

ORIGINAL ARTICLE

Association of *gyrA/B* mutations and resistance levels to fluoroquinolones in clinical isolates of *Mycobacterium tuberculosis*

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To evaluate the association between mutations in the genes *gyrA/B* and resistance levels to fluoroquinolones in clinical isolates of *Mycobacterium tuberculosis*, a total of 80 ofloxacin-resistant isolates collected in 2009 by the Shanghai Municipal Centers for Disease Control and Prevention were studied. The minimum inhibitory concentration (MIC) of ofloxacin, moxifloxacin and gatifloxacin for each isolate was determined using the microscopic observation drug susceptibility assay. Sequencing was used to identify mutations in the quinolone resistance-determining region (QRDR) of the *gyrA* and *gyrB* genes. In total, 68 isolates had mutations in *gyrA*, three isolates had mutations in *gyrB*, six isolates had mutations in both *gyrA* and *gyrB*, and three isolates had no mutations. Two common mutations in *gyrA*, the D94G and D94N mutations, were associated with higher-level resistance to all three fluoroquinolones than two other common mutations (A90V and D94A). Understanding the relationship between MICs and mutations in ofloxacin-resistant isolates will facilitate the optimization of the use of new-generation fluoroquinolones to treat patients with ofloxacin-resistant tuberculosis (TB).

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INTRODUCTION

Fluoroquinolones, including the older-generation drug, ofloxacin, and the newer-generation drugs, gatifloxacin and moxifloxacin, are second-line anti-tuberculosis agents.¹ These agents are used in the treatment of drug-resistant tuberculosis, particularly for multi-drug-resistant tuberculosis (TB), which is defined as resistance to at least isoniazid and rifampicin.¹ Fluoroquinolones inactivate *Mycobacterium tuberculosis* by binding to gyrase-DNA complexes and inhibiting DNA replication.² The widespread and erroneous use of fluoroquinolones in TB treatment has led to the recent emergence of resistance.³

M. tuberculosis acquires resistance to fluoroquinolones mainly through mutations in conserved regions, referred to as quinolone resistance-determining regions (QRDRs), of the *gyrA* and *gyrB* genes, which encode DNA gyrase.^{2,4,5} DNA gyrase contains a drug-binding pocket called the quinolone-binding pocket (QBP), which consists of both amino acid residues and DNA nucleotides.^{6,7} Mutations in the two genes change the structure of the QBP and may lead to broad cross-resistance to all fluoroquinolones.^{8,9} The most frequent mutations in clinical isolates are found at codons 90 (A90V), 91 (S91P) and 94 (D94G, D94A, D94N and D94Y) of *gyrA*.^{10–12} Mutations of codons 500, 538, 539 and 540 in *gyrB* are also related to resistance to fluoroquinolones.^{13,14} The altered amino acids encoded by these gene mutations are all located within the QBP and interact directly with

quinolones.^{9,13} Because every generation of fluoroquinolones has the same drug targets, cross-resistance among fluoroquinolones is common. However, the resistance levels of each isolates against individual drugs are variable. In general, the minimum inhibitory concentrations (MICs) of newer generation fluoroquinolones are much lower than those of older generation fluoroquinolones.¹⁵ Thus, the newer-generation fluoroquinolones are reported to retain efficacy in the treatment of ofloxacin-resistant cases.^{15–17}

Despite current knowledge, the association between resistance levels and different mutations remains debatable. Some studies have shown that mutations in codon 94 in *gyrA* are related to higher-level resistance, whereas mutations in codon 91 of *gyrA* may be associated with lower-level resistance.^{10–12} However, a study by Cui and colleagues suggested that there may be no significant association between *gyrA* mutations and the levels of resistance to ofloxacin.¹⁸ Thus, this association is unclear and requires further exploration. As molecular techniques have been increasingly applied to the rapid diagnosis of drug resistant *M. tuberculosis*,¹⁹ clarifying the fluoroquinolone resistance levels of *M. tuberculosis* that carry different *gyrA* and *gyrB* mutations will help optimize therapeutic regimens in clinical TB treatment.

To explore the association between resistance levels to fluoroquinolones and different mutations in *M. tuberculosis*, we determined the MICs of ofloxacin, moxifloxacin and gatifloxacin on 80 ofloxacin-resistant isolates and identified the mutations in *gyrA/B*.

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MATERIALS AND METHODS

Bacterial isolates

A total of 1960 *M. tuberculosis* isolates were collected by the Shanghai Municipal Centers for Disease Control and Prevention in 2009. The identification of these *M. tuberculosis* isolates was performed by conventional biochemical and polymerase chain reaction (PCR) tests.²⁰ Among the isolates, 458 were identified as phenotypically resistant to at least one first-line anti-TB drug (isoniazid, rifampicin, ethambutol or streptomycin) by the proportion method on Lowenstein–Jensen medium with the following concentrations: isoniazid (0.2 mg/L), rifampin (40 mg/L), ethambutol (2 mg/L) and streptomycin (4 mg/L). The susceptibility of these 458 isolates to ofloxacin was further tested at a breakpoint concentration of 2 mg/L on Lowenstein–Jensen medium using the proportion method. From these analyses, 80 ofloxacin-resistant isolates were identified and selected for further study. *M. tuberculosis* H37Rv (ATCC 27294) was included as the reference strain.

Determination of MICs of ofloxacin, moxifloxacin and gatifloxacin

The MICs of ofloxacin (Sigma-Aldrich Co., St Louis, MO, USA), moxifloxacin (TRC Co., Toronto, Canada) and gatifloxacin (Sigma-Aldrich Co.) for the 80 isolates were determined using the microscopic observation drug susceptibility assay as recommended at <http://www.modsperu.org/>.^{21,22} The growth of *M. tuberculosis* was observed microscopically as the formation of characteristic strings and tangles. The validity of microscopic observation drug susceptibility for determining fluoroquinolone resistance has been demonstrated previously.²² Ofloxacin was tested at concentrations of 0.5, 1.0, 2.0, 4.0, 8.0, 16.0 and 32.0 mg/L; gatifloxacin and moxifloxacin were tested at concentrations of 0.125, 0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 mg/L. MICs were determined visually as the minimum drug concentration that prevented the growth of cord structures. The cutoff concentrations of resistance to ofloxacin, moxifloxacin and gatifloxacin were 2.0 mg/L, 0.5 mg/L and 0.5 mg/L, respectively.^{22,23}

DNA extraction

A loopful of *M. tuberculosis* culture from Lowenstein–Jensen medium was transferred to a 1.5 mL tube that contained 400 µL of TB lysis buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA (pH 8.0)) and heat inactivated at 85 °C for 30 min. The tube was then centrifuged at 10 000 g at 4 °C for 10 min. The supernatant was transferred to a fresh 1.5-mL tube and stored at –20 °C until use.

Sequencing of the QRDRs of the gyrA and gyrB genes

The primers for PCR amplification were newly designed using *gyrA* and *gyrB* sequences (GenBank accession number L27512) encompassing the QRDRs of these two genes (forward, 5'-CAG CTA CAT CGA CTA TGC GA-3' and reverse, 5'-GGG CTT CGG TGT ACC TCA T-3' for *gyrA*; forward, 5'-CGT AAG GCA CGA GAG TTG GT-3' and reverse, 5'-ATC TTG TGG TAG CGC AGC TT-3' for *gyrB*). The basic 50-µL amplification reaction mixture contained 3 µL DNA, 20 µL 2×PCR MIX (TianGen, Beijing, China), 10 µM PCR primer mixture (Sangon, Shanghai, China) and 17 µL distilled water. The following steps were performed: one denaturation cycle at 94 °C for 10 min, 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. Both PCR products were 320 base pairs in size. They were purified and sequenced by Sunnybio (Shanghai, China). Mutations of *gyrA* and *gyrB* were identified by aligning sequences of resistant isolates to the sequence of *M. tuberculosis* strain H37Rv using CLUSTAL W (European Bioinformatics Institute).

Statistical analysis

The association of *gyrA* mutations with MIC levels was analyzed using the Kruskal–Wallis test and Mann–Whitney *U* test in the Statistical Package for Social Sciences version 12.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS

To study the association between mutations and resistance levels, we determined the MICs of the three fluoroquinolones for the *M. tuberculosis* isolates and identified associated mutations in the *gyrA/B* genes. The profiles of these isolates (Table 1) show that the MIC₅₀ values of ofloxacin, moxifloxacin and gatifloxacin was 4 mg/L, 1 mg/L and 0.5 mg/mL, respectively. Seventy-seven isolates (96.25%, 77/80) carried mutations in the QRDRs of *gyrA/B* (Table 1); of those, 68 (88.3%, 68/77) had mutations in *gyrA* (at codons 88, 89, 90, 91 and 94); three isolates had mutations in *gyrB*; and the remaining six isolates had mutations in both the *gyrA* and *gyrB* genes. Based on the cutoff concentrations used, only one isolate carried no mutation in *gyrA* or *gyrB* and showed susceptibility to gatifloxacin and resistance to ofloxacin and moxifloxacin. All of the other isolates had broad cross-resistance to all three of the drugs.

A total of 61 (76.25%, 61/80) isolates carried the four most frequent mutations in *gyrA*: A90V (*n*=18), D94A (*n*=13), D94G (*n*=18) and D94N (*n*=12). The MIC distributions of fluoroquinolones for *M. tuberculosis* with these mutations are shown in Figure 1. To determine whether there is any correlation between the MICs of the fluoroquinolones and the different mutations in the isolates, the Kruskal–Wallis test was applied. The results showed that the MIC of each drug was significantly associated with the mutations (ofloxacin: $\chi^2=26.339$, $P<0.001$; moxifloxacin: $\chi^2=29.454$, $P<0.001$; gatifloxacin: $\chi^2=21.408$, $P<0.001$). These results strongly suggest that the *gyrA* mutations are associated with the different MICs of fluoroquinolones. To further explore this association, we applied pair-wise comparisons among these four mutations using the Mann–Whitney *U* test. We noticed that the D94G and D94N mutations were associated with significantly higher MICs of all three drugs than were the A90V and D94A mutations (Table 2 and Figure 1).

DISCUSSION

In this study, we present the phenotypic resistance profiles of 80 *M. tuberculosis* isolates and confirmed cross-resistance among different generations of fluoroquinolones. The obtained profiles indicated that the bactericidal activities of moxifloxacin and gatifloxacin are greater than the bactericidal activity of ofloxacin. The *gyrA* mutations D94G and D94N were associated with higher MICs than were the A90V and D94A mutations.

Resistance to fluoroquinolones in *M. tuberculosis* has been predominantly attributed to *gyrA* mutations.^{10,12} In this study, we found high mutation frequencies in codons 90 (18/80, 22.5%) and 94 (46/80, 57.5%). It was striking that for three different drugs, isolates with the A90V and D94A mutations showed a lower level of resistance, whereas isolates carrying the D94G and D94N mutations showed a higher level of resistance. Previous studies have indicated that fluoroquinolones inhibit the activity of the DNA gyrase complex by binding to the QBP; the binding process is shape-dependent, and mutations in the QRDRs modify the geometry of the QBP.^{8,9} We speculate that the higher resistance of *M. tuberculosis* carrying the D94G and D94N mutations relative to *M. tuberculosis* carrying the other two mutations is a result of larger QBP geometric changes, leading to greater instability. However, we noticed that this correlation was not absolute; several

Table 1 The mutation profiles and the fluoroquinolone MICs of the 80 ofloxacin-resistant *M. tuberculosis* isolates

Substitution			MIC (mg/L)			Total number with this mutation (%)	MIC ₅₀ (mg/L)		
<i>gyrA</i>	<i>gyrB</i>	<i>n</i>	OFX	MOX	GAT		OFX	MOX	GAT
G88A (GGC→GCC)	WT ^a	1	8.0	1.0	1.0	1 (1.25%)	8.0	1.0	1.0
D89G (GAG→AAG)	WT	1	8.0	2.0	0.5	1 (1.25%)	8.0	2.0	0.5
A90V (GCG→GTG)	WT	1	2.0	1.0	0.25	18 (22.50%)	4.0	1.0	0.5
		1	2.0	1.0	0.5				
		1	2.0	1.0	1.0				
		1	4.0	0.5	0.5				
		1	4.0	1.0	0.25				
		8	4.0	1.0	0.5				
		1	4.0	1.0	1.0				
		1	4.0	2.0	1.0				
		1	8.0	1.0	1.0				
		1	8.0	2.0	1.0				
A90V (GCG→GTG)	E498Q (GAA→CAA)	1	4.0	0.5	0.5	1 (1.25%)	4.0	0.5	0.5
A90V (GCG→GTG)	G551R (GGG→AGG)	1	2.0	0.5	0.5	2 (2.50%)	3.0	0.75	0.5
		1	4.0	1.0	0.5				
A90V (GCG→GTG)	N538S (AAC→AGC)	1	2.0	0.5	0.5	1 (1.25%)	2.0	0.5	0.5
S91P (TCG→CCG)	WT	1	2.0	1.0	0.5	1 (1.25%)	2.0	1.0	0.5
D94A (GAC→GCC)	WT	3	2.0	0.5	0.5	13 (16.25%)	4.0	1.0	0.5
		1	2.0	0.5	0.25				
		1	2.0	1.0	0.5				
		1	2.0	1.0	1.0				
		4	4.0	1.0	0.5				
		1	4.0	1.0	1.0				
		1	8.0	1.0	0.5				
		1	8.0	2.0	1.0				
		1	4.0	0.5	0.5				
		1	4.0	1.0	0.5				
D94G (GAC→GGC)	WT	1	4.0	0.5	0.5	18 (22.50%)	8.0	2.0	1.0
		1	4.0	1.0	0.5				
		2	4.0	1.0	1.0				
		1	4.0	4.0	1.0				
		4	8.0	2.0	0.5				
		4	8.0	2.0	1.0				
		3	8.0	2.0	2.0				
		1	16.0	4.0	2.0				
		1	16.0	4.0	4.0				
		1	8.0	2.0	1.0				
D94G (GAC→GGC)	E498Q (GAA→CAA)	1	8.0	2.0	1.0	1 (1.25%)	8.0	2.0	1.0
D94N (GAC→AAC)	WT	1	2.0	0.5	0.5	12 (15.00%)	8.0	2.0	1.0
		1	4.0	2.0	1.0				
		3	8.0	2.0	1.0				
		2	8.0	2.0	2.0				
		2	8.0	4.0	1.0				
		1	16.0	4.0	2.0				
		1	16.0	8.0	1.0				
		1	32.0	4.0	1.0				
		1	8.0	2.0	2.0				
		1	4.0	1.0	0.5				
D94N (GAC→AAC)	G551R (GGG→AGG)	1	8.0	2.0	2.0	1 (1.25%)	8.0	2.0	2.0
D94Y (GAC→TAC)	WT	1	4.0	1.0	0.5	4 (5.00%)	8.0	2.0	0.5
		1	8.0	2.0	0.5				
		2	8.0	2.0	1.0				
WT	E498Q (GAA→CAA)	1	4.0	1.0	0.5	1 (1.25%)	4.0	1.0	0.5
WT	A543V (GCG→GTG)	1	2.0	0.5	0.25	1 (1.25%)	2.0	0.5	0.25
WT	G551R (GGG→AGG)	1	8.0	2.0	1.0	1 (1.25%)	8.0	2.0	1.0
WT	WT	1	2.0	0.5	<0.125	3 (3.75%)	2.0	2.0	0.5
		1	2.0	2.0	0.5				
		1	4.0	0.5	0.25				
Total						80 (100%)	4.0	1.0	0.5
H37Rv		1	<0.5	<0.125	<0.125	1	<0.5	<0.125	<0.125

Abbreviations: GAT, gatifloxacin; MOX, moxifloxacin; OFX, ofloxacin.

^a Wild type.

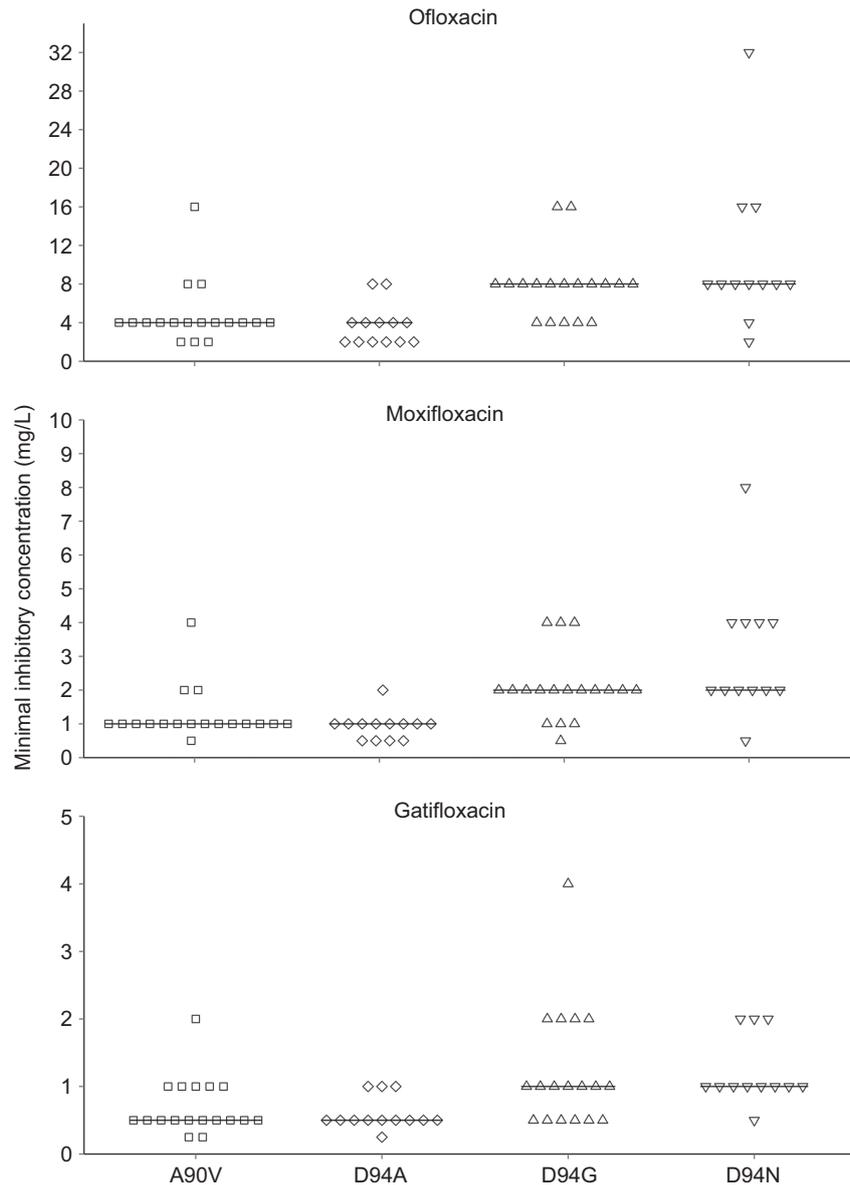


Figure 1 MICs of the three fluoroquinolones for isolates carrying the four most frequent mutations. The transverse lines represent the median MIC for each drug. The D94G and D94N mutations were significantly associated with higher resistance to all three drugs than the A90V and D94A mutations (pair-wise Mann–Whitney *U* test, Table 2).

Table 2 Pair-wise comparison of resistance levels of isolates carrying A90V/D94A/D94G/D94N mutations, using the Mann–Whitney *U* test.

Grouping mutations	Ofloxacin		Moxifloxacin		Gatifloxacin	
	Mann–Whitney <i>U</i>	<i>P</i> value ^a	Mann–Whitney <i>U</i>	<i>P</i> value ^a	Mann–Whitney <i>U</i>	<i>P</i> value ^a
A90V/D94A	86.000	0.168	82.000	0.079	107.500	0.661
A90V/D94G	68.000	0.001	71.000	0.002	92.500	0.019
A90V/D94N	40.500	0.002	31.500	0.001	40.500	0.002
D94A/D94G	33.500	<0.001	31.500	<0.001	55.500	0.008
D94A/D94N	21.500	0.001	14.000	<0.001	19.500	0.001
D94G/D94N	88.000	0.415	74.500	0.158	92.000	0.518

^a *P* value in bold: significant below the level of 0.05.

isolates with mutations in A90V and D94A have an extremely high level of resistance to fluoroquinolones (MIC \geq 16 mg/L). This phenomenon might be associated with additional resistance mechanisms, such as efflux pumps,²⁴ or the genetic background of these isolates.²⁵

Considering the limited number of effective drugs for TB treatment, identifying the resistance levels of individual mutations in *M. tuberculosis* is imperative to ensure that strains with a low-level of resistance are effectively treated with high-dose antibiotics or antibiotics with high bactericidal activity.²⁶ Fluoroquinolones are important second-line drugs for treating multidrug-resistant TB.¹ Although broad-cross resistance within fluoroquinolones has been identified, a recent study showed that the MIC of moxifloxacin was \geq 4.0 mg/L, which is above moxifloxacin's peak serum concentration and indicates possible treatment failure, for only 4% of ofloxacin-resistant isolates.¹⁵ However, in this study, we found that the MIC of moxifloxacin was \geq 4.0 mg/L for 11.25% (9/80) of the isolates (Table 1 and Figure 1).²⁷ Eight isolates carried mutations in D94G or D94N, which were associated with higher resistance to fluoroquinolones; these eight isolates accounted for as many as 25% of all 32 isolates carrying these mutations. Therefore, caution must be taken when using moxifloxacin to treat ofloxacin-resistant cases.

The mutations in *gyrB* (N498Q, A543V and G551R) were located outside the QRDR (codons 500 to 540) and have not been reported to be associated with resistance in earlier studies.^{13,14} However, in the nine isolates with *gyrB* mutations, six also carried additional *gyrA* mutations. Thus, these three *gyrB* mutations may not be implicated in resistance. In fact, the G551R mutation has also been detected in susceptible isolates;¹⁸ therefore, we speculate that G551R may arise from a single-nucleotide polymorphism rather than being a resistance-related mutation.

In conclusion, two common mutations of *gyrA*, D94G and D94N, were significantly associated with higher resistance to fluoroquinolones. The MIC of moxifloxacin was \geq 4.0 mg/L for a considerable proportion of clinical isolates carrying these two mutations, which might lead to treatment failure. Understanding the relationship between MICs and mutations in ofloxacin-resistant TB cases will be helpful when new-generation fluoroquinolones are used for TB treatment.

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