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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

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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

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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.

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