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NOTE

Prevalence of *gyrA* and *B* gene mutations in fluoroquinolone-resistant and -sensitive clinical isolates of *Mycobacterium tuberculosis* and their relationship with MIC of ofloxacin

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The study was done to know the prevalent mutations of *gyrA* and *gyrB* genes, and their significance with drug resistance in clinical isolates of *Mycobacterium tuberculosis*. A total of 100 ofloxacin- (OFX) resistant and 100 OFX-sensitive isolates of *M. tuberculosis* were consecutively selected from routine Tuberculosis laboratory. Drug resistance pattern of these isolates was recorded. MIC of OFX was tested in all these isolates by absolute concentration method. Quinolone resistance determining region (QRDR) of *gyrA* and *gyrB* genes of 320 and 428 bp, respectively, were amplified and sequenced. Sequencing data were analyzed by BLAST on NCBI with reference strain H37Rv. Of 100 OFX-sensitive isolates, 30 were pansusceptible, 28 were monoresistant, 10 were polyresistant and 32 were multidrug resistant (MDR). Among 100 OFX-resistant isolates, 19 were OFX monoresistant, 16 were polyresistant and 65 were MDR. Mutations in *gyrA* and *gyrB* genes were observed in 79% and 5% of OFX-resistant isolates, respectively. Most prevalent mutation was found at codon 94 in QRDR of *gyrA* gene. Double mutations found in *gyrA* gene and in both *gyrA* and *gyrB* genes signifies higher levels of OFX resistance. In one isolate, a substitution at codon 592 (Pro592Ser) was found as a novel mutation outside the QRDR of *gyrB* gene. Our findings support previous studies that the OFX resistance to *M. tuberculosis* is associated with mutations in the QRDR of *gyrA* gene; however, the level of OFX resistance may not be predicted based on the mutation patterns in the *gyrA* gene.
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Emergence of fluoroquinolone (FQ) resistance in *Mycobacterium tuberculosis* is due to the increasing numbers of FQ prescriptions, and the expanded use of these broad-spectrum agents for many infections leads to selective FQ pressure. In *M. tuberculosis,* FQs target DNA gyrase,[1] which consist of two A and two B subunits encoded by the *gyrA* and *gyrB* genes, respectively.[2] The quinolone resistance determining region (QRDR) is a short conserved region within the *gyrA* (codon 74–113)[2] and *gyrB* (codon 500–538)[1] genes. Studies have reported that the majority (approximately 50–90%) of FQ-resistant *M. tuberculosis* isolates carry mutations in the QRDR of the *gyrA* gene,[3,4] and a small number (7%) have mutations in the *gyrB* gene.[5] However, the genetic involvement of some *gyrA* and most *gyrB* gene mutations to FQ resistance in *M. tuberculosis* is not known. Therefore, this study was planned to identify mutations in the *gyrA* and *gyrB* genes of *M. tuberculosis* and assessed their significance in determining the level of FQ resistance.

A total of 100 OFX-resistant and 100 OFX-sensitive isolates of *M. tuberculosis* were consecutively enrolled from the tuberculosis laboratory, Department of Microbiology, King George's Medical University, Lucknow, during August 2012 to July 2013. The drug resistance pattern of five antitubercular drugs streptomycin (SM), isoniazid (INH), rifampicin (RIF), ethambutol (ETH) and ofloxacin (OFX) were recorded. Laboratory quality control for drug susceptibility testing against SM, INH, RIF and EMB by the 1% proportion method is provided by the National JALMA Institute of Leprosy and Other Mycobacterial Diseases, Agra (India).

The OFX MICs were determined in all of these isolates by the absolute concentration method on Lowenstein Jensen (LJ) slants[6] using a range of 1–16 $\mu g\,ml^{-1}$. The standard strain of *M. tuberculosis* H37Rv was tested as control (sensitive at $<1\,\mu g\,ml^{-1}$ levels of OFX). Resistance to OFX was defined as MIC of $>2\,\mu g\,ml^{-1}$. DNA extraction from *M. tuberculosis* isolates was done.[7] The QRDR containing 320 bp of the *gyrA* and the 428 bp of *gyrB* gene was amplified.[2] PCR products were analyzed by electrophoresis on 2% agarose gel. PCR products of the *gyrA* and *gyrB* genes were sequenced using forward and reverse primers on an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA) using BigDye Terminator chemistry (version 3.1) and analyzed by using BLAST with reference strain *M. tuberculosis* H37Rv. The DNA sequencing data reported in this study have been submitted to the NCBI database. The provided accession numbers are KC763836–KC763837, KC777350–KC777357, KC819312–KC819320, KF760464–

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KF760514, KF826728–KF826739, KF826741–KF826744, KC880083–KC880111, KF509920–KF509922, KF879517–KF879568 and KC819321–KC819341. SPSS software (SPSS, Chicago, IL, USA) was used for the statistical analysis. The MICs of OFX-resistant *M. tuberculosis* clinical isolates with different mutations in the *gyrA* gene were compared by the analysis of variance test.

Of the 100 OFX-sensitive isolates, 30 were pansusceptible, 28 were monoresistant, 10 were polyresistant and 32 were multidrug resistant (MDR). Monoresistance to INH was most common 12/28 (43%). Among the 32 MDR isolates, 11 (34.4%) isolates were resistant to all four first-line antitubercular drugs. A total of 73 isolates had an OFX MIC value of $1\,\mu g\,ml^{-1}$ and the remaining 27 isolates had an OFX MIC of $2\,\mu g\,ml^{-1}$. No mutations in the *gyrA*/*B* genes were detected in OFX-sensitive isolates, apart from a S95T mutation in the *gyrA* gene in 72 isolates, which was a natural polymorphism that occurred in both OFX-resistant and OFX-sensitive isolates in this study.

Among the 100 OFX-resistant isolates, 19 were OFX monoresistant, 16 were polyresistant and 65 were MDR. Of the 19 OFX monoresistant isolates, 2 had MICs of $4\,\mu g\,ml^{-1}$, 5 had MICs of $8\,\mu g\,ml^{-1}$, 7 had MICs of $16\,\mu g\,ml^{-1}$ and 5 showed MICs $>16\,\mu g\,ml^{-1}$. Of OFX monoresistant isolates, 17 (89.5%) had mutations in the QRDR of the *gyrA* gene and no mutation was found in remaining 2 isolates. Among the 65 MDR isolates, 22 (34%) isolates were panresistant, 23 (35.4%) were resistant to SM, INH, RIF and OFX, 14 (22%) were resistant to INH, RIF and OFX, and 6 (9.2%) were resistant to INH, RIF, EMB and OFX. Nine MDR isolates had an OFX MICs of $4\,\mu g\,ml^{-1}$, 14 had an OFX MICs of $8\,\mu g\,ml^{-1}$, 35 had an OFX MICs of $16\,\mu g\,ml^{-1}$ and 7 had an OFX MIC $>16\,\mu g\,ml^{-1}$. Mutations in the QRDR of the *gyrA* gene were found in 51 (78.5%) MDR isolates (Table 1).

Among OFX-resistant isolates, the S95T polymorphism was detected in 80 isolates. A total of 79 (79%) isolates had mutations in the QRDR of the *gyrA* gene and 5 (5%) isolates had mutations in the *gyrB* gene, except the S95T mutation in the *gyrA* gene. In rest of the isolates no mutation was found in either *gyrA* or *gyrB* genes. Among the 79 isolates with the *gyrA* gene mutations, 68 (86.1%) had single mutations and 11 (14%) had double mutations in the QRDR. The most common single-nucleotide mutation sites were at codons 90 and 94 with a frequency of 24 (35.3%) and 41 (60.1%), respectively. The most frequent amino acid change (Asp94Gly) was found in 21/41 (51.2%) isolates. Other mutations at codons 88 and 91 were also recorded. The OFX MICs of resistant isolates with mutations ranged from 4 to $>16\,\mu g\,ml^{-1}$. Mutations in the *gyrA* gene and corresponding MICs of the 79 OFX-resistant isolates are shown in Table 2. Of OFX-resistant isolates with double substitutions ($n=11$) in the *gyrA* gene, four (36.5%) isolates had a combination of Ala90Val with Ser91Pro, three (27.3%) had a combination of Ala90Val with Asp94Asn and three (27.3%) had a combination of Ala90Val with Asp94Gly (Table 2). The OFX MICs in isolates with single mutations ($F=1.36$, $P=0.26$) and double mutations ($F=0.25$, $P=0.78$) in the *gyrA* gene showed no significant difference.

Mutations in the *gyrB* gene were observed in 5 of the 100 OFX-resistant isolates. The single-nucleotide mutation sites were in codons 500, 538, 539 (in two isolates) and 592. In one isolate, a substitution at codon 592 (Pro592Ser) was found as a novel mutation outside the QRDR of the *gyrB* gene. All of the isolates showing mutations in the *gyrB* gene also had mutations in the *gyrA* gene. No mutation was observed in the QRDR of the *gyrB* gene in OFX-sensitive isolates. The MIC of OFX-resistant isolates with mutations in the *gyrB* gene ranged from 8 to $>16\,\mu g\,ml^{-1}$.

In the present study, an increase in frequency of OFX resistance was found. These results are consistent with data reported by earlier Indian studies[8,9] and a study from Nigeria,[10] but different than those reported in a study from Rawanda.[11] In this study, no significant difference was found between the different mutation patterns in the *gyrA* gene with the level of OFX resistance in *M. tuberculosis* isolates, which is consistent with a study in East China.[12] However, some studies have shown an association between mutations in the QRDR of

Table 1 Mutations detected in OFX-resistant *M. tuberculosis* isolates and their drug resistance pattern

Drug resistance pattern of OFX-resistant isolates	*Total no. of isolates* (n = 100)	*OFX MIC* ($\mu g\,ml^{-1}$) *on LJ*				*Mutations in the gyrA gene at codon positions*					*No mutation* (n = 21)
		4	*8*	*16*	*>16*	*88*	*90*	*91*	*94*	*Double substitutions*	
OFX monoresistant	19	2	5	7	5		3		13	1	2
Polyresistant (n = 16)											
SM, OFX	4	1	2	1			1		2		1
INH, OFX	6	3	1	1	1		1		2		3
RIF, OFX	2	2					2				
INH, EMB, OFX	1			1							1
SM, INH, OFX	2		1		1		2				
SM, INH, EMB, OFX	1			1					1		
MDR (n = 65)											
INH, RIF, OFX	14	1	7	5	1		6	1	3	1	3
SM, INH, RIF, OFX	23	1	1	17	4	1	3	1	10	4	4
INH, RIF, EMB, OFX	6	1	2	3			1		2	1	2
Panresistant (SM, INH, RIF, EMB, OFX)	22	6	4	10	2		5		8	4	5
Total MDR	65	9	14	35	7	1	15	2	23	10	14
Total	100	17	23	46	14	1	24	2	41	11	21

Abbreviations: EMB, ethambutol; INH, isoniazid; LJ, lowenstein jensen; MDR, multidrug resistant; OFX, ofloxacin; RIF, rifampicin; SM, streptomycin.

Table 2 Mutations in the QRDR of the *gyrA* and *gyrB* genes and OFX MIC profile of *M. tuberculosis*

Codon	No. of OFX-resistant isolates (n = 79)	Nucleotide change	Amino acid change	OFX MIC ($\mu g\,ml^{-1}$)				Frequency (%)
				4	8	16	>16	
88 *gyrA*	1	GGC-GCC	Gly88Ala		1			1/79 (1.3)
90 *gyrA*	24	GCG-GTG	Ala90Val	4	8	10	2	24/79 (30.4)
91 *gyrA*	2	TCG-CCG	Ser91Pro	1		1		2/79 (2.5)
94 *gyrA*	3	GAC-CAC	Asp94His		1	1	1	3/79 (3.9)
	21	GAC-GGC	Asp94Gly	4	4	8	5	21/79 (26.6)
	5	GAC-AAC	Asp94Asn			5		5/79 (6.3)
	7	GAC-GCC	Asp94Ala		3	4		7/79 (8.9)
	5	GAC-TAC	Asp94Tyr	1	1	3		5/79 (6.3)
90 and 91 *gyrA*	4	GCG-GTG, TCG-CCG	Ala90Val, Ser91Pro		1	2	1	4/79 (5.1)
90 and 94 *gyrA*	3	GCG-GTG, GAC-AAC	Ala90Val, Asp94Asn			1	2	3/79 (3.8)
88 and 94 *gyrA*	1	GGC-GCC, GAC-GGC	Gly88Ala, Asp94Gly				1	1/79 (1.3)
90 and 94 *gyrA*	3	GCG-GTG, GAC-GGC	Ala90Val, Asp94Gly		1		2	3/79 (3.8)
90 *gyrA* with 539 *gyrB*	2	GCG-GTG, ACC-AAC	Ala90Val, Thr539ASn		2			2/79 (2.5)
90 *gyrA* with 500 *gyrB*	1	GCG-GTG, GAC-GCC	Ala90Val, Asp500Ala			1		1/79 (1.3)
94 *gyrA* with 592 *gyrB*	1	GAC-GCC, CCG-TCG	Asp94Ala, Pro592Ser		1			1/79 (1.3)
94 *gyrA* with 538 *gyrB*	1	GAC-GGC, AAC-ATC	Asp94Gly, Asn538Ile		1			1/79 (1.3)

Abbreviations: OFX, ofloxacin; QRDR, quinolone resistance determining region.

the *gyrA* gene with FQ resistance in *M. tuberculosis*.[2,13] The relationship between double mutations in the *gyrA* gene and the MICs of OFX-resistant *M. tuberculosis* isolates are not well studied. In our study, the relationship between the OFX MICs and double mutations in the *gyrA* gene showed no significant association.

Studies have reported a mutation at codon 95 (Ser95Thr) as a natural polymorphism that is not related to FQ resistance.[14] The frequency of the *gyrA* gene mutations in this study was found at a lower rate than reported from Russia (83%),[15] but higher than reported from the Guangdong province (73.3%).[3] In earlier studies, mutations at codons 90, 91 and 94 were reported[2,14,16] in FQ-resistant isolates. Matrat *et al.*[17] reported less common involvement at codon 88. Studies from Germany, Taiwan, Russia and Kuwait have reported that Asp94Gly is the most common mutation observed in FQ-resistant *M. tuberculosis* isolates.[15,16] Various studies reported double mutations at codons Ala90Val with Asp94Gly, Ala90Val with Ser91Pro and Ala90Val with Asp94Asn[18] as found in the present study with an another combination, Gly88Ala with Asp94Asn. Some of the OFX-resistant isolates ($n = 21$) did not show any mutation in the *gyrA* gene and may be explained by other mechanisms of FQ drug resistance, such as mutations outside the QRDR, decreased cell permeability of the drug or other mechanisms of resistance, for example, efflux pump.[19]

Studies have reported a small number of FQ-resistant isolates containing mutations in the *gyrB* gene.[12,15,18] Some reported mutations such as Asp500Ala,[5] Asn538Ile[20] and Thr539Asn[12] were identified in OFX-resistant isolates in this study. The Thr539Asn mutation was identified in an OFX-resistant strain from East China, in combination with an Ala90Val *gyrA* gene mutation,[12] which is similar to our findings. Moreover, in this study, all of the isolates showing *gyrB* gene mutations also carried mutations in the *gyrA* gene. In addition, the *gyrB* gene mutations were found in a smaller number of cases, and were not frequent enough to evaluate their significance and association with FQ susceptibility.

In conclusion, our findings support previous studies that the OFX resistance to *M. tuberculosis* is associated with mutations in the QRDR of the *gyrA* gene. However, the level of resistance for OFX-resistant isolates could not be predicted based on the mutation patterns in the *gyrA* gene.

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