Original Article

Re-evaluation of Critical Concentrations of Antituberculosis Fluoroquinolones in the Mycobacteria Growth Indicator Tube 960 System

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Abstract

Background: Fluoroquinolones (FQs) have substantial activity against the Mycobacterium tuberculosis complex (MTBc) by preventing bacterial DNA synthesis through DNA gyrase inhibition. The reference standard for FQ-resistance testing is phenotypic drug-susceptibility testing (pDST) based on growth inhibition of MTBc in drug-containing Mycobacteria Growth Indicator Tube system (MGIT) media at a critical concentration (CC) that differentiates phenotypically wild-type from nonwild-type MTBc and at a clinical breakpoint that identifies strains that will likely still respond to treatment at higher doses. Despite the recent introduction of powerful new TB drugs, highly sensitive detection of clinically defined FQ resistance remains key. **Method:** In this study, we re-evaluated the current WHO-recommended CCs of Lfx (1.0 mg/ml), Mfx (0.25 mg/ml), Gfx (0.25 µg/ml), and the nowadays, obsolete CC of Ofx (2.0 mg/ml) for MGIT, using 147 MTBc isolates with known gyrA and gyrB sequences including both high-and low-level FQ resistance-conferring mutants. We tested a wide range of drug concentrations covering the current and former/obsolete WHO-recommended CCs for FQs and some intermediate concentrations to challenge the current WHO-recommended CCs. **Results:** The specificity of all four CCs was 100%. The sensitivities varied: 92.4% for Ofx and Lfx, 85.7% for Mfx, and 83.2% for Gfx. Lowering the CC of Mfx to 0.125 mg/ml would allow to correctly classify all wild-type and mutant isolates while lowering the CC of Gfx to 0.125 mg/ml would still misclassify some gyrA/gyrB mutants as susceptible. **Conclusion:** Based on our findings, a minimal inhibitory concentration of 0.125 mg/ml on MGIT medium is a more appropriate CC for Mfx and probably also as a surrogate for overall FQ resistance in the MTBc.

Keywords: Critical concentration, fluoroquinolones, mycobacteria growth indicator tube drug-susceptibility testing, *Mycobacterium tuberculosis*

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Introduction

The nalidixic acid derivatives fluoroquinolones (FQs) have substantial *in vitro* activity against the *Mycobacterium tuberculosis* complex (MTBc) by preventing bacterial DNA synthesis through DNA gyrase inhibition.[1,2] Multiple FQs have various *in vitro* and *in vivo* activity levels against the MTBc: fourth-generation FQs such as moxifloxacin (Mfx) and gatifloxacin (Gfx) have lower minimal inhibitory concentration (MICs) compared to the second- and third-generation FQs such as ofloxacin (Ofx) and levofloxacin (Lfx).[1,3,4] FQs are classified as group A drugs for the treatment of multidrug-resistant tuberculosis (MDR-TB), defined as resistant to the first-line drugs rifampicin and isoniazid.[5] Despite the recent widespread use of the other group A drugs, bedaquiline, and linezolid, Mfx, and Lfx remain key in most regimens to treat rifampicin-resistant (RR)/MDR-TB.[6,7] Thus, sensitively detecting the (level of) FQ resistance remains crucial for proper RR/MDR-TB patient management.

FQ resistance is mostly associated with mutations in the quinolone-resistance determining region (QRDR) of the

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gyrA gene (codons 74–113) and to a lesser extent in the *gyrB* gene (codons 461–499).[8-11] However, previous studies have shown that not all phenotypically FQ-resistant isolates carried mutations in the QRDR.[9,12] This compromises the sensitivity and specificity of rapid molecular diagnostic methods that target only the QRDR of gyr*A*/gyr*B* genes such as the Genotype MTBDR*sl* (Bruker, Germany) and the GeneXpert XDR (Cepheid, USA). Thus, the reference standard for FQ-resistance testing remains phenotypic drug-susceptibility testing (pDST) based on the growth inhibition of MTB. The automated Mycobacteria Growth Indicator Tube system (MGIT; Becton Dickinson, USA) has been proposed as the reference method for performing pDST for second-line anti-TB drugs, by exposing the bacilli to a critical concentration (CC) of the drugs.[9,12-16] A reference standard represents the highest level of reference and by definition is the best available method for determining the presence or absence of a condition of interest, thus MGIT960-based pDST for FQs should be able to correctly differentiate phenotypically gyr*A*/gyr*B* wild-type and clinically relevant nonwild-type MTBc strains.[17] In the context of MTBc, the current definition for the CC is the lowest concentration of an anti-TB agent that will inhibit the *in vitro* growth of 99% of phenotypically wild-type isolates.[18] CC values for FQs have been defined for various testing media.[16]

In this study, we re-evaluated the current WHO-recommended CCs for Lfx (1.0 µg/ml) and Mfx (0.25 µg/ml), as well as the now-obsolete CCs for Ofx (2.0 µg/ml) and Gfx (0.25 µg/ml) for the MGIT960 system. Furthermore, we re-evaluated if any of these four FQs may be used as a proxy for MTBc susceptibility to all FQs previously served by ofloxacin.

Materials

Isolates

A total of 147 MTBc isolates with previously published *gyrA* and *gyrB* sequences[1] were included in this study. Among these, 40 were gyr*A*/gyr*B* wild-type or carrying mutations that do not confer resistance to FQs as per the WHO catalog of mutations (Version 1).[19] Of 147, 107 isolates had mutations in the QRDR of gyr*A*/gyr*B* associated with both low and high levels of FQ resistance, all of them resulting in amino acid substitutions, predominantly in codon 94 (62/107, 58%) and codon 90 (28/107, 26%). Of 147 isolates, 90 had data on their lineage (L): 17 L1, 27 L2, 24 L3, and 22 L4.

FQ powders and stock solutions

Stock solutions were prepared for Ofx (Sigma-Aldrich, O8757), Lfx (Sigma-Aldrich, 28266), Mfx (Molekula, 85126158), and Gfx (Sigma-Aldrich, G7298) at 10000 µg/ml in 0.1 N sterile NaOH and stored in aliquots at or below-20°C for 6 months maximum. Aliquots were thawed on the day of use; leftovers were not refrozen. Subsequent working dilutions were made in sterile reverse-osmotic/distilled water.

FQ test concentrations and breakpoints

Initially, we tested two-fold dilutions around the CCs suggested in the WHO guidelines on drug-susceptibility testing from 2008 and 2014,[20] and some intermediate concentrations to challenge these CCs [Table 1]. In 2018, WHO revised the CC of Lfx from 1.5 µg/ml to 1.0 µg/ml, and the revised CC was not included in our initial concentrations tested for Lfx.[16] Therefore, we retested the isolates with an initial Lfx-MIC of 1.12 µg/ml (n = 8) at a single concentration of 1 µg/ml in MGIT 960.

Mycobacteria Growth Indicator Tube system inoculation and reading

One hundred microliter of the appropriate drug solution was added to the drug-containing MGIT tubes to achieve the desired final concentrations as described in Table 1. These tubes were supplemented with 800 µl of OADC (oleic acid, albumin, dextrose, and catalase) and inoculated with 500 µl from an initial MGIT broth culture after 1–2 days of showing positive by the instrument (day 1 to day 2) or diluted 1:5 for day 3 to 5 positive tubes. For each strain, a drug-free control vial was inoculated with a 1:100 dilution of the inoculum to represent 1% of the bacterial population. MGIT tubes were then loaded into the MGIT 960 system for incubation and automated reading.

The MIC was determined to be the lowest concentration at which the growth value was <100 growth units (GU), at the moment, the growth control had reached 400 GU. An invalid test (code ×200 or ×400) was repeated once for that strain and drug. Translation from MIC results to resistant (R) versus susceptible (S) was done as follows: if the MIC >set CC, a strain was declared R and if the MIC ≤set CC, it was considered S.

Quality control

As an internal quality control, the pan-susceptible *MTB* H37Rv reference strain (ITM 2008–03715) was included in the runs on a monthly basis (~every two runs). MIC range of 0.06–0.25 µg/ml was considered acceptable for H37Rv for Mfx and Gfx while an MIC of ≤1 µg/ml was considered acceptable for Lfx.[21,22] In addition, an FQ mono-resistant (ITM number 102197, TB Pannet *in vitro* selected, MIC expected to be ≥1 µg/ml) QC strain, as well

Table 1: Tested concentration in MGIT960 for each of the fluoroquinolones

Drug	Tested concentrations (µg/ml)								
Ofx			0.5	1	1.5	2[a]	4	6	8
Lfx	0.37	0.75	**1.0**[b]	1.12	1.5[c]	3	4.5	6	
Mfx	0.125	0.187	**0.25**[b]	0.5	1	1.5	2		
Gfx	0.125	0.187	**0.25**[b]	0.5	1	1.5	2		

[a]Obsolete WHO recommended CC for Ofx,[20] [b]Latest WHO recommended CCs,[16] [c]Former WHO recommended CC for Lfx.[20]
Ofx: Ofloxacin, Lfx: Levofloxacin, Mfx: Moxifloxacin, Gfx: Gatifloxacin, CC: Critical concentration. Bold text indicates the current WHO-recomended CCs

as the FQ-susceptible MDR (ITM number 2002–01617) and kanamycin-capreomycin-resistant (ITM number 1999–01856) strains were tested for every new batch of FQ stock solution.

Results

Mycobacteria Growth Indicator Tube system-minimal inhibitory concentration results for quality control strains

For H37Rv, the MICs ranged from 0,5–1 µg/ml for Ofx, ≤0,37 µg/ml for Lfx, ≤0,125–0,187 µg/ml for Mfx, and ≤0,125 µg/ml for Gfx, thus meeting the predefined criteria, albeit data are truncated at the lower end. The FQ-susceptible control strains were found susceptible for all FQs, with a MIC within the range of H37Rv. The *in vitro* selected Ofx-resistant control strain was found resistant with ≥1 µg/ml MICs for all four FQs.

Mycobacteria Growth Indicator Tube system-minimal inhibitory concentration results for clinical isolates

The MIC distributions for the four FQs are shown in Table 2 and Figure 1.

Of 147 clinical isolates tested, 145 (98.6%) had a valid final MIC result for Ofx. At 2.0 µg/ml CC,[20] all (40/40) wild-type isolates were correctly identified as susceptible with a MIC range of ≤0.5–1.0 µg/ml, while eight resistance-associated gyr*A*/gyr*B* mutants (8/105, 7.6%) would be misclassified as susceptible to Ofx. Five of these have a mutation in *gyrB* (*Asn499Thr, Asn499Arg, Thr500Ala, Thr500Asn, and Thr500Ile*) and three in *gyrA* (*Asp89Asn, Ala90Val, and Asp94Ala*) [Table 2]. The remaining 97 (97/105, 92.4%) gyr*A*/gyr*B* mutants with a valid MIC result were correctly classified as resistant, with overall higher MICs for *Asp94Asn/Gly/His/Tyr* mutants (combined 48/48 (100%) with MIC ≥4.0 µg/ml; 45/48 (94%) with MIC ≥6.0 µg/ml; and 26/48 (54%) with MIC ≥8.0 µg/ml) compared to MIC values for *Ala90Val/Ser91Pro/Asp94Ala* mutants (combined 49/49 (100%) with MIC ≥4.0 µg/ml, 11/49 (22.4%) with MIC ≥6.0 µg/ml; and only 2/49 (4.1%) with MIC ≥8.0 µg/ml). The sensitivity for the obsolete CC of 2 µg/ml of Ofx to detect FQ resistance was 92.4%, with 100% specificity [Table 3].

Of 147 clinical isolates tested, 143 (97.3%) had a valid final MIC result for Lfx. At 1.0 µg/ml, the current WHO-recommended CC,[16] all (38/38) wild-type isolates were correctly identified as susceptible while eight resistance-associated gyr*A*/gyr*B* mutants (8/105, 7.6%) would be misclassified as susceptible to Lfx [Table 2]. Only six of these eight isolates were the same as the missed Ofx-resistance ones. The remaining gyr*A*/gyr*B* mutants with a valid MIC result (97/105, 92.4%) were correctly classified as resistant, with overall higher MICs for *Asp94Asn/Gly/His/Tyr* mutants (combined 49/49 (100%) with MIC ≥3.0 µg/ml, 28/49 (57%) with MIC ≥4.5 µg/ml, 9/49 (18.3%) with MIC ≥6.0 µg/ml,) compared to MIC values for other mutants (combined 43/48 (89.5%) with MIC ≥1.5 µg/ml, 23/48 (47.9%) with MIC ≥3.0 µg/ml, and 2/48 (4.2%) with MIC ≥4.5 µg/ml). The sensitivity for the current CC of Lfx to detect FQ resistance was 92.4%, with 100% specificity [Table 3], the same as for Ofx.

Of 147 clinical isolates tested, 143 (97.3%) had a valid final MIC result for Mfx. At 0.25 µg/ml CC,[16] all (38/38) wild-type isolates were correctly identified as "susceptible" while 15 resistance-associated gyr*A*/gyr*B* mutants (15/105, 14.3%) would be misclassified as "susceptible" to Mfx [Table 2], which is more than for Ofx and Lfx. Two of these have a mutation in *gyrB* (*Thr500Ile* and *Thr500Asn*) and 13 in *gyrA* (*Ala90Val and Asp94Ala*). The remaining 90 (90/105, 85.7%) gyr*A*/gyr*B* mutants with a valid MIC result were correctly classified as resistant, with overall higher MICs for *Asp94Asn/Gly/His/Tyr* mutants (combined 47/47 (100%) with MIC ≥0.5 µg/ml, 46/47 (98%) with

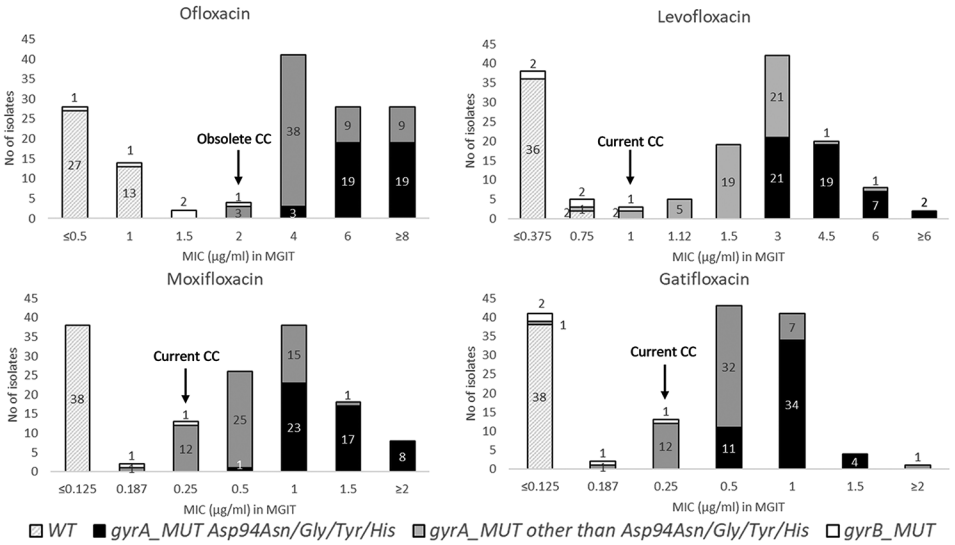

Figure 1: Minimal inhibitory concentration distribution of the drugs tested in relation to the gyr*A*/gyr*B* mutations. CC = Critical concentration, MIC = Minimal inhibitory concentration, MUT = Mutation/s. CC = critical concentration, MIC = minimal inhibitory concentration, MUT = mutation/s

Table 2: Minimal inhibitory concentration distribution of the *Mycobacterium tuberculosis* isolates with final valid phenotypic MGIT960 results, stratified per drug and *gyrA/gyrB* mutation

gyrAB sequence	Total tested	MIC Ofx (μg/ml)									MIC Lfx (μg/ml)									
		≤0.5	1.0	1.5	2.0 (CC)[20]	4.0	6.0	8.0	>8	Invalid	≤0.37	0.75	1.0 (CC)[16]	1.12	1.5	3.0	4.5	6.0	>6	Invalid
Wildtype*	40	27	13	-	-	-	-	-	-	-	36	2	-	-	-	-	-	-	-	2
gyrA mutants	101																			
Asp89Asn	2	-	-	-	**1**	1	-	-	-	-	-	-	**1**	1	-	-	-	-	-	-
Ala90Val	27	-	-	-	**1**	17	7	1	-	1	-	**1**	**1**	3	8	11	1	-	-	2
Ala90Val + Gly512Arg	1	-	-	-	-	1	-	-	-	-	-	-	-	-	1	-	-	-	-	-
Ser91Pro	7	-	-	-	-	6	1	-	-	-	-	-	-	1	1	5	-	-	-	-
Ser91Pro + Gly512Arg	2	-	-	-	-	2	-	-	-	-	-	-	-	-	2	-	-	-	-	-
Asp94Ala	13	-	-	-	**1**	11	1	-	-	-	-	-	-	-	8	5	-	-	-	-
Asp94Asn	7	-	-	-	-	-	-	5	2	-	-	-	-	-	-	1	4	1	1	-
Asp94Gly	34	-	-	-	-	3	18	6	7	-	-	-	-	-	-	18	12	3	1	-
Asp94Gly + Gly512Arg	2	-	-	-	-	-	1	1	-	-	-	-	-	-	-	1	1	-	-	-
Asp94His	1	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	1	-	-
Asp94Tyr	5	-	-	-	-	-	-	3	1	1	-	-	-	-	-	1	2	2	-	-
gyrB mutants	6																			
Asn499Thr	1	-	-	-	**1**	-	-	-	-	-	-	-	**1**	-	-	-	-	-	-	-
Asn499Arg	1	-	**1**	-	-	-	-	-	-	-	**1**	-	-	-	-	-	-	-	-	-
Asn499Ser	1	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	1	-	-
Thr500Ala	1	-	-	**1**	-	-	-	-	-	-	-	**1**	-	-	-	-	-	-	-	-
Thr500Asn	1	-	-	**1**	-	-	-	-	-	-	-	**1**	-	-	-	-	-	-	-	-
Thr500Ile	1	**1**	-	-	-	-	-	-	-	-	**1**	-	-	-	-	-	-	-	-	-
Total	147	28	14	2	4	41	28	16	12	2	38	5	3	8	20	42	20	8	2	4

gyrAB sequence	MIC Mfx (μg/ml)									MIC Gfx (μg/ml)								
	≤0.125	0.187	0.25 (CC)[16]	0.5	1.0	1.5	2.0	>2	Invalid	≤0.125	0.187	0.25 (CC)[16]	0.5	1.0	1.5	2.0	>2	Invalid
Wildtype*	38	-	-	-	-	-	-	-	2	38	-	-	-	-	-	-	-	2
gyrA mutants																		
Asp89Asn	-	-	-	2	-	-	-	-	-	-	-	-	1	1	-	-	-	-
Ala90Val	-	**1**	**9**	10	7	-	-	-	-	**1**	**1**	**7**	18	-	-	-	-	-
Ala90Val + Gly512Arg	-	-	-	1	-	-	-	-	-	-	-	**1**	-	-	-	-	-	-
Ser91Pro	-	-	-	3	3	-	-	-	1	-	-	-	4	3	-	-	-	-
Ser91Pro + Gly512Arg	-	-	-	-	2	-	-	-	-	-	-	-	1	1	-	-	-	-
Asp94Ala	-	-	**3**	7	3	-	-	-	-	-	-	**4**	8	1	-	-	-	-
Asp94Asn	-	-	-	-	1	3	1	2	-	-	-	-	-	4	3	-	-	-

Contd...

Table 2: Contd...

gyrAB sequence	MIC Mfx (µg/ml)								MIC Gfx (µg/ml)							
	≤0.125	0.187	0.25 (CC)[16]	0.5	1.0	1.5	2.0	>2	Invalid	≤0.125	0.187	0.25 (CC)[16]	0.5	1.0	1.5	2.0	>2	Invalid
Asp94Gly	-	-	-	1	19	12	1	1	-	-	-	-	11	22	1	-	-	-
Asp94Gly + Gly512Arg	-	-	-	-	1	1	-	-	-	-	-	-	-	2	-	-	-	-
Asp94His	-	-	-	-	-	-	1	-	-	-	-	-	-	1	-	-	-	-
Asp94Tyr	-	-	-	-	2	1	2	-	-	-	-	-	-	5	-	-	-	-
gyrB mutants																		
Asn499Thr	-	-	-	1	-	-	-	-	-	-	-	-	-	1	-	-	-	-
Asn499Arg	-	-	-	-	-	-	-	-	1	**1**	-	-	-	-	-	-	-	-
Asn499Ser	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	1	-
Thr500Ala	-	-	-	1	-	-	-	-	-	-	**1**	-	-	-	-	-	-	-
Thr500Asn	-	-	**1**	-	-	-	-	-	-	-	-	**1**	-	-	-	-	-	-
Thr500Ile	-	**1**	-	-	-	-	-	-	-	**1**	-	-	-	-	-	-	-	-
Total	38	2	13	26	38	18	5	3	4	41	2	13	43	41	4	-	1	2

*Strains with wild-type *gyrA* and *gyrB* genes or *gyrA/gyrB* mutations that are not associated with resistance to fluoroquinolones (WHO mutation catalogue, Version 1.0),[19] *gyrA/gyrB* mutants misclassified as S by the current WHO recommended (ref) CCs for Lfx, Mfx and Gfx and the obsolete CC for Ofx are boxed with bold text. Ofx: Ofloxacin, Lfx: Levofloxacin, Mfx: Moxifloxacin, Gfx: Gatifloxacin, CC: Critical concentration, MIC: Minimal inhibitory concentration

MIC ≥1.0 µg/ml, 24/47 (51%) with MIC ≥1.5 µg/ml and 8/47 (17%) with MIC ≥2.0 µg/ml) compared to MIC values for other mutants (combined 43/43 (100%) with MIC ≥0.5 µg/ml, 18/43 (41.8%) with MIC ≥1.0 µg/ml, and 2/43 (4.6%) with MIC ≥1.5 µg/ml). The sensitivity of the current CC of Mfx to detect FQ resistance (85.7%) was lower compared to Ofx and Lfx, with 100% specificity [Table 3].

Of 147 clinical isolates tested, 145 (98.6%) had a valid final MIC result for Gfx. At 0.25 µg/ml CC,[16] 100% of (38/38) wild-type isolates were correctly identified as "susceptible" while 18 resistance-associated gyr*A*/gyr*B* mutants (18/107, 16.8%) would be misclassified as "susceptible" to Gfx [Table 2], which is more than for Ofx, Lfx, and Mfx. Four of these have a mutation in *gyrB* (*Thr500Ala*, *Asn499Arg*, *Thr500Asn*, and *Thr500Ile*), 13 in *gyrA* (*Ala90Val and Asp94Ala*), and one had both *gyrA* and *gyrB* mutations (*Ala90Val + Gly512Arg*). The latter showed low-level resistance for Ofx, Lfx, and Mfx [Table 2]. The remaining 89 (89/105, 84.8%) gyr*A*/gyr*B* mutants with a valid MIC result were correctly classified as resistant, with overall higher MICs for *gyrA_Asp94Asn/Gly/His/Tyr* mutants (combined 49/49 (100%) with MIC ≥0.5 µg/ml, 38/49 (77.5%) with MIC ≥1 µg/ml, and 4/49 (8.2%) with MIC 1.5 µg/ml and none above 1.5 µg/ml) compared to MIC values for other mutants (combined 40/40 (100%) with MIC ≥0.5 µg/ml, 8/40 (20.0%) with MIC ≥1.0 µg/ml, and 1/40 (2.5%) with MIC 1.5 µg/ml). The sensitivity of the CC of Gfx to detect FQ resistance (83.2%) was the lowest of all four FQs tested, with 100% specificity [Table 3].

Further, at the lowest concentrations evaluated in this study, Ofx (0.5 µg/ml) and Lfx (0.375 µg/ml) exhibited overlapping MICs of wild-type and *gyrB* mutants, Gfx (0.125 µg/ml) exhibited overlapping MICs of both *gyrA* and *gyrB* mutants with wild types, whereas at 0.125 µg/ml of Mfx, gyr*A*/gyr*B* wild types could be distinguished from the mutants, as the MICs of the gyr*A*/gyr*B* mutants were ≥0.187 µg/ml.

Overall, *gyrB* mutants exhibited lower MICs to all four FQs, with the exception of *Asn499Ser* which showed MIC values at the upper end for all four FQs. The *gyrB_Asn499Thr* and *Thr500Ala* were only detected by Mfx testing, two (*Thr500Asn* and *Thr500Ile)* were missed by all four CCs, and *Asn499Arg*-which did not have a valid MIC for Mfx-was missed by the other three FQs [Tables 2 and 4].

Discussion

FQs are recommended by the WHO for use in the 4-month short-course therapy of drug-sensitive TB, in the treatment of RR TB without FQ resistance, and in salvage regimens with low-level FQ resistance.[6,23] Thus, there is an important need for accurate detection of FQ resistance. The WHO recommends testing the specific FQs used in treatment regimens proposing the MGIT960-based pDST as the reference method.[16] In this study, we re-evaluated the current WHO-recommended CCs for Lfx (1.0 µg/ml) and Mfx (0.25 µg/ml), as well as the now-obsolete CCs for Ofx (2.0 µg/ml) and Gfx (0.25 µg/ml) for

Table 3: Sensitivity and specificity of the obsolete critical concentration for ofloxacin and the current critical concentrations for levofloxacin, moxifloxacin and gatifloxacin along with their 95% confidence intervals

Drug and CC	Resistance associated mutations	Resistant/susceptible by MIC testing in MGIT				
		Susceptible	Resistant	Total	Sensitivity [95% CI]	Specificity [95% CI]
Ofx 2.0 µg/ml	Absent*	40	8	48	92.4 (85.5–96.7)	100.0 (91.2–100.0)
	Present	0	97	97		
	Total	40	105	145		
Mfx 0.25 µg/ml	Absent*	38	15	53	85.7 (77.5–91.8)	100.0 (90.8–100.0)
	Present	0	90	90		
	Total	38	105	143		
Lfx 1.0 µg/ml	Absent*	38	8	46	92.4 (85.5–96.6)	100.0 (90.8–100.0)
	Present	0	97	97		
	Total	38	105	143		
Gfx 0.25 µg/ml	Absent*	38	18	56	83.2 (74.7–89.7)	100.0 (90.8–100.0)
	Present	0	89	89		
	Total	38	81	145		

*Trains with wild-type *gyrA* and *gyrB* genes or *gyrA/gyrB* mutations that are not associated with resistance to fluoroquinolones (WHO Mutation Catalogue, version 1.0).[19] CC: Critical concentration, Ofx: Ofloxacin, Lfx: Levofloxacin, Mfx: Moxifloxacin, Gfx: Gatifloxacin, MIC: Minimal inhibitory concentration, MGIT: Mycobacteria Growth Indicator Tube system

Table 4: Overview of *gyrA/gyrB* mutants classified as susceptible to one or more of the fluoroquinolones tested at the current critical concentrations

CC (µg/ml) Mutants found susceptible to the drug and critical concentration depicted (number of isolates)	Mutants showing resistance to the drug at the depicted critical concentration (number of isolates)				
	Ofx 2.0 µg/mL	Lfx 1.0 µg/mL	Mfx 0.25 µg/mL	Gfx 0.25 µg/mL	None of the four CCs
Ofx 2.0	NA	*gyrA_Asp94Ala* (1) *gyrA_Asp89Asn* (1)	*gyrA_Asp89Asn* (1) *gyrB_Thr500Ala* (1) *gyrB_Asn499Thr* (1)	*gyrA_Asp89Asn* (1) *gyrB_Asn499Thr* (1)	NA
Lfx 1.0	*gyrA_Ala90Val* (1) *gyrA_Asp89Asn* (1)	NA	*gyrA_Asp89Asn* (1) *gyrB_Thr500Ala* (1) *gyrB_Asn499Thr* (1)	*gyrA_Asp89Asn* (1) *gyrB_Asn499Thr* (1)	NA
Mfx 0.25	*gyrA_Ala90Val* (9) *gyrA_Asp94Ala* (2)	*gyrA_Ala90Val* (8) *gyrA_Asp94Ala* (3)	NA	*gyrA_Ala90Val* (3)	NA
Gfx 0.25	*gyrA_Ala90Val* (8) *gyrA_Asp94Ala* (3) *gyrA_ Ala90Val* *gyrB_Gly512Arg* (1)	*gyrA_Ala90Val* (7) *gyrA_Asp94Ala* (4) *gyrA_Ala90Val* + *gyrB_Gly512Arg* (1)	*gyrA_Ala90Val* (2) *gyrA_Asp94Ala* (1) *gyrA_ Ala90Val* + *gyrB_Gly512Arg* (1) *gyrB_Thr500Ala* (1)	NA	NA
All four CCs	NA	NA	NA	NA	*gyrA_Ala90Val* (1) *gyrB_Thr500Asn* (1) *gyrB_Thr500Ile* (1)

Ofx: Ofloxacin, Lfx: Levofloxacin, Mfx: Moxifloxacin, Gfx: Gatifloxacin, CCs: Critical concentrations, NA: Not available

the MGIT960 system. The specificity of the CCs of all four FQs tested was 100%. However, the sensitivity of the CCs varied, with Ofx and Lfx showing the highest sensitivity (92.4%), followed by Mfx 0.25 µg/ml (85.7%) and Gfx 0.25 µg/ml (83.2%).

Discordance between phenotypic and genotypic DSTs for FQs has previously been observed, particularly for low-level FQ resistance-conferring gyr*A*/gyr*B* mutations, and breakpoint artifacts can be a key contributor to such discordances.[24,25] Based on previously published MIC data of clinical isolates, the WHO lowered the CCs of Mfx and Lfx in 2018 to correspond to the epidemiological cutoff (ECOFF), the upper end of the wild-type MIC distribution.[16,26] Our findings suggest that the current CC of Mfx may be above the highest end of the wild-type MIC distribution, with the isolates included in this investigation all falling at least one dilution below the current CC. However, our truncated data in the lower concentration ranges do not allow a clear view of the wild-type MIC distribution. In addition, the isolates included in this study represented only two South Asian countries, Bangladesh, and Pakistan, and 90 isolates with lineage data represent only four MTBc lineages, thus their wild-type MIC distribution

may not represent the global wild-type MIC distribution of MTBc for FQs. Further, in this study, we did not test at least 100 wild-type isolates as per CLSI guidelines. Thus, we were not able to conclude if the current CCs represent the ECOFF.

In line with previous studies, our data also suggest that different mutations in *gyrA* and *gyrB* cause variable levels and patterns of resistance to different FQs.[27-30] While non-Ala codon 94 mutations in the *gyrA* gene showed overall higher MIC increases to all the FQs, the other commonly found *gyrA* mutations such as *Ala90Val* and *Asp94Ala* mutations showed a low-to-moderate increase of the MICs, notably for Mfx and Gfx, leading to false-susceptibility. In addition, three of the five *gyrB* mutants with a valid MIC to Mfx showed resistance while only one of them showed resistance to all four FQs, implying that *gyrB* mutations may have a greater impact on Mfx than the other FQs and highlights the importance of testing the specific FQs used in treatment regimens and further investigating the possible use of Lfx for such mutants. In MGIT, the *gyrB* mutation_*Thr500Asn* has previously been reported to exhibit variable patterns of FQ resistance, however, in our study, this was classified as susceptible by all four FQs with MICs one dilution lower than the current CCs, probably underscoring the lack of reproducibility of pDSTs for the low-level FQ resistance-conferring mutations.[10,27,31]

Despite the fact that now obsolete CC of Ofx and the current CC of Lfx showed the highest sensitivity in this study, they still misclassified mutants, notably the *gyrB* mutations, which may cause clinically relevant resistance at least for Mfx, thus may not be suitable as a surrogate for FQ susceptibility in MTBc. On the other hand, lowering the CC of Mfx to 0.125 µg/ml would allow us to correctly classify all wild-type and mutant isolates while lowering the CC of Gfx to 0.125 µg/ml would still misclassify some gyr*A*/gyr*B* mutants. Based on our findings, 0.125 µg/ml in MGIT medium may be an appropriate CC for Mfx as well as a surrogate for FQ resistance in MTBc.

Ethics clearance

Ethics approval was not required for this laboratory-based study, as anonymized stored clinical isolates were used.

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Conflicts of interest

There are no conflicts of interest.

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