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Wild-Type and Non-Wild-Type *Mycobacterium tuberculosis* MIC Distributions for the Novel Fluoroquinolone Antofloxacin Compared with Those for Ofloxacin, Levofloxacin, and Moxifloxacin

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Antofloxacin (AFX) is a novel fluoroquinolone that has been approved in China for the treatment of infections caused by a variety of bacterial species. We investigated whether it could be repurposed for the treatment of tuberculosis by studying its *in vitro* activity. We determined the wild-type and non-wild-type MIC ranges for AFX as well as ofloxacin (OFX), levofloxacin (LFX), and moxifloxacin (MFX), using the microplate alamarBlue assay, of 126 clinical *Mycobacterium tuberculosis* strains from Beijing, China, of which 48 were OFX resistant on the basis of drug susceptibility testing on Löwenstein-Jensen medium. The MIC distributions were correlated with mutations in the quinolone resistance-determining regions of *gyrA* (*Rv0006*) and *gyrB* (*Rv0005*). Pharmacokinetic/pharmacodynamic (PK/PD) data for AFX were retrieved from the literature. AFX showed lower MIC levels than OFX but higher MIC levels than LFX and MFX on the basis of the tentative epidemiological cutoff values (ECOFFs) determined in this study. All strains with non-wild-type MICs for AFX harbored known resistance mutations that also resulted in non-wild-type MICs for LFX and MFX. Moreover, our data suggested that the current critical concentration of OFX for Löwenstein-Jensen medium that was recently revised by the World Health Organization might be too high, resulting in the misclassification of phenotypically non-wild-type strains with known resistance mutations as wild type. On the basis of our exploratory PK/PD calculations, the current dose of AFX is unlikely to be optimal for the treatment of tuberculosis, but higher doses could be effective.

In 2009, the Chinese State Food and Drug Administration granted marketing approval for the new fluoroquinolone antofloxacin hydrochloride (here referred to as antofloxacin [AFX]), a derivative of levofloxacin (LFX) (1, 2). Its intended uses are for the treatment of (i) acute bacterial exacerbations of chronic bronchitis due to *Klebsiella pneumoniae*, (ii) acute pyelonephritis and cystitis due to *Escherichia coli*, and (iii) wound infection and multiple epifolliculitis due to *Staphylococcus aureus* or coagulase-negative staphylococci (1). However, given that AFX has activity against a wider array of bacterial pathogens and other fluoroquinolones are used for treatment of tuberculosis, we wanted to investigate its *in vitro* activity against *Mycobacterium tuberculosis* strains from China (1). Moreover, we studied the degree of cross-resistance to fluoroquinolones that are already being used to treat tuberculosis (i.e., ofloxacin [OFX], LFX, and moxifloxacin [MFX]) on a phenotypic as well as a genotypic level to assess whether current genotypic drug susceptibility testing (DST) assays could be used to detect resistance to AFX and whether AFX might be an option for the treatment of infections caused by strains that are resistant to these existing fluoroquinolones.

MATERIALS AND METHODS

Study setting and bacterial strains. We studied 126 *M. tuberculosis* complex strains that were collected from the National Clinical Laboratory on Tuberculosis, Beijing Chest Hospital, between January and March 2014 from retreatment patients with presumed multidrug-resistant (MDR) tuberculosis (i.e., resistance to rifampin and isoniazid), which included 45 pansusceptible *M. tuberculosis* strains, 49 MDR *M. tuberculosis* strains, and 17 extensively drug-resistant *M. tuberculosis* strains (i.e., MDR *M. tuberculosis* strains with additional resistance to OFX and amikacin or

capreomycin), as well as 3 strains that were monoresistant to OFX (Sigma-Aldrich, St. Louis, MO, USA), as determined using the absolute concentration method on Löwenstein-Jensen medium (LJ) with 2 μg/ml as the critical concentration (CC). The *M. tuberculosis* laboratory strain H37Rv (ATCC 27294) served as a negative control.

MIC testing. We determined the MICs for OFX, LFX (Sigma-Aldrich, St. Louis, MO, USA), MFX (Bayer Pharmaceutical Corporation, Leverkusen, Germany), and AFX (Anhui Huanqiu Pharmaceutical Co., Hefei, China) using the microplate alamarBlue assay (MABA) in 2-fold dilutions ranging from 16 to 0.032 μg/ml (3, 4). Drug powder was dissolved in 1% NaOH at a concentration of 10 mg/ml, and different aliquots were prepared and stored at −70°C. All the working solutions were freshly prepared before use. All the strains were subcultured onto LJ slopes for 3 weeks. Bacterial suspensions were prepared using 5% (vol/vol) Tween 80 in 0.9% NaCl, and the turbidity was adjusted to a 1 McFarland turbidity

standard. Suspensions were further diluted (1:25) with 7H9 broth. H37Rv was used as a control.

Genotypic analyses. We sequenced the quinolone resistance-determining regions (QRDRs) of *gyrA* (*Rv0006*) and *gyrB* (*Rv0005*) and called mutations relative to the sequence of the H37Rv reference genome (GenBank accession number AL123456.3) using the 2002 numbering for *gyrB* (5–7). We usually sequenced isolates recovered from the drug-free LJ slopes, but where no resistance mutations were found in phenotypically resistant strains, sequencing was repeated with isolates recovered from the OFX-containing LJ slope to detect low-frequency mutations (8, 9). Strains belonging to the East Asian lineage were identified on the basis of RD105 (10).

RESULTS

A total of 92.9% (117/126) of the strains in this study belonged to the East Asian lineage (see Table S1 in the supplemental material) (11). We found that the MIC distributions for all four fluoroquinolones were bimodal (Fig. 1A to D), where the more susceptible of the two distributions represented the phenotypically wild-type distributions, whereas the remaining strains were, by definition, phenotypically non-wild type. Based on visual inspection, we therefore set tentative epidemiological cutoff values (ECOFFs) for MIC determination using the MABA method at 2, 1, 0.5, and 0.25 µg/ml for OFX, AFX, LFX, and MFX, respectively (12). Not all phenotypically wild-type strains were identical genotypically (i.e., all 126 Chinese strains harbored the known *gyrA* S95T mutation that does not correlate with resistance [7, 13]), but after the exclusion of this polymorphism, we found a nearly perfect correlation between the tentative ECOFFs and nonsynonymous mutations in the two subunits of DNA gyrase, encoded by *gyrA* and *gyrB*.

All *gyrA* mutations detected in this study were classical resistance mutations that fell into the QRDR and resulted in an MIC increase above the tentative ECOFF for all four fluoroquinolones (Fig. 1; see also Table S1 in the supplemental material) (7, 14). This was in line with the fact that all *gyrA* mutants tested resistant to OFX on LJ, although retesting of seven strains that were initially discrepant was required to achieve complete agreement (Table 1). In line with a recent systematic review, the D94G and A90V mutations were the most frequent and the second most frequent mutations, respectively, whereas other changes (e.g., G88C) occurred in only a single strain (15). Theoretically, all of these mutations could have been detected with the genotypic DST assays of Hain Lifescience, Nipro, and YD Diagnostics, whereas the assays of AID and Seegene would have missed the two resistant strains with mutations at codon 88 (see Table S1 in the supplemental material) (16–22). In practice, however, some resistance mutations might have been missed, given that the detection limits of these assays, albeit unknown, are almost certainly higher than the critical proportion of 1% (e.g., strain 14140 was heteroresistant, and its D94G mutation was detectable only using Sanger sequencing of an isolate from the drug-containing slope [see Table S1 in the supplemental material]) (23–25).

As expected, *gyrB* mutations were rare and usually coincided with *gyrA* mutations (in 5/6 cases); thus, they did not improve markedly the sensitivity of detecting phenotypically non-wild-type strains (48/49 strains had a *gyrA* mutation) (15). Strain 14117 was the sole exception. It harbored only a *gyrB* mutation (T500N), was found to be susceptible to OFX on LJ, and had MABA MICs that corresponded to the aforementioned ECOFFs for the four respective fluoroquinolones (Table 1). The mutation in question

fell just outside of the *gyrB* QRDR, as defined by Maruri et al. (7), which spans codons 461 to 499, but inside the QRDR based on the findings of Pantel et al. (26), which extends to codon 501. Using the recently developed version 2 of the Hain Lifescience Genotype MTBDR*sl* assay, which covers codons 497 to 502 of *gyrB*, an isolate with this mutation would also have been interpreted to be resistant (22). We therefore repeated DST for this strain, whereupon the MICs for AFX, LFX, and MFX increased by 1 doubling dilution and the strain consequently became phenotypically non-wild-type, whereas the OFX MIC and LJ result remained unchanged (Table 1).

DISCUSSION

The aim of DST is usually to distinguish resistant strains, patients infected with which are likely to fail treatment, from susceptible strains, patients infected with which have a high likelihood of clinical success (an intermediate category is sometimes possible) (27). The clinical breakpoints (known as critical concentrations [CCs] in the tuberculosis field) employed for this purpose should be based on clinical, pharmacokinetic/pharmacodynamic, and, ideally, clinical outcome data, which, for a variety of reasons, are difficult to obtain for tuberculosis drugs (27). As a result, an important aim of DST for the majority of tuberculosis drugs is to distinguish wild-type from non-wild-type strains [i.e., strains with elevated MICs compared with those for strains that (i) have never been exposed to the agent or class of agent in question and (ii) are not intrinsically resistant] using the ECOFF, which represents the highest concentration of the wild-type distribution determined by modern microbiological principles pioneered by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (12, 23, 27–30). In other words, the ECOFF represents the lowest possible CC and some non-wild-type strains might remain treatable, as proposed for MFX, albeit on the basis of limited evidence (i.e., the CC of 2 µg/ml set by the World Health Organization [WHO] is higher than the ECOFF) (9, 29, 31).

Setting conclusive ECOFFs and validating MABA as a method for routine DST were beyond the scope of this study, which would have required a larger number of phylogenetically diverse strains from multiple laboratories and more extensive reproducibility testing, as specified by EUCAST and the International Organization for Standardization (ISO) (12, 28, 32, 33). Nevertheless, our MABA results were sufficiently robust compared with those of LJ DST and the genotypic results to set tentative ECOFFs. Accordingly, AFX had a lower ECOFF than OFX *in vitro* but an ECOFF higher than the ECOFFs of LFX and MFX. All *gyrA* mutations correlated with non-wild-type MICs for all fluoroquinolones. Consequently, clinicians should consider the possibility that the use of AFX to treat infections caused by *E. coli*, *K. pneumoniae*, and staphylococci at the doses currently suggested might result in the selection of fluoroquinolone resistance in *M. tuberculosis* in coinfected patients.

We had only one strain that had a *gyrB* mutation without a mutation in *gyrA*. The fact that four different amino acid changes had been observed at the *gyrB* codon in question (T500A/I/N/P) constitutes a potential signal for drug selection (7, 34, 35). In line with this observation, allelic exchange experiments with T500N in an Erdman background increased the MIC from wild-type levels to the CC for OFX and LFX and just above the CC for MFX (36). The results of the equivalent experiment in an H37Rv background were identical for OFX and LFX, but no increase in MIC was
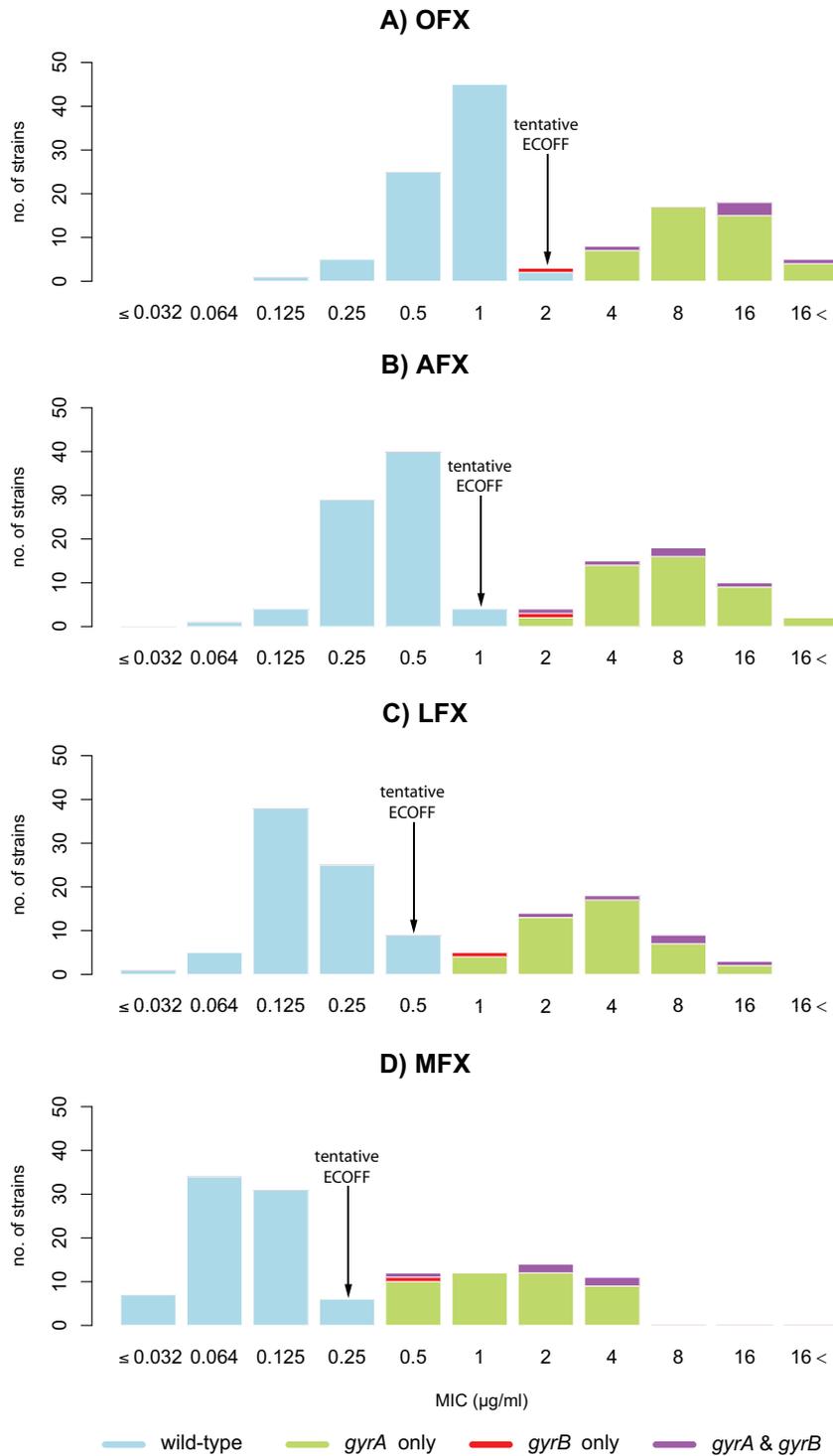
FIG 1 Wild-type and non-wild-type MIC distributions for the four fluoroquinolones under investigation relative to their *gyrA* and *gyrB* genotypes (see Table S1 in the supplemental material). The tentative ECOFF represents the upper limit of the wild-type distribution. All clinical strains, with the exception of H37Rv, harbored the *gyrA* S95T mutation that is known not to confer fluoroquinolone resistance and was consequently excluded from the analysis (13).

observed for MFX (36). In accordance with the results of the *in vitro* selection experiments and the aforementioned allelic exchange experiments, this suggested that the MIC of the strain with *gyrB* T500N was close to the ECOFF, which, due to biological and technical variability (e.g., for reproducibility, the ISO guidelines

allow ±1 dilution of the mode for ≥95% of the results), would likely result in a poor reproducibility of DST (32, 37–39). Irrespective of whether this slightly elevated MIC increases the likelihood of treatment failure, it is possible that it increases the likelihood of selecting for higher levels of fluoroquinolone resistance due to a

TABLE 1 Initial and repeat LJ DST and MABA MIC results for the seven strains for which there was disagreement between the different methods during the initial round of testing[a]

Strain	OFX LJ DST result	MABA MIC (μg/ml)				Genotype[b]	
		OFX	AFX	LFX	MFX	*gyrA*	*gyrB*
14170	**R**	2	0.25	0.125	0.125	WT	WT
	S	0.5	0.5	0.25	0.25		
12657	**R**	2	1	0.5	0.25	WT	WT
	S	1	1	0.5	0.25		
14130	**R**	2	0.5	0.25	0.125	WT	WT
	S	1	1	0.5	0.25		
14132	**R**	0.5	0.5	0.125	0.125	WT	WT
	S	1	0.5	0.5	0.25		
14150	**R**	2	**2**	1	0.5	WT	WT
	S	1	1	0.5	0.25		
14175	**R**	2	0.5	0.25	0.125	WT	WT
	S	0.5	0.5	0.25	0.125		
14198	R	**2**	<u>4</u>	<u>2</u>	<u>1</u>	D94A	WT
	R	<u>8</u>	<u>8</u>	<u>4</u>	<u>2</u>		
14117	S	2	1	0.5	0.25	WT	T500N
	S	2	**2**	**1**	**<u>0.5</u>**		

[a] In each case, the repeat results are shown in Fig. 1 and listed in Table S1 in the supplemental material. MICs above the ECOFF (i.e., phenotypically non-wild-type results) are underlined. All of these discrepancies, which are shown in bold, were resolved upon retesting. In contrast, strain 14117 was retested because the initial MICs and the previous literature suggested that the MICs were close to the ECOFFs, which retesting supported.
[b] Excluding the *gyrA* S95T polymorphism. WT, wild type.

gyrA mutation or a secondary *gyrB* mutation, as observed for streptomycin (36, 40, 41). Larger data sets, ideally with longitudinal samples from the same patients, would be required to clarify this possibility (i.e., to determine in which order *gyrA* and *gyrB* mutations arose in double mutants, such as the five strains observed in this study [Fig. 1; see also Table S1 in the supplemental material]).

Using the published area under the concentration-time curve from time zero to 24 h (AUC_{0-24}) of 47.59 ± 7.85 mg · h/liter for the currently approved dose of AFX (i.e., a 200-mg daily dose following a 400-mg loading dose) and protein binding of 17.5%, the unbound $fAUC_{0-24}$/MIC ratio for the wild-type MICs of 0.064 to 1 μg/ml would range from 613.46 ± 101.19 h to 39.26 ± 6.48 h (42, 43). Although there is no consensus on the precise $fAUC_{0-24}$/MIC ratio that best predicts *in vivo* efficacy, ratios of >100 at the upper end of the wild-type distribution are likely required to maximize clinical success (44, 45). Given that the currently recommended dose of AFX is unusually low (probably because of a narrow clinical indication) compared with the doses of the other fluoroquinolones used to treat tuberculosis, the target $fAUC_{0-24}$/MIC of >100 at an increased dose is likely achievable, but this would have to be evaluated in clinical trials, where side effects would have to be monitored carefully.

Our study also has implications for DST for OFX on LJ. Although the absolute concentration method has not been validated by the WHO for second-line drugs, it is used clinically with the CC

recommended for the proportion method (29). In our case, we employed a CC of 2 μg/ml, which corresponded to the old CC for this drug for the proportion method, which the WHO recently increased to 4 μg/ml, although the rationale for this change is unclear (29). In light of the excellent correlation between the LJ DST results and MABA MICs for all four fluoroquinolones, which is in line with the findings of previous studies, this suggested that the revised CC is likely too high for the absolute concentration method, resulting in non-wild-type strains being misclassified as wild type (46, 47). This, together with prior studies that raised doubts regarding the validity of some CCs, underlined the fact that the WHO should start to apply modern microbiological principles and, crucially, to publish the evidence used to set CCs, as has been the case for EUCAST for many years (12, 27, 39).

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