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

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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 pan-susceptible *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 Huanqi 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.

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 epiploiculitis 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.
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 non-synonymous 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 MTBDRd 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 not 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 would be 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 infected 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/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
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
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 (AUC0–24) 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 [AUC0–24/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 [AUC0–24/ 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 [AUC0–24/ 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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