

Molecular Diagnosis of Fluoroquinolone Resistance in *Mycobacterium tuberculosis*

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As a consequence of the use of fluoroquinolones (FQ), resistance to FQ has emerged, leading to cases of nearly untreatable and extensively drug-resistant tuberculosis. Mutations in DNA gyrase represent the main mechanism of FQ resistance. A full understanding of the pattern of mutations found in FQ-resistant (FQ^r) clinical isolates, and of their proportions, is crucial for improving molecular methods for the detection of FQ resistance in *Mycobacterium tuberculosis*. In this study, we reviewed the detection of FQ resistance in isolates addressed to the French National Reference Center for Mycobacteria from 2007 to 2012, with the aim of evaluating the performance of PCR sequencing in a real-life context. *gyrA* and *gyrB* sequencing, performed prospectively on *M. tuberculosis* clinical isolates, was compared for FQ susceptibility to 2 mg/liter ofloxacin by the reference proportion method. A total of 605 isolates, of which 50% were multidrug resistant, were analyzed. The increase in FQ^r strains among multidrug-resistant (MDR) strains during the time of the study was alarming (8% to 30%). The majority (78%) of the isolates with *gyrA* mutations were FQ^r, whereas only 36% of those with *gyrB* mutations were FQ^r. Only 12% of the FQ^r isolates had a single mutation in *gyrB*. Combined *gyrA* and *gyrB* sequencing led to >93% sensitivity for detecting resistance. The analysis of the four false-positive and the five false-negative results of *gyrA* and *gyrB* sequencing illustrated the actual limitations of the reference proportion method. Our data emphasize the need for combined *gyrA* and *gyrB* sequencing in the investigation of FQ susceptibility in *M. tuberculosis* and challenge the validity of the current phenotype-based approach as the diagnostic gold standard for determining FQ resistance.

Tuberculosis (TB) remains one of the most serious infectious diseases worldwide, causing 1.8 million deaths each year (1). The emergence and transmission of drug-resistant *Mycobacterium tuberculosis* strains further threaten TB control efforts. The treatment of infection due to multidrug-resistant (MDR) *M. tuberculosis* (MDR-TB) (i.e., that which is resistant to isoniazid and rifampin) requires the use of fluoroquinolones (FQ), since it is correlated with good prognosis (2). Unfortunately, the extensive use of FQ has led to the emergence of extensively drug-resistant (XDR) isolates, defined as MDR with resistance to any FQ and at least one of the three injectable second-line drugs (amikacin, kanamycin, or capreomycin). Disease caused by XDR *M. tuberculosis* is associated with very poor treatment outcomes close to the historical outcomes, when no chemotherapy for TB was available (3).

FQ are antibiotics with broad-spectrum antimicrobial activities and are therefore widely used for the treatment of bacterial infections of the respiratory, gastrointestinal, and urinary tracts, as well as of sexually transmitted diseases and chronic osteomyelitis (4). The FQ resistance rates in TB patients have been estimated to lie between 0.15 and 30%, depending on the country (5–8). The rates are higher among patients exposed to FQ prior to the diagnosis of TB, i.e., in cases of “acquired” resistance (6). However, in some countries, especially where FQ consumption is very high, FQ resistance is often due to the transmission of strains that are already FQ resistant (FQ^r) (7, 8).

FQ resistance is defined by the WHO as resistance to 2 mg/liter ofloxacin (OFX) (9). The conventional methods for drug susceptibility testing (DST) involve the primary culture of specimens and isolation of *M. tuberculosis*. This process has a long turnaround time of several weeks. Over the past several years, molecular techniques have been developed for the rapid detection of resistance to antitubercu-

lous agents (10). DNA gyrase, the only target of FQ in *M. tuberculosis*, is a GyrA₂GyrB₂ tetrameric enzyme containing two A and two B subunits encoded by the *gyrA* and *gyrB* genes, respectively. Mutations within the highly conserved region, the so-called quinolone resistance-determining region (QRDR) of *gyrA* and/or *gyrB*, have been reported to be responsible for ≥70% of FQ resistance in *M. tuberculosis*, affecting most commonly the GyrA subunit and less frequently the GyrB subunit as well (11). The vast majority of the published studies are retrospective, and thus, the molecular diagnoses in these studies were made for strains already known to be fluoroquinolone resistant or susceptible. To test the potential of molecular diagnosis in a real-life context, we conducted a 6-year prospective study using combined *gyrA* and *gyrB* sequencing for an initial evaluation of fluoroquinolone susceptibility.

MATERIALS AND METHODS

Bacterial strains and selection of isolates. This prospective study included all isolates for which a genotypic and phenotypic diagnosis of FQ resistance was established between 2007 and 2012 at the French National

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TABLE 1 Multiple substitutions in GyrA and/or GyrB found in fluoroquinolone-resistant and -susceptible clinical *M. tuberculosis* isolates

Substitutions by type	No. of isolates:	
	FQ-R (<i>n</i> = 6)	FQ-S (<i>n</i> = 2)
GyrA		
T80A/A90E	1	
T80A/A90G		2
A90V/D94G	1	
A90V/D94H	1	
A90V/D94N	1	
A90V/D94Y	1	
GyrA+GyrB		
D94G/A543V ^a	1	

^a GyrB A543V corresponds to GyrB A504V in the proposed numbering system (11).

Reference Center for Mycobacteria (NRC). These isolates comprised (i) all MDR strains isolated in France, (ii) some isolates that had been sent to the NRC because of high suspicion of being MDR (and that had been tested for second-line drug susceptibility immediately after isolation but that eventually turned out to be resistant to isoniazid or rifampin only, or even susceptible to both drugs), and (iii) some non-MDR isolates from patients for whom treatment with FQ was considered because of intolerance of or resistance to antituberculous drugs.

Drug susceptibility testing. *In vitro* DST was performed on Lowenstein-Jensen (LJ) medium according to the proportion method (12) with rifampin (40 mg/liter), isoniazid (0.2 and 1 mg/liter), OFX (2 mg/liter), amikacin and capreomycin (40 mg/liter each), and kanamycin (30 mg/liter), and a critical proportion of 1% (9).

DNA sequencing of quinolone resistance-associated genes. DNA sequencing of *gyrA* and *gyrB* was performed using a primary culture of all isolates. Genomic DNA was isolated from bacteria grown on LJ medium. A loopful of culture was suspended in water (500 μ l) and heated at 95°C for 15 min. The DNA used for PCR amplification was obtained by heat shock extraction (1 min at 95°C and 1 min on ice, repeated five times). A volume of 5 μ l was used in the PCRs with the oligonucleotide primers described below. For FQ resistance, the QRDRs of *gyrA* and *gyrB* were

amplified and sequenced using primers PRI8 (5'-YGGTGGRTTCRTTC CYGGCGA-3') and PRI9 (5'-CGCCGCGTGTCTATGCRATG-3') for *gyrA* and primers gyrBa (5'-GAGTTGGTGGCGCGTAAGAGC-3') and gyrBe (5'-CGGCCATCAAGCACGATCTTG-3') for *gyrB* (13).

After amplification, unincorporated nucleotides and primers were removed by filtration with Microcon 100 microconcentrators (Amicon, Inc., Beverly, MA), and the amplicons were sequenced using the BigDye Terminator version 3.1 cycle sequencing kit (Life Technologies), according to the manufacturer's instructions.

The amino acid substitution S95T in GyrA was omitted from the summary tables because it corresponds to a well-known polymorphism that does not confer drug resistance (11).

When more than one substitution was observed in one strain (double or triple substitution), we noted two scenarios: (i) one of the substitutions was observed as a single substitution elsewhere, or (ii) the substitutions were never observed independently of one another. In both scenarios, the substitutions were listed as single and multiple substitutions. The mutations that were never observed independently of one another are noted in the tables. All substitutions in GyrA and GyrB were annotated according to the numbering system of *M. tuberculosis*, in which the QRDR of *M. tuberculosis* GyrA ranges from codon 74 to codon 113, and the QRDR of GyrB ranges from codon 500 to codon 540 (this is the most frequently GyrB numbering system used in the literature [GenBank accession no. CAB02426.1]) (11, 14). The correspondences with the proposed numbering system are given as footnotes in Tables 1 and 2 (11). The implication of DNA gyrase substitutions in FQ resistance noted in the tables corresponds to the results of biochemical studies found in the literature, whose references are noted in the tables.

Statistical tests. Chi-square tests were performed using the Biostat-TGV website (<http://marne.u707.jussieu.fr/biostatgv/>). The results were considered significant at a *P* value of <0.05.

RESULTS

Drug susceptibility pattern of the isolates included in this study.

A total of 605 *M. tuberculosis* isolates obtained from clinical specimens were sent to the NRC during 2007 to 2012 for the diagnosis of FQ resistance. The number of isolates studied rose every year, from 71 in 2007 to 137 in 2012, but the proportion of MDR isolates remained the same (*ca.* 50%) (Table 3). Most of the isolates

TABLE 2 Substitutions in GyrB found in fluoroquinolone-resistant and -susceptible clinical *M. tuberculosis* isolates

Substitution in GyrB ^a	No. of isolates by resistance type				% of total substitutions in GyrB	Implication in FQ resistance in <i>M. tuberculosis</i> demonstrated in the literature (reference[s])
	FQ-R		FQ-S			
	MDR	Non-MDR	MDR	Non-MDR		
S470I			1		4.5	ND ^b
D473N				1	4.5	No (35)
P478A			1	1	9.0	No (35)
S486F			1		4.5	No (35)
S486Y	2				9.0	ND
D500A			1		4.5	Yes (14)
D500N	1				4.5	Yes (32)
A506G				1	4.5	No (35)
E540V	3		1		18.5	Yes (14, 15)
A543V	2				9.0	Yes (32)
A547V				1	4.5	No (35)
G551R			1		4.5	No (35)
G559A				4	18.5	No (35)
Total	8	0	6	8	100	

^a GyrB S470, D473, P478, S486, D500, A506, E540, A543, A547, G551, and G559 in the most frequently used numbering system (CAB02426.1) correspond to GyrB S431, D434, P439, S447, D461, A467, E501, A504, A508, G512, and G520 in the proposed numbering system (11), respectively.

^b ND, not demonstrated.

TABLE 3 Pattern of phenotypic drug susceptibility of the 605 *M. tuberculosis* isolates included in this study

Resistance profile ^a	No. of isolates (no. of FQ-R isolates) in:						Total no. of isolates (no. of FQ-R isolates)
	2007	2008	2009	2010	2011	2012	
INH-S+RIF-S	19 (0)	20 (0)	20 (0)	25 (0)	23 (0)	25 (1)	132 (1)
INH-R, non-MDR	15 (0)	16 (0)	27 (0)	27 (0)	38 (1)	28 (0)	151 (1)
RIF-R, non-MDR	1 (0)	6 (1)	3 (1)	4 (0)	4 (0)	7 (0)	25 (2)
MDR	36 (3)	48 (6)	38 (10)	40 (9)	58 (15)	77 (23)	297 (66)
XDR	2	2	5	5	6	11	31
Total	71	90	88	96	123	137	605 (70)

^a INH, isoniazid; RIF, rifampin; S, susceptible; R, resistant; MDR, multidrug resistant; XDR, extensively drug resistant (all XDR strains are included within the MDR strains).

(78%) were resistant to isoniazid or rifampin or both: 297 (49%) were resistant to both isoniazid and rifampin (i.e., MDR or XDR), and 176 (29%) were resistant either to rifampin ($n = 25$) or isoniazid ($n = 151$). Phenotypic OFX resistance was found in 70 (12%) of the 605 isolates. Among the OFX-resistant (OFX^r) isolates, most (66/70 [94%]) were MDR, while one was resistant to isoniazid but susceptible to rifampin, two were resistant to rifampin but susceptible to isoniazid, and one was susceptible to both isoniazid and rifampin.

The percentage of OFX^r isolates included in this study rose significantly, from 4% in 2007 to 18% in 2012 ($P = 0.003$), mainly due to the increase in OFX^r isolates among the MDR isolates (from 8% in 2007 to 30% in 2012).

Amino acid substitutions in GyrA. Eighty-one substitutions in the QRDR of GyrA were observed in 74 (12%) of the isolates (Table 4). Out of these, 63 isolates harboring 69 substitutions were MDR (including 24 XDR), seven isolates harboring seven substitutions were resistant to either rifampin or isoniazid, and four isolates harboring five substitutions were susceptible to both isoniazid and rifampin. The amino acid substitutions were located at six different positions: T80, G88, A90, S91, D94, and Q101 (Table 4). The most common substitution occurred at position D94 ($n =$

37 [46%]) and resulted in 13 D94G, 13 D94A, four D94N, four D94H, and three D94Y substitutions. The second most common substitution occurred at position A90 ($n = 21$) and resulted in 18 A90V substitutions, two A90G substitutions, and one A90E substitution. The A90G and A90E substitutions were systematically associated with other substitutions in GyrA (Table 1). The third most common substitution was T80A ($n = 16$). The remaining substitutions were S91P ($n = 5$), G88C ($n = 1$), and Q101E ($n = 1$).

Seven isolates had double substitutions in GyrA: three harbored T80A plus A90E/G substitutions, and four harbored A90V plus D94G/H/N/Y substitutions (Table 1).

Amino acid substitutions in GyrB. Substitutions in the QRDR of GyrB were observed in 22 (4%) of the 605 isolates (Table 2). Out of these 22 isolates, 14 were MDR (including seven XDR), six were resistant to either rifampin or isoniazid, and two were susceptible to both isoniazid and rifampin. The amino acid substitutions were located at 11 positions. No substitution was significantly more frequent than any other; there were four G559A, four E540V, three S486F/Y, two P478A, two D500A/N, and two A543V substitutions, and one each of the S470I, D473N, A506G, A547V, and G551R substitutions (Table 2).

TABLE 4 Substitutions in GyrA found in fluoroquinolone-resistant and -susceptible clinical *M. tuberculosis* isolates^a

Substitution in GyrA	No. of isolates by resistance type				% of total substitutions in GyrA	Implication in FQ resistance in <i>M. tuberculosis</i> demonstrated in the literature (reference[s])
	FQ-R		FQ-S			
	MDR	Non-MDR	MDR	Non-MDR		
T80A	1 ^b		7 + 1 ^b	6 + 1 ^b	19.9	No (32, 33)
G88C	1				1.2	Yes (34)
A90E ^d	1				1.2	ND ^c
A90G ^d			1	1	2.4	No (32, 33)
A90V	14 + 4 ^b				22.2	Yes (33)
S91P	5				6.2	Yes ^e
D94A	13				16.1	Yes ^e
D94G	10 + 1 ^b	2			16.1	Yes (33)
D94H	3 + 1 ^b				4.9	Yes (33)
D94N	2 + 1 ^b	1			4.9	Yes ^e
D94Y	2 + 1 ^b				3.7	Yes ^e
Q101E				1	1.2	ND
Total	60	3	9		9	100

^a Seven isolates harbored 2 substitutions.

^b Isolate harboring two substitutions.

^c ND, not demonstrated.

^d Substitution systematically associated with other GyrA substitutions (see Table 1 for details).

^e Considered to be implicated in FQ resistance despite the absence of unequivocal demonstration, since it is frequently observed in FQ-R strains (11) and is included in the MTBDRsl probe assay.

As opposed to what was observed for GyrA, no isolate harbored more than one substitution in GyrB. However, one isolate showed a substitution in both GyrA (D94A) and GyrB (A543V) (Table 1).

Distribution of DNA gyrase mutations regarding the susceptibility to FQ. Among the isolates included in this study, 535 were OFX susceptible (OFX^s) and 70 were OFX^r (Table 3). The distributions of substitutions in GyrA and GyrB according to the FQ sensitivities of the isolates are detailed in Tables 2 and 4. Among the 74 isolates with a substitution(s) in GyrA, 16 (harboring 18 substitutions) were OFX^s, whereas the majority were OFX^r ($n = 58$ [78%], harboring 63 substitutions). Among the 22 isolates with substitutions in GyrB, more isolates were OFX^s ($n = 14$ [64%], harboring 14 substitutions) than OFX^r ($n = 8$ [36%], harboring eight substitutions). Among the OFX^r isolates, one isolate harbored a substitution in both GyrA (D94A) and GyrB (A543V). The proportion of substitutions in GyrB associated with FQ resistance was significantly lower than that in GyrA (36% versus 78%, $P = 0.0002$).

Surprisingly, no substitution in GyrA or GyrB was found in five of the 70 (7%) OFX^r isolates, which were mainly MDR isolates ($n = 4$). For these isolates, the proportion of colonies growing on OFX-containing LJ medium was close to the breakpoint given by the proportion method for four isolates (1%, 1%, 2%, and 5% mutants) and higher for one isolate (proportion of colonies growing on OFX-containing LJ medium, 25%). The sequencing of *gyrA* and *gyrB* was therefore also performed on these colonies growing on OFX-containing LJ medium, and substitutions were found in four isolates, with GyrA D94G/Y substitutions in three isolates and a GyrB N538K substitution in one isolate. No substitutions in GyrA or GyrB were found in the remaining isolate, although it harbored the highest proportion of colonies growing on OFX-containing LJ medium (25%).

On the other hand, 30 OFX^s isolates harbored 18 substitutions in GyrA and/or 14 substitutions in GyrB. Among these 30 strains, two strains harbored substitutions known to be implicated in FQ resistance (GyrB D500A and GyrB E540V) (14, 15), and another two strains harbored substitutions whose implications in FQ resistance are not known (GyrA Q101E and GyrB S470I) (Tables 2 and 4).

Substitutions implicated in FQ resistance were more frequent in MDR than in non-MDR isolates (42 versus 2%, $P < 0.01$).

DISCUSSION

Knowing the epidemiology of DNA gyrase mutations is of primary importance for the design of a reliable approach for diagnosing FQ resistance. The previously published studies in this field of investigation have some limitations: (i) most studies evaluated the performance of molecular diagnosis for FQ^r isolates only (11, 16–18), (ii) most studies were performed on retrospectively chosen isolates with a high prevalence of FQ resistance (most were >50%) (11), and (iii) only half of the studies included sequencing of *gyrA/gyrB* (11, 16–30). To our knowledge, the present study is the largest study published that was performed prospectively, apart from that by Hu et al. (28), which was performed prospectively and included fewer isolates selected from a population in which the prevalence of FQ resistance was similar to that studied here (14 versus 12%).

The first important finding of our study was the increase in the proportion of FQ^r isolates among the MDR-TB isolates, from 8% in 2007 to 30% in 2012 ($P = 0.07$). This increase is due to the

marked increase in the number of MDR-TB cases observed in France in the last years in patients born in the former Soviet Union (6 cases in 2007, 10 in 2008 and 2009, 11 in 2010, 21 in 2011, and 47 in 2012) (31). Incidentally, a recent study performed in the Russian Federation reported an FQ resistance rate as high as 62% in consecutive MDR-TB cases (26). This major increase is a challenge to the existing health care facilities in the management of MDR-TB and XDR-TB.

The second major result of our study is that FQ^r isolates harbored more substitutions in GyrA or GyrB than is generally reported in the literature (93% versus an average of 81% in the 13 studies providing both *gyrA* and *gyrB* sequence data; $P = 0.02$) (Tables 2 and 4) (11, 16–30). Among these substitutions found in the FQ^r isolates, we observed a higher proportion in GyrB than was previously published (11.5% versus an average of 3%) (11). In our study, substitutions in GyrB at position E540 were the most frequently found, followed by S486Y and A543V and D500N (Table 2). The overall geographical distribution of the substitutions in GyrB has been reported to be highly variable (11). The reason for this variability remains unclear. It could be speculated that these substitutions that are most likely phenotypically silent simply occur more frequently in certain geographical regions. The variability might also be due to selection bias, since the vast majority of studies were made retrospectively. Regarding the substitutions in GyrA, those at position D94 were the most frequent, followed by A90V and S91P (Table 4), as previously reported (11, 16–30).

We analyzed the performance of the DNA sequencing strategy for detecting FQ resistance compared to that of the proportion method, using OFX (2 mg/liter), which is considered the reference method. We took into account epidemiological and biochemical studies on the implication of DNA gyrase substitutions in FQ resistance (14, 15, 32–35) (Tables 2 and 4). In our population, in which the prevalence of FQ resistance was 12%, the sensitivity, specificity, and positive and negative predictive values were the 92.9%, 99.2%, 94.2%, and 99.1%, respectively. The sensitivity and specificity were similar to those previously published (11, 15–29).

Analyzing our results closely, i.e., the four false-positive and the five false-negative results, we see a very complex picture in which the sole presence or absence of mutations does not allow a prediction of susceptibility or resistance, and it challenges the current phenotype-based approach as the gold standard for FQ resistance detection while the potential of molecular methods is still under evaluation (36, 37). Indeed, among the 4 false-positive results, two strains harbored substitutions in GyrB (D500A and E540V) known to be implicated in low-level FQ resistance, and two strains harbored substitutions whose implication in FQ resistance is not known (GyrA Q101E and GyrB S470I). Among the 5 false-negative results, all strains harbored a percentage of mutants of $\leq 25\%$, and the sequencing of colonies growing on OFX-containing LJ medium revealed the presence of substitutions well known to be implicated in FQ resistance for 4 strains (GyrA D94G/Y and GyrB N538K).

The questionable ability of existing phenotypic and PCR sequencing DST to properly classify strains as susceptible or resistant was also raised for other antituberculous agents. For example, regarding rifampin, some *rpoB* mutations entailing low-level resistance that is not detected by conventional phenotypic DST have a negative impact on treatment efficacy (36, 38).

Finally, it is of crucial importance to determine whether low-level resistance not detected by phenotypic methods has an impact

on treatment efficacy and whether the phenotypic and/or molecular DST methods might be able to detect these resistance levels. The answer to both questions seems to be yes, considering (i) *in vitro* studies showing that the MICs of moxifloxacin or gatifloxacin, the most active fluoroquinolones against *M. tuberculosis*, may be lower than their peak serum levels for some ofloxacin-resistant strains of *M. tuberculosis* (14–16, 20–22, 37), (ii) a murine model whose results support current WHO recommendations to use moxifloxacin when there is resistance to early generation fluoroquinolones, such as ofloxacin, when moxifloxacin MICs of ≤ 2 mg/liter (39, 40), and (iii) recent clinical data regarding the excellent outcome of the Bangladesh regimen used to treat XDR-TB patients, including among patients with low-level gatifloxacin resistance, whereas the relapses and death were observed among patients with high-level gatifloxacin resistance (41).

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