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DRUG DISCOVERY AND RESISTANCE

Correlating Minimum Inhibitory Concentrations of ofloxacin and moxifloxacin with *gyrA* mutations using the genotype MTBDR*sl* assay

Priti Kambli [a], Kanchan Ajbani [a], Meeta Sadani [a], Chaitali Nikam [a], Anjali Shetty [a], Zarir Udwadia [b], Timothy C. Rodwell [c], Antonino Catanzaro [c], Camilla Rodrigues [a, *]

[a] *Section Microbiology, Department of Laboratory Medicine, P.D. Hinduja National Hospital and Medical Research Center, Mumbai, India*
[b] *Section Pulmonology, Department of Medicine, P.D. Hinduja National Hospital and Medical Research Center, Mumbai, India*
[c] *University of California, San Diego, Department of Medicine, San Diego, CA, USA*

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SUMMARY

Objective: To correlate *gyrA* mutations found on the Genotype MTBDR*sl* assay in *Mycobacterium tuberculosis* (*MTB*) isolates with Minimum Inhibitory Concentrations (MICs) to the fluoroquinolones compounds ofloxacin (OFX) and moxifloxacin (MXF).
Methods: MICs for OFX and MXF were ascertained for 93 archived clinical *MTB* isolates that showed *gyrA* mutations at Ala90Val, Ser91Pro, Asp94Ala, Asn/Tyr, Gly and His. Thirty fluoroquinolones susceptible isolates as determined by presence of all wild-type *gyrA* bands on the Genotype MTBDR*sl* assay were also included.
Results: *gyrA* mutations at Ala90Val ($n = 25$), Ser91Pro ($n = 6$), Asp94Ala ($n = 4$), Asp94Asn/Tyr ($n = 13$), Asp94Gly ($n = 42$) and Asp94His ($n = 3$) were observed. Isolates with mutations at Ala90Val or Ser91Pro had MIC_{90} of 4.0 μg/ml and 1.0 μg/ml for OFX and MXF, respectively, and isolates with mutations at Asp 94Ala, Asn/Tyr, Gly and His had MIC_{90} of 8.0 μg/ml, and 2.5 μg/ml for OFX and MXF, respectively.
Conclusions: *MTB* MICs were found to be consistently lower for MXF than for OFX among isolates with the same *gyrA* mutation (e.g. Ala90Val). The majority of *MTB* isolates containing mutations at Asp94Ala, Asn/Tyr, Gly and His in *gyrA* were associated with a moderate level of resistance to MXF (MIC = 2.5 μg/ml), although 3 isolates with the mutations Asp94Asn/Tyr/Gly were associated with a high level of resistance to both fluoroquinolones (MXF MICs = 5.0–8.0 μg/ml, OFX MICs = ≥10.0 μg/ml).

1. Introduction

Drug resistance in *Mycobacterium tuberculosis* (*MTB*) is a major public health issue. The global burden of tuberculosis (TB) is high [1]. There is growing concern regarding the rise and spread of multi- and extensively drug-resistant TB (M/XDR-TB) [2]. The recent appearance of untreatable, or totally drug-resistant, TB (TDR-TB) has further underscored the urgent need for efficient and effective TB control efforts [3]. Currently, clinical diagnosis of drug-resistant TB relies upon slow growth culture and drug susceptibility testing (DST) of *MTB* isolates via liquid culture Mycobacteria Growth Indicator Tube (MGIT) platforms, yielding phenotypic resistance profiles of these isolates [4,5]. Molecular diagnosis of M/XDR-TB, in contrast, is based on the rapid detection of genetic mutations known to confer resistance to the given drugs of interest [7]. Unfortunately, both phenotypic DST at critical concentrations as well as molecular diagnostic methods are qualitative, giving rise to clinical uncertainties regarding the optimal drug dosing required to treat patients who may have different levels of resistance to the different TB treatment drugs [8,9].

Fluoroquinolones represent an effective class of bactericidal second-line drugs for the treatment of MDR-TB [1,6]. However, phenotypic resistance to these compounds have been shown to be heterogeneous in *MTB* isolates, varying from low level resistance (suggesting that infections might still be treated effectively with increased concentrations of, or alternative, fluoroquinolones), to high-level resistance (where *MTB* cannot be cleared with any fluoroquinolones) [10]. Although phenotypic DST with fluoroquinolones specific Minimum Inhibitory Concentrations (MICs) is a valid way to estimate fluoroquinolones resistance, it is a

* Corresponding author. P.D. Hinduja Hospital & Medical Research Centre, Veer Sarvarkar Marg, Mahim, Mumbai 400016, India. Tel.: +91 22 24447795; fax: +91 22 24442318.
E-mail address: dr_crodrigues@hindujahospital.com (C. Rodrigues).

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somewhat inefficient means of characterizing drug resistance due to the time required for such methodology [8] and the laboratory limitations of most low-resource clinical settings. Genetic diagnostic technologies have the ability to overcome these barriers. Correlating specific mutations conferring drug resistance with specific MICs for given drug classes are essential before successful implementation of these technologies become a reality.

There are additional clinical benefits in clearly defining the relationship between resistance-associated mutations found in the *gyrA* gene and fluoroquinolones phenotypic resistance profiles. When patients with MDR-TB are compared to patients with MDR-TB that have additional fluoroquinolones resistance, those with fluoroquinolones resistance appear to have a more serious form of disease, in that treatment success becomes less common, and the risk of developing XDR-TB increases [1]. Cross-resistance to the fluoroquinolones is frequent and fluoroquinolones resistance in *MTB* is usually associated with mutations in the conserved quinolone resistance-determining region (QRDR) of *gyrA*, particularly at codons 90 and 94 [12–15]. Previous studies have confirmed that the presence of these different mutations in *MTB* genes correlate with different levels of resistance [11], and we sought to further explore this relationship, considering a MICs range for susceptible *MTB* of 0.0625–0.25 μg/ml for moxifloxacin (MXF) and 0.5–2.0 μg/ml for ofloxacin (OFX) [16–18].

In this study, we estimated the fluoroquinolones MICs of 123 clinical *MTB* isolates and correlated those MICs with specific *gyrA* mutations on the Genotype MTBDR*sl* assay found within the isolates to determine whether these commonly occurring mutations were associated with different levels of fluoroquinolones resistance.

2. Materials and methods

2.1. Study population

The study was performed at the Mycobacteriology Laboratory of the P. D. Hinduja National Hospital (PDHNH) and Medical Research Centre (MRC), a tertiary care hospital in Mumbai, India with a referral bias towards non-responders [19]. The study was approved by the Institutional Review Board (IRB) of Hinduja Hospital. Written consent was waived for all participants as the study was carried out on 123 archived isolates for which both Genotype MTBDR*sl* assay and DST at the WHO approved critical concentrations had been performed previously.

2.2. Phenotypic DST

Quantitative drug susceptibility testing was conducted using MGIT960:

Preparation of Drugs: A stock solution of OFX (Sigma Aldrich 08757) was prepared by dissolving the drug in 0.1 N NaOH. MXF (Sigma Aldrich 32477) was prepared by dissolving the compound in distilled water. Both drugs were filtered, further diluted in distilled water, and stored at −80 °C for up to 6 months. Preparation of the inoculum, as well as inoculation and incubation were performed as per manufacturer instructions (Becton Dickinson Diagnostic System, Sparks, MD) [20].

2.3. Selection of drug concentrations for MICs

Six MICs were selected in order to fully define the phenotypic resistance profiles of the *gyrA MTB* isolates. A critical concentration of 2.0 μg/ml was used for OFX and 0.25 μg/ml was used for MXF, in accordance with WHO recommendations [21]. For estimation of OFX MICs, two concentrations below the critical concentration (0.5 and 1.0 μg/ml), and three concentrations above the critical concentration (4.0, 8.0 and 10.0 μg/ml), were utilized. For estimation of MXF MICs, two concentrations below the critical concentration (0.0625and 0.125 μg/ml), and three concentrations above the critical concentration (0.5, 1.0 and 2.5 μg/ml) were used. For MXF, if any isolate demonstrated resistance at 2.5 μg/ml, then we also tested those isolates ($n = 3$) at three additional higher concentrations 5, 8 and 10 μg/ml.

2.4. MIC_{50} and MIC_{90} calculation

In this study, MIC_{50} was defined as the fluoroquinolones drug concentration that inhibited 50% of the isolates from each mutation group and MIC_{90} was defined as the fluoroquinolones drug concentration that inhibited 90% of the Isolates from each mutation group. MIC_{50} and MIC_{90} were calculated as follows:

MIC_{50} = no. of isolates (n) × 0.5 and MIC_{90} = no. of isolates (n) × 0.9.

2.5. Genotype MTBDR*sl* assay

A 123 consecutive archived isolates for which both Genotype MTBDR*sl* assay and DST at the WHO approved critical concentrations had been performed previously were selected.

Genotype MTBDR*sl* assay was processed as follows:

DNA extraction was performed on all decontaminated patient samples using a GenoLyse kit (Hain Lifescience, Nehren, Germany). Multiplex polymerase chain reaction (PCR) amplification was then conducted utilizing biotinylated primers. PCR was performed with the following cycling conditions: Initial Denaturation 95 °C/15 min, [Denaturation 95 °C/30 s, Annealing 58 °C/2 min, (10 cycles)], [Denaturation 95 °C/25 s, Annealing 53 °C/40 s, Extension 70 °C/40 s (30 cycles)], and Final Extension 70 °C/8 min. Reverse Hybridization/Genotype MTBDR*sl* assay was performed as per manufacturer instructions (Hain Lifescience, Nehren, Germany) [22].

2.6. Pyrosequencing (PSQ)

Thirty representative isolates of the 123 *MTB* isolates were sequence confirmed by PSQ. These isolates were selected so as to encompass four isolates of each *gyrA* mutations, on the Genotype MTBDR*sl* assay i.e. MUT1, MUT2 and 16 isolates from MUT3 i.e. MUT3A, MUT3B, MUT3C and two isolates of MUT 3D. Four isolates with wild-type characterization were also pyrosequenced.

PSQ consisted of a modified PCR amplification followed by PSQ reaction using one set of *MTB*-specific *gyrA*primers. Reagents from the Hot Start Taq kit and deoxynucleoside triphosphate (dNTP) mixtures (Qiagen, Valencia, CA) were used in the PCR master mix. Each PCR reaction contained 2.5 μL of extracted isolate DNA and 22.5 μL of PCR master mix, comprised of: 1 × PCR buffer, 2.5 mM $MgCl_2$, 0.96 mMdNTP mixture, 1 × Q-solution, 0.5 μM primer, and 1 U of HotStartTaq. The PCR reaction included: initial activation of the Hot Start Taq at 95 °C for 15 min, 50 cycles of amplification at 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 20 s, and final extension at 72 °C for 5 min. PyroMark Q96 reagents were then employed for PSQ, utilizing the sequence analysis mode of the Pyro Mark Q96 ID system (Qiagen, Valencia, CA) [23].

2.7. Statistical analysis

A Kruskal–Wallis test (GraphPad Prism 6 One-way ANOVA) was performed to evaluate whether different level of resistance is associated with the different *gyrA* mutations.

3. Results

Of the 123 *MTB* isolates evaluated in this study, 93 were OFX-resistant isolates, of which 75 were MDR with additional resistance to the fluoroquinolones; two were monoresistant to the fluoroquinolones and 16 were XDR and 30 isolates were fluoroquinolones susceptible. Eighty-eight were resistant to both MXF and OFX at the critical concentrations of 0.25 and 2.0 μg/ml, respectively.

Agreement between genotypic Genotype MTBDR*sl* assay and phenotypic susceptibility assay (using critical concentrations) was 95% for MXF and 100% for OFX. PSQ confirmed that, of the 30 representative isolates, four isolates with an absence of the wild-type2 band and presence of the MUT1 band via Genotype MTBDR*sl* assay corresponded to a 90GCG-GTG mutation; four isolates with an absence of the wild-type band and presence of a MUT2 band corresponded to a 91TGC-CCG mutation; 16 isolates with an absence of the WT3 band and presence of a MUT3A, MUT3B or MUT3C band corresponded to 94GAC-GCC, 94GAC-AAC/TAC or 94GAC-GGC mutations, respectively; two isolates with the absence of a WT3 band and presence of a MUT3D band corresponded to a 94GAC-CAC mutation; and four isolates with Wild-type bands on Genotype MTBDR*sl* assay were confirmed to be wild type via PSQ.

Results of MIC_{50} and MIC_{90} for *gyrA* mutations are summarized in Table 1. Isolates harboring the mutations Ala90Val and Ser91Pro were found to have a MIC_{90} of 4.0 μg/ml for OFX and 1.0 μg/ml for MXF, whereas Asp94Ala, Asp94Asn/Tyr, Asp94Gly and Asp94His were found to have a MIC_{90} of 8.0 μg/ml for OFX and 2.5 μg/ml for MXF (Table 1). Three isolates had MICs > 2.5 μg/ml for MXF. We performed additional MXF MICs testing on these three isolates utilizing the higher drug concentrations 5, 8 and 10 μg/ml. We observed that two of isolates harboring Asp94Asn/Tyr mutations had MICs of 5.0 μg/ml while one isolate with an Asp94Gly mutation had an MIC of 8.0 μg/ml for MXF.

To determine the correlation between fluoroquinolones MICs and *gyrA* mutations a Kruskal Wallis test was conducted. The results revealed that the level of resistance to OFX and MXF was significantly associated with specific *gyrA* mutations. For MXF, low level of resistance was seen with Ala90Val ($n = 25$) and Ser91Pro ($n = 6$), Moderate level of resistance was seen with Asp94Ala ($n = 3$), Asp94Asn/Tyr ($n = 11$), Asp94Gly ($n = 32$) and Asp94His ($n = 2$) and high level were seen with Asp94Asn/Tyr ($n = 2$) and Asp94Gly ($n = 1$). For OFX, Moderate level of resistance was seen with Ala90Val ($n = 22$) and Ser91Pro ($n = 5$) and high level of resistance was seen with Asp94Ala ($n = 3$), Asp94Asn/Tyr ($n = 13$), Asp94Gly ($n = 42$) and Asp94His ($n = 3$). OFX did not show low level of resistance (Figure 1). For OFX, the Kruskal Wallis statistic was 64.66, p value < 0.0001 and for MXF it was 60.10, p value < 0.0001.

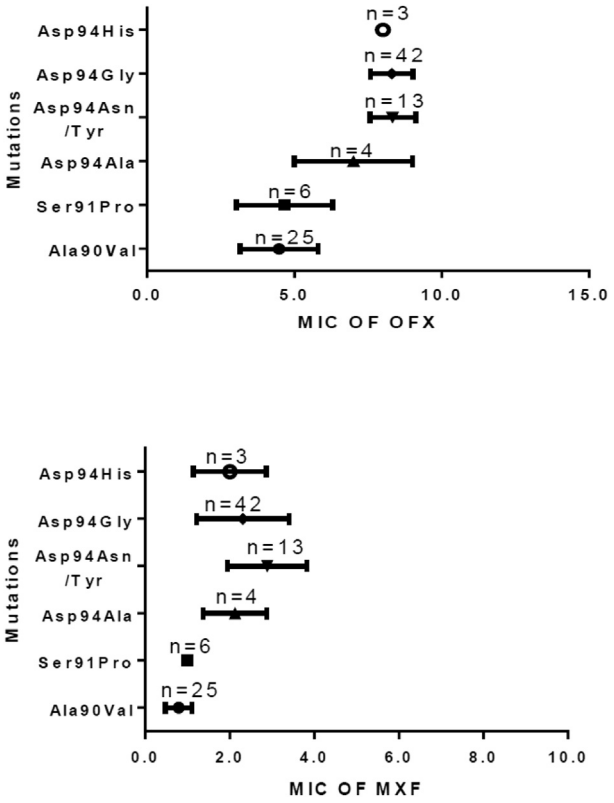

Figure 1. Different gyrA mutations with levels of resistance for OFX and MXF.

Table 1
*gyr*A mutations and MICs of MXF and OFX for wild-type isolates and isolates harboring mutations.

Nucleotide changes	Genotype MTBDR*sl* assay	Mutation	Total no. (%)	No. of isolates	MIC		MIC range		MIC_{50}		MIC_{90}	
					OFX (μg/ml)	MXF (μg/ml)	OFX (μg/ml)	MXF (μg/ml)	OFX (μg/ml)	MXF (μg/ml)	OFX (μg/ml)	MXF (μg/ml)
GCG-GTG	MUT1	Ala90Val	25 (26.9)	13	4.0	1.0	4.0–8.0	0.25–1.0	4.0	1.0	4.0	1.0
				4	4.0	0.5						
				3	8.0	1.0						
				5	4.0	0.25						
TGC-CCG	MUT2	Ser91Pro	6 (6.5)	5	4.0	1.0	4.0–8.0	1.0	4.0	1.0	4.0	1.0
				1	8.0	1.0						
GAC-GCC	MUT3A	Asp94Ala	4 (4.3)	3	8.0	2.5	4.0–8.0	1.0–2.5	8.0	2.5	8.0	2.5
				1	4.0	1.0						
GAC-AAC/TAC	MUT3B	Asp94Asn/Tyr	13 (13.9)	10	8.0	2.5	8.0–>10.0	2.5–5.0	8.0	2.5	8.0	2.5
				1	10.0	2.5						
				1	>10.0	5.0						
				1	10.0	5.0						
GAC-GGC	MUT3C	Asp94Gly	42 (45.2)	26	8.0	2.5	8.0–>10.0	1.0–8.0	8.0	2.5	8.0	2.5
				9	8.0	1.0						
				6	10.0	2.5						
				1	>10.0	8.0						
GAC-CAC	MUT3D	Asp94His	3 (3.2)	1	8.0	1.0	8.0	1.0–2.5	8.0	2.5	8.0	2.5
				2	8.0	2.5						
WT	WT	WT	30	26	0.5	0.0625	0.5–2.0	0.0625–0.25				
				2	1.0	0.125						
				2	2.0	0.25						

4. Discussion

There was a clear correlation between the various *gyrA* mutations observed with Genotype MTBDR*sl* assay and the levels of phenotypic resistance seen to OFX and MXF by MGIT. Our results indicated that the MICs for wild type *MTB* isolates were clearly different from MICs for isolates harboring different mutations. *gyrA* mutations associated with fluoroquinolones resistance were found in 93 of the 123 *MTB* isolates that were evaluated. In these 93 fluoroquinolones resistant isolates, we observed seven unique mutations at Ala90Val, Ser91Pro, Asp94Ala, Asp94Asn/Tyr, Asp94Gly and Asp94His.There was a significant correlation between the fluoroquinolones MICs and the *gyrA* mutation position.

In present study, 88% (22/25) and 100% (25/25) of isolates with the mutation Ala90Val had MICs at or above the critical concentrations of 4.0 μg/ml and 0.25–1.0 μg/ml for OFX and MXF, respectively. Similarly, 83% (5/6) and 100% (6/6) of isolates with a Ser91Pro mutation showed an MIC of 4.0 μg/ml and 1.0 μg/ml for OFX and MXF, respectively. A previous study reported, similar findings with a MIC of 4.0 μg/ml for OFX and 1.0 μg/ml for MXF for the mutations Ala90Val and Ser91Pro [18].

MICs ranging from 8.0 to ≥10.0 μg/ml for OFX were seen in 100% (42/42) of isolates with Asp94Gly mutations, in accordance with other studies reporting 94% (16/17) of the isolates with the mutation having MICs ranging from 8.0 to 10.0 μg/ml [18]. MICs of 2.5 μg/ml for MXF were seen in 76% (32/42) of isolates with an Asp94Gly mutation. A previous study reported that, 82% (14/17) of the isolates had a MICs of 2.0 μg/ml for MXF [18]. For other Asp94Asn/Tyr mutations, we found a MIC_{50} of 2.5 μg/ml for MXF and 8.0 μg/ml for OFX. This has been noted for *gyrA* mutation with similar MIC_{50} of 2.0 μg/ml for MXF and 8.0 μg/ml for OFX [24].

While most *gyrA* mutations appear to confer cross-resistance to OFX and MXF, we found clear evidence that the MIC_{50} associated with specific *gyrA* mutations were sufficiently different enough, between OFX and MXF to be clinically relevant. For example, the MIC_{50} for the mutations Ala90Val and Ser91Pro were 1.0 μg/ml for MXF, but 4.0 μg/ml for OFX; while isolates with the mutation Asp94Gly had an MIC_{50} of 2.5 μg/ml for MXF whereas for OFX it was 8.0 μg/ml. These findings confirm results reported in a similar study [24].

The maximum serum concentrations (C_{max}) achievable in humans for MXF is 4.3 μg/ml with daily oral dosing of 400 mg, with a half-life range of 9–12 h; whereas the C_{max} for OFX is 4.0 μg/ml with daily oral dosing of 400 mg, with a half-life of 4–5 h [16,18]. C_{max} of MXF/OFX are similar but the MICs for OFX are consistently almost double those for MXF for any of primary *gyrA* mutations that are found in fluoroquinolones-resistant isolates, MXF apparently has superior pharmacokinetics and pharmacodynamics (PK/PD) compared to OFX (MXF: $C_{max}/MIC = 9$, $AUC_{24}/MIC = 96$ after a daily dose of 400 mg. OFX: $C_{max}/MIC = 2$, AUC24/MIC = 24 after a daily dosing of 400 mg) [16]. It is notable that, despite the presence of resistance-associated mutations in the *gyrA* gene, most of the MXF MIC levels were below the peak serum concentration while the OFX MICs levels were closer to the peak serum concentration. For example, 20% (5/25) isolates with an Ala90Val mutation had MXF MICs at 0.25 μg/ml. For these same isolates, the MICs for OFX was 4.0 μg/ml, or two fold higher than the critical concentration. Structural differences between the two fluoroquinolones can explain the phenotypic differences between the two drugs. The MICs of MXF could be the result of the drug's structure, as it contains a methoxy group at the 8- position and a diazabicyclonyl ring moiety with an S, S configuration at the 7- position. This large hydrophobic moiety at C7 does not allow for efflux the drug [18].

Additionally, as many of the *gyrA* mutations evaluated in our study had MICs below the C_{max} for MXF, it is possible that certain *gyrA* mutations, detectable by rapid molecular diagnostics, may be used to predict MICs that are above the critical concentration for resistance, but still potentially clinically treatable through increased MXF dosing.

We propose that low level of resistance for MXF (with MIC of ≤1 μg/ml) may be treated with normal or higher doses of MXF. Whether moderate level of resistance (with MIC of 2.5 μg/ml) can be treated with higher doses of MXF will require a clinical outcome trial. High level of resistance (with MICs of 5.0–8.0 μg/ml) perhaps cannot be treated with any of the fluoroquinolones (Figure 1).

We believe that this study has important clinical implications. First, it emphasizes the need for performing phenotypic DST for MXF at 2 critical concentrations especially in regions where, MXF is used in the management of MDR TB. Secondly given the superior PK/PD characteristics of MXF, as majority of mutations (including those at *gyrA* 94) showed an MIC of 2.5 μg/ml with moderate level of resistance, clinical outcome studies assessing the efficacy of MXF in these groups are urgently warranted in high MDR TB endemic regions.

5. Conclusions

While it is well known that certain *gyrA* mutations in *MTB* confer phenotypic resistance to both MXF and OFX, it appears that the MICs conferred by many *gyrA* mutations (eg. Ala90Val) are consistently lower for MXF than for OFX. This observation has significant clinical relevance and suggests that MXF will have better PK/PD characteristics than OFX for most *gyrA* mutations. In our study, the majority of *MTB* isolates with the mutations Asp94Ala, Asp94Asn/Tyr, Asp94Gly and Asp94His were associated with a moderate level of resistance to MXF (MIC = 2.5 μg/ml), while three isolates with the mutations Asp94Asn/Tyr or Gly were associated with a high level of resistance to both MXF and OFX (MXF MICs = 5.0–8.0 μg/ml, OFX MICs = ≥10.0 μg/ml). Our results suggest that clinical *MTB* isolates shown to have the *gyrA* mutations Ala90Val and Ser91Pro have the potential to be rapidly diagnosed and treated with standard or increased MXF dosing. However, mutations at 94 *gyrA* with moderate level of resistance will require clinical outcomes in MDR and XDR patients with *MTB* isolates harboring theses mutations.

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