Analysis of mutations in the gyrA and gyrB genes and their association with the resistance of Mycobacterium tuberculosis to levofloxacin, moxifloxacin and gatifloxacin

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The purpose of the present study was to analyse mutations in the gyrA and gyrB genes of Mycobacterium tuberculosis and define the possible correlation between these mutations and resistance to levofloxacin (LVX), moxifloxacin (MFX) and gatifloxacin (GAT), based on their MICs. One hundred and forty-two M. tuberculosis clinical isolates were collected from pulmonary tuberculosis patients in the Moscow region. All M. tuberculosis strains were tested for drug susceptibility to rifampicin and isoniazid using the BACTEC MGIT 960 System and to ofloxacin (OFX) using the absolute concentration method on solid Lowenstein–Jensen slants. All in all, 68 strains were selected at random (38 strains were resistant and 30 were susceptible to OFX) for further analysis using the TB-BIOCHIP-2 test system and DNA sequence analysis. The MICs of LVX, MFX and GAT for selected strains were determined using the BACTEC MGIT 960 System. Mutations in the gyrA gene were observed in 36 out of 38 (94.7 %) OFX-resistant M. tuberculosis strains. Asn538Asp and Asp500His substitutions in the gyrB gene only were found in two (5.3 %) strains. Twenty-nine out of 30 OFX-sensitive M. tuberculosis strains had no mutations in either gene. One (3.3 %) OFX-sensitive M. tuberculosis strain carried an Arg485His substitution in gyrB. The results of our investigation showed that there is no clear correlation between the type of mutation in the genes gyrA and gyrB, and the MIC levels of LVX, MFX and GAT for resistant strains. Mutations in gyrA and Asn538Asp, and Asp500His substitutions in gyrB were associated with cross-resistance of M. tuberculosis to fluoroquinolones. The substitution Arg485His in gyrB does not confer resistance to LVX, MFX and GAT in M. tuberculosis.

INTRODUCTION

Fluoroquinolones (FQs) are highly active antimicrobial agents, which are widely used in chemotherapy against multidrug-resistant (MDR) tuberculosis (TB). However, the application of ofloxacin (OFX), ciprofloxacin and levofloxacin (LVX) for the treatment of undiagnosed respiratory infections led to the development of resistance in Mycobacterium tuberculosis isolates (Wang et al., 2007). Moreover, the initial occurrences of resistance to FQs were observed in MDR strains and isoniazid (IHN)- and rifampicin (RIF)-susceptible strains isolated from newly diagnosed TB patients (Delgado & Telenti, 1996; Xu et al., 2009).

The most promising drugs undergoing phase III trials are the fourth generation FQs, such as moxifloxacin (MFX) and gatifloxacin (GAT), which demonstrated high in vivo and in vitro activities against MDR strains and OFX-resistant and ciprofloxacin-resistant MDR strains (Zhao et al., 1999; Rodriguez et al., 2002; Poissy et al., 2010; Merle et al., 2012).

Recent studies have shown that resistance to all FQs is due to mutations (single nucleotide polymorphisms, SNPs) not only in the gyrA (320 bp) gene, but also in the gyrB (375 bp) gene, which encode the respective subunits of the DNA topoisomerase gyrase (Takiff et al., 1994; Ginsburg et al., 2003; Shi et al., 2006; Lau et al., 2011). The most common mutations in the gyrA gene are Ala90Val, Asp94 (Gly, Ala, His, Asn or Tyr) and Ser91Pro, the Gly88Cys mutation is found less frequently (Shi et al., 2006; Wang et al., 2008; van Doorn et al., 2008; Feuerriegel et al., 2009). An SNP in one of these four positions leads to FQ resistance in M. tuberculosis in vitro, including resistance to...
M. tuberculosis genes gyrA and gyrB: resistance to FQs

MFX and GAT (Kam et al., 2006; Von Groll et al., 2009). About 7% of strains carry mutations in the gyrB gene (Wang et al., 2007). At present, Arg485 (His, Cys), Ser486Phe, Asp495Asn, Asn533Thr, Asn538 (Thr, Asp), Thr539Pro, Asp500 (Ala, His, Asn), Gly509Ala, Glu540 (Val, Asp) nucleotide substitutions have been described in the quinolone-resistance-determining region of the gyrB gene (Pitaksaikul et al., 2005; Feuerriegel et al., 2009; An et al., 2009). These SNPs occur independently in gyrB, or in combination with mutations in gyrA (Von Groll et al., 2009). High M IC values for FQ-resistant strains are associated with the appearance of at least two mutations in gyrA, the substitutions Asp500 (Asn, His) and Glu540 (Val, Asp) in gyrB or mutations in both genes (An et al., 2009; Von Groll et al., 2009; Cui et al., 2011).

The purpose of our study was to analyse mutations in the gyrA and gyrB genes and define the possible correlation between these mutations and resistance to LVX, MFX and GAT, based on the determination of MICs in the liquid medium Middlebrook 7H9 using the automated BACTEC MGIT 960 System.

METHODS

Bacterial strains. A total of 142 M. tuberculosis clinical isolates were collected from pulmonary TB patients in the Moscow region. Identification of the M. tuberculosis strains was performed by using molecular (identification of insertion sequence IS6110) and microbiological (growth rate, pigmentation, colony morphology, microscopie of Ziehl–Neelsen-stained smears) methods, and biochemical tests (niacin and catalase production, nitrate reduction, sodium salicylate susceptibility) (Order of the Ministry of Health of the Russian Federation, 2003; Siddiqi & Rusch-Gerdes, 2007; Gryadunov et al., 2011). All M. tuberculosis strains were tested for drug susceptibility to INH and RIF using the BACTEC MGIT 960 System and for susceptibility to OFX using the absolute concentration method on solid Lowenstein–Jensen (L–J) slants. A total of 68 strains were selected at random (38 strains were resistant and 30 were susceptible to OFX) for further analysis.

Drug susceptibility testing (DST). First-line DST for INH and RIF was performed using the BACTEC 960 System, according to the manufacturer’s instructions (Siddiqi & Rusch-Gerdes, 2007). DST for OFX was performed using the absolute concentration method on L–J slants with the breakpoint concentration 2 mg l⁻¹, as recommended by the Russian Ministry of Health (Order of the Ministry of Health of the Russian Federation, 2003).

MIC determination. LVX (28266; Sigma-Aldrich), MFX (SRP06644m; Sequoia Research Products) and GAT (SRP01230g; Sequoia Research Products) stock solutions were prepared at 1 mg ml⁻¹ in deionized water, filter-sterilized and were stored at −20 °C. Final concentrations of LVX ranged from 0.125 to 32.0 mg l⁻¹, MFX from 0.063 to 10.0 mg l⁻¹ and GAT from 0.063 to 6.0 mg l⁻¹. Test procedures for the determination of the MICs of LVX, MFX and GAT were those as described by Rüscher-Gerdes et al. (2006) on second-line DST. The strain H37Rv (ATCC 25618) was used as a control. Resistance was defined as an MIC of ≥2 mg l⁻¹ for LVX, ≥0.5 mg l⁻¹ for MFX and GAT.

DNA extraction. An analysis using the TB-BIOCHIP-2 test system for the identification of mutations in gyrA, including DNA extraction, PCR, electrophoresis and hybridization on biochips, was performed as described in the manufacturer’s manual, based on original publications (Antonova et al., 2008; Gryadunov et al., 2011). The TB-BIOCHIP-2 test permits the detection of nine types of mutations in five polymorphic codons (88, 90, 91, 94, 95) of the quinolone-resistance-determining region of the gyrA gene. Codon 95 contains a natural polymorphism, Ser (AGC) to Thr (ACC), which does not lead to FQ resistance in M. tuberculosis.

Sequencing. DNA sequencing of the gyrA and gyrB genes was performed after the DNA was amplified by PCR in the automated sequencer GS Junior (Roche) using the primers gyrAF (5’-CTATGC-AAATTGTCCGATGACTCTGCTTCGTTCCCT3’) and gyrAR (5’-ACTGTTCCCTCCGTCGATTTCCTCCT-3’) for the gyrA gene and gyrBF (5’-AGAGTTTGCTGGGGTAAAGA-3’) and gyrBR (5’-AACACATGCCTCTTGCAG-3’) for the gyrB gene.

RESULTS

We analysed 68 M. tuberculosis strains. Thirty-eight strains were resistant and 30 were susceptible to OFX. Among the 38 OFX-resistant strains, 35 were also resistant to RIF and INH (MDR strains), two were resistant to RIF and one was susceptible to both drugs. Among the 30 susceptible strains, 22 were also susceptible to RIF and INH, three were resistant to RIF and INH, two were resistant to RIF and three were resistant to INH.

Mutations in gyrA were observed in 36 out of 38 (94.7%) OFX-resistant strains. Two (5.3%) OFX-resistant strains carried mutations in gyrB only (Asn538Asp, Asp500His). Twenty-nine out of 30 OFX-susceptible strains displayed no mutations in gyrA gyrB, apart from a Ser95Thr natural polymorphism in gyrA, which was detected in 19 OFX-susceptible and in all OFX-resistant strains. One (3.3%) susceptible strain carried an Arg485His substitution in gyrB. Substitutions in codons 94 (35.3%) and 90 (23.7%) occurred the most frequently. In four out of 36 strains, double substitutions were detected. The spectrum of mutations is shown in Table 1.

LVX, MFX and GAT MICs for the 30 OFX-susceptible strains, including strains with the Arg485His substitution in gyrB, ranged from 0.125 to 1.0 mg l⁻¹, from 0.063 to 0.25 mg l⁻¹ and from 0.063 to 0.125 mg l⁻¹, respectively. LVX, MFX and GAT MICs for the 38 OFX-resistant strains were relatively higher than those obtained for the OFX-susceptible strains (Table 2). The MICs of LVX, MFX and GAT for 14 out of 15 strains with Asp94 mutations (Asp94Gly, Asp94Tyr and Asp94His) were high; however, for strains with the Asp94Ala mutation, they were not. The MIC of LVX for M. tuberculosis strains with the mutation Ala90Val ranged from 2.0 to 8.0 mg l⁻¹ and the MICs of MFX and GAT for these strains ranged from 0.5 to 2.0 mg l⁻¹. LVX, MFX and GAT MICs for strains with double mutations in gyrA (Asp94Gly, Ala90Val) were high, >32.0, 8.0 and 4.0 mg l⁻¹, respectively. At the same time, MICs for strains with double substitutions (Gly88Ala and His70Arg, Asp94Tyr and Ala90Val) were lower (2.0 mg l⁻¹ for LVX, 1.0 mg l⁻¹ for MFX and 0.5–1.0 mg l⁻¹ for
GAT). However, a four- to eightfold decrease in the MFX and GAT MIC values was observed when compared with the MIC of LVX.

Two OFX-resistant and one OFX-susceptible strains carried mutations only in gyrB. LVX, MFX and GAT MICs for a strain with Asn538Asp SNP were 2.0, 0.5 and 1.0 mg l⁻¹, respectively, and the MICs for a strain with Asp500His SNP were 4.0, 0.5 and 1.0 mg l⁻¹, respectively. An OFX-sensitive strain with the Arg485His substitution in the gyrA gene only (Table 2). An OFX-susceptible strain with the mutation Arg485His in gyrB was also susceptible to LVX (MIC=1.0 mg l⁻¹), MFX (MIC=0.25 mg l⁻¹) and GAT (MIC=0.25 mg l⁻¹).

**DISCUSSION**

FQ resistance in *M. tuberculosis* occurs due to mutations mostly in the gyrA gene, rarely in the gyrB gene (Takiff *et al.*, 1994; Pitaksajjakul *et al.*, 2005; Wang *et al.*, 2007; Feuerriegel *et al.*, 2009). On the other hand, Von Groll *et al.* (2009) showed that the substitution Asn533Thr in gyrB is associated with resistance to low concentrations of MFX and GAT, but is not associated with resistance to OFX. In addition, previous studies have shown that the level of LVX resistance in LFX-resistant strains (Yin & Yu, 2010) and the level of OFX resistance in OFX-resistant strains (Cui *et al.*, 2011) had no clear correlation with the type of mutation present in gyrA and gyrB.

In this study all mutations in the gyrA gene were associated with phenotypic resistance to all four FQs and cross-resistance was observed. High-level MICs of LVX (16.0 and ≤32.0 mg l⁻¹) were detected for three (7.9 %) strains. MFX MICs for two (5.3 %) strains were 4.0 and 8.0 mg l⁻¹. GAT MIC for one (2.36 %) strain was 4.0 mg l⁻¹. Asp94 mutations led to high-level MICs of LVX, MFX and GAT for most strains (Kam *et al.*, 2006; Yin & Yu, 2010). Fourteen (36.8 %) strains were resistant to the lowest LVX concentration (2.0 mg ml⁻¹). MFX MIC for seven (18.4 %) strains was 0.5 mg l⁻¹. GAT MIC for 15 (39.5 %) strains was also 0.5 mg l⁻¹ (Table 2). Mutations in the gyrA gene detected using the TB-BIOCHIP-2 test system and the identification of a double substitution, Gly88Ala and His70Arg, in one strain by sequence analysis, which were described by Matrat *et al.* (2006), Mokrousov *et al.* (2008) and Yin & Yu (2010), showed no clear correlation between the levels of resistance in strains resistant to LVX, MFX and GAT, and the type of mutation that coincided with previously reported data (Von Groll *et al.*, 2009; Yin & Yu, 2010; Cui *et al.*, 2011).

Reports concerning the occurrence of FQ-resistant strains with mutations in the gyrB gene have been published (Pitaksajjakul *et al.*, 2005; An *et al.*, 2009; Von Groll *et al.*, 2009; Kim *et al.*, 2011); however, some SNPs (Arg485Leu, Gly551Arg and Gly549Asp) have also been found in FQ-susceptible isolates (El Sahly *et al.*, 2011; Cui *et al.*, 2011). In this study, two OFX-resistant and one OFX-susceptible strains carried SNPs only in the gyrB gene. The three substitutions Arg485His, Asn538Asp and Asp500His have been described as mutations connected to FQ resistance (Pitaksajjakul *et al.*, 2005; Wang *et al.*, 2007; Feuerriegel *et al.*, 2009; An *et al.*, 2009). According to our data, OFX-resistant strains with mutations Asp500His and Asn538Asp in the gyrB gene only were cross-resistant to LVX, MFX and GAT, as were all OFX-resistant strains with SNPs in the gyrA gene only (Table 2). An OFX-susceptible strain with the mutation Arg485His in gyrB was also susceptible to LVX (MIC=1.0 mg l⁻¹), MFX (MIC=0.25 mg l⁻¹) and GAT (MIC=0.25 mg l⁻¹) (Pantel *et al.*, 2011). Other SNPs at this codon, Arg485Cys and Arg485Asn, were detected only in OFX-resistant strains from Uzbekistan, Thailand and Vietnam, and were not detected in the OFX-susceptible strains (Pitaksajjakul *et al.*, 2005; Feuerriegel *et al.*, 2009; Cui *et al.*, 2011).

The results from a study of mutations in the gyrA and gyrB genes of *M. tuberculosis* strains obtained from patients with pulmonary TB in the Moscow region of Russia coincided with those from studies in other countries around the world.

In conclusion, our data showed that mutations in the gyrA gene and the two SNPs Asn538Asp and Asp500His in the gyrB gene are associated with cross-resistance to FQs in *M. tuberculosis*. The mutation Arg485His in the gyrB gene does not confer resistance to OFX, LVX, MFX or GAT. Also, it has been shown that the levels of resistance to three FQs in resistant strains of *M. tuberculosis* are not associated with the type of mutation present in the gyrA and gyrB genes. It is necessary to conduct further research on a large number of strains.
Table 2. Mutations in *M. tuberculosis* genes *gyrA* and *gyrB*, and the MICs of LVX, MFX and GAT

<table>
<thead>
<tr>
<th><em>gyrA</em> mutation</th>
<th><em>gyrB</em> mutation</th>
<th>LVX MIC (mg l⁻¹)</th>
<th>Cut-off MIC for resistance</th>
<th>LVX No. of strains</th>
<th>MFX MIC (mg l⁻¹)</th>
<th>Cut-off MIC for resistance</th>
<th>MFX No. of strains</th>
<th>GAT MIC (mg l⁻¹)</th>
<th>Cut-off MIC for resistance</th>
<th>GAT No. of strains</th>
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<td>4 2 3</td>
<td>≥ 0.5</td>
<td>0.25 0.5 1 2 4</td>
<td>7 1 1</td>
<td>≥ 0.5</td>
<td>0.25 0.5 1 2 4</td>
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<td>2 3 1</td>
<td>≥ 0.5</td>
<td>0.25 0.5 1 2 4</td>
<td>6</td>
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<td>1 12</td>
<td>≥ 0.5</td>
<td>0.25 0.5 1 2 4</td>
<td>1 7 5</td>
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<td>0.25 0.5 1 2 4</td>
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<td>1 2 4 8</td>
<td>1</td>
<td>≥ 0.5</td>
<td>0.25 0.5 1 2 4</td>
<td>1</td>
<td>≥ 0.5</td>
<td>0.25 0.5 1 2 4</td>
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<td>1 2 4 8</td>
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<td>≥ 0.5</td>
<td>0.25 0.5 1 2 4</td>
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<td>0.25 0.5 1 2 4</td>
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<td>1</td>
<td>&lt; 0.5</td>
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<td>&lt; 0.5</td>
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<td>≥ 0.5</td>
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<tr>
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<td>≥ 0.5</td>
<td>0.25 0.5 1 2 4</td>
<td>1</td>
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</table>
of *M. tuberculosis* strains to study the role of mutations in the *gyrB* gene in the development of resistance to FQs.

**ACKNOWLEDGEMENTS**

We wish to thank the staff of the Centralized Mycobacteriological Laboratory of the Moscow Scientific and Clinical Antituberculosis Center for supplying bacterial isolates and Mr Danila V. Zimenkov from the Engelhardt Institute of Molecular Biology for performing sequence analysis.

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