Correlating Minimum Inhibitory Concentrations of ofloxacin and moxifloxacin with gyrA mutations using the genotype MTBDRsl 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

Objective: To correlate gyrA mutations found on the Genotype MTBDRsl 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 MTBDRsl 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 MIC50 of 4.0 µg/ml and 1.0 µg/ml for OFX and MXF, respectively, and isolates with mutations at Asp94Ala, Asn/Tyr, Gly and His had MIC50 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].

Conclusion: 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).

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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 risk of developing XDR-TB increases [1].

In this study, we estimated the fluoroquinolones MICs of 123 clinical MTB isolates and correlated those MICs with specific gyrA mutations on the Genotype MTBDRsl 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 by the Institutional Review Board (IRB) of Hinduja Hospital. Written consent was waived for all participants as the study was carried out by the Institutional Review Board (IRB) of Hinduja Hospital.

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 (0.0625 and 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. MIC50 and MIC90 calculation

In this study, MIC50 was defined as the fluoroquinolones drug concentration that inhibited 50% of the isolates from each mutation group and MIC90 was defined as the fluoroquinolones drug concentration that inhibited 90% of the isolates from each mutation group. MIC50 and MIC90 were calculated as follows:

\[ \text{MIC} = \text{no. of isolates} \times \text{MIC} \]

2.5. Genotype MTBDRsl assay

A 123 consecutive archived isolates for which both Genotype MTBDRsl assay and DST at the WHO approved critical concentrations had been performed previously were selected. Genotype MTBDRsl 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 using 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 MTBDRsl 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 MTBDRsl 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 MgCl2, 0.96 mM dNTP 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 Q66 reagents were then employed for PSQ, utilizing the sequence analysis mode of the Pyro Mark Q66 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.