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ORIGINAL RESEARCH

Establishing Sitafloxacin Epidemiological Cut-off Values (ECOFFs) for Clinical Bacterial Isolates

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Objective: To establish the epidemiological cut-off values (ECOFFs) of sitafloxacin against *Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, Acinetobacter baumannii, Staphylococcus aureus, Enterococcus faecalis, Enterococcus faecium*, and *Streptococcus pneumoniae.*

Methods: We collected 2264 clinical isolates from five different labs located in four cities in China. The minimum inhibitory concentrations (MICs) and inhibition zone diameters of sitafloxacin for all isolates were determined by using the broth microdilution method (BMD) and the disk diffusion method according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. MIC ECOFFs were determined using ECOFFinder software, with the 99% calculated ECOFF selected as the initial value. Zone diameter ECOFFs were determined via the visual estimation method. Whole-genome sequencing was performed on *E. coli* strains exhibiting overlapping MICs between wild-type (WT) and non-wild-type (NWT) groups to analyze resistance mechanisms.

Results: Sitafloxacin MICs ranged from 0.002 to 64 mg/L, while inhibition zone diameters ranged from 6 to 45 mm across the nine species. MIC ECOFFs were determined as 0.032, 0.064, 0.125, 0.5, 0.064, 0.125, 0.5, 0.25, and 0.125 mg/L for *E. coli, K. pneumoniae, P. mirabilis* (tentative ECOFF), *P. aeruginosa, A. baumannii, S. aureus, E. faecalis, E. faecium*, and *S. pneumoniae*, respectively. Except for *S. pneumoniae*, MICs of the other eight species showed a high correlation with zone diameters ($|r| > 0.8$, $P < 0.0001$). Consequently, the zone diameter ECOFFs were established as 26, 25, 24, 24, 25, 26, 21, and 22 for *E. coli, K. pneumoniae, P. mirabilis, P. aeruginosa, A. baumannii, S. aureus, E. faecalis*, and *E. faecium*, respectively.

Conclusion: We established MIC and zone diameter ECOFFs for sitafloxacin against the nine species listed above. The MIC ECOFF for *P. mirabilis* was classified as tentative. For *S. pneumoniae*, the correlation between zone diameters and MICs was insufficient to establish a zone diameter ECOFF.

Keywords: sitafloxacin, ECOFF, MIC, zone diameter, BMD, disk diffusion

Introduction

The escalating prevalence of antibiotic-resistant bacterial strains constitutes a critical global health threat, driving clinical and diagnostic laboratories to prioritize the development of novel antimicrobial therapies.[1,2] Among fluoroquinolones, antibiotics such as levofloxacin remain widely prescribed in clinical practice. Nevertheless, their extensive application has been accompanied by a concerning rise in resistance phenotypes. This investigation aims to establish the

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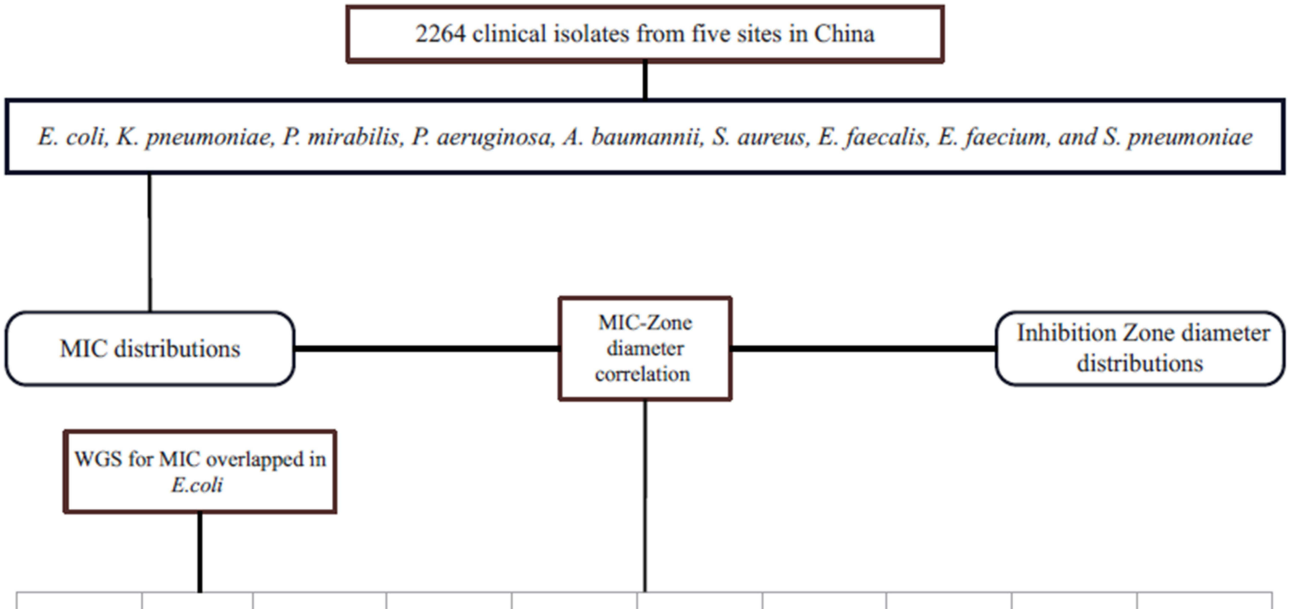
Graphical Abstract



	E. coli	*K. pneumoniae*	*P. mirabilis*	*P. aeruginosa*	*A. baumannii*	*S. aureus*	*E. faecalis*	*E. faecium*	*S. pneumoniae*
MIC ECOFFs (mg/L)	0.032	0.064	0.125	0.5	0.064	0.125	0.5	0.25	0.125
Inhibition Zone diameter ECOFFs (mm)	26	25	24	24	25	26	21	22	-

epidemiological cutoff values (ECOFFs) for sitafloxacin - a third-generation fluoroquinolone - through systematic analysis of susceptibility data from multicenter clinical isolates.

Sitafloxacin, a synthetic broad-spectrum 8-chloro-fluoroquinolone, exhibits in vitro activity against both Gram-positive and Gram-negative bacteria.[3] It inhibits DNA gyrase and topoisomerase IV, enzymes essential for bacterial DNA replication and transcription.[4] The N-1-cyclopropyl substitution in sitafloxacin enhances its antibacterial activity,[5] resulting in superior bacteriostatic effects compared to other quinolones such as levofloxacin, moxifloxacin and ciprofloxacin.[6–9]

First developed in Japan in 1988,[10] sitafloxacin was approved for clinical use in 2008 and later introduced to the Chinese market in 2019. It is indicated for infections caused by *Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, Acinetobacter baumannii, Staphylococcus aureus, Enterococcus faecalis, Enterococcus faecium, Streptococcus pneumoniae, Mycobacterium*, and *Helicobacter pylori*.[6,11–17] However, clinical evidence for its efficacy in treating pneumonia and urinary tract infections remains limited,[18–20] and no clinical breakpoint or ECOFFs have been established in China.

In 2002, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) defined ECOFFs as the highest minimum inhibitory concentration (MIC) for bacteria or fungi lacking phenotypically detectable acquired resistance mechanisms.[21] This threshold distinguishes wild-type (WT) from non-wild-type (NWT) populations and serves as a provisional guideline for clinical treatment until clinical breakpoint are validated.[22] To date, ECOFFs have been established for numerous antibiotics and pathogens in Europe.[23–25]

Despite increasing clinical use of sitafloxacin, neither its ECOFFs nor clinical breakpoints have been defined. Consequently, physicians currently rely on extrapolated data from other quinolones with established breakpoints and

limited clinical reports to guide empirical use – a practice that lacks precision. This study aims to address this gap by establishing ECOFFs for sitafloxacin against common clinical strains.

The broth microdilution method (BMD), recommended by both the Clinical and Laboratory Standard Institute (CLSI) and EUCAST for antimicrobial susceptibility testing (AST), remains the gold standard. However, the disk diffusion method offers greater practicality in routine clinical settings. Validating the correlation between these two methods is therefore critical to ensure the reliability of disk diffusion results for sitafloxacin.

To our knowledge, neither EUCAST nor CLSI has established clinical breakpoints or ECOFFs for sitafloxacin. This study therefore seek to determine ECOFFs for sitafloxacin against prevalent Gram-negative and Gram-positive bacterial species.

Methods

Bacterial Isolates and Quality Control Strains

This multicenter study analyzed 2,264 non-duplicate clinical isolates collected from five tertiary hospitals across four Chinese cities between 2017 and 2020. The participating hospitals included Peking Union Medical College Hospital (PU, Beijing; central laboratory), Peking University First Hospital (BD, Beijing), Sir Run Run Shaw Hospital of Zhejiang University (YF, Hangzhou), Huashan Hospital of Fudan University (HS, Shanghai), and Tongji Hospital of Tongji Medical College (WH, Wuhan).

All isolates were obtained from hospitalized patients across various clinical specimens, primarily including sputum, urine, blood, bile, bronchoalveolar lavage fluid (BALF), drainage fluid, abscess, and others. Species identification was performed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), with the following distribution: 250 *A. baumannii*, 253 *E. faecalis*, 205 *E. faecium*, 240 *E. coli*, 250 *K. pneumoniae*, 206 *P. mirabilis*, 353 *P. aeruginosa*, 250 *S. aureus*, and 257 *S. pneumoniae* (Supplementary material, Table S1).

For quality control, 27 external quality control (EQC) isolates (three per species with stratified MIC values) were analyzed as blind samples. Reference strains (*E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 29213, *E. faecium* ATCC 29212, and *S. pneumoniae* ATCC 49619) were included. MICs for all blind samples and control strains were determined in triplicate over three consecutive days across five independent laboratories. Results were validated if ≥2/3 measurements fell within one two-fold dilution of the central laboratory's values.[21]

Antimicrobial Susceptibility Testing

The BMD concentration range of sitafloxacin was from 0.002 to 64 mg/L. The content of sitafloxacin disk (Daiichi Sankyo Company Limited) was 5 μg. In accordance with the EUCAST SOP 11.0,[26] all 2264 isolates were detected by BMD and disk diffusion method in five separate laboratories. Levofloxacin was used as control agent to perform experiments on BMD and disk diffusion.

Whole-Genome Sequencing

Because of the overlap of WT and NWT MIC distribution of sitafloxacin against *E.coli* strains, we performed whole-genome sequencing to deeply clarify the resistance mechanism distribution on the *E.coli* strains with MICs of 0.032 and 0.064 mg/L. Genomic DNA was extracted with the SDS method.[27] The harvested DNA was detected by the agarose gel electrophoresis and quantified by Qubit® 2.0 Fluorometer (Thermo Scientific). A total amount of 1μg DNA per sample was used as input material for the DNA sample preparations. Sequencing libraries were generated using NEBNext® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. The whole genome of clinical isolates were sequenced using Illumina NovaSeq PE150. The raw data obtained by sequencing (Raw Data) had a certain proportion of low-quality data. In order to ensure the accuracy and reliability of the subsequent information analysis results, the original data must be filtered to obtain valid data (Clean Data). The specific processing steps for genome assembly with Clean Data.

Data Analysis

MICs and inhibition zone diameters from five laboratories were centrally analyzed. According to EUCAST Standard Operating Procedure,[28] MIC ECOFFs of nine species were calculated and determined by ECOFFinder software (www.eucast.org/mic_distributions_and_ecoffs/) and visual estimation in central laboratory, the two sets of data should be consistent or within one dilution difference. Zone diameter ECOFFs were determined by the visual estimation. When it had high correlation between MICs and zone diameters, we can set zone diameter ECOFFs for species.[29]

To explore whether there is a correlation between inhibition zone diameter and MIC ECOFFs, we used PRISM 8.0 software (GraphPad, La Jolla, CA, USA) to analyze the correlation of MICs and zone diameters, linear regression, calculate the P value and R square.

After the whole genome assembly was completed, Resfinder and PointFinder software were used to predict the resistance genes and point mutations of the strain, respectively.[30,31]

Results

MIC Distributions and ECOFFs Establishment

All results of the EQC samples and QC strains were within one dilution step and satisfied the criteria.

MICs against the nine species were distributed in the concentration range of 0.002–64 mg/L (Table S1). The modal MICs of the centers for the same strain were almost always within a dilution factor. The maximum value of MIC of *S. pneumoniae* was 1 mg/L, which was much smaller than the value of the other 8 species. *K. pneumoniae* has the widest MIC distribution, ranging from 0.004 to 64 mg/L. Only the modal MIC of *E. coli* and *E. faecium* produced by the WH lab had a large deviation (Table 1). The modal MIC for all *E. coli* at the five centers was 0.008 mg/L, compared to 1 mg/L for the WH lab. Similarly, WH lab had an *E. faecium* modal MIC of 2 mg/L, but an overall *E. faecium* modal MIC was 0.064 mg/L.

We selected 99% calculated ECOFF as the initially formulated ECOFF value, these were consistent or within one dilution difference with visual estimate ECOFFs (Figure 1). The calculated ECOFF values were relatively small, with the largest being 0.25 mg/L for *P. aeruginosa* and *E. faecalis*.

Among them, we believe that the data of *P. mirabilis* was not amenable to analysis, according to EUCAST SOP10.1,[28] the species was only formulated tentative ECOFF (TECOFF). We analyzed the *P. mirabilis* data of the five centers one by one, and made the MIC distribution (Table 1). Except for YF, the data of the other 4 centers peaked at 0.032 mg/L. While YF was relatively concentrated in the range of 0.032 to 0.5 mg/L, these five concentrations had 8, 7, 6, 5, and 5 MIC value distributions, respectively. Therefore, the data of *P. mirabilis* from YF lab were considered substandard data, and only 4 centers were included in the end.

In the end, we determined that the MIC ECOFFs were 0.032, 0.064, 0.125, 0.5, 0.064, 0.125, 0.5, 0.25, and 0.125 mg/L for *E. coli, K. pneumoniae, P. mirabilis* (TECOFF), *P. aeruginosa, A. baumannii, S. aureus, E. faecalis, E. faecium*, and *S. pneumoniae*, respectively.

At the same time, the MIC ECOFFs of levofloxacin were calculated using the ECOFFinder software. Combined with the visual method, we determined the MIC ECOFFs of levofloxacin to be 0.125, 0.25, 0.25, 2, 0.5, 1, 4, 8, and 2 mg/L for *E. coli, K. pneumoniae, P. mirabilis, P. aeruginosa, A. baumannii, S. aureus, E. faecalis, E. faecium*, and *S. pneumoniae* (Table S2), which is consistent with the data published on the EUCAST website.

Correlation Between MICs and Inhibition Zone Diameters

The distribution of MICs and inhibition zone diameters for the 9 species was shown in Figure 2. Except for *S. pneumoniae*, the other 8 species had Pearson correlation coefficients between MIC values and inhibition zone diameters were $R > 0.8$ ($P<0.0001$) (Table 2), with very strong correlation between two methods. However, the R value of *S. pneumoniae* was 0.46, which meant that the two methods were poorly correlated. Therefore, ECOFF of the *S. pneumoniae* zone diameter cannot be established.

Table 1 MIC Distributions of Five Labs and (T)ECOFFs for Sitafloxacin Against Nine Species

Species/Lab	Number of Isolates With MIC (mg/L)																Total number	Range (mg/L)	Modal MICs (mg/L)	Median (mg/L)	IQRs (mg/L)	99% calculated ECOFFs (mg/L)
	0.002	0.004	0.008	0.016	0.032	0.064	0.125	0.25	0.5	1	2	4	8	16	32	64						
E. coli																						
PU	0	1	24	7	3	7	2	2	0	3	3	4	1	0	0	0	57	0.004–8	0.008	0.016	0.045	
BD	0	0	7	3	3	0	3	6	0	3	3	2	0	0	0	0	30	0.008–4	0.008	0.125	0.984	
YF	1	6	12	1	13	5	0	1	2	6	3	3	1	1	0	0	55	0.002–16	0.032	0.032	0.92	
HS	1	3	5	7	2	5	4	1	3	4	6	2	0	0	0	0	43	0.002–4	0.016	0.064	0.984	
WH	0	0	9	8	4	6	3	5	1	10	7	1	0	1	0	0	55	0.008–16	1	0.125	0.984	
Total	2	10	57	26	25	23	12	15	6	26	22	12	2	2	0	0	240	0.002–16	0.008	0.064	0.992	0.032
K. pneumoniae																						
PU	0	0	0	10	30	3	0	0	0	0	1	4	0	2	0	0	50	0.016–16	0.032	0.032	0	
BD	0	0	2	13	13	4	0	7	1	0	4	5	0	0	1	0	50	0.008–32	0.016; 0.032	0.032	0.234	
YF	0	0	0	27	7	3	0	2	2	0	0	0	0	2	6	1	50	0.016–64	0.016	0.016	0.234	
HS	0	1	3	14	4	1	6	6	0	2	3	1	4	5	0	0	50	0.004–16	0.016	0.125	1.984	
WH	0	0	0	14	17	1	3	2	2	1	6	3	1	0	0	0	50	0.016–8	0.032	0.032	0.484	
Total	0	1	5	78	71	12	9	17	5	3	14	13	5	9	7	1	250	0.004–64	0.016	0.032	0.234	0.064
P. mirabilis																						
PU	0	0	0	6	20	4	3	5	2	6	2	3	0	0	0	0	51	0.016–4	0.032	0.032	0.468	
BD	0	0	0	2	11	9	0	4	2	7	1	1	0	0	0	0	37	0.016–4	0.032	0.064	0.468	
YF	0	0	2	1	8	7	6	5	5	3	0	1	0	0	0	0	38	0.008–4	0.032	0.25	0.218	
HS	0	0	0	0	10	1	1	4	8	4	3	3	2	0	3	0	39	0.032–32	0.032	0.5	1.968	
WH	0	0	0	4	15	2	3	5	4	5	2	0	1	0	0	0	41	0.016–8	0.032	0.064	0.468	
Total	0	0	2	13	64	23	13	23	21	25	8	8	3	0	3	0	206	0.008–32	0.032	0.125	0.468	0.125[a]
P. aeruginosa																						
PU	0	0	0	0	0	1	16	22	9	9	1	0	0	0	0	0	58	0.064–2	0.25	0.25	0.375	
BD	0	0	0	0	1	4	23	8	4	3	5	2	0	0	0	0	50	0.032–4	0.125	0.125	0.375	
YF	0	0	1	0	4	8	43	6	1	2	3	3	4	0	0	0	75	0.008–8	0.125	0.125	0.125	
HS	0	0	0	0	1	13	21	7	10	3	4	4	0	0	0	1	64	0.032–64	0.125	0.125	0.375	
WH	0	0	0	0	1	13	59	21	2	3	1	3	1	2	0	0	106	0.032–16	0.125	0.125	0.125	
Total	0	0	1	0	7	39	162	64	26	20	14	12	5	2	0	1	353	0.008–64	0.125	0.125	0.125	0.5
A. baumannii																						
PU	0	0	0	22	14	3	0	0	0	0	0	3	3	5	0	0	50	0.016–16	0.016	0.032	0.016	
BD	0	0	0	13	17	5	1	0	0	2	9	3	0	0	0	0	50	0.016–4	0.032	0.032	0.984	
YF	0	0	9	22	2	2	0	0	1	2	8	3	1	0	0	0	50	0.008–8	0.016	0.016	0.984	
HS	0	0	2	16	9	7	0	0	0	1	11	4	0	0	0	0	50	0.008–4	0.016	0.032	1.984	
WH	0	0	0	7	22	9	0	0	0	3	8	1	0	0	0	0	50	0.016–4	0.032	0.032	0.032	
Total	0	0	11	80	64	26	1	0	1	8	36	14	4	5	0	0	250	0.008–16	0.016	0.032	0.984	0.064

(*Continued*)

Table 1 (Continued).

Species/Lab	Number of Isolates With MIC (mg/L)																Total number	Range (mg/L)	Modal MICs (mg/L)	Median (mg/L)	IQRs (mg/L)	99% calculated ECOFFs (mg/L)
	0.002	0.004	0.008	0.016	0.032	0.064	0.125	0.25	0.5	1	2	4	8	16	32	64						
S. aureus																						
PU	0	0	0	11	19	7	2	0	0	5	4	0	1	1	0	0	50	0.016–16	0.032	0.032	0.093	
BD	0	0	0	1	19	13	6	0	0	4	5	1	0	0	1	0	50	0.016–32	0.032	0.064	0.093	
YF	0	0	1	9	18	7	0	1	3	5	4	1	0	1	0	0	50	0.008–16	0.032	0.032	0.468	
HS	0	0	1	12	23	2	0	0	4	6	0	0	2	0	0	0	50	0.008–8	0.032	0.032	0.048	
WH	0	0	0	1	16	21	1	0	2	5	2	2	0	0	0	0	50	0.016–4	0.064	0.064	0.032	
Total	0	0	2	34	95	50	9	1	9	25	15	4	3	2	1	0	250	0.008–32	0.032	0.032	0.093	0.125
E. faecalis																						
PU	0	0	0	1	1	13	17	6	1	7	4	0	0	0	0	0	50	0.016–2	0.125	0.125	0.186	
BD	0	0	0	0	0	0	22	14	2	4	6	2	0	0	0	0	50	0.125–4	0.125	0.25	0.375	
YF	0	0	0	0	1	11	21	3	1	5	8	0	0	0	0	0	50	0.032–2	0.125	0.125	0.875	
HS	0	0	0	0	0	10	26	4	0	4	7	2	0	0	0	0	53	0.064–4	0.125	0.125	0.125	
WH	0	0	0	0	0	8	25	4	1	2	8	2	0	0	0	0	50	0.064–4	0.125	0.125	0.375	
Total	0	0	0	1	2	42	111	31	5	22	33	6	0	0	0	0	253	0.016–4	0.125	0.125	0.375	0.5
E. faecium																						
PU	1	1	1	0	2	10	4	3	10	6	2	1	1	0	0	0	42	0.002–8	0.064; 0.5	0.25	0.436	
BD	0	0	0	0	4	11	4	3	3	1	4	8	1	0	0	0	39	0.032–8	0.064	0.25	1.936	
YF	0	0	1	8	13	10	3	0	1	3	6	2	1	0	0	0	48	0.008–8	0.064	0.064	0.468	
HS	0	0	0	1	4	15	5	3	0	2	8	4	0	1	0	0	43	0.016–16	0.064	0.125	1.936	
WH	0	0	0	0	4	6	4	4	0	2	7	1	1	4	0	0	33	0.032–16	2	0.25	1.936	
Total	1	1	2	9	27	52	20	13	14	14	27	16	4	5	0	0	205	0.002–16	0.064	0.125	1.936	0.25
S. pneumoniae																						
PU	0	0	0	0	10	35	4	1	0	0	0	0	0	0	0	0	50	0.032–0.25	0.064	0.064	0	
BD	0	0	0	0	20	30	0	0	0	0	0	0	0	0	0	0	50	0.032–0.064	0.064	0.064	0.032	
YF	0	0	1	3	39	3	0	3	1	0	0	0	0	0	0	0	50	0.008–0.5	0.032	0.032	0	
HS	0	1	0	4	42	7	2	0	0	1	0	0	0	0	0	0	57	0.004–1	0.032	0.032	0	
WH	0	0	0	2	25	22	1	0	0	0	0	0	0	0	0	0	50	0.016–0.125	0.032	0.064	0.032	
Total	0	1	1	9	136	97	7	4	1	1	0	0	0	0	0	0	257	0.004–1	0.032	0.032	0.032	0.125

Notes: a: *Proteus mirabilis* - We thought the data of YF was not amenable to analysis, resulting in only 4 distributions, and only a tentative ECOFF.

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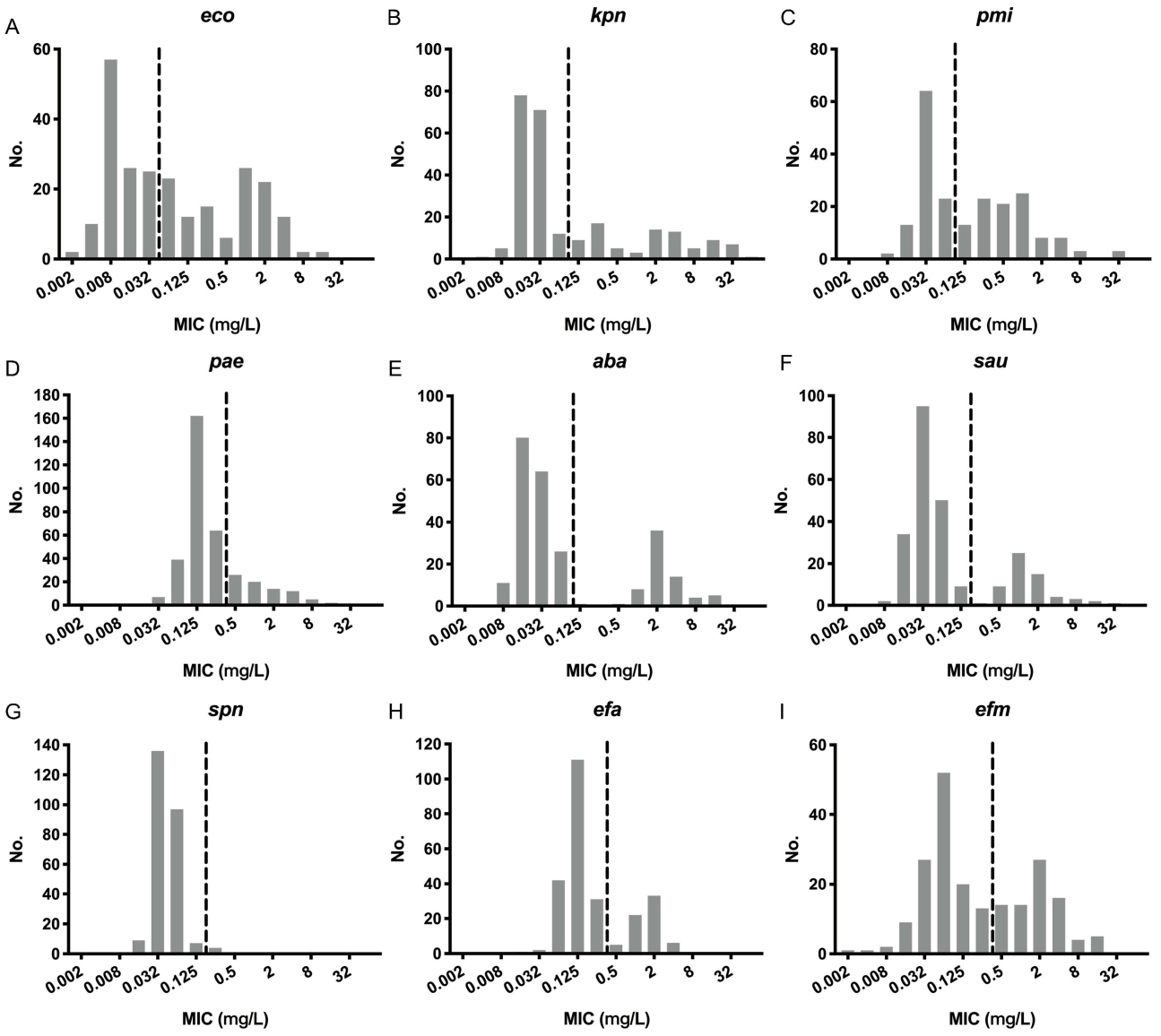

Figure 1 (**A–I**): The MIC distributions of *E. coli, K. pneumoniae, P. mirabilis, P. aeruginosa, A. baumannii, S. aureus, E. faecalis, E. faecium*, and *S. pneumoniae*, respectively. The dotted lines represent estimated ECOFFs of each species using ECOFFinder.

Inhibition Zone Diameter Distributions and ECOFFs Establishment

As shown in Figure 2, we visually estimated the zone diameter for 8 species except *S. pneumoniae*. Zone diameter ECOFFs were that *E. coli* and *S. aureus* was 26 cm, *K. pneumoniae* and *A. baumannii* was 25 cm, *P. mirabilis* and *P. aeruginosa* was 24 cm, *E. faecium* was 22 cm, *E. faecalis* was 21 cm.

Resistance Mechanisms Distribution in E. Coli Strains with Overlapped MICs Between WT and NWT Group

The combination of ECOFFinder and visual inspection led to the determination of a MIC ECOFF value of 0.032 mg/L for *E. coli*. As can be seen from Figure 1A, the number of *E. coli* with MIC values of 0.032 and 0.064 mg/L (close to calculated ECOFF) were very similar (25 and 23 strains, respectively), which indicate an overlap between WT and NWT strains in these two MIC distributions. To deeply investigate the resistance mechanisms distribution, these two groups of strains were selected for whole-genome sequencing to detect sitafloxacin resistance-related genes (*qnr*) or mutations (*gyrA*/*parC*/*parE*).

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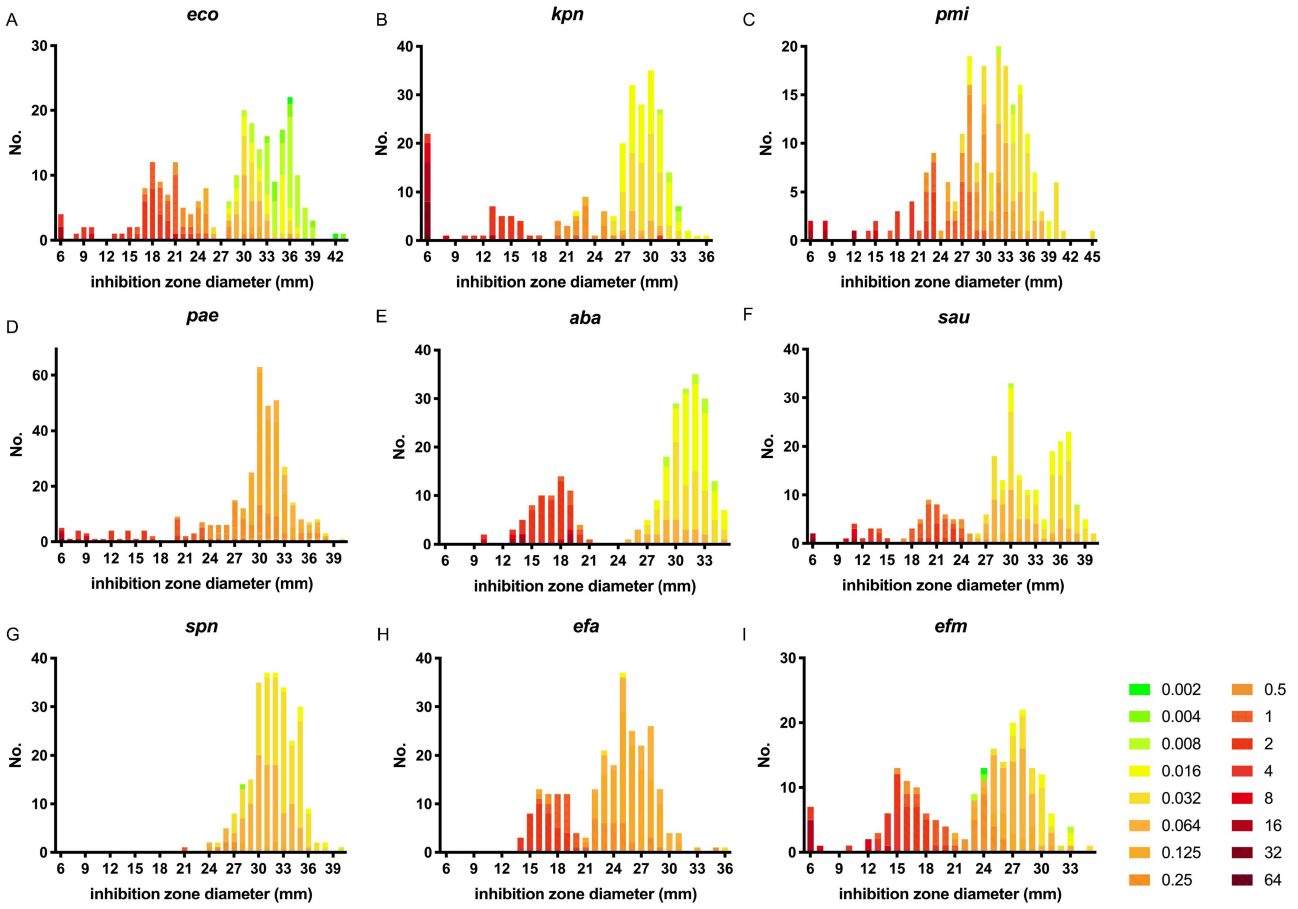

Figure 2 (**A–I**): Distribution of inhibition zone diameters and MICs (mg/L) of *E. coli, K. pneumoniae, P. mirabilis, P. aeruginosa, A. baumannii, S. aureus, E. faecalis, E. faecium*, and *S. pneumoniae*, respectively.

Twelve-point mutations associated with quinolone resistance in whole genomes were detected from 52.1% (25/48) of *E. coli* strains by using Pointfinder software. The mutations identified were *gyrA p.S83L, gyrA p.D87N, gyrA p.D87A, parC p.S57T, parC p.S80I, parC p.E84V, parE p.I529L, parE p.S458A, parE p.S458T, parE p.I355T, parE p.L416F*, and *parE p.P439S*. Among the bacteria with a MIC of 0.032 mg/L, 48.0% (12/25) of the strains had at least one mutation, with *gyrA p.S83L* being the most common mutation detected in up to 40.0% (10/25) of strains. In addition, *gyrA p.D87N* and *parC p.S80I* were also commonly detected at frequencies of 16.0% (4/25) and 12.0% (3/25), respectively. Among the

Table 2 The Correlation of MIC Values and Inhibition Zone Diameters for 9 Species

Species	Total Number	R	P value	Liner Regression Equation[a]
E. coli	240	−0.93	<0.0001	Y = −0.39X + 7.18
K. pneumoniae	250	−0.95	<0.0001	Y = −0.40X + 6.62
P. mirabilis	206	−0.81	<0.0001	Y = −0.32X + 6.91
P. aeruginosa	353	−0.89	<0.0001	Y = −0.24X + 4.65
A. baumannii	250	−0.94	<0.0001	Y = −0.44X + 8.49
S. aureus	250	−0.88	<0.0001	Y = −0.31X + 5.59
E. faecalis	253	−0.83	<0.0001	Y = −0.33X + 5.73
E. faecium	205	−0.92	<0.0001	Y = −0.39X + 6.80
S. pneumoniae	257	−0.46	<0.0001	Y = −0.13X - 0.28

Notes: a: Y is log2 (MIC), X is the inhibition diameter (mm).

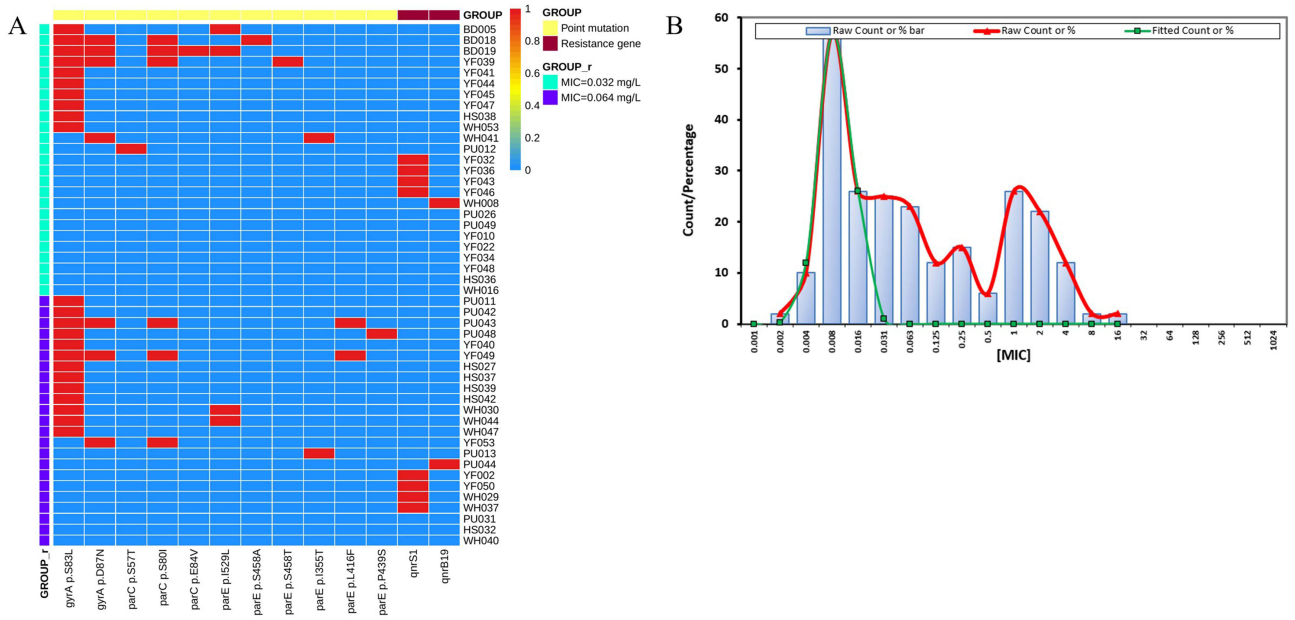

Figure 3 (**A**) Distribution of resistance mechanisms of *E. coli* with MIC of 0.032 and 0.064 mg/L in 48 strains. (**B**) ECOFFinder calculated the ECOFF value of *E. coli*.

bacteria with a MIC of 0.064 mg/L, mutation sites were detected in 65.2% (15/23) of strains, with *gyrA p.S83L, gyrA p.D87N*, and *parC p.S80I* being the most common mutations detected at frequencies of 56.5% (13/23), 8.7% (2/23), and 13.0% (3/23), respectively (Figure 3A).

Resfinder software detected two sitafloxacin resistance-related genes, *qnrS1* and *qnrB19*, from the whole genomes of 20.8% (10/48) of *E. coli*. Among the 25 strains with a MIC of 0.032 mg/L, 4 strains carried *qnrS1* gene and 1 strain carried *qnrB19* gene. In 23 strains with a MIC of 0.064 mg/L, 4 *qnrS1* gene and 1 *qnrB19* gene-carried isolates were also detected (Figure 3A).

Overall, 17 strains of *E. coli* with MIC of 0.032 mg/L carried resistance mechanisms, and 8 strains did not carry any resistance mechanisms (neither resistance genes nor point mutations). Among *E. coli* with MIC of 0.064 mg/L, 20 strains carried resistance mechanism, and only 3 strains did not carry any quinolone–resistance determinants. The resistance mechanism distribution exhibited statistical difference between the two groups ($P = 0.003$).

Discussion

Nowadays, the widespread use of antibiotics has led to a large increase in the number of various drug-resistant strains, which has increased the difficulty of clinical treatment. As a result, new antibiotics are constantly being developed to combat drug-resistant strains with their better antimicrobial activity. Sitafloxacin is a new fluoroquinolone drug, which has good antibacterial activity against gram-negative bacteria and positive bacteria.[32]

From the data on the EUCAST website, the MIC (T)ECOFF of levofloxacin for these 9 strains was 0.125, 0.25, 0.25, 2, 0.5, 1, 4, 8, and 2 mg/L for *E. coli, K. pneumoniae, P. mirabilis, P. aeruginosa, A. baumannii, S. aureus, E. faecalis, E. faecium*, and *S. pneumoniae*, respectively.[33] In comparison, the MIC (T)ECOFF of levofloxacin are larger than the sitafloxacin established in this study. This demonstrates that sitafloxacin exhibits more potent antibacterial activity compared to levofloxacin. The introduction of a methoxy group (–OCH_3) at the C-8 position in sitafloxacin significantly enhances its affinity and stability for DNA gyrase. This modification enables the drug to bind more securely to the β-subunit, reducing the dissociation rate of the enzyme and thereby prolonging the duration of inhibition. Compared to the shorter side chain (–$NHCOCH_3$) in levofloxacin, sitafloxacin features a longer C-7 substituent (–CF_3–CH_2–$N(CH_3)_2$), which may enhance hydrophobicity, promote penetration of the bacterial outer membrane lipid bilayer, and increase intracellular concentrations.[34]

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In recent years, some domestic reports have also confirmed that sitafloxacin has better efficacy. Li et al evaluated the efficacy and safety of sitafloxacin and levofloxacin in the treatment of complicated urinary tract infections in adults in China. The clinical cure rate and microbiological success rate of sitafloxacin in the treatment of complicated UTI were better than levofloxacin, especially bacterial eradication rate, which was 93.3% (14/15) versus 63.6% (7/11).[7] Similarly, the pharmacokinetic/pharmacodynamic studies of multidrug-resistant bacteria in the model of sitafloxacin in dynamic urinary tract infection showed significantly lower MIC values than levofloxacin for drug-resistant *Escherichia coli, Pseudomonas aeruginosa*, and *Enterococcus faecium*.[35] Shi et al studied the comparison of the activity of sitafloxacin, levofloxacin, and moxifloxacin on *S. aureus* in vitro. The MIC of sitafloxacin was about 4 concentration gradients smaller than levofloxacin and 1–2 concentration gradients smaller than moxifloxacin.[9] So sitafloxacin exhibited strong bactericidal activity against organisms resistant to other fluoroquinolones. Because sitafloxacin had not yet developed a clinical breakpoint, it was important to establish ECOFF first.

The distribution of sitafloxacin MIC and zone diameter was relatively wide, ranging from 0.002 to 64 mg/L and from 6 to 45 mm, respectively. The 99% calculated ECOFFs was one concentration gradient smaller than the verified ECOFFs, with *P. mirabilis, P. aeruginosa*, and *E. faecalis*. Among *P. aeruginosa* and *E. faecalis*, we may believe that visual results of visual estimate method were more inclined to 0.5 mg/L than 0.25 mg/L.

Regarding *P. mirabilis*, the MIC ECOFF ECOFFinder calculated was 0.064 mg/L. However, we thought that the data of YF was not amenable to analysis about *P. mirabilis*. In addition to YF, the *P. mirabilis* data of the other four labs had obvious peak. However, the YF data did not show obvious quantitative changes from 0.032–0.5 mg/L concentration gradient, and the changes of YF enrollment *P. mirabilis* could not be judged. This may be related to the resistance mechanism of the enrolled strains, or it may be that laboratory staff readings were biased.

Among the 9 species in this study, the results of *P. aeruginosa* were the most ideal from the experimental data obtained. Its MIC distribution was symmetrical with fewer NWT strains. The presence of an obvious resistance mechanism may not be evident from the MIC distribution.

As shown in Figure 1, there were 2 peaks of different sizes in the distribution of *Escherichia coli* MIC. It may be because *Escherichia coli* enrolled showed 2 different resistance mechanisms. The molecular mechanisms of fluoroquinolone resistance, including plasmid-mediated quinolone resistance (PMQR) along with mutation in the quinolone-resistance determining regions (QRDRs) of *gyrA, gyrB, parC* and *parE*.[36–38] The clinically common extended-spectrum *β*-lactamase-producing (ESBL) *Enterobacteriaceae* also have more than one-third resistance to fluoroquinolones.[39]

Furthermore, the analysis results of whole genome drug resistance information showed a similar situation of carrying drug resistance genes and point mutations for the two groups of *E. coli* with MIC values of 0.032 and 0.064 mg/L. The strains with MIC values less than or equal to the ECOFF value were WT strains and did not carry any resistance mechanism. In this study, only 8 out of the 25 *E. coli* strains with MIC values of 0.032 mg/L did not carry drug resistance mechanisms. It was possible that some of the low-tolerance strains in the NWT strains crossed with the WT strains near the ECOFF value (Figure 3B). We followed EUCAST SOP 10.2 as described, the WT distribution is visually assessed to be a log-normal distribution.

Except for *S. pneumoniae*, all other species showed a high correlation between MIC and zone diameter ($R > 0.8$, $P < 0.0001$). Because it was more convenient to operate the disk diffusion method in the microbiology laboratories, we recommend the disk diffusion method to test the resistance for 8 strains other than *S. pneumoniae* to sitafloxacin. Most of the MIC values obtained by our enrolled *S. pneumoniae* distributed in concentrations of 0.032 and 0.064 mg/L, and the largest MIC value was 1 mg/L. The ECOFF of *S. pneumoniae* MIC were 0.125 mg/L, so the number of NWT in the enrolled strains was very small, and it was not good to analyze the reasons for the poor consistency of the results of the two methods of MIC and disk diffusion method from the level of resistance genes. In conclusion, we did not recommend the disk diffusion method in clinical microbiology laboratories to detect the tolerance of *S. pneumoniae* to sitafloxacin.

In summary, we established MIC and zone diameter ECOFFs for sitafloxacin against *E. coli, K. pneumoniae, P. mirabilis, P. aeruginosa, A. baumannii, S. aureus, E. faecalis, E. faecium*, and *S. pneumoniae*. The MIC ECOFF of the above nine strains were 0.032, 0.064, 0.125, 0.5, 0.064, 0.125, 0.5, 0.25, and 0.125 mg/L, respectively. The zone diameter ECOFF of the above nine strains were 26, 25, 24, 24, 25, 26, 21.22, and NA. The MIC ECOFF for *P. mirabilis* remains tentative pending further validation. For *S. pneumoniae*, MICs and zone diameters showed weak correlation, preventing the establishment of zone diameter ECOFFs.

Data Sharing Statement

The WGS data of 48 *E.coli* to are available in the China National Center for Bioinformation (https://ngdc.cncb.ac.cn) under project number PRJCA023379.

Ethical Approval

This study complies with the Declaration of Helsinki and was approved by the Health Research Ethics Board of Peking Union Medical College Hospital (No. S-K1240).

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no conflict of interest.

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