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Full-length paper

Mycobacterium tuberculosis wild-type and non-wild-type MIC distributions for the novel fluoroquinolone antofloxacin compared with ofloxacin, levofloxacin, and moxifloxacin

Running title: *M. tuberculosis* MIC distributions for antofloxacin

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Keywords: *Mycobacterium tuberculosis*, antofloxacin, ofloxacin, levofloxacin, moxifloxacin, epidemiological cut-off value

Abstract

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

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non-wild-type range MICs for AFX as well as ofloxacin (OFX), levofloxacin (LFX), and moxifloxacin (MFX) using the microplate alamar blue assay of 126 clinical *Mycobacterial tuberculosis* strains from Beijing (China), of which 48 were OFX resistant based on 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 than LFX and MFX based on the tentative epidemiological cut-off values (ECOFFs) determined in this study. All strains with non-wild-type MICs to 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 for OFX for Löwenstein-Jensen that was recently revised by the World Health Organization might be too high, resulting in misclassification of non-wild-type strains with known resistance mutations as wild-type. Based on 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.

Introduction

In 2009, the Chinese State Food and Drug Administration granted marketing approval for the new fluoroquinolone antofloxacin hydrochloride (hereafter referred to as antofloxacin (AFX)), a derivative of levofloxacin (LFX) (1, 2). Its intended uses are: (a) acute bacterial exacerbations of chronic bronchitis due to *Klebsiella pneumoniae*, (b) acute pyelonephritis and cystitis due to *Escherichia coli*, and (c) 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* 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 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 to treat strains that are resistant to these existing fluoroquinolones.

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59 **Methods**

60 *Study setting and bacterial strains*

61 We studied 126 *M. tuberculosis* complex strains that were collected from the National Clinical
62 Laboratory on Tuberculosis, Beijing Chest Hospital between January and March 2014 from
63 retreatment patients with presumed multidrug-resistant (MDR) tuberculosis (i.e. resistance to
64 rifampicin and isoniazid), which included 45 pan-susceptible, 49 MDR, and 17 extensively drug-
65 resistant tuberculosis strains (i.e. MDR with additional resistance to OFX and amikacin or
66 capreomycin), as well as 3 strains that were mono-resistant to OFX (Sigma-Aldrich, St. Louis, MO,
67 USA), as determined using the absolute concentration method on Löwenstein-Jensen (LJ) with 2
68 µg/ml as critical concentration. The *M. tuberculosis* laboratory strain H37Rv (ATCC 27294) served as
69 negative control.

70

71 *MIC testing*

72 We determined the MICs for OFX, LFX (Sigma-Aldrich, St. Louis, MO, USA), MFX (Bayer Pharmaceutical
73 Corporation, Leverkusen, Germany), and AFX (Anhui Huanqiu Pharmaceutical Co., Hefei, China) using
74 the microplate alamar blue assay (MABA) in two-fold dilutions ranging from 16 to 0.032 µg/ml (3, 4).
75 Drug powder was dissolved in 1% NaOH at the concentration of 10 mg/ml, different aliquots were
76 prepared and stored at -70°C. All the working solutions were freshly prepared before use. All the
77 strains were sub-cultured onto LJ slopes for 3 weeks. Bacterial suspensions were prepared using 5%
78 (vol/vol) Tween 80 in 0.9% NaCl and the turbidity was adjusted to 1 McFarland turbidity standard.
79 Suspensions were further diluted (1:25) with 7H9 broth. H37Rv was used as control.

80

81 *Genotypic analyses*

82 We sequenced the quinolone resistance determining regions (QRDR) of *gyrA* (*Rv0006*) and *gyrB*
83 (*Rv0005*) and called mutations relative to the H37Rv reference genome (AL123456.3) using the 2002
84 numbering for *gyrB* (5-7). We usually sequenced from the drug-free LJ slopes, but where no
85 resistance mutations were found in phenotypically resistant strains, sequencing was repeated from
86 the OFX-containing LJ slope to detect low-frequency mutations (8, 9). Strains belonging to the East
87 Asian lineage were identified based on the RD105 (10).

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Results

92.9% (117/126) of the strains in this study belonged to the East Asian lineage (Table S1) (11). We found that the MIC distributions for all four fluoroquinolones were bimodal (Figures 1A-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 cut-off 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 near 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 (Figure 1 and Table S1) (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 and second most frequent mutations, respectively, whereas other changes (e.g. G88C) only occurred in a single strain (15). Theoretically, all of these mutations could have been detected with the genotypic DST assays by Hain Lifescience, NIPRO, and YD Diagnostics, whereas the assays by AID and Seegene would have missed the two resistant strains with mutations at codon 88 (Table S1) (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 only detectable using Sanger sequencing from the drug-containing slope (Table S1)) (23-25).

As expected, *gyrB* mutations were rare and usually coincided with *gyrA* mutations (in 5/6 cases) and thus did not improve the sensitivity of detecting phenotypically non-wild-type strains markedly (48/49 strains had a *gyrA* mutation) (15). Strain 14117 was the sole exception. It only harbored a *gyrB* mutation (T500N) and 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).

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The mutation in question fell just outside of the *gyrB* QRDR as defined by Maruri et al., which spans codons 461 to 499, but inside the QRDR based on Pantel et al., which extends to codon 501 (7, 26). Using the recently developed version 2 of the Hain Lifescience Genotype MTBDR*sl* assay, which covers the codons 497 to 502 of *gyrB*, this mutation would have also been interpreted as resistant (22). We therefore repeated DST for this strain, whereupon the MICs for AFX, LFX, and MFX increased by one doubling dilution and 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, which are likely to fail treatment, from susceptible strains, 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/pharmacodynamics, 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 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 as 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 based on 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 was beyond the scope of this study, which would have required 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 LJ DST and the genotypic results to set tentative ECOFFs. Accordingly, AFX had a lower ECOFF than OFX *in vitro*, but higher than LFX and MFX. All *gyrA*

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mutations correlated with non-wild-type MICs to all fluoroquinolones. Consequently clinicians should consider the possibility that the use of AFX to treat *E. coli*, *K. pneumoniae* and staphylococci, at the doses currently suggested, might result in selection of fluoroquinolone resistance in *M. tuberculosis* in co-infected patients.

We only had 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 of 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 a H37Rv background were identical for OFX and LFX, but no increase in MIC was observed for MFX (36). In accordance with *in vitro* selection experiments and the aforementioned allelic exchange results, this suggested that the MIC of *gyrB* T500N was close to the ECOFF, which, due to biological and technical variability (e.g. the ISO guidelines allow for the reproducibility of ±1 dilution of the mode for ≥95% of the results), would likely result 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* or a secondary *gyrB* mutation, as observed for streptomycin (36, 40, 41). Larger datasets, 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 (Figure 1 & Table S1)).

Using the published $AUC_{0\text{-}24}$ of 47.59±7.85 mg·h/L for the currently approved dose of AFX (i.e. 200 mg daily dose following a 400 mg loading dose) and limited protein binding data of 17.5%, the $fAUC_{0\text{-}24}$/MIC ratio for the wild-type MICs of 0.064-1 µg/ml would range between 613.46±101.19 and 39.26±6.48 h (42, 43). Although there is no consensus on the precise $fAUC_{0\text{-}24}$/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 other fluoroquinolones used to treat tuberculosis, the target of $fAUC_{0\text{-}24}$/MIC>100 at increased dosing is

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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 that 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 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). In fact, a CC of 4 µg/ml is also likely too high for the proportion method, as shown by Coeck et al. (48). 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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Conflicts of interest

Anhui Huanqiu Pharmaceutical Co. provided AFX for this study, but this work was designed, conducted, and analyzed independently of the company. T. S. is a member of the EUCAST subgroup on antimycobacterial susceptibility testing. J. P., S. J. P. and C. U. K. have collaborated with Illumina Inc. on a number of scientific projects. J. P. has received funding for travel and accommodation from Pacific Biosciences Inc. and Illumina Inc. S. J. P. has received funding for travel and accommodation from Illumina Inc. C. U. K. is a consultant for the Foundation for Innovative New Diagnostics and was a technical advisor for the Tuberculosis Guideline Development Group of the World Health Organization. The Bill & Melinda Gates Foundation and Janssen Pharmaceutica covered C. U. K.'s travel and accommodation to present at meetings. The European Society of Mycobacteriology awarded C. U. K. the Gertrud Meissner Award, which is sponsored by Hain Lifescience.

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382 **Table 1**

383 Initial and repeat LJ DST and MABA MIC results for the seven strains for which there was
384 disagreement during the initial round of testing between the different methods (in each case, the
385 repeat results are shown in Figure 1 and listed in Table S1). MICs above the ECOFF (i.e. non-wild-type
386 results) are underlined. All of these discrepancies, which are shown in bold, resolved upon retesting.
387 By contrast, 14117 was retested because the initial MICs and the previous literature suggested that
388 the MICs were close to the ECOFFs, which retesting supported.

389

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

390 [a]Excluding the *gyrA* S95T polymorphism.

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Figure 1

Wild-type and non-wild-type MIC distributions for the four fluoroquinolones under investigation relative to their *gyrA* and *gyrB* genotypes (Table S1). 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 FQ resistance and was consequently excluded from the analysis (13).

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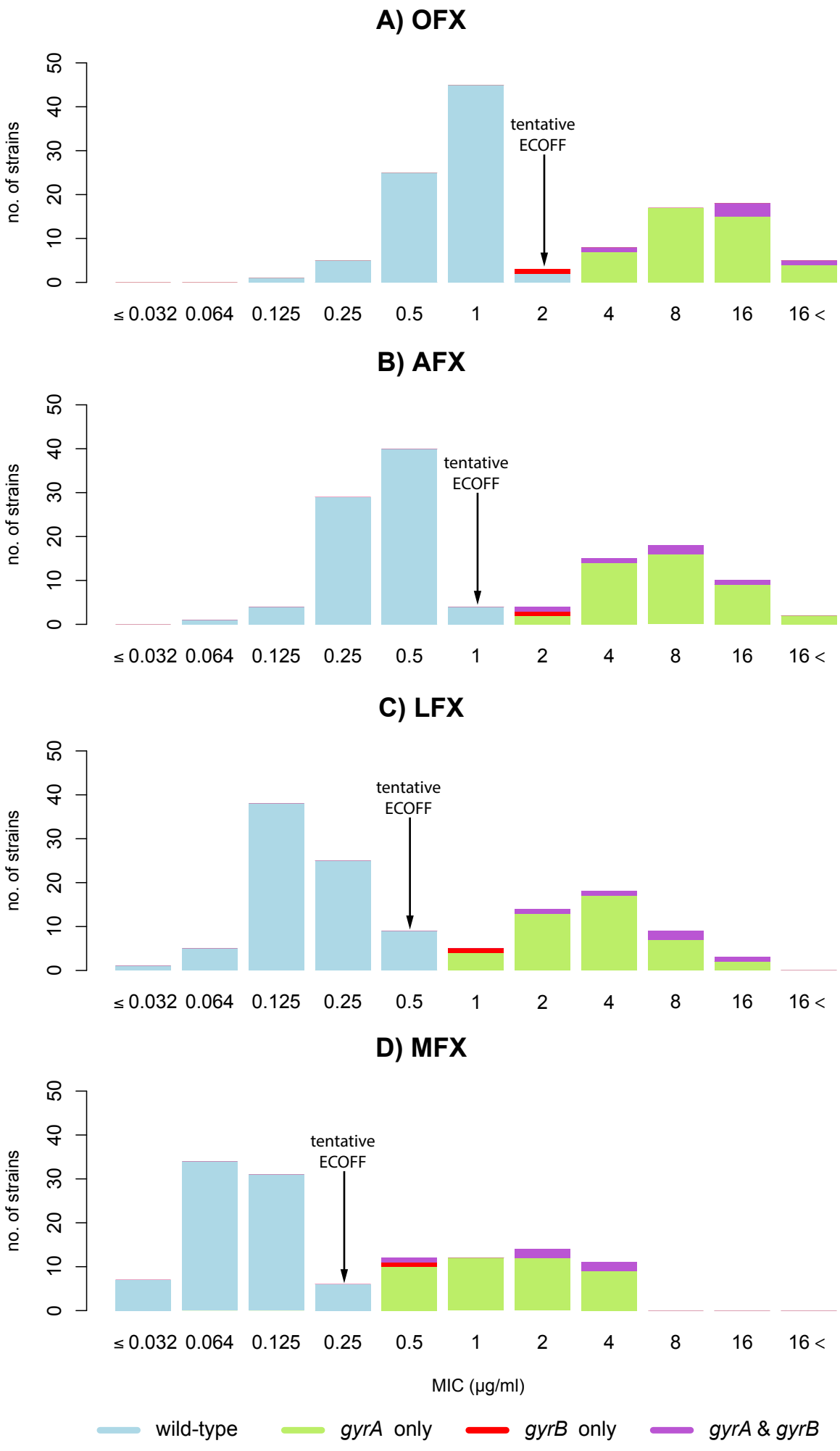

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