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Fluoroquinolone Resistance in *Mycobacterium tuberculosis* and Mutations in *gyrA* and *gyrB*▽

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This study evaluated cross-resistance of *Mycobacterium tuberculosis* strains to ofloxacin, moxifloxacin, and gatifloxacin and investigated the presence of mutations in *gyrA* and *gyrB*. Fluoroquinolone susceptibilities were determined for 41 *M. tuberculosis* strains by the proportion method on 7H11, and MICs were determined by the resazurin microtiter assay. Forty strains shared the same resistance results for the three fluoroquinolones. However, one strain, with an Asn-533 → Thr mutation in *gyrB*, was susceptible to ofloxacin but resistant to moxifloxacin and gatifloxacin.

Drug resistance is a major threat for the control of tuberculosis (TB). Multidrug-resistant (MDR) *Mycobacterium tuberculosis* strains resistant to at least rifampin (rifampicin) and isoniazid and extensively drug resistant TB caused by *M. tuberculosis* strains that in addition to being MDR are resistant to any fluoroquinolone and to at least one of three injectable second-line drugs, amikacin, kanamycin, or capreomycin, have now been reported worldwide (25).

Ofloxacin, ciprofloxacin, and levofloxacin have been used as second-line drugs for the treatment of drug-resistant TB or in patients with intolerance to one of the first-line drugs (24). A new generation of fluoroquinolones is under clinical evaluation, and these are being proposed as first-line regimens for shortening the duration of treatment (2, 12, 17). Moxifloxacin and gatifloxacin are promising drugs undergoing phase III trials (27), having previously shown favorable pharmacokinetic activity and low MICs against *M. tuberculosis* (5, 9, 14).

The main target of fluoroquinolones in *M. tuberculosis* is the DNA gyrase, encoded by *gyrA* and *gyrB* (26). Mutations in two short regions known as "quinolone resistance-determining regions" have been associated with fluoroquinolone resistance in *M. tuberculosis* (20). Mutations in Ala-90 and Asp-94 of *gyrA* have been the most commonly found (1, 7, 16). We looked for mutations *gyrA* and *gyrB* in a panel of fluoroquinolone-resistant *M. tuberculosis* strains and evaluated the possible correlation between gene mutations and resistance to ofloxacin, moxifloxacin, and gatifloxacin based on their MICs.

A total of 41 *M. tuberculosis* clinical isolates were studied. Twenty-six strains were MDR. Fresh subcultures were prepared on Löwenstein-Jensen medium and kept at 37°C for no longer than 4 weeks. Moxifloxacin (Bayer, Brussels, Belgium), gatifloxacin (Lupin Ltd., India), and ofloxacin (Sigma-Aldrich, Bornem, Belgium) stock solutions were prepared at 1 mg/ml in 0.1 N NaOH, filter sterilized, and stored at −20°C for less than 2 months. DNA was obtained from a loopful of culture resuspended in 200 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]), heat inactivated at 100°C for 10 min, and centrifuged at 10,000 × *g* at 4°C for 20 min. Two microliters of supernatant was used for amplification of the *gyrA* and *gyrB* genes using the primers (oligo 101) GyraseB-sense (5′-TAAGAGCGCCACC GACATCGGTGGATTG-3′) (positions 1404 to 1431 of *gyrB*) and (oligo 102) GyraseA-reverse (5′-GATGAAATCGACTG TCTCCTCGTCGATTTCCC-3′) (positions 428 to 459 of *gyrA*). The PCR product was 1,231 bp in size and was used as a template for the sequencing reaction, using the primers oligo 101 for the sense strand and (oligo 103) GyraseAB-Seq (5′-G TCGATTTCCCTCAGCATCTCCATC-3′) (positions 414 to 438 of *gyrA*) for the reverse strand. Cycling parameters were 5 min at 94°C, followed by 39 cycles of 45 s at 94°C, 45 s at 66°C, and 1 min at 72°C, followed by elongation at 72°C for 10 min. PCR products were sequenced with an automatic DNA sequencer (Applied Biosystems 3730 DNA analyzer). The ClustalX (version 1.83.1) and Genedoc (version 2.100) software programs were used for analysis. Mutations in *gyrA* and *gyrB* were identified using BLAST by comparison with the *M. tuberculosis* strain H37Rv sequence (GenBank accession no. NC_000962). Drug susceptibility testing was performed by the proportion method (PM) on 7H11 agar according to the standard procedure (11) with breakpoint concentrations of 2 mg/liter for ofloxacin and 0.5 mg/liter for moxifloxacin and gatifloxacin (18). MIC determinations were performed by using the resazurin microtiter assay as described previously (10).

Table 1 shows the mutations in *gyrA* or *gyrB* correlated with the MICs obtained by resazurin microtiter assay and the drug resistance profiles of the strains determined by the PM on 7H11 agar. Out of 41 strains, 16 were susceptible and 25 resistant to ofloxacin. For moxifloxacin and gatifloxacin, 15 were susceptible and 26 resistant by as determined the proportion method. As determined by sequencing of *gyrA* and *gyrB*, 10 strains had an Ala-90 → Val mutation, four strains had an Asp-94 → Gly mutation, three strains (each) had Asp-94 → Tyr or Asp-94 → Ala, and one strain (each) had Asp-94 → Asn

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TABLE 1. Mutations in *gyrA* and *gyrB*, MICs determined by REMA, and susceptibility profiles of *M. tuberculosis* strains determined by PM[a]

Strain	Mutation in:		MIC (mg/liter) of:			Susceptibility to drug by PM		
	gyrA	*gyrB*	OFX	MXF	GFX	OFX	MXF	GFX
00-0715	Ala-90 → Val	None	4	0.5	0.5	R	R	R
03-0738[b]	Ala-90 → Val	None	4	0.25	0.25	R	R	R
02-2934[b]	Ala-90 → Val	None	4	0.25	0.25	R	R	R
01-1647[b]	Ala-90 → Val	None	4	0.5	0.5	R	R	R
01-0930[b]	Ala-90 → Val	None	4	0.5	0.5	R	R	R
08-0757[b]	Ala-90 → Val	None	8	1	1	R	R	R
08-0785[b]	Ala-90 → Val	None	8	2	1	R	R	R
08-0790[b]	Ala-90 → Val	None	4	1	0.5	R	R	R
08-0791[b]	Ala-90 → Val	None	4	0.5	0.25	R	R	R
08-0797[b]	Ala-90 → Val	None	4	1	0.5	R	R	R
99-1914[b]	Asp-94 → Gly	None	8	2	1	R	R	R
02-1975[a]	Asp-94 → Gly	None	8	2	1	R	R	R
01-2614[b]	Asp-94 → Gly	None	16	2	1	R	R	R
01-2230[b]	Asp-94 → Gly	None	8	2	1	R	R	R
08-0744[b]	Asp-94 → Tyr	None	8	1	1	R	R	R
08-0810[b]	Asp-94 → Tyr	None	8	2	1	R	R	R
08-0821[b]	Asp-94 → Tyr	None	8	4	1	R	R	R
04-0649	Asp-94 → Asn	None	16	4	1	R	R	R
02-2399[b]	Asp-94 → Ala	None	4	0.5	0.25	R	R	R
02-0319[b]	Asp-94 → Ala	None	4	0.5	0.25	R	R	R
01-0172[b]	Asp-94 → Ala	None	4	0.5	0.25	R	R	R
04-0519[b]	Ala-90 → Val + Asp-94 → Asn	None	>32	>8	>4	R	R	R
08-0787	Asp-94 → His	Δ of codons 678 and 679	4	2	1	R	R	R
08-0745	Asp-89 → Asn	None	4	2	1	R	R	R
02-1234[b]	None	Asn-533 → Thr	1	0.5	0.5	S	R	R
01-2834[b]	None	None	4	1	0.5	R	R	R
08-0794[b]	Thr-80 → Ala	None	0.5	≤0.125	≤0.062	S	S	S
08-0815	Thr-80 → Ala + Ala-90 → Gly	None	≤0.25	≤0.125	≤0.062	S	S	S
08-0773	Pro-8 → Ala	None	0.5	≤0.125	≤0.062	S	S	S
08-0769	Putative promoter	None	1	≤0.125	0.125	S	S	S
08-0771	Putative promoter	None	1	≤0.125	0.125	S	S	S
08-0746[b]	None	None	0.5	≤0.125	≤0.062	S	S	S
08-0762	None	None	0.5	≤0.125	≤0.062	S	S	S
08-0764	None	None	0.5	≤0.125	≤0.062	S	S	S
08-0774	None	None	≤0.25	≤0.125	≤0.062	S	S	S
08-0777[b]	None	None	0.5	≤0.125	≤0.062	S	S	S
08-0779[b]	None	None	0.5	≤0.125	≤0.062	S	S	S
08-0781	None	None	≤0.25	≤0.125	≤0.062	S	S	S
08-0782	None	None	0.5	≤0.125	0.125	S	S	S
08-0789	None	None	0.5	≤0.125	≤0.062	S	S	S

[a] OFX, ofloxacin; MXF, moxifloxacin; GFX, gatifloxacin; Δ, deletion; R, resistant; S, susceptible.
[b] MDR strain.

or Asp-89 → Asn. Additionally, one strain had an Asp-94 → His mutation in *gyrA* and a deletion of the codons 678 and 679 in *gyrB*, and another had a double point mutation, Ala-90 → Val plus Asp-94 → Asn, in *gyrA*. One additional finding was a strain with no mutation in *gyrA* or *gyrB* but resistant to the three drugs and another strain with an Asn-533 → Thr mutation in *gyrB* resistant to moxifloxacin and gatifloxacin but susceptible to ofloxacin. Strains with a Thr-80 → Ala ($n = 1$) or Pro-8 → Ala ($n = 1$) mutation, a Thr-80 → Ala plus Ala-90 → Gly double mutation ($n = 1$), or a mutation in the *gyrA* putative promoter ($n = 2$), as well as all wild-type strains ($n = 10$), were susceptible to the three drugs.

Cross-resistance among fluoroquinolones is of concern because they have a mode of action different from that of the classical first-line anti-TB drugs. Having been used widely for other infectious diseases, they are even available without prescription in several countries, increasing the burden of selective pressure and compromising their efficacy in the treatment of TB. In the present study, moxifloxacin and gatifloxacin were more active than ofloxacin, showing strong cross-resistance with the latter, as previously reported (19, 20). Twenty-four of the 26 moxifloxacin- and gatifloxacin-resistant strains had a mutation in *gyrA*, mostly at positions Ala-90 and Asp-94, corroborating previous reports (1, 7, 16, 21). This high frequency

allows considering this region as a candidate marker for fluoroquinolone resistance detection and a possible indicator of an extensively drug-resistant case (3, 23). Mutations in *gyrB* have been less commonly found in *M. tuberculosis*, with Asp-495, Arg-516, and Asn-533 reported previously (6, 16). Herein we have shown that an Asn-533 → Thr mutation in *gyrB* was associated with resistance to moxifloxacin and gatifloxacin but interestingly not to ofloxacin. We can speculate that the absence of resistance to ofloxacin could be related to the fact that *gyrB* could be having more interaction with common structures of moxifloxacin and gatifloxacin, such as the methoxy derivative at C-8 or the methyl piperazine ring at position C-7, which are absent in ofloxacin. This finding stresses the need to look for *gyrB* mutations when determining resistance to moxifloxacin and gatifloxacin. Furthermore, one strain (08-0787) presented an Asp-94 → His mutation in *gyrA* and a deletion of the codons 678 and 679 in *gyrB*, while another strain (04-0519) had a double mutation, Ala-90 → Val and Asp-94 → Asn, that was related to high-level resistance, which is in agreement with previous reports, where multiple mutations are associated with high-level resistance (20). One strain without mutation in *gyrA* or *gyrB* was resistant to all three drugs. This situation has been reported previously with fluoroquinolone resistance (4, 7, 8) and is possibly explained by a mutation outside of the regions sequenced or another molecular alteration decreasing the accumulation of the drug inside the cell, such as an efflux mechanism (13, 15, 22).

In conclusion, we found almost full cross-resistance to ofloxacin, moxifloxacin, and gatifloxacin among strains, with the exception of one strain, with the mutation Asn-533 → Thr in *gyrB*, which was resistant to moxifloxacin and gatifloxacin but still susceptible to ofloxacin. Further studies are needed to confirm these findings among large numbers of clinical isolates of *M. tuberculosis*.

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We have no conflict of interest to declare.

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