study among 231 DR-TB samples, 135 cases were Pre-XDR TB. Most common gene mutation associated with low level fluoroquinolone resistance was gyrA WT2 MUT1 A90V in 39/135(28.8%) and high-level fluoroquinolone resistance was associated most with gyrA WT3 MUT3C D94G in 77/135(57%)

mutation. In this context, the World Health Organization (WHO) recommends that all patients with confirmed rifampicin-resistant tuberculosis be promptly initiated on a MDR-TB treatment regimen, even in the absence of complete drug-susceptibility testing results. LPA provide a rapid means of identifying additional drug resistance thereby enabling the early initiation of appropriate and individualized therapy.

The high prevalence of fluoroquinolone (FQ) resistance observed among MDR/RR-TB cases in this study reflects the rapid emergence of resistance to this critical class of anti-tubercular drugs. This trend is largely attributable to the widespread and often indiscriminate use of FQs for a variety of infections. The findings highlight a concerning rise in FQ resistance, which threatens the effectiveness of currently WHO recommended MDR-TB treatment regimens such as all-oral short 6-month BPaLM regimen (bedaquiline, pretomanid, linezolid, and moxifloxacin), moxifloxacin which is FQ is included as part of the core drug combination for eligible patients.

Given the substantial burden of FQ resistance identified among first line drug resistant TB cases, LPA represents a highly valuable diagnostic tool for detection of second-line drug resistance and associated genetic mutations, offering a short turnaround time that facilitates early, accurate treatment decisions and optimized combination therapy particularly in high-burden settings such as ours, where access to advanced diagnostic infrastructure may be limited.

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