

Surveillance of Fluoroquinolone-Resistant Clinical Isolates of *Pseudomonas aeruginosa*

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ABSTRACT

Ciprofloxacin (CPFX) and pazufloxacin (PZFX) have strong antibacterial activity against *Pseudomonas aeruginosa*. We investigated the sensitivity of *P. aeruginosa* to CPFX and PZFX in 373 strains isolated from inpatients (321 strains) and outpatients (52 strains) during September 2010 to September 2011 at Toho University Ohashi Medical Center. The percentage of CPFX-non-susceptible (≥ 3.91 $\mu\text{g/mL}$) among inpatients was 22.4%, but that among outpatients was 1.9%. As the major resistance mechanism to fluoroquinolones in *P. aeruginosa* involves modification of type II topoisomerases (DNA gyrase and topoisomerase IV), we examined mutations in the quinolone-resistance-determining regions (QRDRs) of *gyrA* and *parC* of *P. aeruginosa* isolates. Among the 373 isolates, 73 isolates had reduced CPFX-susceptibility and 88 had reduced PZFX-susceptibility. Sequencing of *gyrA* and *parC* revealed base substitutions that resulted in amino acid replacements in QRDR of *GyrA* in 70 *P. aeruginosa* isolates, while Thr83Ile (in *GyrA*) and Ser87Leu (in *ParC*) substitutions were found in 12 strains. These replacements were clearly associated with reduced susceptibility to CPFX and PZFX. However, we also found strains with high MICs to quinolones without mutations in either *gyrA* or *parC*. We then investigated the effect of efflux pumps in CPFX-resistance in these isolates. In the presence of an efflux pump inhibitor, MIC values in 12 of 66 strains decreased to $1/2^3$. We also sequenced genes related to overexpression of efflux pumps, viz., *mexZ*, *mexR*, and *nfxB*. Eight of the strains without mutations in QRDRs had a mutation in *mexZ*, 7 strains had a mutation in *mexR*, but no mutation was identified in *nfxB*.

Keywords: *Pseudomonas aeruginosa*; Fluoroquinolone Resistant; Type II Topoisomerases; Efflux Pump

1. Introduction

Pseudomonas aeruginosa, a ubiquitous Gram-negative soil organism, is widely distributed in nature. *P. aeruginosa* is noted for its metabolic versatility and its exceptional ability to colonize a wide variety of environments, and also for its intrinsic resistance to a wide variety of antimicrobial agents. Such characteristics result in a higher prevalence of this organism in hospital environments, where it is a clinically significant pathogen causing opportunistic infections and nosocomial outbreaks [1, 2].

P. aeruginosa typically infects injured, burned, immune deficient, or immunocompromised patients and causes a variety of infections. The bacterium has various virulence determinants, such as adhesins [3], toxins [4], capsules [5], and biofilms [6], and is also frequently resistant

to many commonly used antibiotics [7]. Though many strains of *P. aeruginosa* are susceptible to cephalosporins, carbenicillin, gentamicin, tobramycin, colistin, polymyxin, and fluoroquinolones, strains resistant to those antibiotics are increasing in medical care facilities and isolation of the multidrug-resistant strain have been reported [8-12].

One of the challenges in managing *P. aeruginosa* infections is an inherent resistance mechanism, referred to as intrinsic resistance. Because of its virulence and the limited choices of effective antimicrobial agents, treatment of *P. aeruginosa* infection is often complicated. The present study aimed to study the antimicrobial susceptibility patterns of *P. aeruginosa* isolated from outpatients and inpatients, using the agar dilution method, and by defining mutations in *gyrA*, *parC*, *mexZ*, *mexR* and *nfxB*, which contribute to fluoroquinolone resistance, in these isolates.

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2. Materials and Methods

2.1. Antibacterial Agents and Bacterial Strains

Ciprofloxacin, meropenem, and amikacin were purchased from Wako Pure Chemical Industries, Ltd., Japan, and pazufloxacin was purchased from Toyama Chemical Co., Ltd., Japan.

In this study, 373 clinical isolates of *P. aeruginosa* were collected during September 2010 to September 2011 at Toho University Ohashi Medical Center. Of these, 321 isolates of *P. aeruginosa* were obtained from inpatients with respiratory tract infections, urinary tract infections, surgical site infection, gastrointestinal tract infections, or bacteremia, whereas the remaining 52 isolates were obtained from outpatients with tympanitis, bacterial skin infections, respiratory tract infections, or urinary tract infections. All the *P. aeruginosa* strains isolated were maintained in Microbank™ (Pro-Lab, Toronto, Canada) and kept at -80°C.

2.2. Determination of MIC

MICs were determined by the standard agar dilution assay, according to guidelines of the National Committee for Clinical Laboratory Standards (NCCLS), using Mueller-Hinton (MH) agar (Becton, Dickinson and Company, USA). Strains preserved on Microbank™ were inoculated into 2 mL of MH broth and incubated at 37°C for 18 - 20 h. After dilution with MH broth, 2 µL of the bacterial suspension containing 10⁴ CFU was spotted onto drug-containing agar plates. The plates were incubated at 37°C for 24 h. The MIC was defined as the lowest drug concentration that prevented visible growth of bacteria.

2.3. Statistical Analysis

Mann-Whitney's U-test was used to evaluate the rela-

tionship between the distribution of MIC of CPFX, MEPM, PZFX and AMK in the strains of *P. aeruginosa* isolated from outpatients and inpatients.

2.4. PCR Amplification and DNA Sequence Determination and Analysis

The strains were incubated in MH broth at 37°C overnight, and 1 mL of the culture was centrifuged (15,000 rpm, 10 min, at 4°C). A sterilized toothpick was used to lightly touch the precipitate, and cells on the toothpick were suspended in 25 µL of PCR mixture: KOD FX 0.5 U (TOYOBO Co., Ltd., Japan), 0.3 µM of each primer, 0.4 mM of each dNTP. Primers used for PCR are listed in **Table 1**. The amplification procedure comprised denaturation at 95°C for 2 min, followed by 40 cycles, each consisting of denaturation for 10 s at 98°C, annealing for 30 s at 55°C, and polymerization for 30 s at 74°C. Amplified DNA was directly sequenced by the dideoxy chain termination method, employing a Thermo Sequenase II dye terminator cycle sequencing kit (Amersham Pharmacia Biotech Inc., NJ, USA), BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Inc., CA, USA) according to the manufacturer's protocol. The products were automatically analyzed in a model ABI 3500 DNA autosequencer (Applied Biosystems, Inc.). DNA sequences have been deposited in DDBJ (Accession numbers: AB753299-AB753440).

2.5. Impact of Efflux Pump Inhibitor (EPI) on the MICs of Ciprofloxacin

Strains of *P. aeruginosa* of which the ciprofloxacin MIC exceeded 3.91 µg/mL were inoculated on agar plates containing various concentrations of ciprofloxacin, with or without 20 µg/mL of the efflux pump inhibitor, phenylalanine arginyl-β-naphthylamide (Sigma-Aldrich, MO, USA). MICs of ciprofloxacin in the presence of EPI were

Table 1. Primers for amplification and sequencing of *gyrA*, *parC*, *mexZ*, *mexR* and *nfxB* genes.

Gene	Nucleotide sequence (5' to 3')	Nucleotide position	Accession No.
<i>gyrA</i>	<u>GTTTTCCAGTCACGACGTTGAGTCCTATCTCGACTACGCGAT</u>	320 - 341	AAG06556
	AGTCGACGGTTTCCTTTTCCAG	676 - 697	
<i>parC</i>	<u>GTTTTCCAGTCACGACGTTGCGAGCAGGCCTATCTGAACTAT</u>	214 - 235	AAG08349
	GAAGGACTTGGGATCGTCCGGA	496 - 517	
<i>mexZ</i>	<u>TGTA AACGACGGCCAGTCCAGGAAAACCAAAGAGGAA</u>	5 - 24	AAF14521
	CAGGAAACAGCTATGACCCAGCAACAGGTAGGGAGA ACT	601 - 622	
<i>mexR</i>	<u>TGTA AACGACGGCCAGTATGAACTACCCCGTGAATCCC</u>	1 - 21	AAG03813
	CAGGAAACAGCTATGACCGGTTGCGCGGCCAGGCACTGGTCGAGGAGA	399 - 429	
<i>nfxB</i>	<u>TGTA AACGACGGCCAGTATGACCCTGATTTCCCATGA</u>	1 - 20	AAG07988
	CAGGAAACAGCTATGACCACCAGGGTGATGAACAG	439 - 456	

Underline: 5'-end attached oligonucleotides for sequencing; GTTTTTCCAGTCACGACGTTG, M13M4 Forward oligonucleotide; TGTA AACGACGGCCA GT, M13(-21) Forward oligonucleotide; CAGGAAACAGCTATGAC, M13 Reverse oligonucleotide.

determined after overnight incubation of isolates at 37°C.

3. Results and Discussion

3.1. Antibiogram Pattern of *Pseudomonas aeruginosa*

The results of MH agar-based antibiogram pattern study of the strains of *P. aeruginosa* isolated from inpatients (n = 321) and outpatients (n = 52) is shown in **Figure 1**. The MIC range of CPFZ in the strains isolated from outpatients, except for 1 strain, was between <0.06 and 1.95 µg/mL, but that of strains isolated from inpatients was between <0.06 and >62.5 µg/mL (**Figure 1(a)**). Thus, strains from outpatients were sensitive to CPFZ, except for 1 strain that was isolated from an outpatient who had received ofloxacin treatment for chronic otitis media. The percentage of CPFZ-non-susceptible strains (≥ 3.91 µg/mL) among inpatients was 22.4%, but that among outpatients was 1.9%.

The MIC range of the strains from outpatients for PZFX was between 0.06 and 7.81 µg/mL, except for 1 strain, and that from inpatients was between <0.06 and 62.5 µg/mL (**Figure 1(c)**).

The MIC ranges of the strains from outpatients and inpatients for MEPM were between <0.49 and 7.81 µg/mL, and between <0.49 and >125 µg/mL, respectively. The percentage of MEPM-non-susceptible strains (≥ 15.6

µg/mL) among inpatients was 30.5%, but there were no MEPM-non-susceptible strains among outpatients (**Figure 1(b)**).

The MIC ranges of the strains from outpatients for AMK was between <0.49 and 15.62 µg/mL, and that from inpatients was between <0.49 and 62.5 µg/mL. The percentage of AMK-non-susceptible strains (≥ 31.25 µg/mL) from inpatients was 5.6%, but no AMK-non-susceptible strains were found among outpatients (**Figure 1(d)**).

Our results indicated that most of the strains isolated from outpatients were susceptible to CPFZ and PZFX, but 22.4% of the strains isolated from inpatients were resistant to CPFZ; strains isolated from outpatient were susceptible to MEPM, but 30.5% of the strains isolated from inpatients were resistant to MEPM (**Figure 1(b)**). Similarly, strains isolated from outpatients were susceptible to AMK, but 5.6% of the strains isolated from inpatients were resistant to this antimicrobial agent (**Figure 1(d)**).

Statistical analysis of the distribution of MIC of CPFZ, MEPM, PZFX and AMK in the strains of *P. aeruginosa* isolated from outpatients and inpatients showed significant differences ($P < 0.05$) in each antibiotics. Surveys of antimicrobial susceptibilities in clinical isolates of *P. aeruginosa* in 2004 and 2008 in Japan showed that 22% of the 90 isolates collected from 13 medical facilities

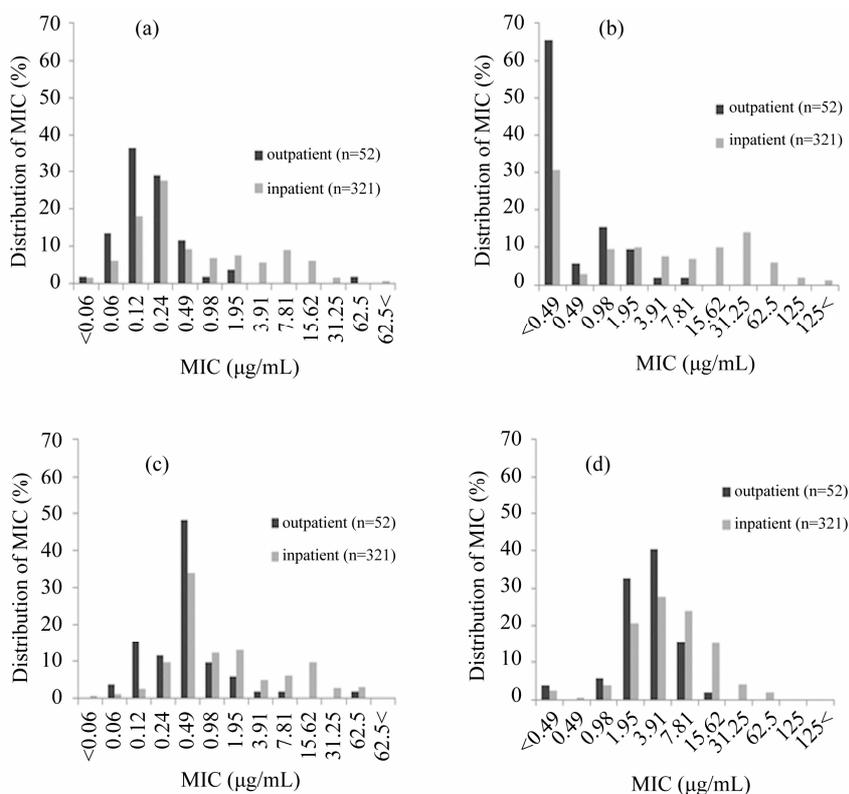


Figure 1. Distribution of MIC of CPFZ (a); MEPM (b); PZFX (c); and AMK (d) in outpatients and inpatients.

were resistant to CPFEX, while 22% were resistant to MEPM [13]; furthermore, 42.9% of 207 isolates from 1 medical facility were resistant to CPFEX, while 12.6% were resistant to MEPM [14]. In another study, among 188 strains isolated from 139 cases in a number of medical facilities, 24% were resistant to CPFEX, whereas 18% were resistant to MEPM [15]. The differences in resistance rates among the present study and the surveys mentioned above could reflect differences in clinical usage of antimicrobial agents and periods of administration, etc.

3.2. Relationship between MIC and Mutations in *gyrA* and *parC*

As mentioned above, 22.4% of the *P. aeruginosa* strains isolated from inpatients were not susceptible to CPFEX; we then set out to identify mutations in *gyrA* and *parC* that may underlie this CPFEX-resistance. Strains of *P. aeruginosa* repeatedly isolated from the same patient, or strains with MICs exceeding 3.91 $\mu\text{g/mL}$ for CPFEX or PZFX were selected. Mutations in *gyrA* and *parC* of 131 strains were analyzed by PCR and DNA sequencing.

As shown in **Figure 2(a)**, we found no mutations in the PCR-amplified *gyrA* and *parC* fragments from 52 of 60 strains with MICs less than 3.91 $\mu\text{g/mL}$ for CPFEX. The remaining 8 strains had a mutation only in *gyrA*; these mutations resulted in a Thr83Ile substitution in *GyrA* in 4 strains, an Asp87Gly substitution in 3 strains, and an Asp87Asn substitution in 1 strain. Nine of 46 strains with a MIC range from ≥ 3.91 $\mu\text{g/mL}$ to < 15.6 $\mu\text{g/mL}$ for CPFEX had no mutation in *gyrA* and *parC*, whereas 35 of these 46 strains had a mutation in only *gyrA*. Two strains had mutations in both *gyrA* and *parC*. Two of 25 strains with MICs for CPFEX of ≥ 15.6 $\mu\text{g/mL}$ had no mutation, 15 of these 25 strains had a mutation only in *gyrA*, and 8 strains had mutations in both *gyrA* and *parC*.

A similar analysis was performed in strains with MICs for PZFX of less than 3.91 $\mu\text{g/mL}$, ≥ 3.91 $\mu\text{g/mL}$ to < 15.6 $\mu\text{g/mL}$, and ≥ 15.6 $\mu\text{g/mL}$ (**Figure 2(b)**). No strains with MICs of less than 3.91 $\mu\text{g/mL}$ for PZFX had mutations in *gyrA* and *parC*. In the 27 strains with a MIC range of ≥ 3.91 $\mu\text{g/mL}$ to < 15.6 $\mu\text{g/mL}$ for PZFX, 7 had no mutations in *gyrA* or *parC*, 16 strains had a mutation only in *gyrA*, and 4 strains had mutations in both *gyrA* and *parC*. Two of 52 strains with MICs for PZFX of 15.6 $\mu\text{g/mL}$ or more had no mutations, 42 of the 52 strains had a mutation only in *gyrA*, and 8 strains had mutations in both *gyrA* and *parC*.

We found no CPFEX- or PZFX-resistant strain with a mutation only in *parC*. In Gram-positive cocci, mutations in *parC* are seen commonly in fluoroquinolone-resistant strains, and it is well-known that these mutations precede

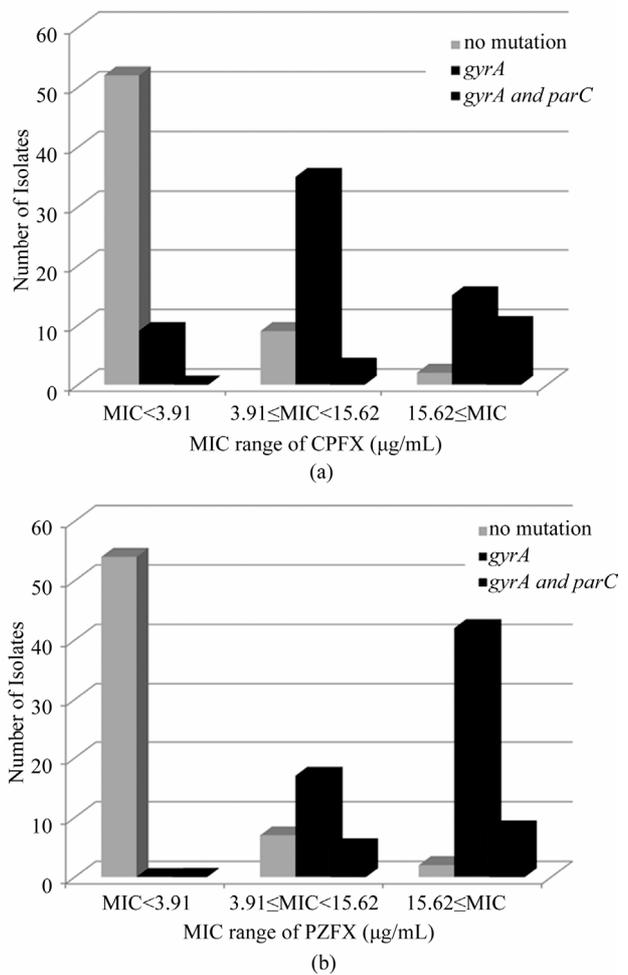


Figure 2. The relation with MICs of CPFEX (a) or PZFX (b) and mutations in *gyrA* and *parC* genes.

mutations in *gyrA* [16,17]. The inverse, *i.e.*, that mutations in *gyrA* precede mutations in *parC*, is typically seen in Gram-negative bacteria. In this study, CPFEX-resistant strains had mutations in *gyrA*, while some strains had mutations in both *gyrA* and *parC*, similar to the finding in fluoroquinolone-resistant Gram-negative bacteria. Moreover, the strains with mutations in both *gyrA* and *parC* had higher MICs for CPFEX [18,19].

3.3. Impact of Efflux Pump Inhibition on MICs of Ciprofloxacin

Interestingly, we found strains with high MICs for fluoroquinolones without mutations in either *gyrA* or *parC*. These higher CPFEX-resistant strains were assumed to have other fluoroquinolone-resistance mechanisms, such as an overproduction of efflux pumps. To investigate this possibility, we used phenylalanine arginyl β -naphthylamide ($\text{PA}\beta\text{N}$), which inhibits efflux pumps in *P. aeruginosa*. [20]. **Table 2** shows the effect of $\text{PA}\beta\text{N}$ on the MICs of CPFEX-resistant strains. Twelve of 66 CPFEX-

Table 2. Impact of efflux pump inhibitor on the MICs of ciprofloxacin.

Strain No.	Amino acid substitutions		MIC of CPFX ($\mu\text{g/mL}$)	
	<i>GyrA</i>	<i>ParC</i>	Without EPI	With EPI
368	Thr83Ile, Asp87Asn	Ser87Leu	>62.5	31.25
348	Thr83Ile, Asp87Asn	Ser87Leu	>62.5	15.6
28	Thr83Ile	Ser87Leu	62.5	15.6
12, 34, 186	Thr83Ile	Ser87Leu	31.25	7.81
63, 64	Thr83Ile	Ser87Leu	15.6	15.6
53	Thr83Ile	Ser87Leu	15.6	7.81
54	Thr83Ile	Ser87Leu	15.6	3.91
36	Thr83Ile	Ser87Leu	7.81	7.81
109, 225	Thr83Ile	-	31.25	3.91
280	Thr83Ile	-	15.6	7.81
44, 49, 121, 125, 147, 167, 168, 197, 228	Thr83Ile	-	15.6	3.91
190, 291, 292	Thr83Ile	-	15.6	1.95
74	Thr83Ile	-	7.81	7.81
35, 41, 57, 78, 116, 154, 176, 214, 235, 250, 254, 255, 258, 328	Thr83Ile	-	7.81	3.91
122, 187, 232, 238, 251, 263, 268, 270, 272, 275, 276	Thr83Ile	-	7.81	1.95
199	Thr83Ile	-	7.81	<0.49
353, 357	Asp87Asn	-	3.91	0.98
286	-	-	15.6	1.95
297	-	-	15.6	0.98
162	-	-	3.91	1.95
118, 136, 143, 144, 145	-	-	3.91	0.98
148, 152, 156	-	-	3.91	0.49

resistant strains showed an 8-fold decrease in CPFX-MIC in the presence of PA β N. Strains 109 and 225 had an alteration only in *GyrA* (Thr83Ile), and the MICs for CPFX of these strains (31.25 $\mu\text{g/mL}$) were higher than those of strains with the same *GyrA* alteration. However, MICs of these strains were decreased to 3.91 $\mu\text{g/mL}$ in the presence of PA β N. Similar MIC decreases were observed in strains with no alterations in *GyrA* and *ParC*. Therefore, overexpression of efflux pumps appears to contribute to the higher CPFX-resistance in these 12 strains.

3.4. Mutations in *gyrA*, *parC*, *mexZ*, *mexR* and *nfxB* in Clinical Isolates from Inpatients

As mentioned above, not only mutations in DNA topoisomerase II, but also those in genes regulating efflux pumps could contribute to CPFX-resistance in *P. aeruginosa*. We sequenced *gyrA*, *parC*, *mexZ*, *mexR*, and *nfxB* in 120 *P. aeruginosa* strains isolated from inpatients. The sequences were compared with the corresponding sequences of *P. aeruginosa* PAO1 (Pseudomonas Ge-

nome Database, <http://www.pseudomonas.com/>). The isolates were grouped into 12 groups on the basis of the amino acid substitutions found in *GyrA*, *ParC*, *MexZ*, *MexR*, and *NfxB* as shown in **Table 3**. No mutation were found in these genes in 52 strains (Group I), but MICs of CPFX in 11 of these strains were ≥ 3.91 $\mu\text{g/mL}$. Strains with mutations in *mexZ*, or *mexZ* and *mexR*, were sensitive to 3.91 $\mu\text{g/mL}$ CPFX. The mutation most frequently found in *mexZ* was a single base substitution that resulted in a missense mutation (C490T; 15 of 27 strains). Other mutations identified in *mexZ* were single base deletions leading to frame-shifts after Glu114 and Glu146, and 5-bp deletions leading to frame-shifts following Ala21 or Gly148. However, when comparing the MIC ranges of Groups V and VII, the contribution of these *mexZ* mutations to CPFX-resistance was not significant.

Amino acid substitutions in *MexR* were found in 31 of 120 strains; the Val126Glu substitution was found in 30 of 31 strains, and 1 strain carried substitutions Val126-Glu and Ser92Asn. As for *mexZ* mutations, the *mexR* mutations found in this study did not have a significant

Table 3. Alterations in *GyrA*, *ParC*, *MexZ*, *MexR* and *NfxB* subunits and correlation of MICs of ciprofloxacin.

Group, subunit	No. of isolates	No. of isolates for which MICs ($\mu\text{g/mL}$) were:												
		<0.12	0.12	0.24	0.49	0.98	1.95	3.91	7.81	15.6	31.25	62.5	>62.5	
I, no alteration	52	1	9	4	4	10	13	9		2				
II, <i>MexZ</i>	1					1								
III, <i>MexZ</i> and <i>MexR</i>	7				4	3								
IV, <i>GyrA</i> (position 87)	6					1	3	2						
V, <i>GyrA</i> (position 83)	15							1	14					
VI, <i>GyrA</i> (83) and <i>MexR</i>	13								3	8	2			
VII, <i>GyrA</i> (83) and <i>MexZ</i>	14						1		9	4				
VIII, <i>GyrA</i> (83), <i>MexZ</i> and <i>MexR</i>	2								1	1				
IX, <i>GyrA</i> (83), <i>ParC</i> and <i>MexR</i>	7								1	4	2			
X, <i>GyrA</i> (83), <i>ParC</i> and <i>MexZ</i>	1										1			
XI, <i>GyrA</i> (83 and 87), <i>ParC</i> , <i>MexR</i> and <i>NfxB</i>	1												1	
XII, <i>GyrA</i> (83 and 87), <i>ParC</i> , <i>MexZ</i> , <i>MexR</i> and <i>NfxB</i>	1												1	

effect on CPF_X-resistance. Two strains had an 11-bp deletion in *nfxB* leading to a frame-shift following Val35; these strains also had other alterations affecting *GyrA*, *ParC*, *MexZ*, and *MexR*.

Amino acid substitutions in quinolone resistance-determining region (QRDR) of *GyrA* (Thr83Ile or Asp87Asn) were found in 60 of 120 isolates; moreover, the Thr83Ile substitution was the mutation most frequently reported previously in CPF_X-resistant clinical isolates of *P. aeruginosa* [21,22]. Fifteen strains in Group V with an amino acid substitution in QRDR of *GyrA* (Thr83Ile) were resistant to 3.91 $\mu\text{g/mL}$ CPF_X, but 2 of 6 strains in Group IV with another amino acid substitution in QRDR of *GyrA* (Asp87Asn) were resistant to 3.91 $\mu\text{g/mL}$ CPF_X.

The only amino acid substitution found in QRDR of the *ParC* subunit of topoisomerase IV, another target of CPF_X, was Ser87Leu; this alteration was always found along with a *GyrA* alteration (Groups IX-XII). These results suggested that CPF_X-resistance in *P. aeruginosa* is mainly mediated through alterations in *GyrA*, and that alterations in *ParC* are secondary, as reported previously [23]. Alterations in *ParC*, *MexZ*, *MexR*, or *NfxB* in addition to mutation in *GyrA* (Thr83Ile) increased the MICs for CPF_X, although the number of strains with multiple alterations was limited.

Our results revealed that multiple alterations in *GyrA*, *ParC*, *MexZ*, *MexR*, and *NfxB* were associated with a higher-level resistance to CPF_X than that caused by a single alteration in *GyrA*. Although 2 strains in Group I (**Table 3**), in which we could not identify mutation in QRDRs of *gyrA*, *parC*, or the efflux pump regulator genes *mexZ*, *mexR*, and *nfxB*, were resistant to >7.81 $\mu\text{g/mL}$ CPF_X, the MICs of CPF_X in strains 286 and 297 decreased from 15.6 $\mu\text{g/mL}$ to 1.95 or 0.98 $\mu\text{g/mL}$ in the

presence of PA β N, respectively (**Table 2**). A similar effect of PA β N on the MICs of Group III strains, which had a *GyrA* (Thr83Ile) alteration, but no alterations in efflux pump regulators, was observed in 9 of 14 strains. These results suggested that over expression of other active efflux systems or decreased permeability could contribute to CPF_X-resistance in these strains [24].

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