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Correlation between GyrA Substitutions and Ofloxacin, Levofloxacin, and Moxifloxacin Cross-Resistance in *Mycobacterium tuberculosis*

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The newer fluoroquinolones moxifloxacin (MXF) and levofloxacin (LVX) are becoming more common components of tuberculosis (TB) treatment regimens. However, the critical concentrations for testing susceptibility of *Mycobacterium tuberculosis* to MXF and LVX are not yet well established. Additionally, the degree of cross-resistance between ofloxacin (OFX) and these newer fluoroquinolones has not been thoroughly investigated. In this study, the MICs for MXF and LVX and susceptibility to the critical concentration of OFX were determined using the agar proportion method for 133 isolates of *M. tuberculosis*. Most isolates resistant to OFX had LVX MICs of >1 μg/ml and MXF MICs of >0.5 μg/ml. The presence of mutations within the *gyrA* quinolone resistance-determining regions (QRDR) correlated well with increased MICs, and the level of LVX and MXF resistance was dependent on the specific *gyrA* mutation present. Substitutions Ala90Val, Asp94Ala, and Asp94Tyr resulted in low-level MXF resistance (MICs were >0.5 but ≤2 μg/ml), while other mutations led to MXF MICs of >2 μg/ml. Based on these results, a critical concentration of 1 μg/ml is suggested for LVX and 0.5 μg/ml for MXF drug susceptibility testing by agar proportion with reflex testing for MXF at 2 μg/ml.

It is estimated that one-third of the world's population is infected with *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), and 5% to 10% of infected persons will become ill with active disease necessitating a complicated treatment regimen. Patients with fully susceptible TB are generally treated with a combination of first-line drugs, including rifampin, isoniazid, ethambutol, and pyrazinamide for 6 months (1). Intolerance or acquisition of resistance to one or more of the first-line drugs requires deviation from the standard treatment regimen, and second-line drugs, including the injectables amikacin (AMK), kanamycin (KAN), and capreomycin (CAP) and one of a number of fluoroquinolone (FQ) derivatives, may be needed to successfully treat the patient.

FQs are a class of broad-spectrum, synthetic antibiotics effective against many different bacterial infections such as those of the urinary and respiratory tracts, including infections by *M. tuberculosis*. Early FQs had a limited spectrum of activity; however, two of the newest FQ derivatives, moxifloxacin (MXF) and levofloxacin (LVX) (the levo isomer of ofloxacin [OFX]), exhibit superior bactericidal activity against a wide range of organisms, have longer half-lives, and are less toxic than older FQs (2, 3). Studies have demonstrated the effectiveness of these new FQs against *M. tuberculosis* (4, 5), with comparatively low MICs (MXF MIC < 0.5 μg/ml) (1, 6–9). Although clinical resistance to FQs has been reported, estimating the global burden of FQ-resistant TB is difficult given that drug susceptibility testing (DST) is not performed in many areas and testing for FQ resistance is typically undertaken only when resistance to first-line drugs is detected. However, in 2013, the World Health Organization (WHO) estimated that 9% of the 480,000 new cases of multidrug-resistant tuberculosis (MDR TB) (showing resistance to at least rifampin and isoniazid) were actually extensively drug-resistant (XDR) TB (i.e., MDR TB with added resistance to at least 1 second-line injectable drug and an FQ) (10). FQ resistance in *M. tuberculosis* has thus far been attributed to acquisition of mutations within specific, defined regions of the genes encoding the subunits of DNA gyrase, *gyrA* and *gyrB*, referred to as quinolone resistance-determining regions (QRDR) (11–13). Previous studies have reported that up to 96% of *M. tuberculosis* isolates resistant to OFX harbor a mutation in the QRDR of *gyrA*, with changes in codons 90 and 94 predominating (11, 13–16). Moreover, specific GyrA substitutions may result in differing levels of FQ resistance (14, 15, 17).

As MXF and LXF become more popular treatment options, an evaluation of cross-resistance between these drugs and the current class representative, OFX, is crucial. A limited number of published reports have appraised cross-resistance among these drugs, and it appears that resistance to OFX may not correlate with complete cross-resistance to MXF or LVX (15, 17–20). FQs often represent one of the last lines of defense in the fight against drug-resistant TB; the possibility that strains resistant to OFX may be susceptible to another FQ presents a valuable treatment option. The WHO has recognized this potential and recommended the use of MXF to treat certain XDR TB patients in spite of resistance to OFX (21).

In this study, we evaluated 133 clinical *M. tuberculosis* isolates for susceptibility to OFX at the critical concentration and determined MICs for LVX and MXF by the method of agar proportion to assess the degree of cross-resistance among these drugs. Based

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on our findings, we suggest concentrations appropriate for DST of MXF and LVX. Additionally, we correlated MICs with specific mutations within the *gyrA* QRDR and determined that although there is some variability, certain substitutions lead to low-level FQ resistance while others are more likely to result in higher levels of resistance. Data presented in this study could be used to design molecular tests that not only detect FQ resistance but also predict the level of resistance based on the specific mutation identified, consequently enabling rapid formulation of patient-specific treatment regimens.

MATERIALS AND METHODS

Isolate selection. One hundred thirty-three clinical isolates of *M. tuberculosis* collected between 2000 and 2010 were selected for this study from the CDC Division of Tuberculosis Elimination, Laboratory Branch culture collection. Isolates were selected based on one of the following criteria: FQ-resistant clinical isolates described in a previous study (22), FQ-resistant clinical isolates added to the Laboratory Branch *M. tuberculosis* culture collection between the end of the previous study and the initiation of this study, and a selection of FQ-susceptible isolates collected between 2000 and 2010. All isolates had been previously tested for susceptibility to ciprofloxacin (CIP), OFX, or both by agar proportion.

Modified agar proportion test. The MICs of MXF and LVX were determined by the modified agar proportion method according to standardized procedures (23). Briefly, isolates were inoculated from frozen stocks into Middlebrook 7H9 broth supplemented with 10% albumin-dextrose-catalase (ADC; BD Biosciences), 0.05% Tween 80 (Sigma), and 0.4% glycerol and incubated at 37°C until turbidity was approximately that of a McFarland standard of 1.0. Cultures were diluted 1:100 in sterile, deionized water containing 0.01% Tween 80, and 100 μl was dispensed onto quadrants of Middlebrook 7H10 solid medium supplemented with 10% oleic acid-ADC (OADC) and 0.4% glycerol containing no drug, 2 μg/ml OFX, or MXF or LVX at 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, or 16.0 μg/ml. The MIC was defined as the minimum concentration of drug that inhibited growth of >99% of the bacterial population compared to the no-drug control quadrant. Resistance to OFX was defined as >1% growth in the presence of 2 μg/ml OFX compared to the no-drug control quadrant. MXF MIC determination and OFX susceptibility testing were repeated exactly as described above for isolates with phenotypic and genotypic discordance.

DNA sequencing. The QRDR of *gyrA* was sequenced for all isolates either during a prior work (22) or as part of the current study using the techniques and primers previously described (22). The *gyrB* QRDR was sequenced for isolates resistant to FQ, but with a wild-type (WT) *gyrA* using methods previously detailed (22) with slight modifications. Briefly, the *gyrB* region of nucleotides 381 to 989 encompassing the QRDR was amplified from 2 μl of culture using primers gyrBSF and gyrBSR (12). Amplicons were diluted 1:10 and used as the templates for sequencing reactions using the primers described above. Sequences were generated by standard techniques and analyzed using DNASTAR Lasergene 8 SeqMan software.

RESULTS

OFX resistance and MXF and LVX MICs. The isolates included in this study were part of an archived collection maintained by our laboratory as frozen stocks, and their susceptibility to CIP and/or OFX had been determined previously. Depending on the date of receipt of particular study isolates, original FQ susceptibility testing may have been done using CIP, OFX, or both. For the current study, we initially chose 96 FQ-resistant and 51 FQ-susceptible isolates from our collection (2000 to 2010), and we achieved viable cultures from 133 isolates.

Isolates were retested for susceptibility to OFX at its critical concentration (2 μg/ml), and MICs of LVX and MXF were determined using the agar proportion method. Eighty-five isolates were resistant and 48 were susceptible to OFX (Table 1), and MICs for MXF and LVX ranged from <0.25 to 16 μg/ml (Tables 2 and 3). The majority (81/85, 95.3%) of OFX-resistant isolates exhibited an MIC of >0.5 μg/ml for MXF, while most (45/48, 93.8%) OFX-susceptible isolates had an MIC of ≤0.5 μg/ml (see Table S1 in the supplemental material). All OFX-resistant isolates exhibited an MIC of >1.0 μg/ml for LVX, while most (47/48, 97.9%) of the OFX-susceptible isolates had an LVX MIC of ≤1.0 μg/ml (see Table S1 in the supplemental material). Therefore, a high degree of cross-resistance exists between strains resistant to OFX (2 μg/ml), LVX (MIC > 1.0 μg/ml), and MXF (MIC > 0.5 μg/ml) (see Table S1 in the supplemental material).

TABLE 1 OFX susceptibility results for isolates according to GyrA QRDR substitutions

Substitution(s)	No. of isolates		
	Total	Resistant	Susceptible
Ala90Val	24	24[b]	0
Asp89Asn	1	1	0
Asp89Gly	1	0	1[c]
Asp94Ala	12	11	1[c]
Asp94Tyr	2	2	0
Asp94Asn	11	11[b]	0
Asp94Gly	25	25[b]	0
Gly88Cys	1	1	0
Ser91Pro	1	1	0
Ala90Val, Asp94Gly	1	1	0
Ser91Pro, Asp94Gly	1	1[a]	0
Ala90Val, Ser91Pro	1	1[b]	0
Ala90Val, Asp94Gly, Gly88Cys	1	1	0
None (WT)	51	5[d]	46
Total	133	85	48

[a] When OFX susceptibility testing was repeated, initial results are presented.
[b] A *gyrA* mutation was identified in one isolate in this category during repeat sequencing of isolates previously classified as WT.
[c] The initial DST result was susceptible, but the repeat test result was resistant.
[d] All isolates have *gyrB* mutations.

Effects of mutations in *gyrA* on MIC. Specific mutations within the QRDR region of *gyrA* have been shown to be associated with resistance to FQs (11, 12, 14, 15). The *gyrA* QRDR was sequenced for all isolates either as part of this study or during a previous study (22), and the sequences were analyzed in the context of MIC data to determine the correlation, if any, between specific mutations within the *gyrA* QRDR and FQ resistance. Of 133 isolates, 82 contained a mutation within the *gyrA* QRDR (Tables 1, 2, and 3). All mutations had been previously associated with resistance to FQs (11, 13–15, 24). Mutations occurred most frequently at *gyrA* codons for Ala90 and Asp94 (95.1%, 78/82), where numerous amino acid substitutions were detected. Other isolates had substitutions at Gly88, Asp89, and Ser91. Three isolates appeared to have double substitutions in GyrA, while one isolate harbored three GyrA substitutions. All 82 isolates with *gyrA* QRDR mutations were OFX resistant either on initial (80 isolates) or repeat (2 isolates) testing, while the majority had MICs of >0.5 μg/ml for MXF (80/82 97.6%) and MICs of >1 μg/ml for LVX (81/82, 98.7%) (Tables 2 and 3).

Interestingly, the degree of resistance to MXF and LVX varied depending on the loci and the specific amino acid change

TABLE 2 MXF MICs according to GyrA QRDR substitutions

Substitution(s)	No. of isolates							
		With MXF MIC (μg/ml) of:						
	Total	≤0.25	0.5	1	2	4	8	16
Ala90Val	24			13[a]	10	1		
Asp89Asn	1					1		
Asp89Gly	1			1[b]				
Asp94Ala	12		1	7[c]	1	2	1	
Asp94Tyr	2			1	1			
Asp94Asn	11					6[a]	4	1
Asp94Gly	25		1[a]		2	12	8	2
Gly88Cys	1						1	
Ser91Pro	1				1			
Ala90Val, Asp94Gly	1					1		
Ser91Pro, Asp94Gly	1					1		
Ala90Val, Ser91Pro	1				1[a]			
Ala90Val, Asp94Gly, Gly88Cys	1						1	
None (WT)	51	35	12	2[d]	1[e]	1[e]		
Total	133	35	14	24	17	25	15	3

[a] A *gyrA* mutation was identified in one isolate in this category during repeat sequencing of isolates previously classified as WT.
[b] Strain MLB 38.
[c] This group includes strain MLB 55.
[d] One of these isolates has a *gyrB* mutation.
[e] All isolates have *gyrB* mutations.

(Tables 2 and 3). For example, two of the four substitutions identified at Asp94, Asp94Asn and Asp94Gly, conferred high-level resistance (MXF MIC ≥ 2.0 μg/ml and LVX MIC ≥ 4.0 μg/ml). However, the remaining two Asp94 substitutions identified, Asp94Tyr and Asp94Ala, generally resulted in lower MICs for MXF and LVX (Tables 2 and 3). Compared to isolates with mutations elsewhere within the *gyrA* QRDR, isolates with Ala90Val substitutions consistently exhibited the lowest levels of resistance to MXF, with 23 of 24 (95.8%) of these isolates having a MIC of ≤2 μg/ml (Table 2). This study included only one isolate each with amino acid substitutions Asp89Asn, Asp89Gly, Gly88Cys, and Ser91Pro, making it difficult to draw conclusions regarding the level of resistance associated with these substitutions. However, isolates bearing Asp89Asn, Gly88Cys, and Ser91Pro substitutions would be considered resistant to LVX at a critical concentration of 1.0 μg/ml and MXF at 0.5 μg/ml, which is noteworthy, and isolates harboring Gly88Cys or Asp89Asn also exhibited an MXF MIC of >2.0 μg/ml (Tables 2 and 3).

Identification of *gyrB* mutations in resistant isolates with WT *gyrA*. Ten OFX-, LVX-, and/or MXF-resistant isolates were initially characterized as harboring a WT *gyrA* QRDR (Table 4). Although less frequent than mutations in *gyrA*, mutations within *gyrB* have also been shown to confer resistance to FQs (12, 14, 25, 26). The QRDR of *gyrB* was sequenced for each of these isolates,

TABLE 3 LVX MICs according to GyrA QRDR substitutions

Substitution(s)	No. of isolates							
		With LVX MIC (μg/ml) of:						
	Total	≤0.25	0.5	1	2	4	8	16
Ala90Val	24				7	15[a]	2	
Asp89Asn	1					1		
Asp89Gly	1			1				
Asp94Ala	12				6	4	2	
Asp94Tyr	2					1	1	
Asp94Asn	11					1	9[a]	1
Asp94Gly	25				1[a]	1	18	5
Gly88Cys	1						1	
Ser91Pro	1				1			
Ala90Val, Asp94Gly	1						1	
Ala90Val, Ser91Pro	1						1[a]	
Ala90Val, Asp94Gly, Gly88Cys	1				1			
Ser91Pro, Asp94Gly	1						1	
None (WT)	51	12	29	5	4[b]	1[b]		
Total	133	12	29	6	20	24	36	6

[a] A *gyrA* mutation was identified in one isolate in this category during repeat sequencing of isolates previously classified as WT.
[b] Isolates have a *gyrB* mutation.

TABLE 4 Isolates with discordant phenotypic and genotypic results[a]

Isolate	OFX susceptibility (%)[c]	MIC (μg/ml)		Substitution	
		MXF	LVX	GyrA[d]	GyrB
WT *gyrA* QRDR based on initial sequence results but resistant to OFX, MXF, and/or LVX					
MLB 159	R (100)	1	4	None (WT)	Asp500His
MLB 105	R (100)	0.5	2	None (WT)	Asp500Asn
MLB 200	R (25)	0.5	2	None (WT)	Asp500Asn
MLB 269	R (100)	2	2	None (WT)	Asn538Asp
MLB 99	R (25)	4	2	None (WT)	Asn538Lys
MLB 138	R (100)	1	4	Ala90Val	None (WT)
MLB 141	R (100)	2	2	Ala90Val, Ser91Pro	None (WT)
MLB 149	R (50)	4	8	Asp94Asn	None (WT)
MLB 151	R (10)	0.5	2	Asp94Gly	None (WT)
MLB 268[b]	S	1	1	None (WT)	None (WT)
	R (25)	1	ND	None (WT)	None (WT)
	S	<0.25	ND	None (WT)	None (WT)
Mutant *gyrA* QRDR but susceptible to OFX, MXF, and/or LVX					
MLB 38[b]	S	1	1	Asp89Gly	None (WT)
	R (12.5)	2	ND	Asp89Gly	None (WT)
	S	0.5	ND	Asp89Gly	None (WT)
MLB 55[b]	S	1	4	Asp94Ala	None (WT)
	R(10)	0.5	ND	Asp94Ala	None (WT)
MLB 198[b]	R (10)	0.5	2	Asp94Ala	None (WT)
	R (50)	0.5	2	Asp94Ala	None (WT)

[a] Results from individual tests are presented on separate rows. R, resistant; S, susceptible; ND, not determined.
[b] OFX susceptibility testing (2 μg/ml) and MXF MIC determination were repeated multiple times for these isolates.
[c] Percentage of cells that were resistant compared to no-drug control.
[d] GyrA substitutions identified when isolates were grown on 2 μg/ml OFX.

and mutations were identified in five isolates (Table 4). Interestingly, in a recent report GyrB substitution Asp500Asn was shown to confer resistance to OFX and LVX but not MXF (12). The two isolates in this study with the Asp500Asn substitution demonstrate a similar resistance profile.

Discordant isolates. In some instances, patients with TB may harbor more than one distinct bacterial population with possibly different susceptibility profiles (11, 27). This appears to be particularly true in the case of FQ resistance (28–31). It is possible that the remaining five FQ-resistant isolates with reportedly WT *gyrA* and *gyrB* QRDRs actually consist of wild-type susceptible *M. tuberculosis* mixed with a proportion of mutant cells that is below the level of detection by conventional sequencing. To address this possibility, isolates were grown in the presence or absence of 2 μg/ml OFX to select for the resistant population, and *gyrA* QRDR was sequenced from four colonies from each condition for each isolate. Mutations were identified within the *gyrA* QRDR in four isolates (Table 4). The original DST for the remaining isolate, strain MLB 268, indicated susceptibility to OFX and LVX (MIC = 1 μg/ml) but resistance to MXF (MIC = 1 μg/ml). Additional DSTs were performed in an attempt to resolve the discordance, but mixed results persisted (Table 4).

gyrA mutations were detected in three isolates determined to be susceptible to OFX, LVX, and/or MXF (0.5 μg/ml) (Table 4). Sequencing, OFX DST, and MXF MIC assays were repeated, and the presence of *gyrA* mutations was confirmed for all isolates. Two isolates demonstrated variable DST and MIC results upon repeat testing, suggesting heterogeneous mixtures of resistant and susceptible cells. The repeat DST and MIC results for strain MLB 198 were identical to the original results.

To further investigate the notion of mixed populations of FQ-resistant and -susceptible isolates within individual samples, we made serial dilutions of three isolates that original sequencing reported as WT *gyrA* but that were resistant to OFX on the basis of agar proportion results (strains MLB 141, 149, and 151) and one susceptible control strain (H37Rv). Dilutions were plated on 7H10 medium with and without OFX (2 μg/ml). As expected, the control strain failed to grow on OFX. However, the test strains had growth on OFX plates that equated to 7% (MLB 141), 5.7% (MLB 149), or 0.04% (MLB 151) of colonies that grew in the absence of drug. *gyrA* mutations were identified when colonies grown in the presence of drug served as the template for sequencing reactions, indicating heteroresistance.

MLB 187 presents an interesting case. Sequencing of *gyrA* from strain MLB 187 revealed the presence of mixed C/T peaks within the Ala90 codon, suggesting a mixed population of WT and Ala90Val substitution-encoding alleles at this locus. Isolates with Ala90Val substitutions typically have MXF MICs of <2 μg/ml, but this isolate had an MIC of 8 μg/ml. To investigate the cause of this unexpectedly elevated MIC, we cultured this isolate on medium containing OFX and sequenced *gyrA* from four colonies. Surprisingly, we now identified a combination of three substitutions, Ala90Val, Asp94Gly, and Gly88Cys, in three of four colonies tested. The fourth colony had only the Gly88Cys and Asp94Gly GyrA substitutions. In all cases, the mutant allele was found in combination with the WT allele as indicated by mixed peaks on the chromatogram. We grew MLB 187 on medium containing no drug or MXF at 0.25, 0.5, 1.0, 2.0, 4.0, or 8.0 μg/ml and sequenced *gyrA* from two colonies grown under each condition. In the absence of drug, Ala90Val was the only detectable substitution,

TABLE 5 Mutations present in MLB 187 culture grown with OFX or at different MXF concentrations[a]

Substitution	Nucleotide change	No drug	MXF (μg/ml)						OFX (μg/ml)
			0.25	0.5	1	2	4	8	2
Ala90Val	GCG→GTG	+[b]	+[b]	−	−	−	−	−	+[b]
Asp94Gly	GAC→GGC	−	+[b]	+[b]	+[b]	−	−	−	+[b]
Gly88cys	GGC→TGC	−	+[b]	+[b]	+[b]	+	+	+	+[b]

[a] +, mutation/substitution is present; −, mutation/substitution is absent.
[b] Mixture of mutant and WT alleles.

whereas the population appeared to shift from Ala90Val to Asp94Gly and finally to Gly88Cys with increasing concentrations of MXF (Table 5). MLB 187 appears to demonstrate the influence of drug concentration on the process of mutations becoming "fixed" within a genome with successively higher concentrations of drug selecting for populations of bacteria harboring mutation(s) conferring resistance to those concentrations (32).

DISCUSSION

In 2013, an estimated 9 million persons had TB worldwide (10). Although it is complicated, successful treatment of all TB patients is critical not only to protect their lives but also to stop the spread of this disease and thereby protect the lives of many others. Regrettably, 3.6% of newly diagnosed and 20% of previously treated TB cases are likely to be MDR TB, which makes successful treatment even more complex and necessitates the use of second-line drugs such as the FQs (33). Compared to first-line drugs, current second-line drugs are more expensive, more toxic, and more difficult to obtain reliably. Furthermore, when second-line drugs are required, treatment time is extended up to 2 years and cure rates drop to 50% (33). Acquisition of resistance to an FQ and an injectable drug in addition to rifampin and isoniazid results in XDR TB. An estimated 9% of MDR cases are in reality XDR TB (10). A recent study reported an increase in the percentage of MDR isolates in France that were FQ resistant from 8% in 2007 to 30% in 2012 (16). The need for better treatment options is indisputable. As new or improved drugs become available, the degree of cross-resistance with currently used therapies must be evaluated and protocols for susceptibility testing must be determined. Also important is the identification of mechanisms of resistance and, if possible, the development of molecular methods for predicting resistance.

Two newer FQs, LVX and MXF, have shown promise against TB. However, studies describing the appropriate concentrations for evaluating susceptibility to these drugs are limited. For *M. tuberculosis*, phenotypic drug susceptibility is evaluated by testing at critical concentrations defined as the lowest concentration of drug at which 95% of wild-type organisms not previously exposed to drug are inhibited. CLSI document M24-A2 (23) and the WHO document entitled "*Policy Guidance on Drug Susceptibility Testing (DST) of Second-Line Antituberculosis Drugs*" (34) recommend a critical concentration of 2 μg/ml for determination of *M. tuberculosis* susceptibility to OFX in Middlebrook 7H10 or 7H11. CLSI document M24-A2 advises critical concentrations of 1 μg/ml and 0.5 μg/ml for LVX and MXF, respectively (23). Importantly, CLSI notes that the MXF recommendations are based on a small number of studies. Moreover, while the study that is the basis for the CLSI MXF critical concentration recommendation is well done and thorough, its purpose was to investigate the MIC range for susceptible clinical isolates, and it included only two resistant isolates and no genotypic information (7).

In this work, we determined MXF and LVX MICs and OFX susceptibility for 133 clinical isolates. Since the critical concentration for OFX is well established, we compared MXF and LVX MICs to OFX susceptibility data. In general, MICs for MXF were less than those for LVX (Tables 2 and 3). Forty-five of 48 (93.75%) OFX-susceptible isolates had an MIC of ≤0.5 μg/ml for MXF. Repeat DST and sequencing of the three isolates that were not MXF susceptible suggested that they are actually heterogeneous mixtures of resistant and susceptible cells. Eighty-one (95.3%) of 85 OFX-resistant isolates had an MXF MIC of >0.5 μg/ml. The Asp500Asn substitution within GyrB has been shown to confer resistance to OFX and LVX but not MXF (12) and was identified in two isolates (MLB 105 and MLB 200) that demonstrated this pattern of susceptibility. Repeat DST of the other two OFX-resistant isolates with an MXF MIC of ≤0.5 μg/ml (MLB 151 and 198) suggested mixed cultures containing susceptible and resistant bacteria. Likewise, 47 of 48 (97.9%) OFX-susceptible isolates had an MIC of ≤1.0 μg/ml for LVX. The single isolate with an MIC of >1 μg/ml for LVX displayed OFX resistance when retested. All OFX-resistant isolates ($n = 85$) had MICs of >1.0 μg/ml for LVX. Based on these combined data, we suggest that the critical concentration for DST by agar proportion should be 0.5 μg/ml for MXF and 1 μg/ml for LVX.

Resistance to FQs is most frequently attributed to mutations within the *gyrA* QRDR and, to a much lesser degree, the *gyrB* QRDR (11, 13–15). We evaluated the correlation between *gyrA* polymorphisms and MXF and LVX MICs. MXF MICs were ≤0.5 μg/ml for 47 of 51 (92.2%) isolates with wild-type *gyrA* QRDRs (Table 2). Resistance-associated mutations within the *gyrB* QRDR were later identified in three of four isolates with WT *gyrA* QRDRs but MXF MICs of >0.5 μg/ml. *gyrA* QRDR mutations corresponded to MXF MICs of >0.5 μg/ml in 80 of 82 (97.6%) isolates, and the two discordant isolates had MICs of >0.5 upon retesting. We determined that 46 of 51 (90.2%) isolates with WT *gyrA* QRDRs had MICs for LVX of ≤1 μg/ml (Table 3). Mutations within the *gyrB* QRDR associated with FQ resistance were identified in all five discordant isolates (Table 4). Eighty-six isolates had an MIC of >1 for LVX. Each of these isolates contained a mutation within either the *gyrA* or *gyr*B QRDR (Table 3). Therefore, the presence of mutations within *gyrA* or *gyrB* correlates with resistance to MXF and LVX at the above-described critical concentrations.

Others have reported that specific mutations within the *gyrA* QRDR result in different FQ MICs (14, 15, 35, 36). We analyzed MICs associated with each *gyrA* allele detected in this study population and found that certain *gyrA* mutations were good predictors of low-level (0.5 μg/ml ≤ MIC < 2.0 μg/ml) or high-level

(MIC > 2.0 μg/ml) MXF resistance. Substitutions Asp94Asn and Asp94Gly typically resulted in high-level resistance, while substitutions Asp94Ala, Asp94Tyr, and Ala90Val generally resulted in low-level MXF resistance. Incidentally, Ala90Val and Asp94Ala are among the most frequently identified GyrA substitutions (47.6% of isolates in our study with QRDR mutations had one of those two substitutions) (11, 13–15). The single Asp89Gly and Ser91Pro isolates and both Asp94Tyr isolates demonstrated low-level resistance. In light of these findings, we propose a second breakpoint for MXF resistance at 2 μg/ml to discriminate between low-level (0.5 μg/ml < MIC ≤2 μg/ml) and high-level (MIC > 2 μg/ml) resistance by agar proportion. With the exception of one outlier (MLB 268), all isolates susceptible to 2 μg/ml OFX with WT *gyrA* and *gyrB* were also susceptible to 0.5 μg/ml MXF (see Table S1 in the supplemental material). On the other hand, approximately one-half of the isolates resistant to OFX were susceptible to MXF at 2.0 μg/ml despite the presence of *gyrA* QRDR mutations (see Table S1 in the supplemental material). It is not uncommon for multiple degrees of resistance to exist for a particular antibiotic. For example, both low and high levels of resistance to isoniazid, streptomycin, and kanamycin have been reported for *M. tuberculosis* (37). As seen here, different levels of resistance often correlate with different mutations/mechanisms of resistance (38, 39).

Low-level resistance to MXF could be clinically meaningful. Many pharmacokinetic studies have measured the levels of MXF achieved during therapy and reported levels in serum of 3.1 to 4.7 mg/liter (mean) depending on dose and coadministration of other drugs (6, 15, 36, 40, 41). Thus, it seems that 2 μg/ml, the suggested critical concentration for defining low-level MXF resistance, would be achievable *in vivo*. However, the relevance of concentrations in serum for predicting effectiveness in TB treatment is debated. During the course of TB, a large part of the bacterial burden can be found within the lung in granulomas. Consequently, the ability of a drug to achieve therapeutic concentrations within the granuloma is critical. One recent study measured the levels of four different drugs in rabbits in sera, lung tissue, and granulomas (42). Different drugs had vastly different abilities to concentrate in tissue, granulomas, and sera. The relationship between concentration in serum and concentration in granulomas differed for each drug. Remarkably, MXF was shown to preferentially localize within the granuloma, achieving peak tissue concentrations as high as 2,485 ng/g (42). In the present study, many isolates with *gyrA* QRDR mutations had MICs for MXF between 0.5 and 2 μg/ml (Tables 3 and 4). A similar phenomenon is observed in the case of LVX, with some isolates exhibiting low-level resistance to LVX (1 μg/ml < MIC ≤ 8 μg/ml), while others demonstrate higher levels of resistance (MIC > 8). However, because the MICs of MXF are lower overall than those of LVX and the pharmacokinetic studies that measured drug concentration in granulomas did not include LVX, we focused on MXF. Many of the isolates that were resistant to OFX and LVX would be defined as low-level MXF resistant and therefore likely susceptible to 2.0 μg/ml MXF. The majority of these isolates have mutations within the *gyrA* and *gyrB* QRDRs. Patients infected with *M. tuberculosis* strains bearing these mutations might now have a treatment option that was previously unrecognized. However, if OFX is used as a class representative for the FQs or if MXF is tested only at 0.5 μg/ml, this option will remain unidentified and a potentially effective drug will be excluded from treatment. A report detailing treatment of an individual with MDR TB who had a GyrA Ala90Val substitution but was treated with high-dose MXF and achieved cure supports this hypothesis (43).

During the course of this study, we found a small number of isolates with seemingly discordant phenotypic and genotypic results that upon further investigation appeared to be due to heterogeneous *gyrA* alleles within a single specimen. Heterogeneous populations of *M. tuberculosis* from clinical specimens, especially when FQ resistance is involved, have been reported (27, 28, 44, 45). Heterogeneous infections may be due to emerging resistance or coinfection by two distinct strains. In order to determine whether discrepant isolates were mixed populations, we grew the isolates in the presence of drug to select for the resistant population and then sequenced *gyrA*. The identification of mutations within sequences generated under these conditions suggests that three of these isolates were composed of a mixture of predominantly WT and a small amount of mutant (<10%) alleles. Based on our experience, conventional sequencing is able to detect only alleles present in greater than 25% of the population. It is therefore not surprising that mutations were not detected during the original sequencing. It is important to remember that conventional MIC testing does not measure the MIC of individual cells but of the entire population present in the inoculum. The presence of even small numbers of resistant cells may result in an increase in the MIC. Furthermore, individual cells purified from that population and tested independently may yield different MIC/susceptibility results.

Data on cross-resistance among MXF, LVX, and the older FQ OFX are somewhat limited. In this study, we demonstrated a high degree of cross-resistance between LVX and OFX at the suggested testing concentrations. We also showed a high degree of cross-resistance between >0.5-μg/ml MXF and LVX and OFX. MICs were lower overall for MXF than for OFX and LVX. Based on these data, we concur with the current recommendations of a critical concentration of 1 μg/ml for LVX and suggest 0.5 μg/ml for MXF. However, some *gyrA* QRDR mutations (encoding substitution Ala90Val, Asp94Ala, or Asp94Tyr) correlated with resistance to suggested and established critical concentrations for LVX and OFX but did not result in cross-resistance to MXF at 2 μg/ml. We therefore agree with the findings of others that 2 μg/ml is potentially a valuable second breakpoint for differentiating between low- and high-level resistance to MXF and suggest reflex testing at 2 μg/ml when low-level MXF resistance is detected.

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