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Original Article

Characterization of gyrA and gyrB mutations and fluoroquinolone resistance in *Mycobacterium tuberculosis* clinical isolates from Hubei Province, China

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ABSTRACT

Objective: The study aimed to investigate gyrA and gyrB mutations in *Mycobacterium tuberculosis* (MTB) clinical strains from 93 patients with pulmonary tuberculosis in Hubei Province, China, and analyze the association between mutation patterns of the genes and ofloxacin resistance level.
Results: Among 93 MTB clinical isolates, 61 were ofloxacin-resistant by the proportion method, and 32 were ofloxacin-susceptible MDR-TB. No mutation in the gyrB gene was found in any MTB strains. In the 61 ofloxacin-resistant isolates, 54 mutations were observed in the gyrA gene. Only one mutation in the gyrA gene was found in ofloxacin-susceptible MDR-TB isolates. In this study, the mutation patterns of gyrA involved seven patterns of single codon mutation (A90V, S91P, S91T, D94N, D94Y, D94G or D94A) and two patterns of double codons mutation (S91P & D94H, S91P & D94A). The ofloxacin minimal inhibitory concentrations (MICs) of three patterns of single codon mutations in the gyrA gene (codons 94, 90 and 91) showed a statistically significant difference ($p < 0.0001$).
Conclusions: The gyrA mutations at codons 90, 91 and 94 constitute the primary mechanism of fluoroquinolone resistance in MTB, and mutations at codon 91 in the gyrA gene may be associated with low-level resistance to ofloxacin.
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Introduction

Drug resistance of *Mycobacterium tuberculosis* is a major threat for the control of tuberculosis. Emergence of multidrug resistant tuberculosis (MDR-TB) and extensively drug resistant tuberculosis (XDR-TB) will be a challenge in the tuberculosis epidemic and will seriously impact therapeutic effect. According to a WHO report,[1] an estimated 390,000-510,000 cases of MDR-TB emerged globally in 2008. Among all incident TB cases globally, 3.6% were estimated to be MDR-TB. Almost 50% of MDR-TB cases worldwide were estimated to occur in

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China and India. In 2008, MDR-TB caused an estimated 150,000 deaths all over the world.

Fluoroquinolones are potent second-line drugs recommended to treat MDR-TB.[2] However, fluoroquinolone resistance among *Mycobacterium tuberculosis* strains is ever more prevalent.[3,4] In China, fluoroquinolones have been widely used for tuberculosis treatment and served routinely as monotherapy for the empirical treatment of numerous respiratory infections. Thus, China may be one of the countries with the highest rate of fluoroquinolone abuse and resistance.[5] The patients suffering from MDR-TB would have a poor prognosis once *Mycobacterium tuberculosis* becomes resistant to ofloxacin.[6] It has been reported that the new generation of fluoroquinolones, such as moxifloxacin and gatifloxacin, showed favorable pharmacokinetic activity and lower minimum inhibitory concentrations (MIC) against *Mycobacterium tuberculosis*.[7,8] The main target of fluoroquinolones in *Mycobacterium tuberculosis* is the DNA gyrase, encoded by gyrA and gyrB.[9] Mutations in two short regions known as quinolone-resistant determining regions (QRDRs) have been associated with fluoroquinolone resistance in *Mycobacterium tuberculosis*.

In the present study, we sequenced the QRDR in gyrA and gyrB genes of the MTB isolates from Hubei Province, China, and determined the MICs of ofloxacin, ciprofloxacin, moxifloxacin, and gatifloxacin. Further, we analyzed the possible correlation between gene mutations and resistance level to ofloxacin.

Methods

Bacterial isolates

A total of 93 MTB clinical isolates were collected from patients with pulmonary tuberculosis in Hubei Province, China from June 2009 to October 2010. These patients were epidemiologically unlinked. All MTB strains were identified by biochemical methods and real-time PCR detection based on primer derived from IS6110 (DAAN Gene – China). The ofloxacin drug susceptibility test was performed by the proportion method (PM) on Lowenstein-Jensen (LJ) medium according to the standard procedure, with the breakpoint concentration of 2 µg/mL for ofloxacin.[10] Sixty-one ofloxacin-resistant MTB isolates were selected and 32 ofloxacin-susceptible MDR-TB strains were also selected at random. *Mycobacterium tuberculosis* H37Rv (ATCC27294) was used as the reference control.

Determination of minimum inhibition concentrations (MICs) of four fluoroquinolones

The four fluoroquinolones included were: ofloxacin and ciprofloxacin (Sigma-Aldrich Co. – St Louis, USA), gatifloxacin (National Institutes for Food and Drug Control – China), and moxifloxacin (Bayer – Germany). Ciprofloxacin stock solutions were prepared at 1 mg/mL in distilled water; the three other fluoroquinolones were prepared at 1 mg/mL in 0.1 mmol/L NaOH, and sterilized using a 0.22 µm polycarbonate membrane filter (Corrighwohill, Co. Cork – Ireland). The stock solutions were stored at –70°C in small aliquots for up to six months. Frozen drug solutions were thawed once and then discarded.

MIC determinations of four quinolones were performed by using the resazurin colorimetric assay as described previously.[11] The final concentrations ranged from 0.06 µg/mL to 8 µg/mL. The MIC was determined by eyesight as the minimum drug concentration that prevented the colour change in resazurin solution.

DNA extraction

A loopful of MTB culture from LJ medium was transferred to a 1.5-mL tube that contained 1.0 mL sterilized water. The tube was centrifuged at 10,000×g for 5 min. The supernatant was discarded and the sediment was resuspended in 400 µL of TB lysating buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA [pH 8.0]), heat inactivated at 85°C for 30 min, and centrifuged at 10,000×g at 4°C for 10 min. The supernatant was transferred to another 1.5-mL tube and preserved at –70°C until use.

PCR amplication of gyrA and gyrB genes

The primers for PCR amplification were designed with reference to gyrA and gyrB gene sequences (GenBank accession number L27512). The gyrA gene was amplified with the forward primer (5-ATCGAGCAGGAGATGCAG-3) and reverse primer (5-CGTCGTAGTTAGGGATGAAA-3). The gyrB gene was amplified using the forward primer (5-GTTTGAAGCCAACCCCACC-3) and reverse primer (5-TGAACCGGAACAACAACGT-3). The basic 30-µL amplification reaction mixture containing 1.5 µL DNA, 15 µL 2×PCR MIX (MBI Fermentas – Lithuania), 1.5 µL 2.5 µM PCR primer mixture (Sangon – Shanghai, China) and 12 µL distilled water. Cycling parameters were 5 min at 94°C, followed by 30 cycles of 45 s at 94°C, 30 s at 56°C and 50 s at 72°C, followed by elongation at 72°C for 10 min. The PCR products of gyrA and gyrB were 415 bp and 470 bp in size, respectively.

Sequencing of QRDR of gyrA and gyrB genes

PCR products were purified with UNIQ-10 column purification kit (Sangon – Shanghai, China) and then sequenced with an automatic DNA sequencer (Applied Biosystems 3130xl DNA analyzer – USA). Mutations in gyrA and gyrB were identified using BLAST by comparison with *Mycobacterium tuberculosis* strain H37Rv sequence (GenBank accession number L27512).

Statistical analysis

The Statistical Package for Social Sciences (SPSS) version 12.0 software (SPSS Inc. – Chicago, IL, USA) was used for the statistical analysis. The MICs of ofloxacin-resistant MTB clinical isolates with different mutations in the gyrA gene were compared by the analysis of variance (ANOVA) test.

Results

Characterization of gyrA and gyrB mutations in ofloxacin-sensitive and -resistant MTB isolates

Among the 93 MTB clinical isolates, 32 were ofloxacin-susceptible and 61 were ofloxacin-resistant by the proportion

Table 1 - Mutations in gyrA and MICs determined by the resazurin colorimetric assay of 61 ofloxacin-resistant MTB isolates

Strain isolates	Mutation in gryA	MIC (µg/mL) of			
		OFX	CIP	GAT	MOX
19-7335	A90V	4	2	0.5	0.5
1-6974	A90V	4	4	0.25	0.5
5224	A90V	4	2	0.5	0.5
4462-3243	A90V	8	4	0.5	1
4706-4548	A90V	2	1	0.25	0.25
4740-4650	A90V	4	1	0.25	0.25
4802-4217	A90V	4	4	0.5	0.5
26-8519	A90V	2	1	0.125	0.125
9089	A90V	4	2	0.25	0.25
XN24	A90V	2	1	0.25	0.25
20-7378	S91T	2	1	0.25	0.125
4848-6374	S91P	2	1	0.25	0.25
9054	S91P	2	1	0.25	0.25
5304-1	S91P&D94H	≥ 16	≥ 16	4	≥ 16
19-8620	S91P&D94A	≥ 16	≥ 16	8	≥ 16
F10386	D94N	4	2	0.5	0.5
5357-2	D94N	8	≥ 16	1	2
5375-1	D94N	2	1	0.125	0.25
4-7542	D94N	4	4	0.5	1
4410-2971	D94N	8	4	1	2
4-8283	D94N	≥ 16	8	2	2
5419	D94Y	8	8	0.5	1
4722-4743	D94Y	4	4	0.5	1
5363-1	D94Y	4	4	0.5	1
H59	D94Y	8	4	0.5	1
5416-1	D94G	4	4	0.25	0.25
17-7696	D94G	8	8	0.5	1
5240	D94G	8	4	1	2
5384	D94G	4	4	0.25	0.25
5342	D94G	8	4	0.5	0.5
5252-1	D94G	8	4	0.5	0.5
4542-3759	D94G	4	4	0.5	1
4686-4517	D94G	4	4	0.5	1
4702-4495	D94G	4	4	0.5	1
4795-6140	D94G	4	2	0.25	0.5
4868-6474	D94G	8	8	1	2
4897-6660	D94G	4	4	0.5	1
10-7928	D94G	8	4	0.5	1
5275-1	D94G	4	4	0.5	0.5
5288	D94G	4	4	0.25	0.5
5310-1	D94G	8	8	1	2
5330	D94G	4	2	0.5	0.5
YC29	D94G	≥ 16	8	1	2
YC38	D94G	8	8	2	2
YC41	D94G	16	8	2	4
YC43	D94G	8	8	1	1
9044	D94G	≥ 16	8	1	2
XN5	D94G	8	4	0.5	1
XN15	D94G	8	4	0.5	1
26-8700	D94G	≥ 16	≥ 16	4	4
4863-6412	D94G	4	2	0.25	0.25
5359-2	D94A	8	≥ 16	0.5	1
5441-2	D94A	4	4	0.5	0.5
YC60	D94A	4	2	0.5	1
8-7138	none	2	2	0.25	0.25
5-7427	none	2	1	0.25	0.25
5409-2	none	4	2	0.5	1
4435-3100	none	4	4	0.25	0.5
4862-6411	none	8	4	0.5	2
4927-6955	none	4	4	0.5	1
5296-1	none	8	4	1	2

No mutation was detected in gyrB gene.
OFX, ofloxacin; CIP, ciprofloxacin; GAT, gatifloxacin; MOX, moxifloxacin.

method on LJ medium. Among the 32 ofloxacin-susceptible MDR isolates, only one isolate had a D94N mutation, which had the ofloxacin MIC of 2 µg/mL, while the rest had no mutations. Among the 61 ofloxacin-resistant isolates, no mutation in gyrB was found, but 88.5% (54) had mutations observed in the gyrA gene. The results of mutations in gyrA and MICs of four fluoroquinolones, determined by the resazurin colorimetric assay for the 61 ofloxacin-resistant isolates, are presented in Table 1. The 54 ofloxacin-resistant MTB isolates with gyrA mutation are summarized in Table 2. From Table 2, 26 had a D94G mutation, six had a D94N mutation, four had a D94Y mutation, three had a D94A mutation, 10 had an A90V mutation, two had a S91P mutation, and one had a S91T mutation. Additionally, two strains with ofloxacin MIC ≥ 16 µg/mL had double mutations, one with S91P&D94H, and the other with S91P&D94A. A S95T mutation in gyrA occurred in all MTB strains. No mutation in gyrB was detected in any of the MTB strains.

Association of the patterns of gyrA mutations and ofloxacin MICs profile of ofloxacin-resistant MTB isolates

The patterns of gyrA and the MICs of 54 ofloxacin-resistant MTB mutants were shown in Table 2. The ofloxacin MICs of isolates with mutations at codon 94 ranged from 2 µg/mL to 16 µg/mL, with a median of 8 µg/mL. The ofloxacin MICs of isolates with mutations at codon 90 ranged from 2 µg/mL to 8 µg/mL, with a median of 4 µg/mL. The ofloxacin MICs of isolates with mutations at codon 91 were all 2 µg/mL, with a median of 2 µg/mL. The difference in the ofloxacin MICs of four mutation patterns with different amino acid mutations at codon 94 (D94N, D94Y, D94G, D94A) in the QRDR of gyrA gene showed no statistical significance ($p = 0.755$). The ofloxacin MICs of three patterns with different single codon mutations in the gyrA gene (codons 94, 90 and 91) were analyzed by ANOVA, which showed a statistically significant difference among the three groups ($p < 0.0001$).

MICs of four fluoroquinolones

The MICs of four fluoroquinolones of 61 ofloxacin-resistant MTB strains were determined by the resazurin colorimetric assay. The MIC_{50} and MIC_{90} values of the strains were summarized in Table 3. The MIC_{50} and MIC_{90} values for gatifloxacin and moxifloxacin were four to eight times lower than those for ofloxacin and ciprofloxacin.

Table 3 - MIC_{50} and MIC_{90} of four fluoroquinolones for 61 ofloxacin-resistant MTB strains

Fluoquinolone	MIC_{50}	MIC_{90}	Range of MIC
Ofloxacin	4	8	2 ~ ≥ 16
Ciprofloxacin	4	8	1 ~ ≥ 16
Gatifloxacin	0.5	1	0.125 ~ 8
Moxifloxacin	1	2	0.125 ~ ≥16

Discussion

In the present study, gyrA mutations were predominantly found to occur in codons 90, 91, and 94, largely corroborating the findings of other studies.[12-14] The strains isolated from Beijing, China, showed more double mutations,[5] which indicated that mutations in the gyrA gene might vary in different regions. All MTB isolates had no mutation in the gyrB gene, which is in agreement with previous studies demonstrating that fluoroquinolone resistance of MTB are mostly attributed to the mutations of the gyrA gene, whereas gyrB mutations are rare.[12,15,16] In the ofloxacin-resistant MTB clinical isolates, 54 of 61 (88.5%) had mutations in the QRDR of the gyrA gene, and the other seven strains had no mutations in the gyrA and gyrB genes, a finding consistent with what has been previously reported on fluoroquinolone resistance.[17] Other

Table 2 - Patterns of gyrA mutations and ofloxacin MICs profile of ofloxacin-resistant MTB isolates

Total No. of isolates	Codon mutation patterns of gyrA	Nucleotide change	No. of OFX-resistant isolates MICs (µg/mL)			
			2	4	8	16
26	D94G	GAC→GGC		11	11	4
6	D94N	GAC→AAC	1	2	2	1
4	D94Y	GAC→TAC		2	2	
3	D94A	GAC→GCC		2	1	
10	A90V	GCG→GTC/GTG	3	6	1	
2	S91P	TCG→CCG	2			
1	S91T	TCG→ACG	1			
1	S91P&D94H	TCG→CCG&GAC→CAC				1
1	S91P&D94A	TCG→CCG&GAC→GCC				1

OFX, ofloxacin.

resistance mechanisms, such as mutations in areas of gyrA or gyrB outside of the QRDR, decreased cell-wall permeability to the drug,[18] and efflux pumps,[19,20] probably accounting for the fluoroquinolone resistance in other fluoroquinolone-resistant isolates without gyrA and gyrB mutations. Further studies are needed. The total mutation frequency of the gyrA gene in this study is similar to those of Shanghai (89.5%)[12] and Russia (83%),[13] but higher than those of the Guangdong province (73.3%)[16] and Taiwan (50%).[3]

Previous data suggested a four to 16-fold increase in MICs among laboratory mutants with a single gyrA mutation, compared to those of the parent strains, while the MICs increased 32-fold or more for mutants with double mutations.[21] In the present study, we reported two patterns of double mutations in two MTB clinical isolates; one with S91P&D94H, and the other with S91P&D94A. Previous reports on double mutations mainly focused on codons 90 and 94.[14,21,22] The two MTB clinical isolates with double mutations had ofloxacin MIC ≥ 16 μg/mL, and it indicated that these two patterns of double mutation were associated with high-level resistance, which is consistent with previous reports, where multiple mutations are associated with high-level resistance.[22]

Some studies have shown that the level of drug resistance of MTB was linked to gene mutations. Hauck et al.[23] found that most rpoB gene mutations, such as codons 531 and 526, were correlated with high-level resistance to rifampin, and a lower level of resistance was associated with mutations at codons 511, 516, 533. Mutations at codon 315 of the katG gene were associated with high-level resistance to isoniazid, whereas mutations in the inhA gene or its promoter region were associated with low-level resistance to isoniazid.[24] In the present study, we confirmed that mutations of gyrA constituted the primary mechanism of fluoroquinolone resistance in MTB; an analysis of relationship between different codon mutations in the gyrA gene and the MICs of ofloxacin-resistant MTB isolates by ANOVA showed a statistically significant difference. This suggested that mutations at codon 91 might develop low-level resistance to ofloxacin. Our report differed from the results of the study by Cui,[12] which might be due to the different origin of the MTB isolates. Cheng[17] also observed that the MICs of ofloxacin in mutations at codon 91 were lower than those at other codons such as 90 or 94.

The newer generation of fluoroquinolones, such as moxifoxacin and gatifoxacin, was found to exhibit better activities than the other fluoroquinolones. Our results also showed that the MICs of moxifoxacin or gatifoxacin were 4- to 8-fold lower than those of ofloxacin or ciprofloxacin. For moxifoxacin and gatifoxacin, their superior activities have been attributed to the special structure-activity relationship of the C-8-methoxy and C-8-halogen substitution in the chemical structure of the prototype fluoroquinolones.[25,26] These new members with substitutions can have stronger bacteriostatic and lethal activities against MTB, as shown by their lower MICs even among the gyrA mutants.[14,15] In addition, acquired resistance to these new drugs would be expected to develop less readily, as reflected by their lower mutant prevention concentrations.[27] Recently, a murine model study showed that moxifloxacin was active against MTB strains with low-level fluoroquinolone resistance and reduced the mortality associated with MTB strains with intermediate resistance.[28] Moxifloxacin and gatifloxacin could be used to treat low-level ofloxacin-resistant MDR-TB. Clinical trials should be conducted to confirm this observation.

In conclusion, we confirm that mutations of gyrA codons 90, 91 and 94 constituted the primary mechanism of fluoroquinolone resistance in MTB and indicate that mutations at codon 91 in gyrA gene might be associated with low-level resistance of ofloxacin.

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Conflict of interest

All authors declare to have no conflict of interest.

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