Original Article

Phenotypic and genotypic characterization of levofloxacin- and moxifloxacin-resistant *Mycobacterium tuberculosis* clinical isolates in southern China

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Background: Levofloxacin (LVX) and Moxifloxacin (MXF) are the cornerstones for treatment of multidrug-resistant tuberculosis (MDR-TB). China is one of the highest MDR- and fluoroquinolones (FQ)-resistant TB burdens countries. DNA gyrase encoded by *gyr* genes is the main target of FQ in *Mycobacterium tuberculosis* (MTB). The prevalence and molecular characterization of LVX- and MXF-resistant MTB strains from southern China were examined in this study.

Methods: Drug susceptibility testing (DST) of 400 MTB clinical isolates was evaluated by proportion method on Löwenstein-Jensen (LJ) medium against ten drugs. The sequencing of entire *gyrA* and *gyrB* genes and multiplex PCR were performed to distinguish the prevalence of mutant types in Beijing and non-Beijing genotypes.

Results: Three hundred and twenty-one out of four hundred (80.25%) drug-resistant isolates (resistant > one drug) were categorized as 83/321 (25.80%) MDR, 174/321 (54.20%) pre-XDR and 64/321 (19.93%) XDR-MTB. Overall, 303/400 (75.75%) LVX- and 292/400 (73.00%) MXF-resistant (R) MTB strains were identified. Two hundred seventy-one out of three hundred and three (89.43%) resistant strains carried mutations in *gyrA* and 91/303 (30.03%) in *gyrB*. Interestingly, 18 novel mutations were detected in *gyrA* and *gyrB* genes. Mutations at (A90, D94) and (T500, G510, G512) frequently existed in QRDR(s) of *gyrA* and *gyrB* respectively in 286/400 (71.50%) $LVX^{R}MXF^{R}$ strains. The novel mutations in- and out-side the QRDR of *gyrA* (L105R, A126E, M127K, D151T, V165A) and *gyrB* (D461H, N499S, G520A) increased the sensitivity and consistency of genotypic tests. Notably, 25 $LVX^{R}MXF^{R}$ strains were found with unknown resistance mechanisms.

Conclusions: Mutations in QRDR(s) were concomitantly associated with Beijing and non-Beijing genotypes. The prevalence of resistance and cross-resistance between LVX and MXF in MTB isolates from southern China was immensely higher than other countries. Our valuable findings provide the substantial implications to improve the reliability of genotypic diagnostic tests relying on potential resistance conferring mutations in entire *gyr* genes.

Keywords: *Mycobacterium tuberculosis* (MTB); levofloxacin (LVX); moxifloxacin (MXF); cross-resistance; susceptibility testing; novel mutations

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Introduction

Tuberculosis (TB) is a ninth major cause of morbidity and mortality around the globe that is ranked higher than HIV. Among the 10 million TB cases in 2017, an estimated 558,000 people developed resistance to rifampicin (the most effective first-line drug), of which 82% had multidrug-resistant tuberculosis (MDR-TB) (1). Fluoroquinolones (FQ), the broad-spectrum antimicrobial agents with bactericidal activity against *Mycobacterium tuberculosis* (MTB) are used as second-line drugs (2). Currently, the third- and fourth-generation FQ [levofloxacin (LVX) and moxifloxacin (MXF)] showing high *in vitro* and *in vivo* activities are extensively used for the treatment of MDR-TB (defined as resistant to at least isoniazid and rifampicin) (2-4).

China has around 22% contribution to the global burden of MDR-TB and the highest prevalence of FQ-resistant MTB (5). This serious epidemic of drug-resistant TB is associated with poor treatment outcome in MDR-TB and extensive practice of FQ for the treatment of undiagnosed respiratory bacterial infections (5,6), which further cause the emergence of pre-extensively drug-resistant (pre-XDR) and extensively drug-resistant (XDR) TB. Pre-XDR-TB is defined as MDR-TB associated with resistance to FQ or a second-line injectable (e.g., kanamycin, amikacin, or capreomycin), but not both while XDR-TB is defined as MDR-TB with additional resistance to any FQ and a second-line injectable drug (7). The earlier reports from different regions of China showed the various proportions of XDR among MDR-TB; 6.28% in Beijing (8), 12.6% in Shanghai (9), 12.8% in Xinjiang (10), 20% in Shandong province (11).

In line with older FQ agents, the two new agents (LVX and MXF) inhibit DNA gyrase (a type II topoisomerase composed of α and β subunits), restricting the cell's capacity for DNA replication and transcription (12,13). Mutations in *gyrA* and *gyrB* genes, particularly in the quinolone resistance-determining region (QRDR) of *gyrA* (codons 74 to 113) and *gyrB* (codons 500 to 540), are the main reason of FQ resistance in MTB (14). The substitutions in these two genes alter the structure of the quinolone-binding pocket (QBP) and may widely cause the cross-resistance among FQ. GyrA mainly involves in breaking and reuniting of DNA, whereas GyrB plays a role in ATPase activity (15). Generally, *gyrA* is considered as the most promising target of FQ and most of potential inhibitors for TB are developed against this target.

Numerous molecular-based diagnostic methods to detect *gyrA* and *gyrB* mutations in QRDR have been developed, but the sensitivity for predicting the prevalence of phenotypic FQ resistance is highly inconsistent, ranging from 80.5% in Shanghai (16), 89.7% in Beijing (17), 87.5% in France (18), 90.6% in Germany (19) and 75.6% was in Vietnam (20). Most commonly observed resistance conferring mutations in *gyrA* are A90V, S91P, and D94 (A, N, G, H, Y) while the mutation G88C is rarely detected (4,21). Similarly, R485 (H, C), S486F, D495N, T500 (A, H, N), G509A, N533T, N538 (T, D), T539P, E540 (V, D) amino acid substitutions have been frequently noticed in the *gyrB* gene (22). However, ~60% of FQ-resistant MTB isolates without known mutations in QRDR of *gyrA* and *gyrB* compromises the sensitivity and specificity (2,12). Although LVX and MXF are used in treating XDR-TB patients (23), but their roles are not fully certain due to bacillary baseline and cross-resistance against the FQ (24).

In addition, most of studies infrequently report the cumulative variances in the molecular-based test performance and cross-resistance conflicting the sensitivity and specificity across the new generation FQ agents (25-27). To improve the accuracy of molecular diagnosis for precise treatment of TB patients, there is an urgent need of detailed analyses assessing the entire open reading frames of the *gyr* genes for clinically relevant readout to accumulate the persuasive evidence that various mutations interaction in/out-side the QRDR can significantly affect the independent or cross-resistance between LVX and MXF in MTB clinical strains. The current study aimed to investigate the prevalence of genetic mutations in entire *gyr* genes and their association with independent or cross-resistance to LVX and MXF in MTB clinical strains from southern China.

Methods

Ethical Statement

The current study was conducted in accordance with WHO guidelines and approved by the Ethics Committee of Guangzhou Chest Hospital (GZXK-2016-015).

Collection of MTB clinical isolates

In this study, 400 MTB clinical isolates (resistant to ≥1 or more drug) were collected during 2016–2018 from TB patients at Guangzhou Chest Hospital, The Central Hospital of Southern China for TB. The demographic data were obtained from the electronical medical record and the species were identified using a commercial MPB64 monoclonal antibody assay (Genesis, Hangzhou, China) (28). MTB wild-type reference strain (H37Rv ATCC27294^{T}319) was used as a control in this study.

Drug susceptibility testing (DST)

The DST of 400 MTB clinical strains was performed in line with WHO guidelines using a proportion method on Löwenstein-Jensen (LJ) medium against 10 anti-TB drugs with the following critical concentrations (μg/mL); isoniazid 0.2, rifampicin 40.0, streptomycin 4.0, ethambutol 2.0, MXF 1.0, LVX 2.0, amikacin 30.0, rifabutin 20.0, prothionamide 40.0 and para-aminosalicylic acid 1.0 (29-32). The serially diluted bacterial suspension of MTB strains were inoculated onto LJ slants with and without drugs and incubated at 37 ℃ for 42 days to read up the results. A strain was considered resistant to a tested drug when MTB growth rate is ≥1% compared with the drug-free control (29,32).

gyrA and gyrB genes amplification and sequencing analysis

DNA extracts were prepared from pure and freshly grown MTB colonies as previously described (33). A loopful of MTB colonies was suspended into 500 μL of Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and heated up to 80 ℃ for 20 minutes. Finally, the crude DNA was used as a template for PCR amplification. The entire open reading frames of *gyrA* (product size =2,849 bp) and *gyrB* (product size =2,336 bp) including their (≥170 bp) 5'upstream region to (≥170 bp) 3'downstream region were amplified in 400 MTB clinical strains using the newly designed primers in this study (*gyrA*F: 5'–TGCGTCAAGTGACGCTGGAC–3', *gyrA*R: 5'-ACGGTCGACCAAGCCATCCG-3'), (*gyrB*F: 5'-TATGCCGGACGTCGGGACGC-3', *gyrB*R: 5'-TCATCGCATAGTCGATGTAG-3'). PCR products were observed on 0.7% to 1.0% agarose gels, purified by PCR purification kit (Qiagen, Hilden, Germany) and sent to BGI (Guangzhou, China) for Sanger sequencing. The sequencing data were aligned with the standard MTB H37Rv reference sequence (GenBank accession number NC_000962) using CLC Sequence Viewer; version 7.7.1 (Qiagen).

PCR based detection of Beijing and non-Beijing genotypes

PCR based detection method was used to differentiate Beijing genotype and non-Beijing genotype MTB strains as previously described (34,35), a region spanning genes *Rv2816* to *Rv2819* and part of *Rv2820* is missing in all Beijing genotype of MTB strains. So, the primer set of BJ-F: 5'-ACCGAGCTGATCAAACCCG-3' and BJ-R: 5'-ATGGCACGGCCGACCTGAATGAACC-3' was used to amplify 239-bp fragment containing region specific part of *Rv2819* and part of *Rv2820*. Whereas, Non-Beijing genotype MTB strains were detected by amplification of 539-bp PCR fragment using primer set of NBJ-F: 5'-GATCGCTTGTTCTCAGTGCAG-3' and NBJ-R: 5'-CGAAGGAGTACCACGTGGAG-3' covering the region specific for *Rv2819*. The PCR products were observed on 2% agarose gels.

Statistical analysis

Phenotypic drug susceptibility testing was used as the reference for evaluation. Genotypic LVX and MXF resistance was defined as the prevalence of resistance conferring non-synonymous mutations in- and out-side the QRDR of *gyrA* and *gyrB* genes. The data was processed statistically to measure the sensitivity, specificity, accuracy, odd ratio and 95% confidence interval (CI) by using MEDCALC® statistical software (URL: https://www.medcalc.org/calc/diagnostic_test.php; https://www.medcalc.org/calc/odds_ratio.php). P<0.05 was considered statistically significant.

Results

Demographic characteristics

Of the 400 studied MTB clinical isolates, 281 (70.25%) were from male patients and 119 (29.75%) were from female patients. Age-wise analysis exhibited that MTB isolates

Table 1 Demographic characteristics of 400 MTB clinical isolates

Characteristics	No. of clinical isolates (%)		OR (95% CI)	P value	No. of clinical isolates (%)		OR (95% CI)	P value
	LVX^R (n=303)	LVX^S (n=97)			MXF^R (n=292)	MXF^S (n=108)		
Gender								
Male	211 (69.63)	70 (72.16)	0.88 (0.53–1.46)	0.63	197 (67.46)	79 (73.14)	0.76 (0.46–1.24)	0.27
Female	92 (30.36)	27 (27.83)	1.00 (Ref.)	–	95 (32.53)	29 (26.85)	1.00 (Ref.)	–
Age group								
<20	12 (3.96)	13 (13.40)	0.47 (0.20–1.14)	0.09	7 (2.39)	22 (20.37)	0.18 (0.07- 0.47)	0.0004
20–40	79 (26.07)	41 (42.26)	1.00 (Ref.)	–	83 (28.42)	49 (45.37)	1.00 (Ref.)	–
40–60	121 (39.93)	34 (35.05)	1.84 (1.08–3.15)	0.02	118 (40.41)	30 (27.77)	2.32 (1.36–3.96)	0.0020
>60	91 (30.03)	9 (9.27)	5.24 (2.40–11.46)	<0.0001	84 (28.76)	7 (6.48)	7.08 (3.03–16.54)	<0.0001
Treatment history								
New case	94 (31.02)	43 (44.32)	1.00 (Ref.)	–	99 (33.90)	51 (47.22)	1.00 (Ref.)	–
Retreated	209 (68.97)	54 (55.67)	1.77 (1.10–2.82)	0.01	193 (66.09)	57 (52.77)	1.74 (1.11–2.73)	0.01

P<0.05.

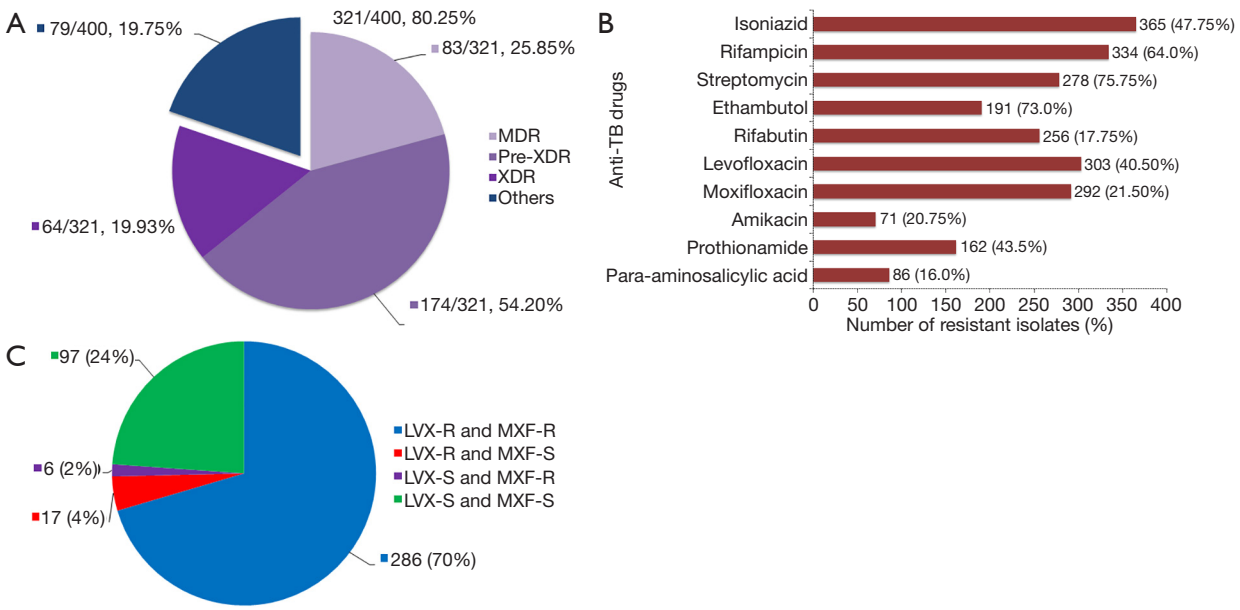

Figure 1 (A) Prevalence of MDR-, pre-XDR- and XDR-TB strains in 400 *Mycobacterium tuberculosis* clinical isolates; (B) drug susceptibility testing against ten anti-tuberculosis drugs; (C) LVX and MXF resistance pattern in *Mycobacterium tuberculosis* clinical isolates.

belonged to patients age range of 18–86 years with a mean age of 42.7 years. In addition, majority of TB patients 263/400 (65.75%) listed in this study were retreated whereas 137/400 (34.25%) were registered as new TB cases. The detailed demographic characteristics are explained in *Table 1*.

Drug susceptibility testing outcome

DST of 400 MTB clinical isolates categorized the 321/400 (80.25%) drug-resistant isolates (which have resistance against more than one drug) as 83/321 (25.80%) MDR, 174/321 (54.20%) pre-XDR and 64/321 (19.93%) XDR-MTB strains. While the remaining 79/400 (19.75%) MTB isolates had random or distinct pattern of drug resistance (mono- and poly-resistant strains) (*Figure 1A*). Of the total isolates, 303/400 (75.75%) LVX^R, and 292/400 (73.00%) MXF^R MTB isolates were identified in this study (*Figure 1B*). These isolates were observed with the

pattern of 286/400 $LVX^{R}MXF^{R}$, 17/400 $LVX^{R}MXF^{S}$, 6/400 $LVX^{S}MXF^{R}$ and 97/400 $LVX^{S}MXF^{S}$ MTB strains (*Figure 1C*). Overall, in this study MTB isolates were resistant to at least one or more drugs as there was no any isolate was included which was fully susceptible to all tested drugs.

Association of gyrA and gyrB mutations with LVX- and MXF-resistance pattern

Molecular diagnosis was performed for both susceptible and resistant MTB clinical isolates. The mutations identified in *gyrA* and *gyrB* genes are summarized in *Table 2*. Among the 286/400 $LVX^{R}MXF^{R}$ strains, 254/286 (88.81%) strains carried missense mutations in entire *gyrA* gene including 5 novel mutations and 237/254 (93.30%) of them were found with mutations only in QRDR of *gyrA*. The mutations A90V, D94N, D94G and D94A were frequently detected in *gyrA* QRDR. However, the new mutations (A126E, M127K) in MTB strains without QRDR mutations were found to be involved in resistance against both LVX and MXF.

Similarly, 78/286 (27.27%) $LVX^{R}MXF^{R}$ MTB strains were identified with missense mutations in *gyrB* gene in which 71 strains harbored *gyrB* mutations along with the mutations in *gyrA* gene, whereas 7 $LVX^{R}MXF^{R}$ strains possessed D461H, N499S, T500N, G510A and G512R mutations only in *gyrB* gene with $gyrA^{wt}$. Total 7 novel mutations were detected in *gyrB* gene in this study. In addition, with other novel non-synonymous mutations in *gyrB*, the new mutations D461H and N499S were found almost at the QRDR of *gyrB* which seems to be a very interesting phenomena of resistance in $LVX^{R}MXF^{R}$ MTB strains. *gyrB* mainly carried missense mutations at codons D79N, T500A, G510A and G512R in $LVX^{R}MXF^{R}$ strains.

Among the 17/400 $LVX^{R}MXF^{S}$ strains, 10/17 (58.82%) strains detected with multiple mutations in- and outside the QRDR of *gyrA* while 7/17 (41.17%) strains had only a novel mutation at L105R in QRDR. Besides, A204V+D94G mutations frequently existed in *gyrA* in this pattern of resistant strains. Likewise, 6/17 $LVX^{R}MXF^{S}$ *gyrA* mutations containing strains were also noticed with a novel mutation R446L accompanied by mutations at A504T and G510A in *gyrB* gene.

Of the 6/400 $LVX^{S}MXF^{R}$ strains, *gyrA* carried only novel mutations at D151T and V165A while *gyrB* showed G512R, D515G and R631S mutations in 7/400 $LVX^{S}MXF^{R}$ strains. Notably, in the current study, broad cross-resistance was noticed between LVX and MXF in 254/286 (88.81%) $LVX^{R}MXF^{R}$ MTB strains by single or multiple mutations in *gyrA* and/or *gyrB* gene(s). The non-synonymous mutations in *gyrB* conferred cross-resistance between LVX and MXF alone or in a combination of *gyrA* gene, it enlightened the role of *gyrB* in development of resistance to FQ.

The $LVX^{S}MXF^{S}$ strains had $gyrB^{wt}$ or synonymous mutations (T37T, L568L), while *gyrA* was found with non-synonymous mutation (E21Q, S95T and G668D) in all strains. Interestingly, overall 18 novel mutations were detected in both genes. Two hundred and forty-four out of two hundred seventy-seven (88.08%) and 59/91 (64.83%) resistant strains harbored mutations only in the QRDRs of *gyrA* and *gyrB* respectively. Whereas, 33 strains carried *gyrA* and 38 strains carried *gyrB* mutations outside the QRDRs among all FQ-resistant isolates in this study. Compared to the *gyrA* gene, *gyrB* had (≤60%) less mutations which strengthen the discernment about *gyrA* to account as a potential target of FQ in MTB. However, the most interestingly, 25/286 $LVX^{R}MXF^{R}$ MTB strains showed mutations at positions (E21Q, S95T and G668D) in the *gyrA* which were not associated with resistance. The same mutations were also present in all susceptible strains that indicates the existence of unknown resistance mechanisms in FQ^{R} MTB strains.

Association of the Beijing and non-Beijing genotypes with gyrA and gyrB mutations

The distribution of different mutation types in *gyrA* and *gyrB* genes between Beijing and non-Beijing genotypes of MTB isolates were summarized in *Table 2*. Total 317/400 (79.25%) MTB isolates belonged to the Beijing genotype, while 83/400 (20.75%) were from non-Beijing genotypes. Among the total Beijing genotype strains, 225/317 (70.97%) isolates harbored mutations in *gyrA*. Fifty-two out of eighty-three (62.65%) non-Beijing genotypes also carried mutations in *gyrA*. The calculated P value was 0.14 for total *gyrA* mutations. The three mutations (E21Q, S95T and G668D) in *gyrA* were found in all MTB isolates of Beijing and non-Beijing genotypes. Moreover, 75/317 (23.65%) MTB isolates with *gyrB* mutations belonged to Beijing genotype whereas 16/83 (19.27%) were from non-Beijing genotype. The P value was 0.39 for total *gyrB* mutations. Statistical analysis revealed no significant difference in prevalence of *gyrA* and *gyrB* mutations between Beijing and non-Beijing genotypes. In the same way, there was also no significant difference was found in the distribution of the mutant *gyrA* and *gyrB* types between Beijing and non-Beijing genotypes.

Table 2 Sequencing outcome of *gyrA* and *gyrB* genes versus Beijing and non-Beijing genotypes in MTB strains

Genes	Resistance pattern		Mutations		No. of strains	No. of isolates with different genotypes (n=400)		P value
	LVX	MXF	Nucleotide change	Amino acid		Beijing (n=317)	Non-Beijing (n=83)	
gyrA	R	R	C269T	A90V	59	51	8	0.14
			T271C	S91P	11	7	4	0.20
			G280T	D94Y	14	10	4	0.46
			G280C	D94H	19	14	5	0.54
			G280A	D94N	26	21	5	0.84
			A281G	D94G	78	67	11	0.11
			A281C	D94A	30	25	5	0.56
			C367T, G280A	P123S$^{\#}$, D94N	2	2	0	0.85
			G376A	A126E$^{\#}$	4	4	0	0.55
			T380A	M127K$^{\#}$	2	2	0	0.85
			G439T, A281C	Q369*, D94A	2	0	2	0.05
			C1862T, A281G	P621L$^{\#}$, D94G	3	3	0	0.68
			C2268G, C269T	D756E$^{\#}$, A90V	4	2	2	0.58
	R	S	G63T, T271C	E21H$^{\#}$, S91P	3	2	1	0.59
			T314G	L105R$^{\#}$	7	5	2	0.60
			C611T, A281G	A204V$^{\#}$, D94G	5	3	2	0.30
			G2426C, G280C	G809A$^{\#}$, D94H	2	2	0	0.85
	S	R	G451T	D151T$^{\#}$	3	2	1	0.59
			T494C	V165A$^{\#}$	3	3	0	0.68
	S	S	G66A	Q22Q	1	1	0	0.88
			G591A	L197L	1	1	0	0.88
			C2268T	D756D	1	0	1	0.13
Total						225 (70.97)	52 (62.65)	0.14
gyrB	R	R	G235A, C1499A	D79N$^{\#}$, T500N	11	7	4	0.20
			G503A	G168E$^{\#}$, G512R	7	6	1	0.67
			G1381C	D461H$^{\#}$	2	2	0	0.85
			G1337T, A1498G	R446L$^{\#}$, T500A	5	3	2	0.30
			A1496G	N499S$^{\#**\phi}$	3	2	1	0.59
			A1496G, A1498G	N499S$^{\#}$, T500A	4	4	0	0.55
			A1498G	T500A	10	9	1	0.41
			C1499A	T500N$^{**\phi}$	9	7	2	0.91
			G1529C	G510A$^{**\phi}$	12	10	2	0.72
			G1534A	G512R$^{**\phi}$	13	11	2	0.62
			G1559C	G520A$^{\#}$	2	2	0	0.85
	R	S	G1337T, G1529C	R446L$^{\#}$, G510A	3	3	0	0.68
			G1510A	A504T	3	3	0	0.68
	S	R	A1544G	D515G	4	4	0	0.55
			C1891A	R631S$^{\#}$, G512R	3	2	1	0.59
	S	S	C111T	T37T	8	5	3	0.25
			G1704A	L568L	4	3	1	0.83
Total					Total	75 (23.65)	16 (19.27)	0.39

25 LVX^{R}MXF^{R} MTB strains had (E21Q, S95T and G668D) mutations in the *gyrA* alike all susceptible strains, which are natural polymorphisms, not associated with resistance. These mutations are excluded from the table. Only non-synonymous mutations were included during analysis. The (**) and ($^{\phi}$) mutations in *gyrB* were also present in *gyrA^{wt}* resistant strains. P<0.05. $^{\#}$, indicates the novel mutations in this study. LVX, Levofloxacin. MXF, Moxifloxacin.

Table 3 Evaluation of phenotypic drug susceptibility testing and genotypic analysis—LVX

Genes	Fragments	LVX^R isolates, n=303		LVX^S isolates, n=97		Sensitivity (95% CI)	Specificity (95% CI)	Accuracy (95% of CI)
		Non-synonymous mutations (%)	WT or synonymous mutations (%)	Non-synonymous mutations (%)	WT or synonymous mutations (%)			
gyrA	QRDR + OR	265 (87.45)	38 (12.54)	0 (0.0)	97 (100.0)	87.46 (83.19–90.97)	100 (96.27–100)	90.50 (87.19–93.19)
	OR#	6 (1.98)	297 (98.01)	0 (0.0)	97 (100.0)	1.98 (0.73–4.26)	100 (96.27–100)	25.75 (21.53–30.33)
	$gyrA^E$	271 (89.43)	32 (10.56)	0 (0.0)	97 (100.0)	89.44 (85.42–92.66)	100 (96.27–100)	92.0 (88.89–94.46)
gyrB	QRDR + OR	82 (27.06)	221 (72.93)	0 (0.0)	97 (100.0)	27.06 (22.14–32.44)	100 (96.27–100)	44.75 (39.81–49.77)
	OR#	2 (0.66)	301 (99.33)	0 (0.0)	97 (100.0)	0.66 (0.08–2.36)	100 (96.27–100)	24.27 (20.60–29.28)
	$gyrB^E$	84 (27.72)	219 (72.27)	0 (0.0)	97 (100.0)	27.72 (22.76–33.13)	100 (96.27–100)	45.30 (40.30–50.27)
All*	$gyrA^E + gyrB^E$	278 (91.74)	25 (8.25)	0 (0.0)	97 (100.0)	91.75 (88.06–94.59)	100 (96.27–100)	93.75 (90.91–95.91)

Sensitivities and specificities of *gyrA* and *gyrB* genes in LVX^R strains alone. $gyrA^E$, $gyrB^E$: entire open reading frame of gene covering all resistant strains with mutations in OR and QRDR + OR. LVX^R, Levofloxacin-resistant; MXF^R, Moxifloxacin-resistant; LVX^S, Levofloxacin-susceptible; MXF^S, Moxifloxacin-susceptible; QRDR, quinolone resistance-determining region; OR, open reading frame outside the QRDR of gene; QRDR + OR, indicates all resistant strains either with single mutations only in QRDR or resistant strains with multiple mutations in QRDR + OR. #, indicates the resistant strains with single mutations only outside the QRDR (excluding QRDR); *, combined evaluation of all resistance conferring mutants in both genes.

Evaluation of LVX^R and MXF^R TB by DNA sequencing

Phenotypic diagnostic veracity was compared with molecular diagnosis. Considering the phenotypic data as a reference, detection of mutation in the QRDR of *gyrA* for LVX^R exhibited the sensitivity 87.46% and specificity 100%, however when the entire gene is evaluated the sensitivity increased up to 89.44% while the specificity remained unchanged. The sensitivity of QRDR mutations in *gyrB* was 27.06% while the entire *gyrB* gene showed slightly increased sensitivity of 27.72% with the same specificity 100%. When both genes were evaluated together, the sensitivity and accuracy increased up to 91.75% and 93.75% respectively (*Table 3*). The sensitivity of *gyrA* QRDR was 84.93% in MXF^R strains but the entire *gyrA* gene showed the increased sensitivity of 89.04% with consistent specificity of 100%. The sensitivity of *gyrB* QRDR in MXF^R strains was 28.42% but it was increased to 29.11% in entire *gyrB* gene. The combined evaluation of *gyrA* and *gyrB* enhanced the sensitivity to 91.44 % with 100% specificity (*Table 4*). Detection of mutations in *gyrA* and *gyrB* to identify cross-resistance in LVX^RMXF^R strains revealed a total sensitivity of 91.26% and a specificity of 100% (*Table 5*).

Discussion

FQ have been extensively used in China for more than a decade not only for treatment of MDR-TB but also for numerous bacterial infections. The exploitation of FQ drastically increased the number of FQ-resistant TB patients in China (5). In conformity with this theory, our data revealed a very high prevalence of FQ resistance particularly in southern China. To the best of our knowledge, this study comprises the highest collection of M/pre-X/X-DR MTB clinical isolates 321/400 (80.25%) from southern China. A total of 303/400 (75.75%) LVX^R and 292/400 (73.00%) MXF^R MTB isolates were identified in this study which were considerably higher than the range of 50–70%, obtained from the studies conducted in Taiwan (2), Morocco (14), China (36), Korea (37) and Belgium (38).

The high prevalence of LVX^R and MXF^R in pre-XDR and XDR *M. tuberculosis* isolates strongly underscores the need for more accurate susceptibility testing method to provide the guidance for appropriate MDR-TB regimens (39). DNA gyrase mutations were found to be associated with resistance or cross-resistance to the new generation of quinolones. The prevalence of *gyrA* mutations in quinolone-resistant clinical isolates varies across the countries, ranging

Table 4 Evaluation of phenotypic drug susceptibility testing and genotypic analysis—MXF

Genes	Fragments	MXF^R isolates n=292		MXF^S isolates n=108		Sensitivity (95% CI)	Specificity (95% CI)	Accuracy (95% of CI)
		Non-synonymous mutations (%)	WT or synonymous mutations (%)	Non-synonymous mutations (%)	WT or synonymous mutations (%)			
gyrA	QRDR + OR	248 (84.93)	44 (15.06)	0 (0.0)	108 (100.0)	84.93 (80.30–88.83)	100 (96.64–100)	89.0 (85.52–91.89)
	OR#	12 (4.10)	280 (95.89)	0 (0.0)	108 (100.0)	4.11 (2.14–7.07)	100 (96.64–100)	30.0 (25.55–4.75)
	$gyrA^E$	260 (89.04)	32 (10.95)	0 (0.0)	108 (100.0)	89.04 (84.88–92.38)	100 (96.64–100)	92.0 (88.89–94.46)
gyrB	QRDR + OR	83 (28.42)	209 (71.57)	0 (0.0)	108 (100.0)	28.42 (23.32–33.97)	100 (96.64–100)	47.75 (42.76–52.77)
	OR#	2 (068)	290 (99.31)	0 (0.0)	108 (100.0)	0.68 (0.08–2.45)	100 (96.64–100)	27.50 (23.18–32.16)
	$gyrB^E$	85 (29.10)	207 (70.89)	0 (0.0)	108 (100.0)	29.11 (23.96–34.69)	100 (96.64–100)	48.25 (43.26–53.27)
All*	$gyrA^E + gyrB^E$	267 (91.43)	25 (8.56)	0 (0.0)	108 (100.0)	91.44 (87.62–94.38)	100 (96.64–100)	93.75 (90.91–95.91)

Sensitivities and specificities of *gyrA* and *gyrB* genes in MXF^R strains alone. $gyrA^E$, $gyrB^E$: entire open reading frame of gene covering all resistant strains with mutations in OR and QRDR + OR. LVX^R, Levofloxacin-resistant; MXF^R, Moxifloxacin-resistant; LVX^S, Levofloxacin-susceptible; MXF^S, Moxifloxacin-susceptible; QRDR, quinolone resistance-determining region; OR, open reading frame outside the QRDR of gene; QRDR + OR, indicates all resistant strains either with single mutations only in QRDR or resistant strains with multiple mutations in QRDR + OR. #, indicates the resistant strains with single mutations only outside the QRDR (excluding QRDR); *, combined evaluation of all resistance conferring mutants in both genes.

Table 5 Evaluation of phenotypic drug susceptibility testing and genotypic analysis—LVX & MXF

Genes	Fragments	LVX^RMXF^R isolates, n=286		LVX^SMXF^S isolates, n=97		Sensitivity (95% CI)	Specificity (95% CI)	Accuracy (95% of CI)
		Non-synonymous mutations (%)	WT or synonymous mutations (%)	Non-synonymous mutations (%)	WT or synonymous mutations (%)			
gyrA	QRDR + OR	248 (86.71)	38 (13.28)	0 (0.0)	97 (100.0)	86.71 (82.22–90.42)	100 (96.27–100)	90.08 (86.64–92.88)
	OR#	6 (2.09)	280 (97.94)	0 (0.0)	97 (100.0)	2.10 (0.77–4.51)	100 (96.27–100)	22.52 (22.52–31.63)
	$gyrA^E$	254 (88.81)	32 (11.18)	0 (0.0)	97 (100.0)	88.81 (84.57–92.22)	100 (96.27–100)	91.64 (88.41–94.22)
gyrB	QRDR + OR	76 (26.57)	210 (73.42)	0 (0.0)	97 (100.0)	26.57 (21.55–32.09)	100 (96.27–100)	45.17 (40.11–50.31)
	OR#	2 (0.0)	284 (99.30)	0 (0.0)	97 (100.0)	0.70 (0.08–2.50)	100 (96.27–100)	25.85 (21.53–30.54)
	$gyrB^E$	78 (27.27)	208 (72.72)	0 (0.0)	97 (100.0)	27.27 (22.20–2.83)	100 (96.27–100)	45.69 (40.62–50.83)
All*	$gyrA^E + gyrB^E$	261 (91.25)	25 (8.74)	0 (0.0)	97 (100.0)	91.26 (87.37–94.26)	100 (96.27–100)	93.47 (90.51–95.73)

Sensitivities and specificities of *gyrA* and *gyrB* genes of combined LVX^RMXF^R strains. $gyrA^E$, $gyrB^E$: entire open reading frame of gene covering all resistant strains with mutations in OR and QRDR + OR. LVX^R, Levofloxacin-resistant; MXF^R, Moxifloxacin-resistant; LVX^S, Levofloxacin-susceptible; MXF^S, Moxifloxacin-susceptible; QRDR, quinolone resistance-determining region; OR, open reading frame outside the QRDR of gene; QRDR + OR, indicates all resistant strains either with single mutations only in QRDR or resistant strains with multiple mutations in QRDR + OR. #, indicates the resistant strains with single mutations only outside the QRDR (excluding QRDR); *, combined evaluation of all resistance conferring mutants in both genes.

from 50% to 90% (5,40-43). In this study, we found ~90% of prevalence of *gyrA* mutations among the (271/303) LVX^R and (260/292) MXF^R strains which is noticeably higher than the studies from Beijing (68%), Shanghai (76%), Russia (83%), Taiwan (50%), Tunisia (50%) and New York (67%) (40,41,44-47).

In our study, 244/278 (87.76%) resistant strains had mutations in QRDR of *gyrA* with the most frequent mutations occurring at A90V, D94N, D94G and D94A. The mutations at D94 (D94G, D94A, D94N, D94H, D94Y) were the most detected mutations in QRDR. The D94 residue, that uphold the water-magnesium ion bridge with a conserved C3/C4 keto acid moiety of quinolones, plays a significant role to stabilize the quinolone molecule in the QBP, an amino acid substitution at 94 position will aggrandize the deleterious effect of the binding between quinolones and DNA gyrase (48,49). D94 mutations were found to be associated with higher level of phenotypic resistance, as it might be the reason of positive epistasis between D94 mutations and the mutations eliciting rifampin-resistance to develop MDR-TB to XDR-TB (3,24,42,50). In addition, the mutations in *gyrA* conferred resistance to 254/286 (88.81%) LVX^RMXF^R, 17 LVX^RMXF^S and 6 LVX^SMXF^R MTB strains which indicates the resilient behavior of *gyrA* mutations in independent or cross-resistance between LVX and MXF.

Several studies have demonstrated that less number of mutations are found in *gyrB* gene in FQ-resistant MTB strains. We found 91 LVX^R and/or MXF^R MTB clinical strains with missense mutations in *gyrB* and 84/91 (92.30%) of them also had mutations in *gyrA* gene. However, it is interesting to note that 7 LVX^RMXF^R MTB strains were detected with D461H, N499S, T500N, G510A and G512R mutations in *gyrB* gene only without any mutation in *gyrA*. Two of them (D461H and N499S) are newly identified in this study. In agreement with the previous studies, T500A, G510A and G512R mutations were at high-level of mutation rate in *gyrB* of LVX^RMXF^R MTB strain (2,5). Furthermore, 78/91 (85.71%) strains with *gyrB* mutations showed resistance to LVX and MXF similar to earlier reports of cross-resistance between older generation of FQ in MTB (3,4,14,51). The *gyrB* mutation rate was ≤30% in (84/303) LVX^R and (85/292) MXF^R strains in our study which was markedly higher than previously published studies from Russia 12.5% (40), Shanghai 15.5% (47) and other parts of China 5.0% (33).

Based on the present data, non-synonymous novel nucleotide substitutions were also detected outside the QRDR(s) of *gyrA* (A126E, M127K, D151T, V165A) and *gyrB* (D461H). These novel nucleotide substitutions were found without commonly existed mutations in QRDR(s) which showed their possible involvement in the development of LVX and/or MXF resistance. The repetition of phenotypic and genotypic testing methods in two independent experiments produced the same results in these strains. However, further molecular study will provide the new insight about the role of these novel non-synonymous nucleotide substitutions.

Moreover, the mutations in the QRDR(s) of *gyrA* and *gyrB* genes are widely accepted as the key reason for FQ resistance, so, we also identified the novel mutations (L105R) in QRDR(s) of *gyrA* and (N499S, G520A) in *gyrB* for the first time. On the other hand, alike previous studies no resistance conferring non-synonymous mutations were detected in *gyrB* gene in LVX^SMXF^S strains (52). However, in these strains *gyrA* gene showed non-synonymous mutations (E21Q, S95T and G668D), but it has been proved that these non-synonymous mutations have no association with FQ resistance and thus considered as nonfunctional polymorphisms (52).

Generally, MTBDRsl (Hain Lifescience, Nehren, Germany) assay, identifies only the most common mutations found in the QRDR of *gyrA* (16,17). Whereas in our study, compared with the phenotypic data, the sensitivities for identifying LVX^R or MXF^R strains were detected by genotypic analysis of entire *gyrA* and *gyrB* genes. The combined evaluation of novel and commonly existed resistance conferring mutations in entire open reading frames of *gyrA* and *gyrB* genes rather than sequencing only QRDR(s) improved the sensitivities up to 91.75% and 91.44% respectively with the 100% specificity which were higher than the sensitivities measured in previous studies from Taiwan: 67.7% (2), Vietnam: 75.6% (20), France: 87% (18) and Beijing: 89.7% (17). The cross-resistance between LVX and MXF demonstrated the sensitivity of 91.26% and specificity of 100%. This study shows the ≥90% concordance between phenotypic and genotypic diagnosis. Our findings enlighten that inclusion of entire open reading frames of *gyrA* and *gyrB* in rapid molecular susceptibility testing will facilitate acquiring more accurate and dependable data of FQ resistance in MDR-TB. Total 18 novel non-synonymous mutations (11 *gyrA* + 7 *gyrB*) are identified in our study which have never been reported in earlier studies.

Most importantly, 25 $LVX^{R}MXF^{R}$ strains were identified with (E21Q, S95T and G668D) mutations in *gyrA* accompanied by $gyrB^{wt}$. These mutations were recognized as natural polymorphisms, not related with FQ resistance (52) which indicates the existence of unknown resistance mechanisms. However, alternative resistance mechanisms including an active drug efflux pump and reduced cell wall permeability to the drug to confer FQ resistance were suggested in previous studies but further molecular analysis will explore the new insights in FQ resistant MTB clinical strains (2,5).

Moreover, in prior studies from Russia, Taiwan, Tunisia and Vietnam, Beijing genotype was considerably found to be associated with FQ resistance (12,40,45). On the contrary, in our large number of MTB clinical isolates from southern China, no statistically significant prevalence of FQ resistance and mutant types were observed specifically bound with Beijing genotype, our findings are in line with previously published studies form China (5,33). The mutations in *gyrA* and *gyrB* were concomitantly distributed in Beijing and non-Beijing genotypes. The geographical discrepancy in prevalence of FQ resistant strains might be one of the promising reasons of varied profiles of mutant types and the extensive use of FQ for patients having bacterial infections might be another contributing factor for high emergence of drug resistance among MTB strains from different regions (23).

Conclusions

In conclusion, this is the first report which highlights the rising prevalence of independent and cross-resistance between LVX and MXF, commonly used for treatment of MDR-TB in southern China that is relatively higher than other countries. Genotypic analysis revealed the 18 novel mutations in addition to the highest prevalence rate of A90V, D94N, D94G and D94A mutations in *gyrA* as well as T500A, G510A and G512R mutations in *gyrB* in $LVX^{R}MXF^{R}$ MTB strains. Our findings also demonstrated that mutations in QRDR(s) of gyrase genes are the most predominant mechanism accounting for LVX^{R} and MXF^{R} TB. However, the newly identified mutations in- and outside the QRDR of *gyrA* (L105R, A126E, M127K, D151T, V165A) and *gyrB* (D461H, N499S, G520A) increased the sensitivity and the consistency of molecular diagnostic tests for rapid and more accurate diagnosis of FQ resistant strains. Mutations located in QRDR(s) were simultaneously associated with Beijing and non-Beijing genotypes. Most importantly, 25 $LVX^{R}MXF^{R}$ MTB strains without any known resistance-conferring mutations in *gyrA* and *gyrB* genes indicate the existence of unknown resistance mechanism which should be comprehensively investigated in forthcoming studies to explore the new potential target of FQ in MTB.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The current study was conducted in accordance with WHO guidelines and approved by the Ethics Committee of Guangzhou Chest Hospital (GZXK-2016-015).

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