ORIGINAL ARTICLE

Concordance Between Phenotypic Resistance to Fluoroquinolones and *gyrA* Mutations among Rifampin-Resistant Isolates of *Mycobacterium Tuberculosis* Complex from Pakistan

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Abstract

Background
Fluoroquinolones (FQ) are the cornerstone of treatment for Rifampicin Resistant (RR TB). Here we investigate whether FQ resistance detected by line probe assay (LPA) shows good concordance with phenotypic susceptibility testing.

Methods
Mycobacterium tuberculosis isolates were collected from clinical samples received in clinical Microbiology Laboratory, Aga Khan University, Karachi between January 2016 and February 2017. RR TB isolates were grown in culture and those who were resistant to ofloxacin (OFX) were selected for the present study. Minimum inhibitory concentrations (MICs) were performed for levofloxacin (LEV), moxifloxacin (MXF) and OFX using pre-prepared frozen plates (Thermo Fisher Scientific Inc., 194 Waltham, MA, USA). DNA was extracted using Genolyse® extraction kit (HAIN life sciences, Germany). For the detection of mutations, line probe assay Genotype MTBDRsl version 2 (HAIN Lifesciences, Germany) was used.

Results
From the total of 51 MDR TB stains that were included, majority of patients (n=29, 56.9%) were from Punjab province, 35.3% from Sindh province (n=18), and 5.9 %(n=3), and 1.9% (n=1) from Khyber Pakhtunkhwa and Baluchistan respectively. Concordance between genotypic resistance detection by LPA, and the phenotypic resistance detection by MICs to FQ (any one of OFX, LEV, or MXF) was observed in 84.3% of the isolates (n=43). The most common mutation identified was D94G in the *gyrA* gene in 50.9% of isolates (n=26). No *gyrB* mutations were detected. MIC testing showed high level of cross resistance between LEV/OFX and MFX MICS, with only 11/51 (21.6%) LEV/OFX resistant strains demonstrating MFX MICS of <1 µg/ml.

Conclusion
LPA method is a rapid and reliable method to identify resistance to FQ in MTB. However, for determination of susceptibility to individual FQs, further testing should be performed via phenotypic methods for confirmation.

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Keywords
Rifampin resistant, *Mycobacterium tuberculosis*, Line probe assay, gyrA, Fluoroquinolones

Introduction
Tuberculosis, caused by *Mycobacterium tuberculosis* complex (MTB), is a significant cause of morbidity and mortality throughout world. As of the year 2018, it is estimated that 10 million people got infected with the infection and from them 1.5 million people died[1] Mortality rates are higher for rifampin resistant tuberculosis (RR TB)[2]. In Pakistan, the incidence of RR TB in 2018 was 13 cases per 100 000 population[1], resulting in a high expected mortality burden. Fluoroquinolones (FQ) remain the main treatment option for RR TB.[3] Detection of fluoroquinolone resistance through rapid tests such as line probe assay (LPA) is valuable to direct therapy in such patients, and to identify genetic mutations which confer resistance to all antituberculous fluoroquinolones.

DNA gyrase subunit A and B changes in the fluoroquinolone resistance determining gene (QRDR) brings about resistance. Studies have shown multiple types of changes in the *gyrA* and *gyrB* mutation are correlated to the fluoroquinolone resistance.[4,5] However, the concordance of genetic drug resistance markers with the minimum inhibitory concentrations of FQ among clinical isolates from Pakistan has not been widely studied. Minimum inhibitory concentrations (MICs) of levofloxacin/ofloxacin (LEV/OFX) and moxifloxacin (MFX) are necessary to optimize therapeutic regimens used for RR TB.[3] Critical concentrations for these drugs by MGIT may also be used or even genotypic methods. Here, we have correlated the FQ MIC of LEV/OFX and MFX with *gyrA* and *gyrB* mutations in randomly selected RR MTB strains from Pakistan.

Materials & Method
Clinical strains of MTB isolated from samples received at the Clinical Microbiology Laboratory of the Aga Khan University in Karachi were included in the study. Culture was performed on pulmonary and extra pulmonary specimens with methods

described previously.[6] Briefly, culture was set up using *Mycobacterial* Growth Indicator Tube (MGIT) and 7H10 (Middlebrook) agar after digestion-decontamination 5 %N-acetyl-L-cysteine (NALC)/ (Sodium hydroxide) NaOH high speed centrifugation.

The susceptibility testing was carried out using the agar proportion method, as described previously.[6] Rifampin and ofloxacin resistance was determined using final concentrations of 1 µg/ml of rifampin and 2 µg/ml of ofloxacin in 7H10 agar, respectively. n=51 randomly selected RR and OFX resistant isolates form the year 2016-2017 were included in the study.

MIC testing for fluoroquinolones: MIC testing was performed for ofloxacin, levofloxacin, and moxifloxacin at concentration ranges of 0.12-8, 0.12-4, and 0.06-4 µg/ml using frozen and dried plates for broth microdilution in 7H9 medium (Thermo Fisher Scientific Inc., 194 Waltham, MA, USA) as part of the Bedaquiline DREAM Bedaquiline Drug Resistance Emergence Assessment in MDR-TB (Bedaquiline DREAM Program) project, as recently described by Kone *et al*.[7] FQ MICs were categorized as susceptible or resistant according to drug susceptibility criteria proposed by the Clinical Laboratory Standards Institute M62.[8] FQ MICs were categorized as susceptible or resistant according to drug susceptibility criteria proposed by the Clinical Laboratory Standards Institute M62 for commercial shorter incubation period liquid media systems[8] The critical concentration for LVX, MXF are 1.5 µg/mL and 0.25 µg/mL respectively. Ofloxacin testing is not recommended as it is not used for MDR TB, and specific fluoroquinolone has to be tested. But during the transition period, the recommended critical concentration in liquid system is 2 µg/mL.

Line Probe Assay: DNA was extracted from bacterial growth from isolated colonies that were inoculated into MGIT. The purity of isolates were ensured as these were initially collected from purity plate of agar sensitivity reporting. The DNA extraction was performed using GenoLyse®kit (Hain Lifesciences, Germany) as per manufacturer's instructions (https://www.hain-lifescience.de/en/products/dna-isolation/genolyse.html). The wild type probes used were wild type 1(codon 88), wild type 2(codon 90, 91) and wild type 3(codon 94).

Line Probe Assay was performed using Genotype MTBDR sl 96 version 2(Hain life sciences, Germany)[9] according to manufacturer kit instructions.

Quality control: Susceptible H37Rv MTB strain was used as quality control for all procedures, including agar proportion, broth microdilution MIC testing, and LPA.

Ethical review: The study approved by the ethical review committee of Aga Khan University, Karachi (no: 2019-2178-7005), and exempted from patient informed consent. All isolates used were delinked from patient identifiers and used.

Results

Description of isolates: Of the 51 RR MTB isolates, a total of n= 29 were isolated from samples received from Punjab province, n=18 from Sindh province, n=3 from Khyber Pakhtunkhwa, and n=1 from Balochistan province.

The age range from which samples were collected include patients from 6 years to a maximum age of 75 years of age. The median age range was of 30 years (interquartile range of 23.5- 44). There were a total of 4 extra pulmonary (3 pus and 1 cervical lump) and remaining were from pulmonary sites (n=41 sputum and n=6 bronchial wash). The male-female ratio was 30:21.

Correlation of FQ phenotypic resistance by MIC with genotypic resistance by LPA:

All isolates tested by MIC were resistant to at least 1 FQ tested. Concordance between genotypic resistance detection by LPA, and phenotypic resistance detection by MICs to FQ (any one of OFX, LEV, or MXF) was observed in 84.3% isolates (n=43). Of the 51 isolates, n=48/51 were resistant to LEV and n=49 were resistant to MXF. The OFX MIC=1 was seen in 3 isolates, OFX MIC 2-4 in n=4, OFX MIC =8 in n=44.

No mutations were detected in 8 (15.7%) isolates. Based on MIC results, the concordance between genotypic resistance detection by LPA and OFX resistance at 2 µg/mL was 87.5% (42 of 48 isolates), while for LEV at ≥1 µg/mL concordance was 89.6% (43 of 48 isolates), and for MXF at ≥ 1 µg/mL concordance was 86.7% (39 of 45 isolates).

In 4 isolates (7.8%), genotypic resistance was detected only on the basis of the absence of wild type probe binding, while specific mutations were identified in 39 isolates (76.5%). These specific mutations corresponded to the detected mutation in 33 (84.6%) isolates, while in 6 (15.4%) isolates the results were considered to be due to possible heteroresistance in the MTB strain. Heteroresistance in MTB isolates is a well know phenomenon, defined as the coexistence of different subpopulations with varying genetic resistance mechanisms within one strain.[10]

Asp94Ala substitution (D94G) was the most frequently observed mutation among these isolates, detected in 26 of 51 isolates (51%).[11] Table 1 shows the results of line probe assay on 51 study isolates, as well as distribution of mutations detected, in relation to MICs of OFX, LEV, MXF.

High level of cross resistance between LEV/OFX and MFX MICS was observed, with only 11/51 (21.6%) LEV/OFX resistant strains demonstrating MFX MICS of <=1 µg/ml.

No specific mutation correlated with MXF MICs of <1 µg/mL;

Table 1: Distributions of mutations and fluoroquinolone MICs in 51 clinical isolates of rifampin-resistant Mycobacterium tuberculosis from Pakistan

Mutation	Aminoacid substitution at locus	Number of isolates MIC (µg/mL)											TOTAL
		OFX			LEV			MXF					
		≤1	2-4	≥8	≤1	2	≥4	0.25	0.5	1	2	≥4	
D94G	Asp94Gly	-	1	22	-	-	23	-	1	5	13	4	23
A90V	Ala90Val	-	2	4	-	-	6	-	2	2	2	-	6
D94A	Asp94Ala	-	-	1	-	-	1	-	-	1	-	-	1
D94N/Y	Asp94Asp/Tyr	-	-	4	-	-	4	-	-	-	1	3	4
S91P	Ser91Pro	-	-	1	-	1	-	-	-	-	1	-	1
D94H	Asp94His	-	-	1	-	-	1	-	-	-	-	1	1
D94G, A90V	Asp94Gly, Ala90Val	-	-	1	-	-	1	-	-	-	1	-	1
D94N/Y, D94G	Asp94Asp/Tyr, Asp94Gly	1	-	1	-	-	2	-	1	-	-	1	2
Absence of WT¶	-	-	-	4	-	-	4	-	-	-	4		4
WT Pattern	-	2	1	5	3	2	3	2	-	-	5	1	8
													51

¶Absence of Wild Type sequence only, no specific mutation detected

in 2 isolates no mutation was detected, while in 4 isolates, D94G (n=1), A90V (n=2) and both D94G with D94N/Y (n=1) were detected.

Discussion

As FQ are the antituberculous agents of choice in the treatment of rifampin resistant tuberculosis, rapid and reliable identification of resistance can further guide treatment regimens. We observed that in RR MTB clinical isolates testing resistant to any one fluoroquinolone, LPA detected resistance in a substantial proportion of isolates. This shows that LPA testing on samples will be able to identify FQ drug resistance and predict phenotypic FQ resistant results in majority of patients. However, susceptibility to individual FQs should be confirmed with phenotypic testing methods on isolates obtained in culture. This is because the line probe assay has been shown to give raise to false resistance if there were synonymous mutations present that could lead to the non- binding of the probes.[12, 13]

We identified D94G to be the most common detected mutation in our study. This mutation is also the most prevalent globally, and has also been reported with high frequency from genomic sequencing databases from Pakistan.[14] The D94G mutation is associated with a low cure rate and higher MICs.[10]

We also observed a high rate absent wild type with no mutations detected. This implies that isolates from Pakistan have additional specific mutations that are not identified by LPA. Genomic

analysis of the QRDR region on these isolates will identify these additional specific mutations.

Heteroresistance in MTB isolates is a well know phenomenon, defined as the coexistence of different subpopulations with varying genetic resistance mechanisms within one strain.[10] LPA is not the ideal method to detect heteroresistance. However, presence of wild type gene mutation along with detection of a mutation signifies the presence of variants which point to heteroresistance. Since we performed LPA on pure MTB isolates in culture, we concluded that the results are due to heteroresistance and not a mixed infection.

Newer generation FQ such as MXF have greater activity against RR MTB strains. The ability of LPA results to predict susceptibility to MFX is therefore of interest. We found a high proportion of FQ resistant isolates to be resistant to MFX(n=49/51, 86.7%), perhaps due to greater population use of MXF. The overall levofloxacin resistance has been shown to be higher among studies from multiple countries including Pakistan i.e. : 87% moxifloxacin (72%) from among 282 tested isolates.[15] Nevertheless, to determine whether a higher dose of MXF can be used in RR MTB infections, more studies are needed with pharmacokinetic and pharmacodynamic data to determine the optimal dosing in correlation with MIC cutoffs.

This study has several limitations. The sample size is small. Clinical isolates were cultured from samples received at a reference laboratory center and therefore likely to have a higher rate of resistance. Isolates selected for the study were resistant to ofloxacin and therefore results do not apply to fluoroquinolone sensitive isolates. This study used the critical concentrations for MGIT for interpretation although this cannot be implemented accurately for clinical application. However, studies have attempted to establish clinical epidemiological cut offs for broth microdilution methods.[16] We also did not perform genome sequencing of isolates to confirm the resistance detected only in the basis of absence of wild type *gyrA*.

Conclusion

This study shows that genotypic testing by line probe assay for concordance between *gyrA* and fluoroquinolone is reliable and can be used to guide therapy in patients with MDR TB. It shows 84.3% concordance with the MICs. A high level of cross resistance between LEV/OFX and MXF MICs was also noted in this study.

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