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ORIGINAL RESEARCH

Mutations in *gyrB* play an important role in ciprofloxacin-resistant *Pseudomonas aeruginosa*

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Xinyuan Feng[1,*]
Zhiqi Zhang[2,*]
Xiaoxia Li[3]
Yan Song[3]
Jianbang Kang[3]
Donghong Yin[3]
Yating Gao[3]
Nan Shi[1]
Jinju Duan[3]

[1]Department of Pharmacy, School of Pharmacy, Shanxi Medical University, Taiyuan, Shanxi, People's Republic of China; [2]Department of Pharmacy, First Hospital of Shanxi Medical University, Taiyuan, Shanxi, People's Republic of China; [3]Department of Pharmacy, Second Hospital of Shanxi Medical University, Taiyuan, Shanxi, People's Republic of China

*These authors contributed equally to this work

Purpose: To investigate the main *molecular* resistance mechanisms to fluoroquinolones (FQs) in *Pseudomonas aeruginosa* and also to investigate the effect of time and concentration on mutations in resistance genes.

Materials and methods: The clinical isolates of *P. aeruginosa* which are sensitive to ciprofloxacin (CIP) or levofloxacin (LEV) were collected. The isolates were incubated with different concentrations of CIP or LEV for 5 days and the minimal inhibitory concentrations (MICs) of CIP, LEV and ofloxacin (OFX) were measured. The MIC of FQs to *P. aeruginosa* was measured by the agar dilution method. FQ resistance determining regions of *gyrA*, *gyrB*, *parC* and *parE* were amplified by PCR, and mutations in four genes were explored using sequence analysis with the Snapgene software. The relative expression levels of two efflux pumps genes (*mexA* and *mexE*) were measured by quantitative reverse transcription PCR.

Results: A total of eleven isolates were collected from the Second Hospital of Shanxi Medical University. Amino acid alterations in *gyrA* and *gyrB* were mainly detected in resistant mutants, and the percentage of strains with amino acid alterations in *gyrB* was significantly higher than that in gyrA ($P<0.001$). MICs of strains with mutations both in gyrA and *gyrB* were not significantly higher than those of strains with mutations only in gyrB ($P>0.05$). No amino acid alterations were detected in genes of *parC* and *parE*. In both *gyrA* and *gyrB*, the number of amino acid alterations increased with incubation time prolonged and increased with increasing incubation concentration.

Conclusion: CIP was more competent than LEV in making *P. aeruginosa* resistant to in vitro selection. Mutations occurring in *gyrB* played an important role in FQ resistance of *P. aeruginosa* in vitro selection.

Keywords: *Pseudomonas aeruginosa*, fluoroquinolones, molecular resistance mechanisms, in vitro

Introduction

Pseudomonas aeruginosa is a clinically important opportunistic pathogen with high pathogenicity and high mortality. A recent report from the National Healthcare Safety Network (NHSN) reported *P. aeruginosa* to be one of the six most common nosocomial pathogens associated with healthcare-associated infections, and the resistance rate of *P. aeruginosa* to multiple drugs (aminoglycosides, extended-spectrum cephalosporin, carbapenems) exceeded 15%.[1] Infections caused by *P. aeruginosa* are difficult to treat due to multiple resistance mechanisms.[2,3] The resistance mechanisms of *P. aeruginosa* include natural resistance, acquired resistance and adaptive resistance.[4,5] These complex and various resistance mechanisms make the infections caused by *P. aeruginosa*

Correspondence: Jinju Duan
Department of Pharmacy, Second Hospital of Shanxi Medical University, No 382, Wuyi Road, Xinghualing District, Taiyuan, Shanxi, People's Republic of China
Tel +86 351 336 5665
Email duanjinju@163.com

life-threatening. Meanwhile, the resistance of *P. aeruginosa* to many antimicrobial drugs is emerging worldwide as a public threat.[6]

Fluoroquinolones (FQs) are an important class of antimicrobial drugs used to treat infections caused by *P. aeruginosa*, among which ciprofloxacin (CIP) or levofloxacin (LEV) is used frequently.[7–9] FOs act by inhibiting the intracellular targets, DNA gyrase and topoisomerase IV for inhibiting DNA replication. DNA gyrase tends to be the primary target in gram-negative bacteria, whereas topoisomerase IV is preferentially inhibited by FQs in gram-positive bacteria.[8,10,11] But the resistance of *P. aeruginosa* to CIP and LEV has emerged. Data from China Antimicrobial Surveillance Network showed that the resistance rate of *P. aeruginosa* to CIP had reached to 14.8% in 2017.[12] Also, a recent report from the NHSN showed that the resistance rate of *P. aeruginosa* to CIP and LEV exceeded 30% within American hospitals.[1] Studying the specific resistance mechanisms of FQs to *P. aeruginosa* is helpful in choosing the appropriate antibiotics, avoiding the emergence of resistant strains and developing new antimicrobial drugs.

In theory, the molecular resistance mechanisms of FQs to *P. aeruginosa* mainly involve gene mutations of *gyrA* and *gyrB* which encode DNA gyrase and *parC* and *parE* which encode topoisomerase IV. But many previous studies revealed that *gyrA* mutations played a crucial role in FQ resistance in *P. aeruginosa* and *parC* mutation was associated with the development of high-level resistance.[7,9,13–17] A few studies reported *gyrB* mutations in FQ-resistant *P. aeruginosa*. Also, the relationship between *gyrB* mutations and drug resistance in the existing literatures is not explicit.[18–20] This raises the question of whether *gyrB* mutations of *P. aeruginosa* play a role in resistance to FQs. To address this, in the current work, we incubated *P. aeruginosa* in vitro and detected the mutations in FQ resistance determining regions (FRDRs) of *gyrA*, *gyrB*, *parC* and *parE*. In addition, during the clinical treatment of infection caused by *P. aeruginosa*, the patients were initially sensitive to FQs, but the sensitivity was reduced after a period of use, resulting in failure of the treatment. Based on this problem, in this study, we set different incubation time periods and concentrations in order to find out the effect of time and concentration on mutations in resistance genes.

Materials and methods

Collection of clinical isolates

Clinical nonrepetitive isolates were collected from the Second Hospital of Shanxi Medical University. All isolates were identified by VITEK-2 Compact system (BioMerieux Italia S.p.A) and were stored in liquid medium of brain heart infusion at –86°C. These isolates and *P. aeruginosa* ATCC15692 were defined as the original strains. ATCC15692 was obtained from the First Hospital of Wuhan and was numbered as PA12 in this study.

P. aeruginosa was isolated from different patients for this research. This research was approved by the ethics committee of the Second Hospital of Shanxi Medical University. We had hidden the patients' information when collecting the isolates. Also, the patients' written informed consent was exempt, which was also approved by the ethics committee.

Measurement of minimal inhibitory concentration (MIC)

The susceptibility of original strains to CIP, LEV and ofloxacin (OFX) was determined by the agar dilution method, with the concentration of each antibacterial agent ranging from 0.0625 to 256 μg/mL. The results of MICs were interpreted according to the American Clinical and Laboratory Standards Institute 2017.[21] Each strain was separately incubated in Mueller–Hinton agars (MHAs; purchased from the British company Oxoid) containing four different concentrations of CIP or LEV. The four incubation concentrations referred to 0.5× MIC, 1× MIC, 2× MIC and 4× MIC, respectively, depending on the MICs of 12 original strains. Each isolate was incubated at each concentration for 5 days. Finally, the strains incubated for 1, 3 and 5 days were stored at –86°C. Similarly, the MICs of strains after incubation were measured by the agar dilution method.

Incubation experiment conducted in vitro

Each original strain was first inoculated in blood culture for recovery and then incubated in a thermotank at 37°C for 18–24 hours. Using cotton swab, a single bacterial colony was picked into a test tube containing normal saline and the bacterial suspensions were adjusted to 3.0 McFarland turbidity standard (MCF). The bacteria were separately incubated on MHAs containing CIP of four different concentrations. The incubation was conducted at 37°C for 1 day, and then the content transferred to the new MHAs containing the same concentration of CIP for the next day. This transfer continued until the incubation reached the fifth day.

The method of incubating strains with LEV was the same as incubating strains with CIP. The strains incubated with CIP were defined as the CIP group, and the strains incubated with LEV as the LEV group.

Conventional PCR amplification and DNA sequencing of FRDRs

The PCR amplification of *gyrA*, *gyrB*, *parC* and *parE* of each strain was performed in a final volume containing the following reaction mixture: Taq PCR Master Mix 25 μL, bacterial suspensions 1 μL (1.5 MCF), forward primer (10 μmol/L) 2 μL, reverse primer (10 μmol/L) 2 μL; finally, sterilized ddH_2O was added and the mixture made up to 50 μL. The PCR primers of the four genes were shown in Table 1. Amplification reaction was performed on a thermal cycler (Lifepro thermal cycler PCR; Bioer, Hangzhou, People's Republic of China) for 30 cycles. The cycle parameters included predegeneration at 95°C for 5 minutes, denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds and elongation at 72°C for 40 seconds. PCR products were electrophoresed on a 1%–1.5% agarose gel, visualized by ethidium bromide staining and photographed under ultraviolet light. The PCR products were sent to Sangon Biotech Co., Ltd (Shanghai, People's Republic of China) for sequencing. The sequencing primers were the same as the PCR primers. The flowchart describing the experimental design is shown in Figure 1.

The base sequences and amino acid sequences in the target regions of the four genes were compared with the PAO1 sequences in the GenBank using the Snapgene software. The DNA sequences were compared with the original nucleotide sequences of *gyrA* (accession number 882880), *gyrB* (accession number 879230), *parC* (accession number 879741) and *parE* (accession number 879897) genes in the GenBank data for PAO1.

Quantitative reverse transcription PCR (RT-PCR)

The expression of two efflux pumps genes (*mexA* and *mexE*) was measured by quantitative RT-PCR. The RT-PCR experiment of *mexA* and *mexE* of *P. aeruginosa* was performed in a final volume containing the following reaction mixture: 2× SG Fast qPCR Master Mix 10 μL, 10 μM forward primer 0.4 μL, 10 μM reverse primer 0.4 μL, a certain amount of template DNA, and finally, PCR-grade water added to make it up to 20 μL. The primers of the two genes are listed in Table 1. The amplification parameters included 40 cycles of predegeneration at 95°C for 3 minutes, denaturation at 95°Cfor 3 seconds, annealing at 60°C for 3 seconds and elongation at 60°C for 3 seconds.

The $\Delta\Delta C_T$ method was used to calculate the relative expression of the two genes, and the results are shown as $2^{-\Delta\Delta CT}$ [$\Delta\Delta C_T = (C_{T\ target\ gene} - C_{TGAPDH})_{experimental\ group} - (C_{T\ target\ gene} - C_{T\ GAPDH})_{control\ group}$]. Measurement of the expression of the two genes in each strain was repeated twice and its mean was recorded in the final results. The GAPDH was used as a reference gene.

Statistical analysis

MICs were described as geometric mean±lg-1(SlgMIC).[22] All data were analyzed by SPSS16.0 software. Effect of the continuous incubation on the MICs to CIP, LEV and OFX was evaluated with the repeated measures ANOVA and least significant difference *t*-test. Measurement data were compared by independent samples *t*-test. The enumerated data were analyzed with chi-squared test. $P<0.05$ was considered statistically significant.

Table 1 Primer sequence used in PCR and RT-PCR

Gene	Primer	Sequence	Expected size (bp)
gyrA	Forward	TGACGGCCTGAAGCCGGTGCAC	418
	Reverse	GCCCACGGCGATACCGCTGGA	
gyrB	Forward	GCGGTGGAACAGGAGATGGGCAAGTAC	510
	Reverse	CTGGCGGAAGAAGAAGGTCAACA	
parC	Forward	CGAGCAGGCCTATCTGAACTAT	357
	Reverse	AGCAGCACCTCGGAATAG	
parE	Forward	CGGCGTTCGTCTCGGGCGTGGTGAAGGA	592
	Reverse	TCGAGGGCGTAGTAGATGTCCTTGCCG	
mexA	Forward	GTTCCCCAACCCGAACAACG	159
	Reverse	ACCTTGTTCTGCGCGTTCAC	
mexE	Forward	GACCGGATCGTCGTGAATGG	142
	Reverse	CCTTCGGTGGTTCGCTGTC	
GAPDH	Forward	CACTCCAGCCGTTTCGAACT	162
	Reverse	CGGCTTGAACACCACCGTAT	

Abbreviation: RT-PCR, reverse transcription PCR.

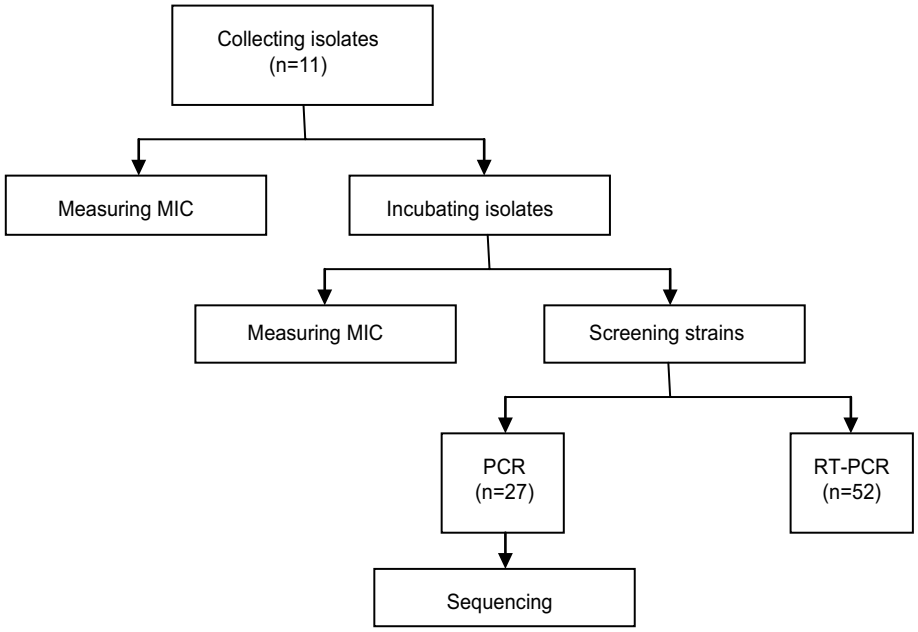

Figure 1 The flowchart describing the experimental design.
Abbreviations: MIC, minimum inhibitory concentration; RT-PCR, reverse transcription PCR.

Results

Collection of clinical isolates

In this study, a total of 11 clinical isolates of *P. aeruginosa* were collected from the Second Hospital of Shanxi Medical University. These 11 isolates were from different specimens such as wound secretion (four isolates), urine (three isolates), blood (two isolates), pleural effusion (one isolate) and ascitic (one isolate). The MICs of the original strains to CIP, LEV and OFX are shown in Table 2.

Table 2 MICs of 12 original strains (μg/mL)

No. of isolates	CIP	LEV	OFX
PA1	0.5	2	8
PA2	0.25	1	0.5
PA3	0.5	2	1
PA4	0.5	2	8
PA5	0.25	0.5	0.25
PA6	0.5	2	4
PA7	0.25	1	2
PA8	0.25	0.125	0.5
PA9	0.125	1	4
PA10	0.25	1	4
PA11	0.0625	0.25	1
PA12	0.25	2	2

Abbreviations: CIP, ciprofloxacin; LEV, levofloxacin; MIC, minimum inhibitory concentration; OFX, ofloxacin.

Incubation experiment conducted in vitro

MICs under different incubation concentrations and days

The 12 strains were separately incubated by four concentrations of CIP for 5 days. One hundred and thirty-five strains were successfully stored, and 9 strains were abandoned because their bacterial colonies were not seen on the culture medium after incubation for 48 hours. The nine strains abandoned included PA3d1, PA3d3, PA3d5, PA7c1, PA7c3, PA7c5, PA8d1, PA8d3 and PA8d5 (b and d represent the incubation concentrations of 2× MIC and 4× MIC, respectively; 1, 3 and 5 represent the incubation time of 1, 3 and 5 days, respectively). Similarly, 114 strains were finally stored after incubating with LEV. The 30 strains abandoned included PA1d1, PA1d3, PA1d5, PA3b1, PA3b3, PA3b5, PA3d1, PA3d3, PA3d5, PA4d1, PA4d3, PA4d5, PA5d1, PA5d3, PA5d5, PA6d1, PA6d3, PA6d5, PA7d1, PA7d3, PA7d5, PA12b1, PA12b3, PA12b5, PA12c1, PA12c3, PA12c5, PA12d1, PA12d3 and PA12d5 (a, b, c and d represent the incubation concentrations of 0.5× MIC, 1× MIC, 2× MIC and 4× MIC, respectively).

MICs of *P. aeruginosa* to CIP, LEV and OFX in the two groups are shown in Tables 3 and 4. According to the breakpoints given in the guidelines of the American Clinical and Laboratory Standards Institute 2017,[21] *P. aeruginosa* was resistant to CIP, LEV and OFX when the MIC was ≥4.00, 8.00 and 8.00 μg/mL, respectively. MICs of *P. aeruginosa* to CIP showed that in two groups, the MICs increased over time under any concentration and reached the maximum

Table 3 MICs of *P. aeruginosa* to CIP, LEV and OFX upon incubation in the CIP group

Drug	Concentration	Time (day)			
		0	1	3	5
CIP	0.5× MIC	0.26±1.86	0.94±1.59	2.83±2.37	4.00±2.19
	1× MIC		1.19±2.08	2.21±1.73	3.56±2.65
	2× MIC		1.30±2.39	4.00±1.71	8.52±2.46
	4× MIC		2.83±2.41	8.57±1.83	13.93±2.49
LEV	0.5× MIC	0.94±2.18	1.33±2.48	4.49±1.64	6.73±1.82
	1× MIC		1.59±2.70	3.56±2.53	6.73±3.16
	2× MIC		2.27±2.25	5.84±2.19	15.02±2.72
	4× MIC		4.00±3.10	14.93±1.99	24.25±2.40
OFX	0.5× MIC	1.78±2.41	2.83±2.72	7.55±3.20	11.31±2.49
	1× MIC		3.17±3.06	8.00±1.81	11.31±2.72
	2× MIC		5.48±2.58	13.24±2.28	21.93±3.11
	4× MIC		8.57±3.17	27.86±2.34	73.52±2.64

Notes: Results are given as G±lg-1(SlgMIC).
Abbreviations: CIP, ciprofloxacin; LEV, levofloxacin; MIC, minimum inhibitory concentration; OFX, ofloxacin.

Table 4 MICs of *P. aeruginosa* to CIP, LEV and OFX upon incubation in the LEV group

Drug	Concentration	Time (day)			
		0	1	3	5
CIP	0.5× MIC	0.26±1.86	1.00±3.14	1.19±2.80	1.19±3.04
	1× MIC		1.37±2.45	1.66±2.28	2.57±2.17
	2× MIC		2.30±2.64	3.25±2.53	4.29±1.99
	4× MIC		5.28±3.52	5.28±2.86	6.96±1.36
LEV	0.5× MIC	0.94±2.18	3.00±2.84	3.36±2.68	3.56±3.12
	1× MIC		4.00±2.40	3.76±2.20	5.48±2.32
	2× MIC		8.00±2.67	7.46±2.14	10.56±2.11
	4× MIC		13.93±2.47	10.56±2.86	16.00±2.89
OFX	0.5× MIC	1.78±2.41	5.34±3.20	7.13±3.36	4.24±3.20
	1× MIC		8.00±2.67	10.96±2.45	8.52±2.59
	2× MIC		13.93±3.07	18.38±2.49	16.00±1.92
	4× MIC		24.25±3.19	24.25±3.19	21.11±1.46

Abbreviations: CIP, ciprofloxacin; LEV, levofloxacin; MIC, minimum inhibitory concentration; OFX, ofloxacin.

when the incubation reached the fifth day; meanwhile, the MICs increased with the incubation concentration and improved during incubation and reached the maximum when the incubation concentration reached 4× MIC. Also, the MICs to LEV and OFX increased over time and increased with incubation concentration. Unlike CIP, the MICs of *P. aeruginosa* to OFX reached the maximum on day 3 under any concentration.

For CIP resistance in *P. aeruginosa*, on day 1, the CIP group remained sensitive under any concentration, but the LEV group became resistant when the concentration in MHAs was increased to 4× MIC. On day 3, the CIP group became resistant under the concentration of 2× MIC, whereas the LEV group became resistant when the concentration in MHAs increased to 4× MIC. On day 5, the CIP group was resistant to CIP under any concentration, but the LEV group became resistant until the concentration in MHAs increased to 2× MIC. All the above results show that although the LEV group became resistant to CIP earlier than CIP group, the LEV group needed a higher concentration to become resistant to CIP than the CIP group over time, that is, the CIP group became resistant to CIP more easily than the LEV group over time. Similarly, the results of MICs to LEV and OFX showed that the CIP group became resistant to the two drugs more easily than the LEV group over time.

Statistical analysis of MICs to CIP, LEV and OFX

Repeated measures ANOVA showed that in MICs to CIP, the interaction between incubation time and group was statistically significant, that is, the trends in the two groups were significantly different over time ($P<0.001$). Further simple effect analysis found that, when the group differed, the MIC increments were different ($P<0.05$). The results above revealed that compared to LEV group, the CIP group's MICs to CIP appeared to increase faster and had a higher increment in vitro selection (shown in Figure 2). Similarly, repeated measures ANOVA showed that in MICs to LEV and OFX, the interaction between incubation time and group was statistically significant, that is, the trends in the two groups were significantly different over time ($P<0.001$). Also, further simple effect analysis proved that compared to the LEV group, the CIP group's MICs to LEV and OFX appeared to have a faster increase and higher increment over time ($P<0.05$).

Resistance rate

The results in Figure 3 show that the resistance rate of *P. aeruginosa* to the three antibiotics increased over time. The CIP group's resistance rates to the three antimicrobial drugs were gradually higher than those of the LEV group. On day 5, the resistance rates of the CIP group for the three drugs were significantly higher than those of the LEV group ($P<0.05$), that is, incubating with CIP made the strains resistant more easily than incubating with LEV.

In summary, in analysis of the three drugs in three aspects – the needed drug concentration to make the strains resistant, the MIC increment and the resistance rate – all the obtained results proved that CIP is more competent than LEV in making the strains resistant to in vitro selection. So, strains incubated with CIP were chosen for the next section.

Conventional PCR amplifying and DNA sequencing

After screening, 27 strains were used for PCR amplification and DNA sequencing, including 4 original strains (PA1, PA10, PA11 and PA12) and 23 strains incubated with CIP (Table 5). By the PCR assay, the presence of *gyrA*, *gyrB*, *parC* and *parE* was detected in 418, 510, 357 and 592 bp, respectively. By comparing the results with the corresponding nucleotide sequences of PAO1 in GenBank, amino acid

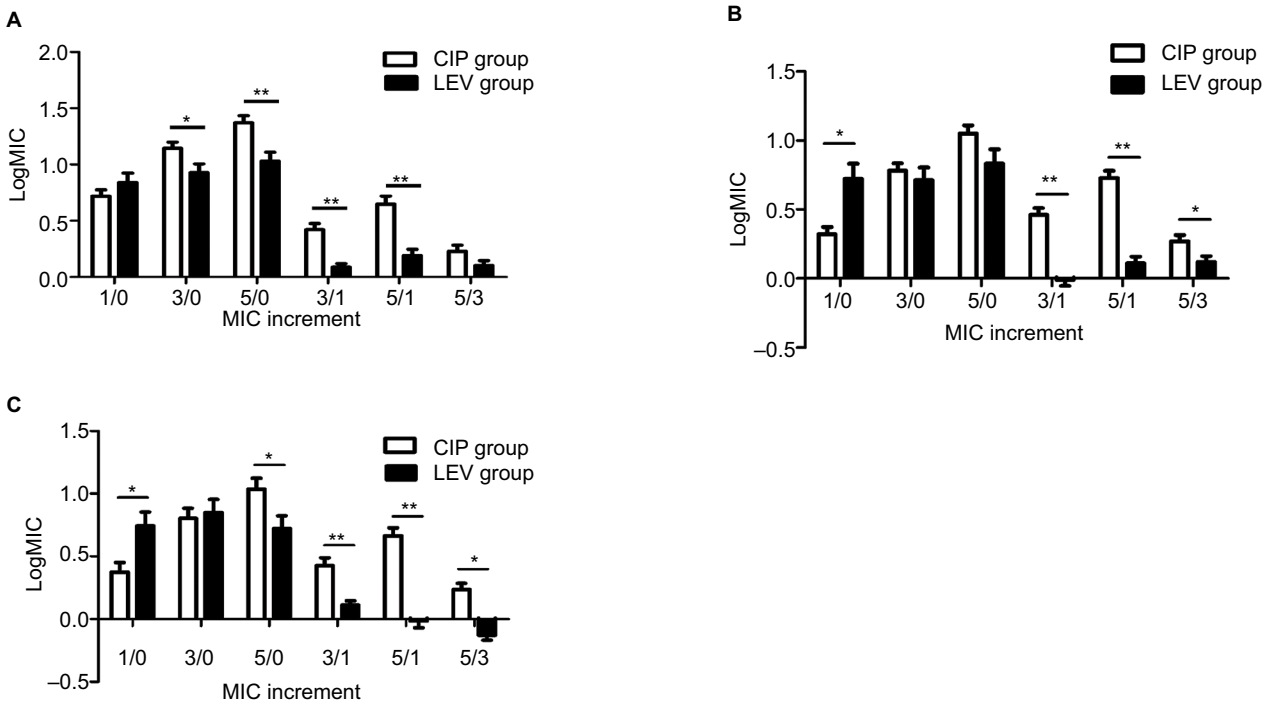

Figure 2 Simple effect analysis of the relationship between groups and MICs to CIP, LEV and OFX.

Notes: (**A**) CIP. (**B**) LEV. (**C**) OFX. 3/0, 5/0: MICs on day 3 or 5 divided by the original MICs, which means the MIC incremental quantity on day 3 or 5. 3/1, 5/1: MICs on day 3 or 5 divided by the MICs on day 1, which means the MIC incremental speed on day 3 or 5. 1/0: MICs on day 1 divided by the original MICs, which means the MIC incremental quantity and speed on day 1. 5/3: MICs on day 5 divided by MICs on day 3, which means the MIC incremental speed on day 5. There is a statistical difference between the CIP group and the LEV group for the same MIC increment and the same drug (*$P<0.05$). There is a statistical difference between the CIP group and the LEV group for the same MIC increment and the same drug (**$P<0.001$).

Abbreviations: CIP, ciprofloxacin; LEV, levofloxacin; MIC, minimum inhibitory concentration; OFX, ofloxacin.

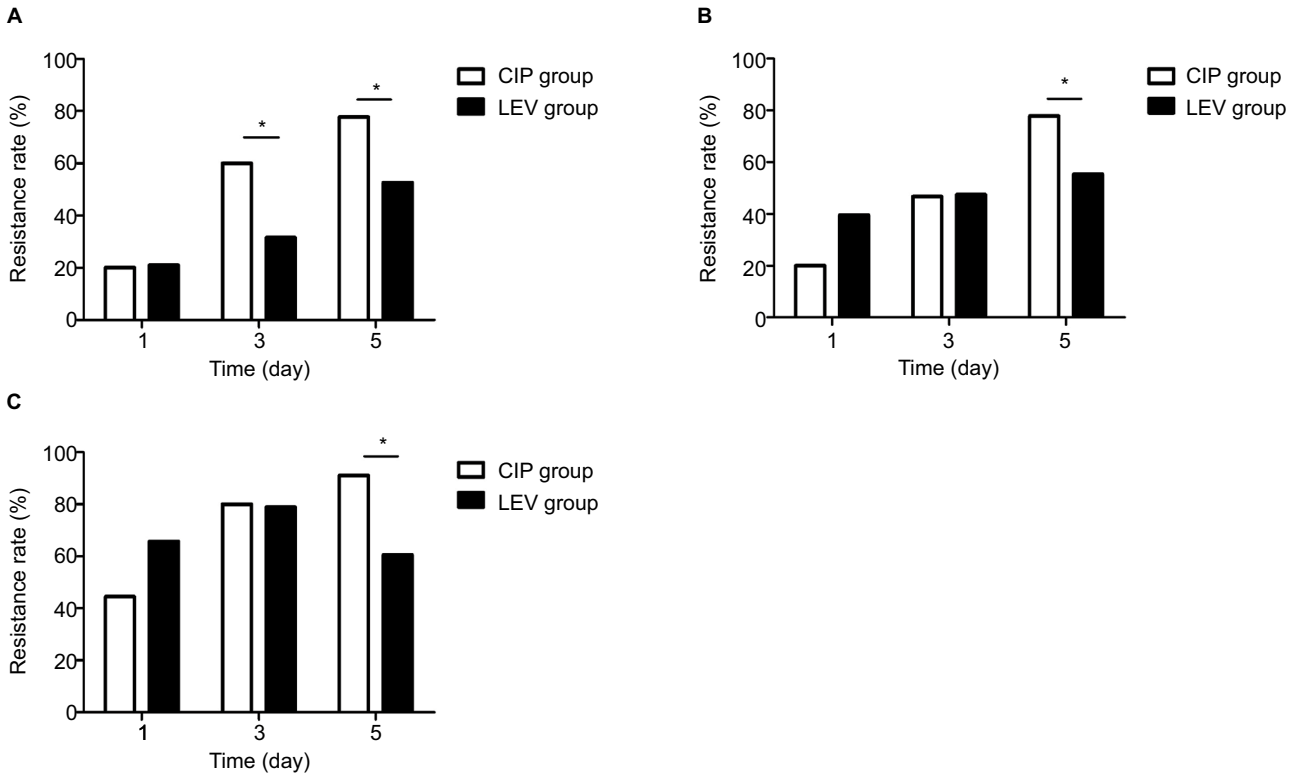

Figure 3 The comparison of resistance rates of strains to CIP, LEV and OFX between the two groups.
Notes: (**A**) CIP. (**B**) LEV. (**C**) OFX. There was a statistical difference between the two groups (*P<0.05).
Abbreviations: CIP, ciprofloxacin; LEV, levofloxacin; MIC, minimum inhibitory concentration; OFX, ofloxacin.

alterations in *gyrA* and *gyrB* were mainly detected in these FQ-resistant strains. No amino acid alterations were detected in the genes *parC* and *parE*. According to the mutant sites of amino acid in *gyrA* and *gyrB*, the results of DNA sequencing are presented in Tables 6 and 7.

Our results showed mutations in *gyrB* were detected on day 1, whereas the mutations in *gyrA* were detected on day 3, that is, *gyrB* had an earlier mutation than *gyrA*. Besides, amino acid alterations in *gyrA* were detected in five strains (21.74%) and amino acid alterations in *gyrB* were detected in 23 strains (100%). The percentage of strains with mutations in *gyrB* was significantly higher than the percentage of strains with mutations in *gyrA* (P<0.001). Noteworthy, five strains had amino acid alterations in both *gyrA* and *gyrB*, but the geometric mean of MICs of these five strains were not significantly higher than that of strains with amino acid alterations only in *gyrB* (P>0.05). What is more, the number of amino acid alterations in *gyrB* (eight alterations) was more than that in *gyrA* (five alterations). All of the above results showed that mutations in *gyrB* played a more important role in FQ resistance to *P. aeruginosa*.

In both *gyrA* and *gyrB*, the number of amino acid alterations increased with time prolonged. Noteworthy, amino acid alteration in *gyrA* in one strain occurred on day 3 under 4× MIC, whereas in other strains it occurred on day 5 at concentrations below 4× MIC, that is, the strains showed *gyrA* mutations earlier when incubated with a higher concentration of CIP. From another perspective, in both *gyrA* and *gyrB*, the number of amino acid alterations increased with increasing incubation concentration. Noteworthy, no amino acid alteration in *gyrA* was found under the concentration of 1× MIC.

The amino acid alterations in *gyrA* mainly occurred at codon 83 (Thr83→Ile). The amino acid alterations in *gyrB* mainly occurred at codon 372 (Ala372→Val, Ala372→Leu), 424 (Ile424→Leu), 464(Leu→Ile) and 483(Glu483→Asp). Further analysis of the data revealed that a total of 23 strains had mutations in *gyrB*, of which 1 strain possessed a single amino acid alteration for Ala 372, 8 strains possessed two amino acid alterations for Ala 372 and Ile 424 and the remaining 14 strains possessed all the four above-mentioned amino acid alterations. In addition, other minor novel alterations were detected in *gyrA* and *gyrB*.

Table 5 MICs of 23 strains used for sequencing (μg/mL)

No. of strain	MICs to			Number of amino acid alteration	
	CIP	LEV	OFX	gyrA	gyrB
PA1d1	4	16	64	–	2
PA1c3	8	16	64	–	2
PA1d3	16	32	64	–	3
PA10d3	4	16	32	–	4
PA1a5	4	8	16	–	2
PA1b5	8	32	32	–	2
PA1d5	32	128	256	–	4
PA10c5	4	8	8	–	4
PA10d5	4	16	32	–	2
PAO1b5	4	8	16	–	4
PAO1c5	8	16	32	–	4
PAO1d5	8	32	64	–	4
PA1c1	2	8	32	–	3
PA10d1	2	8	8	–	2
PAO1d1	16	2	4	–	2
PAO1a3	8	8	2	–	4
PA11d3	4	4	4	–	4
PA10b5	1	8	8	–	2
Geometric mean	5.44±2.30*	12.70±2.51*	20.95±3.45*		
PAO1d3	16	32	64	1	4
PA1c5	16	128	256	1	4
PA10a5	8	16	32	1	2
PA11d5	4	8	16	2	8
PAO1a5	2	8	8	1	4
Geometric mean	6.96±2.47*	21.11±3.19*	36.76±3.79*		

Notes: a, b, c and d represent the incubation concentration of 0.5× MIC, 1× MIC, 2× MIC and 4× MIC, respectively. 1, 3 and 5 represent the incubation time of 1, 3 and 5 days, respectively. For example, PA1d1: the strain of PA1 incubated with CIP for 1 day under the concentration of 4× MIC. *There was no significant difference between the two geometric means for the same drug (*P>0.05).
Abbreviations: CIP, ciprofloxacin; LEV, levofloxacin; MIC, minimum inhibitory concentration; OFX, ofloxacin.

Table 6 Amino acid alterations of *P. aeruginosa* in *gyrA*

Concentration	No. of strain	Time(day)						
		0	1	3	5			
				139	54	83	128	139
	PAO1			Glu(GAG)	Glu(GAG)	Thr(ACC)	Ala(GCC)	Glu(GAA)
0.5× MIC	PA2	–	–	–	–	Ile(ATC)	–	–
	PA12							
1× MIC		–	–	-	-	–	–	–
2× MIC	PA1	–	–	–	Lys(AAG)	–	–	–
4× MIC	PA3	–	–	–	–	–	Thr(ACC)	His(CAT)
	PA12	–	–	His(CAT)	–	–	–	–

Notes: –: no amino acid alteration. Blank: no sequencing.
Abbreviation: MIC, minimum inhibitory concentration.

Efflux pumps expression

A total of 52 strains of *P. aeruginosa* were screened out, including 4 original isolates (PA1, PA10, PA11 and PA12) and 48 strains incubated with CIP. The results showed that the efflux pumps were not highly expressed (Figure 4). Overexpression of efflux pumps was found to be present if the relative expression of *mexA* was higher than 3.00 and the relative expression of *mexE* was higher than 10.00.[23]

Discussion

After incubating for 5 days under different drug concentrations, the effect of incubating with CIP on MICs and

resistance was different from incubating with LEV. For the three drugs (CIP, LEV and OFX), the strains incubated with LEV needed a higher concentration to become resistant than the strains incubated with CIP over time. MICs of strains incubated with CIP showed faster increase over time and the final increment was higher. Also, the resistance rates of strains incubated with CIP became gradually higher than those of strains incubated with LEV over time. After comparisons in three aspects, CIP appeared to have stronger ability to make the strains resistant over time in vitro. This may indicate that LEV is superior to be recommended to treat infection caused by *P. aeruginosa* in terms of resistance patterns demonstrated in this analysis.

MIC refers to the lowest concentration of antimicrobial drugs that inhibit bacterial growth. The mutant prevention concentration (MPC) refers to the drug concentration at which the first-step resistant mutant has zero growth. If the drug concentration surpasses the MPC, the emergence of resistance is expected to be limited. The mutant selection window (MSW) refers to the range of drug concentration between the MIC and MPC, that is, within this window, resistant mutants will be selected under antimicrobial selective pressure.[24–26] The MPCs of *P. aeruginosa* were 2.00 and 8.00 μg/mL for CIP and LEV, respectively.[27,28] In this study, the incubation concentration ranged from 0.03125 to 2 μg/mL and from 0.0625 to 8 μg/mL for CIP and LEV, respectively. Except for the concentration of 0.5× MIC, other incubation concentrations were within the MSW. On one hand, our results showed the MICs increased over time, and resistant mutants had been selected on days 1, 3 and 5. On the other hand, our results showed the MICs increased with increase in the incubation concentration, and resistant mutants had been selected under the concentration of 1× MIC, 2× MIC and 4× MIC. It is worth noting that resistant mutants had also been selected under the concentration of 0.5× MIC, that is, sub-MIC can also be selected for resistant mutants. This has been reported in some literatures.[24,26,29] These results prove that the emergence of resistance was associated with time and the concentration of antimicrobial drugs. This suggests that we should pay attention to the dosage and course of drugs during clinical treatment of infection. In addition, after incubating with CIP or LEV, strains were not only resistant to CIP or LEV, but also resistant to OFX, that is, there was cross-resistance between FQs.[30,31] This indicates that if *P. aeruginosa* is resistant to one drug of the FQs, it may also be resistant to other drugs of the FQs.

In this study, both *mexA* and *mexE* genes were not highly expressed, that is, the FQ resistance to *P. aeruginosa* in these resistant mutants cannot be contributed to efflux pumps. This may be because multidrug resistance was correlated with the overexpression of efflux pumps,[32–34] while all resistant mutants in this study were not multidrug-resistant strains. So, in this study, another molecular resistance mechanism, mutations in target genes, mainly contributed to the FQ resistance to *P. aeruginosa*. Our results showed all mutations were detected in FQ-resistant strains and all point mutations were detected in *gyrA* and *gyrB*. In contrast, no mutations were found in the susceptible strains. These results indicated that mutations in *gyrA* and *gyrB* are associated with the resistance of *P. aeruginosa* to FQs. Meanwhile, our results found no mutations were detected in *parC*, which was not consistent with some studies.[7,9,35] This may be because *P. aeruginosa* is a gram-negative bacterium and the first target of FQ against gram-negative bacteria is DNA gyrase encoded by *gyrA* and *gyrB*.[8,10,11]

Previous studies have reported that major amino acid alterations in *gyrA* frequently occur at codon 83 (Thr83→Ile), and *gyrA* mutations were closely correlated with FQ resistance to *P. aeruginosa*.[7,9,15,16,17,36] A study had reported that amino acid alterations in *gyrB* occurred at codon 467 (Ser467→Phe) and 468 (Gln468→His), but the relationship between the alterations and resistance was not established.[15] These were not completely consistent with our results. Mutations in *gyrA* at codon 83(Thr83→Ile) were also found in our study, but our results showed four amino acid alterations in *gyrB* were mainly detected, including Ala372→Val, Ile424→Leu, Leu464→Ile and Glu483→Asp. Also, our results revealed that mutations in *gyrB* played an important role in FQ resistance to *P. aeruginosa*. But as the mutations in *gyrB* in FQ-resistant *P. aeruginosa* were reported rarely, we have no more data to compare. Also, the relationship between *gyrB* mutations and resistance needs further experiments to be confirmed.

In this study, resistant mutants with *gyrA* had the same amino acid alteration under the subconcentration of 0.5× MIC, whereas under the higher concentrations of 2× MIC and 4× MIC, they had other novel amino acid alterations. For resistant mutants with *gyrB*, the same change was found under lower and higher concentrations. These results indicated that resistant mutants may obtain stable drug resistance under the subconcentration, whereas higher incubation concentrations make mutations in genes of *gyrA* and *gyrB* changeable. This needs further experiments to be confirmed. Some studies reported that resistant strains can be cultured continuously in a medium without antimicrobial drugs to evaluate the stability of resistance.[37] In addition, these minor and rare mutations in *gyrA* and *gyrB* under higher concentrations may increase

Table 7 Amino acid alterations of *P. aeruginosa* in *gyrB*

Concentration	No. of isolate	Time (day)								
		0	1					3		
			372	424	449	464	483	372	424	439
	PAO1		Ala(GCC)	Ile(ATC)	Lys(AAG)	Leu(CTC)	Glu(GAG)	Ala(GCC)	Ile(ATC)	Asn(AAT)
0.5× MIC	PA1	–								
	PA2	–								
	PA12	–						Val(GTG)	Leu(TTG)	
1× MIC	PA1	–								
	PA2	–								
	PA12	–								
2× MIC	PA1	–	Val(GTC)	Leu(TTG)	Arg(AGG)			Val(GTG)	Leu(TTG)	
	PA2	–								
	PA12	–								
4× MIC	PA1	–	Val(GTC)	Leu(TTG)				Val(GTC)		Tyr(TAC)
	PA2	–	Leu(CTG)	Leu(TTG)		Ile(ATT)	Asp(GAC)	Leu(CTG)	Leu(TTG)	
	PA3	–						Leu(CTG)	Leu(TTG)	
	PA12	–	Leu(CTG)	Leu(TTG)		Ile(ATT)	Asp(GAC)	Leu(CTG)	Leu(TTG)	

Notes: –: no amino acid alteration. Blank: no sequencing.
Abbreviation: MIC, minimum inhibitory concentration.

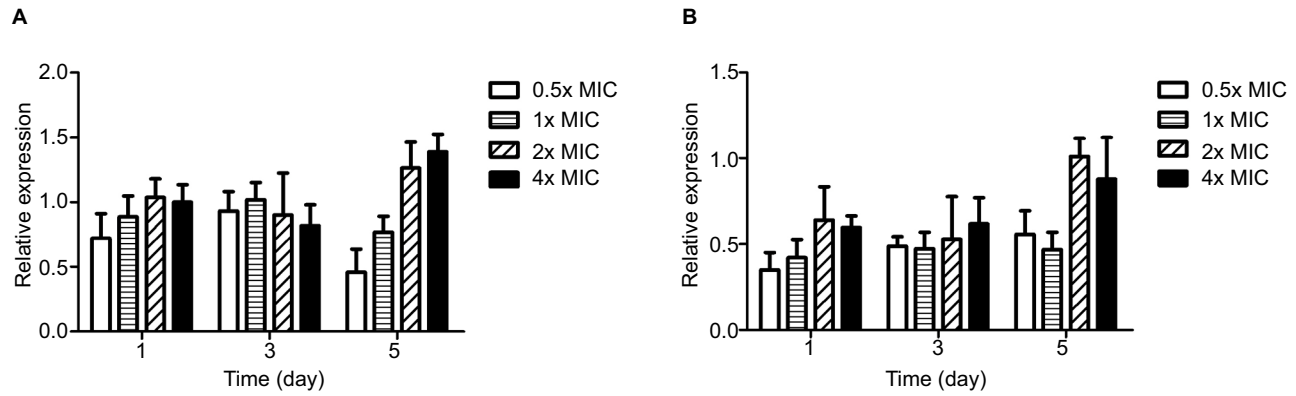

Figure 4 The relative expression of two efflux pumps genes – *MexA* and *MexE*.
Notes: (**A**) *MexA*. (**B**) *MexE*. The efflux pump was highly expressed if the relative expression of *MexA* was higher than 3.00. The efflux pump was highly expressed if the relative expression of *MexE* was higher than 10.00.
Abbreviation: MIC, minimum inhibitory concentration.

the likelihood of resistance or make the strains high-level resistant, which needs further verification.

Conclusion

In this study, CIP appeared to have stronger ability than LEV to make the strains resistant to in vitro selection. The molecular resistance mechanisms of FQs to *P. aeruginosa* were mainly the mutations in *gyrA* and *gyrB*, and the mutations in *gyrB* played a more important role in drug resistance.

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			5							
461	464	483	372	424	464	483	476	484	487	488
Asp(GAC)	Leu(CTC)	Glu(GAG)	Ala(GCC)	Ile(ATC)	Leu(CTC)	Glu(GAG)	Leu(CTG)	Glu(GAA)	Ile(ATC)	Asp(GAC)
			Val(GTC)	Leu(TTG)						
			Val(GTG)	Leu(TTG)						
	Ile(ATT)	Asp(GAC)	Leu(CTG)	Leu(TTG)	Ile(ATT)	Asp(GAC)				
			Val(GTC)	Leu(TTG)						
			Val(GTG)	Leu(TTG)						
			Leu(CTG)	Leu(TTG)	Ile(ATT)	Asp(GAC)				
			Leu(CTG)	Leu(TTG)	Ile(ATT)	Asp(GAC)				
			Leu(CTG)	Leu(TTG)	Ile(ATT)	Asp(GAC)				
			Leu(CTG)	Leu(TTG)	Ile(ATT)	Asp(GAC)				
His(CAC)			Leu(CTG)	Leu(TTG)	Ile(ATT)	Asp(GAC)				
	Ile(ATT)	Asp(GAC)	Val(GTG)	Leu(TTG)						
	Ile(ATT)	Asp(GAC)	Val(GTG)	Leu(TTG)	Ile(ATT)	Asp(GAC)	Trp(TGG)	Lys(AAA)	Phe(TTC)	As(AAC)
	Ile(ATT)	Asp(GAC)	Leu(CTG)	Leu(TTG)	Ile(ATT)	Asp(GAC)				

Disclosure

The authors report no conflicts of interest in this work.

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