Mutations in *gyrA* and *gyrB* among Fluoroquinolone- and Multidrug-Resistant *Mycobacterium tuberculosis* Isolates

Jung-Yien Chien,^{a,b} Wei-Yih Chiu,^{a,b} Shun-Tien Chien,^c Chia-Jung Chiang,^c Chong-Jen Yu,^b Po-Ren Hsueh^{b,d}

Graduate Institute of Clinical Medicine, College of Medicine, National Taiwan University, Taipei, Taiwan^a; Department of Internal Medicine, National Taiwan University Hospital, College of Medicine, National Taiwan University, Taipei, Taiwan^b; Chest Hospital, Ministry of Health and Welfare, Tainan, Taiwan^c; Department of Laboratory Medicine, National Taiwan University Hospital, College of Medicine, National Taiwan University, Taipei, Taiwan^d

In order to correlate the mutations inside the entire *gyrA* and *gyrB* genes with the level of resistance to ofloxacin (OFX) and moxifloxacin (MFX) in isolates of multidrug-resistant *Mycobacterium tuberculosis* (MDR-TB), a total of 111 isolates were categorized into OFX-susceptible (MIC, ≤ 2 $\mu\text{g/ml}$) and low-level (MIC, 4 to 8 $\mu\text{g/ml}$) and high-level (MIC, ≥ 16 $\mu\text{g/ml}$) OFX-resistant isolates and MFX-susceptible (MIC, ≤ 0.5 $\mu\text{g/ml}$) and low-level (MIC, 1 to 2 $\mu\text{g/ml}$) and high-level (MIC, ≥ 4 $\mu\text{g/ml}$) MFX-resistant isolates. Resistance-associated mutations inside the *gyrA* gene were found in 30.2% of OFX-susceptible and 72.5% and 72.2% of low-level and high-level OFX-resistant isolates and in 28.6% of MFX-susceptible and 58.1% and 83.9% of low-level and high-level MFX-resistant isolates. Compared with OFX-susceptible isolates, low-level and high-level OFX-resistant isolates had a significantly higher prevalence of mutations at *gyrA* codons 88 to 94 (17.0%, 65.0%, and 72.2%, respectively; $P < 0.001$) and a higher prevalence of the *gyrB* G512R mutation (0.0%, 2.5%, and 16.7%, respectively; $P = 0.006$). Similarly, compared with MFX-susceptible isolates, low-level and high-level MFX-resistant isolates had a significantly higher prevalence of mutations at *gyrA* codons 88 to 94 (14.3%, 51.6%, and 80.6%, respectively; $P < 0.001$) as well as a higher prevalence of the *gyrB* G512R mutation (0.0%, 0.0%, and 12.9%, respectively; $P = 0.011$). D94G and D94N mutations in *gyrA* and the G512R mutation in *gyrB* were correlated with high-level MFX resistance, while the D94A mutation was associated with low-level MFX resistance. The prevalence of mutations at *gyrA* codons 88 to 94 and the *gyrB* G512R mutation were higher among fluoroquinolone (FQ)-susceptible East Asian (Beijing) and Indo-Oceanic strains than they were among Euro-American strains, implying that molecular techniques to detect FQ resistance may be less specific in areas with a high prevalence of East Asian (Beijing) and Indo-Oceanic strains.

Fluoroquinolones (FQs) are used as second-line drugs for the treatment of tuberculosis (TB). These broad-spectrum antibacterial agents with bactericidal activity against *Mycobacterium tuberculosis* exert their bactericidal effects by inhibiting mycobacterial DNA gyrase activity, which prevents bacterial DNA from unwinding and replicating (1–3).

Moxifloxacin (MFX), a “fourth-generation” FQ, has been shown to have better activity against *M. tuberculosis* than ofloxacin (OFX) and is recommended by the World Health Organization (WHO) for the treatment of multidrug-resistant TB (MDR-TB; defined as resistant to at least two of the most effective antituberculosis drugs, isoniazid and rifampin) (2, 3). Unfortunately, the widespread use of FQs to treat bacterial infections has led to the emergence of FQ-resistant MDR-TB and extensively drug-resistant TB (XDR-TB; defined as MDR-TB resistant to any FQ and a second-line injectable drug) (4, 5), thereby complicating patient care.

Mutations in genes encoding DNA gyrase subunits *gyrA* and *gyrB* are the most common mechanism conveying FQ resistance in TB (6, 7). The most frequent resistance-associated mutations occur in a conserved region of the *gyrA* gene (codons 74 to 113) and, less frequently, the *gyrB* gene (codons 461 to 499), known as the quinolone resistance-determining region (QRDR) (2, 7, 8). However, many previous studies found that up to 60% of FQ-resistant *M. tuberculosis* isolates lack known mutations in the QRDR of *gyrA* or *gyrB* or both (7, 9), which compromises the sensitivity and specificity of molecular testing methods (7).

In MDR-TB, it is unclear whether resistance-associated mutations outside the QRDR of *gyrA* and *gyrB* are associated with FQ resistance (7). In this study, we evaluated the possible association

between the level of resistance to OFX and MFX and mutations in and outside the QRDR of the *gyrA* and *gyrB* genes in *M. tuberculosis* isolates.

MATERIALS AND METHODS

Selection of isolates. This study was conducted at the Chest Hospital, a tertiary TB referral hospital in Taiwan. *M. tuberculosis* was identified by a combination of morphology, the growth rate of the colonies, and biochemical tests (10). Drug susceptibility to first-line anti-TB drugs, including isoniazid (0.2 $\mu\text{g/ml}$), rifampin (1.0 $\mu\text{g/ml}$), and ethambutol (5.0 $\mu\text{g/ml}$), and second-line anti-TB drugs, including streptomycin (2.0 $\mu\text{g/ml}$), kanamycin (6.0 $\mu\text{g/ml}$), and ofloxacin (2.0 $\mu\text{g/ml}$), was tested using modified proportional disk elution methods as described previously (5, 11). This study was approved by the Institutional Review Board of Chest Hospital (B-ER-102-164).

From 2006 to 2011, a total of 55 consecutive OFX-resistant MDR isolates were collected, and we selected one out of every two OFX-susceptible MDR isolates, leading to 56 OFX-susceptible MDR isolates collected. *M. tuberculosis* isolates were preserved at -80°C and were subcultured onto Lowenstein-Jensen (LJ) medium prior to determining MIC

Received 3 May 2015 Returned for modification 27 June 2015

Accepted 10 January 2016

Accepted manuscript posted online 19 January 2016

Citation Chien J-Y, Chiu W-Y, Chien S-T, Chiang C-J, Yu C-J, Hsueh P-R. 2016. Mutations in *gyrA* and *gyrB* among fluoroquinolone- and multidrug-resistant *Mycobacterium tuberculosis* isolates. Antimicrob Agents Chemother 60:2090–2096. doi:10.1128/AAC.01049-15.

Address correspondence to Po-Ren Hsueh, hsporen@ntu.edu.tw.

Copyright © 2016, American Society for Microbiology. All Rights Reserved.

values. MICs were measured by the broth microdilution method using Sensititre microtiter trays (Trek Diagnostic Systems, Cleveland, OH, USA). The range of concentrations tested was 0.25 to 32 $\mu\text{g/ml}$ for OFX and 0.06 to 8 $\mu\text{g/ml}$ for MFX. The results were read at 21 days via the mirror method according to the instructions supplied by the manufacturer. The isolates were categorized into OFX-susceptible (MIC, $\leq 2 \mu\text{g/ml}$) and low-level (MIC, 4 to 8 $\mu\text{g/ml}$) and high-level (MIC, $\geq 16 \mu\text{g/ml}$) OFX-resistant isolates and MFX-susceptible (MIC, $\leq 0.5 \mu\text{g/ml}$) and low-level (MIC, 1 to 2 $\mu\text{g/ml}$) and high-level (MIC, $\geq 4 \mu\text{g/ml}$) MFX-resistant isolates. Genotyping (spoligotyping and 24-locus mycobacterial interspersed repetitive-unit-variable-number tandem-repeat [MIRU-VNTR] genotyping) was performed as previously described (12, 13), and major lineages were determined.

DNA sequencing. The entire *gyrA* and *gyrB* genes were amplified with 8 primer sets, GYRA1 (−43 to 802 bp; forward 5′-GAT GTC TAA CGC AAC CCT GCG TTC GAT and reverse 5′-AGG TAC GAC CGC GGG AAT CCT CTT CTA), GYRA2 (593 to 1,376 bp; forward 5′-CCG ACG CGG TGT TCT GG and reverse 5′-TCG ATT TTG GCC AGG TCG TC), GYRA3 (1,230 to 2,049 bp; forward 5′-GTC GGA GAC CGT CGA TA and reverse 5′-GAA CCT GAT GGA CTG CCC), and GYRA4 (1,883 to 2,678 bp; forward 5′-GCA ACG GGC TGG TGA AAA AG and reverse 5′-GAC CAA GCC ATC CGC ATT C) for the *gyrA* gene, and GYRB1 (−365 to 446 bp; forward 5′-ATC GCC GCA GCG GTT G and reverse 5′-ACC TGA GAC CAC TCG TAC CC), GYRB2 (287 to 1,089 bp; forward 5′-TCG ACG TGG TGA TGA CAC AA and reverse 5′-GCT GAC CTT CAC CGA GAT CA), GYRB3 (939 to 1,744 bp; forward 5′-CGG CAC CCA CGA AGA GG and reverse 5′-ATG CGA ATT CCG GGT CAC TG), and GYRB4 (1,546 to 2,203 bp; forward 5′-GAG TTC GAT ATC GGC AAG CTG and reverse 5′-CCC GTC GCG CAC CTC) for the *gyrB* gene. The sequences were assembled by Sanger sequencing and were compared to the reference strain sequence (H37Rv; GenBank accession number NC_000962.3) using BioEdit software.

Statistical analysis. The sequences from isolates were compared to the reference strain sequence, and the proportions of resistant and susceptible isolates harboring specific mutations were compared using the chi-square test or Fisher's exact test as appropriate. A *P* value of <0.05 was considered to indicate statistical significance; all tests were two tailed. All statistical analyses were performed with the statistical package Stata for Windows (version 11; College Station, Texas).

RESULTS

Of the 111 MDR isolates, 38 (34.2%), 50 (45.0%), and 24 (21.6%) were also resistant to ethambutol, streptomycin, and kanamycin, respectively. Of these, 54 isolates (48.6%) belonged to the East Asian (Beijing) strain, 25 (22.5%) were Euro-American strains, 30 (27.0%) were Indo-Oceanic strains, and the remaining 2 isolates (1.8%) had unique patterns. Of the 111 isolates, 53 (47.7%) were OFX-susceptible isolates, 40 (36.0%) were low-level OFX-resistant isolates, and 18 (16.2%) were high-level OFX-resistant isolates. In addition, 49 (44.1%) were MFX-susceptible isolates, 31 (27.9%) were low-level MFX-resistant isolates, and 31 (27.9%) were high-level MFX-resistant isolates (Table 1).

The sequencing results of the entire *gyrA* and *gyrB* genes and the MIC distributions of OFX and MFX are shown in Table 1. A total of 111 (100%) and 108 (97.3%) isolates had *gyrA* E21Q and S95T natural polymorphisms, respectively. Resistance-associated mutations in *gyrA* were observed in 16/53 (30.2%) OFX-susceptible and 29/40 (72.5%) low-level and 13/18 (72.2%; $P < 0.001$) high-level OFX-resistant strains as well as in 14/49 (28.6%) MFX-susceptible and 18/31 (58.1%) low-level and 26/31 (83.9%; $P < 0.001$) high-level MFX-resistant isolates. In addition, a total of 11 (20.8%) OFX-susceptible and 10 (25.0%) low-level and 8 (44.4%; $P = 0.149$) high-level OFX-resistant strains and 8 (16.3%) MFX-

susceptible and 10 (32.3%) low-level and 11 (35.5%; $P = 0.103$) high-level MFX-resistant strains carried mutations in *gyrB*. Among 29 isolates with *gyrB* mutations, 22 (75.9%) also had mutations in *gyrA*.

Of the 111 isolates, nine isolates (8.1%) had double mutations in the *gyrA* gene, one (0.9%) isolate had double mutations in the *gyrB* gene, and 24 (21.6%) isolates had 2 to 3 mutations in both genes. No mutations in *gyrA* or *gyrB* were found in the remaining 34 (64.2%) OFX-susceptible isolates or in the 9 (22.5%) low-level and 3 (16.7%; $P < 0.001$) high-level OFX-resistant isolates or in the remaining 33 (67.3%) MFX-susceptible isolates or in the 10 (32.3%) low-level and 3 (9.7%; $P < 0.001$) high-level MFX-resistant isolates.

As shown in Table 2, the most prevalent mutations were at codons 88 to 94 of the *gyrA* gene, with mutation at codon 94 being the most prevalent. Compared with OFX-susceptible isolates, low-level and high-level OFX-resistant isolates had a significantly higher prevalence of mutations at *gyrA* codons 88 to 94 (9/53 [17.0%] versus 26/40 [65.0%] and 13/18 [72.2%], respectively; $P < 0.001$) and a higher prevalence of the *gyrB* G512R mutation (0/53 [0.0%] versus 1/40 [2.5%] and 3/18 [16.7%], respectively; $P = 0.006$). Similarly, compared with MFX-susceptible isolates, low-level and high-level MFX-resistant isolates had a significantly higher prevalence of mutations at *gyrA* codons 88 to 94 (7/49 [14.3%] versus 16/31 [51.6%] and 25/31 [80.6%], respectively; $P < 0.001$) and at *gyrA* codon 94 (4/49 [8.2%] versus 10/31 [32.3%] and 24/31 [77.4%]; $P < 0.001$) and a higher prevalence of the *gyrB* G512R mutation (0/49 [0.0%] versus 0/31 [0.0%] and 4/31 [12.9%], respectively; $P = 0.011$). Three (5.7%) OFX-susceptible isolates, four (6.9%) OFX-resistant isolates, two (4.1%) MFX-susceptible isolates, and five (8.1%) MFX-resistant isolates carried mutations only in *gyrB*, and they were not associated with resistance to OFX or MFX.

As shown in Table 2, the D94G mutation, the most common mutation in the *gyrA* gene, was associated with OFX resistance (15/17, 88.2%; $P = 0.001$) and high-level MFX resistance (12/17, 70.6%; $P < 0.001$). Also, we found that the D94N mutation was significantly associated with high-level OFX (5/9, 55.6%; $P = 0.001$) and with high-level MFX resistance (7/9, 77.8%; $P = 0.001$) and that the D94A mutation was associated with low-level MFX resistance (4/6, 66.7%; $P = 0.032$). The mutation at G512R in *gyrB* was associated with high-level OFX (3/4, 72.5%; $P = 0.006$) and MFX resistance (4/4, 100%; $P = 0.011$). Although the *gyrB* mutation G512R was associated with high-level OFX and MFX resistance, 75% (3/4) of the isolates had resistance-associated mutations in *gyrA* (one with a G88C mutation, one with a D94G mutation, and one with a D94N mutation). The remaining one isolate that only had the *gyrB* mutation G512R also had high-level OFX and MFX resistances.

Mutations at codons 88 to 94 of the *gyrA* gene were associated with OFX and MFX resistance in East Asian (Beijing), Euro-American, and Indo-Oceanic strains (Table 3). However, compared with Euro-American strains, OFX- and MFX-susceptible East Asian (Beijing) and Indo-Oceanic strains were more likely to have mutations at codons 88 to 94 of the *gyrA* gene.

Table 4 shows the sensitivity, specificity, positive likelihood ratio (LR+), negative likelihood ratio (LR−), and accuracy of using *gyrA* codon 88 to 94 mutations and the *gyrB* G512R mutation for identifying resistance to OFX and MFX. For OFX, the sensitivity was 69.0% (95% confidence interval [CI], 55.5% to

TABLE 1 MICs of ofloxacin and moxifloxacin established for multidrug-resistant *Mycobacterium tuberculosis* with resistance-associated mutations in the *gyrA* and *gyrB* genes

Gene mutations (codon/nucleotide)		Total no. of isolates	No. of isolates																	
			MIC (µg/ml) of ofloxacin									MIC (µg/ml) of moxifloxacin								
			0.25	0.5	1	2	4	8	16	32	>32	0.06	0.12	0.25	0.5	1	2	4	8	>8
<i>gyrA</i>	<i>gyrB</i>																			
G88C (Ggc/Tgc)	G512R (Ggg/Agg)	1									1									1
A90V (gCg/gTg)		4	1				1	2				1	1		1	1				
A90V (gCg/gTg)	A403S (Gcg/Tcg)	1					1									1				
A90V (gCg/gTg) & A384V (gCa/gTa)	M291I (atG/atC)	2		1			1						1			1				
S91P (Tcg/Ccg)		2	1					1									2			
D94G (GAc/GGc)		13		1	1		1	7	2	1			1	1			3	6	2	
D94G (GAc/GGc)	G512R (Ggg/Agg)	1					1											1		
D94G (GAc/GGc) & P190L (cCg/cTg)		1						1										1		
D94G (GAc/GGc) & A384V (gCa/gTa)	M291I (atG/atC)	2						1	1										2	
D94N (GAc/AAc)		6						3	3								1	3	1	1
D94N (GAc/AAc)	H244N (Cac/Aac)	1							1								1			
D94N (GAc/AAc) & A132S (Gcc/Tcc)	H244N (Cac/Aac)	1						1										1		
D94N (GAc/AAc) & A132S (Gcc/Tcc)	G512R (Ggg/Agg)	1							1										1	
D94A (GAc/GCc)		1					1									1				
D94A (GAc/GCc)	A403S (Gcg/Tcg)	2				1	1									1	1			
D94A (GAc/GCc)	N499T (AAc/ACc)	1							1											1
D94A (GAc/GCc) & A384V (gCa/gTa)		1						1									1			
D94A (GAc/GCc) & A384V (gCa/gTa)	M291I (atG/atC) & A504T (Gcg/Acg)	1								1										1
D94V (GAc/GTc)		2		1		1							1							1
D94H (GAc/CAC)		3				1		2						1			1	1		
D94Y (GAc/TAc)		1							1									1		
A288D (gCc/gAc)		1					1							1						
T335A (Acc/Gcc)		1	1										1							
A384V (gCa/gTa)	M291I (atG/atC)	8	1		1	4		2					1	3	1	2		1		
	D461N (Gac/Aac)	1	1										1							
	I485V (Atc/Gtc)	1					1									1				
	N499D (AAc/GAc)	3				1	1			1					1	1		1		
	A508S (Gcg/Tcg)	1				1											1			
	G512R (Ggg/Agg)	1									1									1
		46	5	10	13	6	4	5	1	2		2	14	9	8	6	4	1	2	
Total		111	10	13	15	15	14	26	11	3	4	2	20	17	10	15	16	17	9	5

80.5%), the specificity was 83.0% (70.2% to 91.9%), the LR+ was 4.1 (2.2 to 7.5), the LR− was 0.4 (0.3 to 0.6), and the accuracy rate was 75.7% (66.6% to 83.3%). For MFX, the corresponding values were 67.7% (54.7% to 79.1%), 85.7% (72.8% to 94.1%), 4.7 (2.3 to 9.6), 0.4 (0.3 to 0.5), and 75.7% (66.6% to 83.3%), respectively. The specificity and LR+ of predicting OFX and MFX resistance in East Asian (Beijing) and Indo-Oceanic strains were slightly lower than those in Euro-American strains.

DISCUSSION

Our study showed that 72.4% of OFX-resistant and 71.0% of MFX-resistant MDR-TB isolates had mutations in the *gyrA* gene, but those mutations were also found in 30.2% of OFX-susceptible and 28.6% of MFX-susceptible isolates. Mutations in the *gyrB* gene were less commonly found in OFX-resistant

(31.0%) and MFX-resistant (33.9%) isolates, but they were found in 20.8% of OFX-susceptible and 16.3% of MFX-susceptible isolates. Mutations at codons 88 to 94 of *gyrA* and the *gyrB* G512R mutation were significantly associated with OFX and MFX resistance; however, among East Asian (Beijing) and Indo-Oceanic strains, OFX- or MFX-susceptible isolates tended to harbor more of those mutations than Euro-American strains.

The most common mutations in the *gyrA* gene that confer resistance in *M. tuberculosis* to fluoroquinolones are the A90V, S91P, and D94 (G, A, H, N, or Y) mutations (2, 7, 8, 14, 15). In this study, we found a rare mutation at codon 94 (D94V) in two isolates. The two isolates were susceptible to OFX, but one showed high-level resistance to MFX. Although some previous studies have shown that the levels of resistance to FQs are not associated with the type of mutation present in the *gyrA* and *gyrB* genes (16),

TABLE 2 Mutations in the *gyrA* and *gyrB* genes and associated levels of ofloxacin and moxifloxacin resistance in multidrug-resistant *Mycobacterium tuberculosis*

Gene mutations (codon/nucleotide)	Ofloxacin				Moxifloxacin			
	No. susceptible (<i>n</i> = 53)	No. resistant		<i>P</i> value	No. susceptible (<i>n</i> = 49)	No. resistant		<i>P</i> value
		Low-level (<i>n</i> = 40)	High-level (<i>n</i> = 18)			Low-level (<i>n</i> = 31)	High-level (<i>n</i> = 31)	
<i>gyrA</i>								
Codon 88–94	9	26	13	<0.001	7	16	25	<0.001
G88C (Ggc/Tgc)	0	0	1	0.074	0	0	1	0.559
A90V (gCg/gTg)	2	5	0	0.112	3	4	0	0.106
S91P (Tcg/Ccg)	1	1	0	0.801	0	2	0	0.152
Codon 94	6	20	12	<0.001	4	10	24	<0.001
D94G (GAc/GGc)	2	11	4	0.002	2	3	12	<0.001
D94N (GAc/AAc)	0	4	5	0.001	0	2	7	0.001
D94A (GAc/GCc)	1	3	2	0.170	0	4	2	0.032
D94H (GAc/CAC)	1	2	0	0.752	1	1	1	1.000
D94V (GAc/GTc)	2	0	0	0.653	1	0	1	1.000
D94Y (GAc/TAc)	0	0	1	0.162	0	0	1	0.559
A132S (Gcc/Tcc)	0	1	1	0.143	0	0	2	0.152
P190L (cCg/cTg)	0	1	0	0.523	0	0	1	0.559
A288D (gCc/gAc)	0	1	0	0.523	1	0	0	1.000
T335A (Acc/Gcc)	1	0	0	1.000	1	0	0	1.000
A384V (gCa/gTa)	7	5	2	1.000	6	4	4	1.000
<i>gyrB</i>								
A227D (gCc/gAc)	1	0	0	1.000	1	0	0	1.000
H244N (Cac/Aac)	0	1	1	0.143	0	1	1	0.310
M291I (atG/atC)	7	4	2	0.923	6	3	4	1.000
A403S (Gcg/Tcg)	1	2	0	0.752	0	3	0	0.041
D461N (Gac/Aac)	1	0	0	1.000	1	0	0	1.000
I485V (Atc/Gtc)	0	1	0	0.523	0	1	0	0.559
Any codon 499	1	1	2	1.000	1	1	2	0.811
N499D (AAc/GAc)	1	1	1	0.565	1	1	1	1.000
N499T (AAc/ACc)	0	0	1	0.162	0	0	1	0.564
A504T (Gcg/Acg)	0	0	1	0.162	0	0	1	0.564
A508S (Gcg/Tcg)	1	0	0	1.000	0	1	0	0.559
G512R (Ggg/Agg)	0	1	3	0.006	0	0	4	0.011
Wild type	34	9	3	<0.001	33	10	3	<0.001

we found that the D94G and D94N mutations in *gyrA* and the G512R mutation in *gyrB* were correlated with high-level MFX resistance, while the D94A mutation was associated with low-level MFX resistance. Similar findings were reported by Nosova et al. and by Kam et al. (15, 17). However, this association is not absolute since the MICs of FQs for isolates with the same mutation vary widely.

Mutations in the *gyrB* gene are less commonly associated with FQ resistance in *M. tuberculosis* (7, 8). We found that 20.8% (11/53) of OFX-susceptible isolates, 16.3% (8/49) of MFX-susceptible isolates, 25.0% (10/40) and 32.2% (10/31) of low-level OFX- and MFX-resistant isolates, respectively, and 44.4% (8/18) and 35.5% (11/31) of high-level OFX- and MFX-resistant isolates, respectively, had mutations in *gyrB*. However, among 29 isolates with *gyrB* mutations, 22 (75.9%) also had mutations in *gyrA*, and 18 (62.1%) isolates concomitantly had mutations at codon 94 in *gyrA*. Among 7 isolates having only *gyrB* mutations, the sole isolate with a *gyrB* G512R mutation had high-level OFX and MFX resistance. However, due to the small numbers of isolates with isolated *gyrB* mutations, no such mutation was significantly independently associated with high-level OFX or MFX resistance.

We found that 24 isolates (21.6%) had 2 to 3 mutations in the *gyrA* gene or the *gyrB* gene or in both genes. Of 24 isolates with multiple mutations, 6 (25.0%) had high-level OFX resistance and 10 (41.7%) had high-level MFX resistance. We also found that the rate of high-level resistance to OFX (25.0%) or MFX (41.7%) among isolates with multiple mutations was similar to that among isolates with a single mutation (22.0% for OFX and 43.9% for MFX). Similar findings were reported by Yin and Yu and Matrat et al. (16, 18).

A substantial proportion of FQ-resistant isolates did not harbor mutations in *gyrA* or *gyrB*. In mycobacteria, FQ resistance can also be conferred by increased FQ efflux or by DNA mimicry in laboratory strains (19, 20). We found that isolates with low-level MFX resistance had fewer mutations in the *gyrA* and *gyrB* genes than isolates with high levels of resistance to MFX. This may indicate that alternative mechanisms of FQ resistance may play a more significant role in conferring low-level MFX resistance (7). This suggests that molecular diagnostic tests that focus on these codons will have lower sensitivity in isolates with low-level FQ resistance.

In this study, mutations at codons 88 to 94 of *gyrA* were found in 17.0% (9/53) of OFX-susceptible isolates and in 14.3% (7/49) of

TABLE 3 Mutations in the *gyrA* and *gyrB* genes and ofloxacin and moxifloxacin resistance in different strains of multidrug-resistant *Mycobacterium tuberculosis*

Gene mutations (codon/nucleotide)	East Asian (Beijing) strain						Euro-American strain						Indo-Oceanic strain					
	Ofloxacin-resistant			Moxifloxacin-resistant			Ofloxacin-resistant			Moxifloxacin-resistant			Ofloxacin-resistant			Moxifloxacin-resistant		
	No. no (n = 29)	No. yes (n = 25)	P value	No. no (n = 25)	No. yes (n = 29)	P value	No. no (n = 9)	No. yes (n = 16)	P value	No. no (n = 10)	No. yes (n = 15)	P value	No. no (n = 15)	No. yes (n = 15)	P value	No. no (n = 14)	No. yes (n = 16)	P value
<i>gyrA</i>																		
Codon 88-94	5	17	<0.001	4	18	0.001	0	10	0.003	0	10	0.001	4	10	0.066	3	11	0.014
G88C (Ggc/Tgc)	0	0		0	0		0	0		0	0		0	1	1.000	0	1	1.000
A90V (gCg/gTg)	0	1	0.463	1	0	0.463	0	3	0.280	0	3	0.250	2	1	1.000	2	1	0.586
S91P (Tcg/Ccg)	1	1	1.000	0	2	0.493	0	0		0	0		0	0		0	0	
Any codon 94	4	15	0.001	3	16	0.001	0	7	0.027	0	7	0.020	2	8	0.05	1	9	0.007
D94G (GAc/GcC)	2	8	0.032	2	8	0.086	0	4	0.260	0	4	0.125	0	3	0.224	0	3	0.228
D94N (GAc/AAc)	0	1	0.463	0	1	1.000	0	2	0.520	0	2	0.500	0	4	1.000	0	4	0.103
D94A (GAc/GcC)	0	3	0.093	0	3	0.240	0	1	1.000	0	1	1.000	1	1	1.000	0	2	0.485
D94H (GAc/CAC)	1	2	0.591	1	2	1.000	0	0		0	0		0	0		0	0	
D94V (GAc/GTc)	1	0	1.000	0	1	1.000	0	0		0	0		1	0	1.000	1	0	0.467
D94Y (GAc/TAc)	0	1	0.463	0	1	1.000	0	0		0	0		0	0		0	0	
A132S (Gcc/Tcc)	0	1	0.463	0	1	1.000	0	1	1.000	0	1	1.000	0	0		0	0	
P190L (cCg/cTg)	0	1	0.463	0	1	1.000	0	0		0	0		0	0		0	0	
A288D (gCg/gAc)	0	0		0	0		0	1	1.000	1	0	0.4	0	0		0	0	
T335A (Acc/Gcc)	0	0		0	0		0	0		0	0		1	0	1.000	1	0	0.467
A384V (gCa/gTa)	1	3	0.326	1	3	0.615	1	1	1.000	1	1	1.000	5	3	0.682	4	4	1.000
<i>gyrB</i>																		
A227D (gCc/gAc)	0	0		0	0		1	0	0.360	1	0		0	0		0	0	
H244N (Cac/Aac)	0	0		0	0		0	1	1.000	0	1	1.000	0	1	1.000	0	1	1.000
M291I (atG/atC)	1	2	0.591	1	2	1.000	1	1	1.000	1	1	1.000	5	3	0.682	4	4	1.000
A403S (Gcg/Tcg)	0	0		0	0		0	1	1.000	0	1	1.000	1	1	1.000	0	2	0.485
D461N (Gac/Aac)	1	0	1.000	1	0	0.463	0	0		0	0		0	0		0	0	
I485V (Atc/Gtc)	0	1	0.463	0	1	1.000	0	0		0	0		0	0		0	0	
N499D (AAc/GAc)	0	0		0	0		1	2	1.000	1	2	1.000	0	0		0	0	
N499T (AAc/ACc)	0	0		0	0		0	1	1.000	0	1	1.000	0	0		0	0	
A504T (Gcg/ACg)	0	1	0.463	0	1	1.000	0	0		0	0		0	0		0	0	
A508S (Gcg/TCg)	1	0		0	1	1.000	0	0		0	0		0	0		0	0	
G512R (Ggg/Agg)	0	3	0.093	0	3	0.240	0	0		0	0		0	1	1.000	0	1	1.000

TABLE 4 Performance of mutation detection (*gyrA* codon 88 to 94 or *gyrB* G512R) for identification of ofloxacin and moxifloxacin resistance (low-level and high-level) in different strains of multidrug-resistant *Mycobacterium tuberculosis*

Agent	Strain	No. of isolates				Performance, % (95% confidence interval)				
		Resistant		Susceptible		Sensitivity	Specificity	LR+	LR–	Accuracy
		Mutation positive	Mutation negative	Mutation positive	Mutation negative					
Ofloxacin	All	40	18	9	44	69.0 (55.5–80.5)	83.0 (70.2–91.9)	4.1 (2.2–7.5)	0.4 (0.3–0.6)	75.7 (66.6–83.3)
	East Asian (Beijing)	18	7	5	24	72.0 (50.6–87.9)	82.8 (64.2–94.2)	4.2 (1.8–9.6)	0.3 (0.2–0.6)	77.8 (64.4–88.0)
	Euro-American	10	6	0	9	62.5 (35.4–84.8)	100.0 (66.4–100.0)	>10.0	0.4 (0.2–0.7)	76.0 (54.9–90.6)
	Indo-Oceanic	10	5	4	11	66.7 (38.4–88.2)	73.3 (44.9–92.2)	2.5 (1.0–6.2)	0.5 (0.2–1.0)	70.0 (50.6–85.3)
Moxifloxacin	All	42	20	7	42	67.7 (54.7–79.1)	85.7 (72.8–94.1)	4.7 (2.3–9.6)	0.4 (0.3–0.5)	75.7 (66.6–83.3)
	East Asian (Beijing)	19	10	4	21	65.5 (45.7–82.1)	84.0 (63.9–95.5)	4.1 (1.6–10.4)	0.4 (0.2–0.7)	74.1 (60.3–85.0)
	Euro-American	10	5	0	10	66.7 (38.4–88.2)	100.0 (69.2–100.0)	>10.0	0.3 (0.2–0.7)	80.0 (59.3–93.2)
	Indo-Oceanic	10	5	4	11	66.7 (38.4–88.2)	73.3 (44.9–92.2)	2.5 (1.0–6.2)	0.5 (0.2–1.0)	70.0 (50.6–85.3)

MFX-susceptible isolates. Surprisingly, those isolates carrying resistance-conferring mutations were susceptible to FQs. Among those susceptible isolates harboring mutations at codons 88 to 94, none were Euro-American strains, but 5 (55.6%) and 4 (44.4%) OFX-susceptible isolates and 4 (57.1%) and 3 (42.9%) MFX-susceptible isolates were East Asian (Beijing) and Indo-Oceanic strains. In addition, there was a limitation because the number of isolates in this study was small, which limited the power to investigate the effect of specific mutations among different lineage types on MICs.

The recommended epidemiological cutoff (ECOFF), which differentiates wild-type and non-wild-type strains, for MFX in Middlebrook 7H10 is 0.5 µg/ml. However, in view of enhanced moxifloxacin activity, two critical concentrations, low level (0.5 µg/ml) and high level (2.0 µg/ml), have been suggested by the World Health Organization since 2012 (21). Previous studies by Sirgel et al. (2) and Zhang et al. (22) have found that mutations at codon 94 in *gyrA* were linked to high-level fluoroquinolone resistance. A recent report by Kambli et al. (23) also supported the view that the majority of isolates with mutations at codon 94 in *gyrA* were associated with a high-level resistance to MFX with MICs of 2.5 mg/ml in 76% (32/42) of isolates with a D94G mutation. In the present study, we found that the D94G and D94N mutations in *gyrA* and the G512R mutation in *gyrB* were correlated with high-level MFX resistance and that high-level MFX resistance was seen in 70.6% (12/17), 77.8% (7/9), and 100% (4/4) of isolates with a D94G mutation of *gyrA*, a D94N mutation of *gyrA*, and a G512R mutation of *gyrB*, respectively. However, inconsistent with the report by Li et al. (24), the D94A mutation was only associated with low-level MFX resistance. Using detection of the presence of *gyrA* D94G and D94N and the *gyrB* G512R mutations, the performance of prediction of high-level MFX-resistance can improve; the sensitivity was 67.7% (48.5% to 82.7%), the specificity was 91.3% (82.3% to 96.1%), the LR+ was 7.7 (3.7 to 16.4), the LR– was 0.2 (0.2 to 0.6), and the accuracy rate was 83.8% (75.6% to 90.1%).

In conclusion, mutations at codons 88 to 94 of *gyrA* and the G512R mutation in the *gyrB* gene were associated with OFX and MFX resistance. The level of resistance was not equally affected by mutations in the two genes. However, some OFX- or MFX-susceptible East Asian (Beijing) and Indo-Oceanic strains also harbored those particular mutations, implying that molecular techniques to detect FQ resistance may be less specific in areas with a high prevalence of those strains.

ACKNOWLEDGMENT

We declare no conflict of interest.

REFERENCES

- Gillespie SH, Billington O. 1999. Activity of moxifloxacin against mycobacteria. *J Antimicrob Chemother* 44:393–395. <http://dx.doi.org/10.1093/jac/44.3.393>.
- Sirgel FA, Warren RM, Streicher EM, Victor TC, van Helden PD, Bottger EC. 2012. *gyrA* mutations and phenotypic susceptibility levels to ofloxacin and moxifloxacin in clinical isolates of *Mycobacterium tuberculosis*. *J Antimicrob Chemother* 67:1088–1093. <http://dx.doi.org/10.1093/jac/dks033>.
- Falzon D, Jaramillo E, Schunemann HJ, Arentz M, Bauer M, Bayona J, Blanc L, Caminero JA, Daley CL, Duncombe C, Fitzpatrick C, Gebhard A, Getahun H, Henkens M, Holtz TH, Keravec J, Keshavjee S, Khan AJ, Kulier R, Leimane V, Lienhardt C, Lu C, Mariandyshev A, Migliori GB, Mirzayev F, Mitnick CD, Nunn P, Nwagboniwe G, Oxlade O, Palmero D, Pavlinac P, Quelapio MI, Raviglione MC, Rich ML, Royce S, Rusch-Gerdes S, Salakaia A, Sarin R, Sculier D, Varaine F, Vitoria M, Walson JL, Wares F, Weyer K, White RA, Zignol M. 2011. WHO guidelines for the programmatic management of drug-resistant tuberculosis: 2011 update. *Eur Respir J* 38:516–528. <http://dx.doi.org/10.1183/09031936.00073611>.
- Centers for Disease Control and Prevention. 2006. Notice to readers: revised definition of extensively drug-resistant tuberculosis. *MMWR Morb Mortal Wkly Rep* 55:1176.
- Lai CC, Tan CK, Huang YT, Chou CH, Hung CC, Yang PC, Luh KT, Hsueh PR. 2008. Extensively drug-resistant *Mycobacterium tuberculosis* during a trend of decreasing drug resistance from 2000 through 2006 at a Medical Center in Taiwan. *Clin Infect Dis* 47:e57–e63. <http://dx.doi.org/10.1086/591702>.
- Chernyaeva E, Fedorova E, Zhemkova G, Korneev Y, Kozlov A. 2013. Characterization of multiple and extensively drug resistant *Mycobacterium tuberculosis* isolates with different ofloxacin-resistance levels. *Tuberculosis (Edinb)* 93:291–295. <http://dx.doi.org/10.1016/j.tube.2013.02.005>.
- Maruri F, Sterling TR, Kaiga AW, Blackman A, van der Heijden YF, Mayer C, Cambau E, Aubry A. 2012. A systematic review of gyrase mutations associated with fluoroquinolone-resistant *Mycobacterium tuberculosis* and a proposed gyrase numbering system. *J Antimicrob Chemother* 67:819–831. <http://dx.doi.org/10.1093/jac/dkr566>.
- Wang JY, Lee LN, Lai HC, Wang SK, Jan IS, Yu CJ, Hsueh PR, Yang PC. 2007. Fluoroquinolone resistance in *Mycobacterium tuberculosis* isolates: associated genetic mutations and relationship to antimicrobial exposure. *J Antimicrob Chemother* 59:860–865. <http://dx.doi.org/10.1093/jac/dkm061>.
- Devasia R, Blackman A, Eden S, Li H, Maruri F, Shintani A, Alexander C, Kaiga A, Stratton CW, Warkentin J, Tang YW, Sterling TR. 2012. High proportion of fluoroquinolone-resistant *Mycobacterium tuberculosis* isolates with novel gyrase polymorphisms and a *gyrA* region associated with fluoroquinolone susceptibility. *J Clin Microbiol* 50:1390–1396. <http://dx.doi.org/10.1128/JCM.05286-11>.

10. Murray PR, Baron EJ. 2007. Manual of clinical microbiology, 9th ed. ASM Press, Washington, DC.
11. Clinical and Laboratory Standards Institute. 2011. Susceptibility testing of mycobacteria, nocardiae, and other aerobic actinomycetes; approved standard—2nd ed. CLSI document M24-A2. Clinical and Laboratory Standards Institute, Wayne, PA.
12. Kamerbeek J, Schouls L, Kolk A, van Agterveld M, van Soolingen D, Kuijper S, Bunschoten A, Molhuizen H, Shaw R, Goyal M, van Embden J. 1997. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. J Clin Microbiol 35:907–914.
13. Supply P, Allix C, Lesjean S, Cardoso-Oelemann M, Rusch-Gerdes S, Willery E, Savine E, de Haas P, van Deutekom H, Roring S, Bifani P, Kurepina N, Kreiswirth B, Sola C, Rastogi N, Vatin V, Gutierrez MC, Fauville M, Niemann S, Skuce R, Kremer K, Loch C, van Soolingen D. 2006. Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*. J Clin Microbiol 44:4498–4510. <http://dx.doi.org/10.1128/JCM.01392-06>.
14. Takiff HE, Salazar L, Guerrero C, Philipp W, Huang WM, Kreiswirth B, Cole ST, Jacobs WR, Jr, Telenti A. 1994. Cloning and nucleotide sequence of *Mycobacterium tuberculosis gyrA* and *gyrB* genes and detection of quinolone resistance mutations. Antimicrob Agents Chemother 38: 773–780. <http://dx.doi.org/10.1128/AAC.38.4.773>.
15. Nosova EY, Bukatina AA, Isaeva YD, Makarova MV, Galkina KY, Moroz AM. 2013. Analysis of mutations in the *gyrA* and *gyrB* genes and their association with the resistance of *Mycobacterium tuberculosis* to levofloxacin, moxifloxacin and gatifloxacin. J Med Microbiol 62:108–113. <http://dx.doi.org/10.1099/jmm.0.046821-0>.
16. Yin X, Yu Z. 2010. Mutation characterization of *gyrA* and *gyrB* genes in levofloxacin-resistant *Mycobacterium tuberculosis* clinical isolates from Guangdong Province in China. J Infect 61:150–154. <http://dx.doi.org/10.1016/j.jinf.2010.05.001>.
17. Kam KM, Yip CW, Cheung TL, Tang HS, Leung OC, Chan MY. 2006. Stepwise decrease in moxifloxacin susceptibility amongst clinical isolates of multidrug-resistant *Mycobacterium tuberculosis*: correlation with ofloxacin susceptibility. Microb Drug Resist 12:7–11. <http://dx.doi.org/10.1089/mdr.2006.12.7>.
18. Matrat S, Veziris N, Mayer C, Jarlier V, Truffot-Pernot C, Camuset J, Bouvet E, Cambau E, Aubry A. 2006. Functional analysis of DNA gyrase mutant enzymes carrying mutations at position 88 in the A subunit found in clinical strains of *Mycobacterium tuberculosis* resistant to fluoroquinolones. Antimicrob Agents Chemother 50:4170–4173. <http://dx.doi.org/10.1128/AAC.00944-06>.
19. Takiff HE, Cimino M, Musso MC, Weisbrod T, Martinez R, Delgado MB, Salazar L, Bloom BR, Jacobs WR, Jr. 1996. Efflux pump of the proton antiporter family confers low-level fluoroquinolone resistance in *Mycobacterium smegmatis*. Proc Natl Acad Sci U S A 93:362–366. <http://dx.doi.org/10.1073/pnas.93.1.362>.
20. Hegde SS, Vetting MW, Roderick SL, Mitchenall LA, Maxwell A, Takiff HE, Blanchard JS. 2005. A fluoroquinolone resistance protein from *Mycobacterium tuberculosis* that mimics DNA. Science 308:1480–1483. <http://dx.doi.org/10.1126/science.1110699>.
21. WHO. 2014. Companion handbook: to the WHO guidelines for the programmatic management of drug resistant tuberculosis. World Health Organization, Geneva, Switzerland.
22. Zhang Z, Lu J, Wang Y, Pang Y, Zhao Y. 2014. Prevalence and molecular characterization of fluoroquinolone-resistant *Mycobacterium tuberculosis* isolates in China. Antimicrob Agents Chemother 58:364–369. <http://dx.doi.org/10.1128/AAC.01228-13>.
23. Kambli P, Ajbani K, Sadani M, Nikam C, Shetty A, Udawadia Z, Rodwell TC, Catanzaro A, Rodrigues C. 2015. Correlating minimum inhibitory concentrations of ofloxacin and moxifloxacin with *gyrA* mutations using the genotype MTBDRsl assay. Tuberculosis (Edinb) 95:137–141. <http://dx.doi.org/10.1016/j.tube.2014.11.003>.
24. Li J, Gao X, Luo T, Wu J, Sun G, Liu Q, Jiang Y, Zhang Y, Mei J, Gao Q. 2014. Association of *gyrA/B* mutations and resistance levels to fluoroquinolones in clinical isolates of *Mycobacterium tuberculosis*. Emerg Microbes Infect 3:e19. <http://dx.doi.org/10.1038/emi.2014.21>.