

Mutations in the *gyrA*, *parC*, and *mexR* genes provide functional insights into the fluoroquinolone-resistant *Pseudomonas aeruginosa* isolated in Vietnam

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Introduction: *Pseudomonas aeruginosa* has many mechanisms of resistance to fluoroquinolones. The main mechanism is to change the effect of two enzymes that open the DNA helix – the enzyme DNA gyrase (*gyrA*) and the topoisomerase IV (*parC*). In addition, mutations that render the MexAB-*oprM* pump (*mexR*) dysfunctional, leading to its overexpression, also enhance resistance to fluoroquinolones. In this study, we aim to detect point mutations of *gyrA*, *parC*, and *mexR* genes that are predicted to be associated with fluoroquinolone resistance in 141 fluoroquinolone-resistant clinical isolates of *P. aeruginosa* isolated in Vietnam during 2013–2016.

Methods: We tested minimum inhibitory concentrations (MICs) of fluoroquinolone antibiotics in 141 clinical isolates of *P. aeruginosa* using the VITEK 2 Compact System, followed by PCR assay, to detect and clone the fluoroquinolone resistance-determining region (FRDR) of *gyrA*, *parC*, and *mexR*. Point mutations were analyzed through Sanger sequencing, and the correlation between genetic mutations and phenotypic resistance of 141 clinical isolates was undertaken.

Results: Fluoroquinolone-resistant substitution mutations such as Ile for Thr83 and Met for Thr133 in *gyrA*, Leu for Ser87 in *parC*, and Val for Glu126 in the repressor of *mexR* were mainly detected. Comparative analytical data indicated that amino acid alterations within the *gyrA* and *parC* genes are highly associated with resistance to ciprofloxacin (CIP) and levofloxacin (LEV) in the isolates, whereas alterations in the efflux regulatory *mexR* gene are not highly consistent with resistance in these isolates. Moreover, fluoroquinolone-resistant clinical isolates of *P. aeruginosa* were mainly isolated from pus and sputum specimens.

Conclusion: In clinical isolates of *P. aeruginosa*, a high correlation was observed between MICs of CIP and LEV and alterations in *gyrA* and *parC* genes. However, mutations occurring in *mexR* did not highly correlate with the antibiotic resistance of the bacterium.

Keywords: fluoroquinolone-resistant *Pseudomonas aeruginosa*, fluoroquinolone resistance-determining region, FRDR, mutation, *gyrA*, *parC*, *mexR*

Introduction

Pseudomonas aeruginosa is a gram-negative bacterium and a clinically significant pathogen that causes opportunistic infections – particularly nosocomial infections such as pneumonia, wound infections, sepsis, and gastrointestinal infections.^{1,2} The bacterium is highly resilient in the external environment, causing major infections and severe clinical symptoms due to virulence factors involved in the infective process. Carbapenems, aminoglycosides, and fluoroquinolones are the three classes of important antibiotics that are used as potent agents for the treatment of *Pseudomonas*

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infections.^{3–5} However, excessive use of broad-spectrum antibiotics has resulted in the emergence of highly resistant strains of *P. aeruginosa*. Data from previous studies indicate that multidrug-resistant *P. aeruginosa* strains can tolerate carbapenems, aminoglycosides, and fluoroquinolone drugs. As a result, the potency of these antibiotics against serious infections of *P. aeruginosa* gets diminished.^{4,6–9}

The fluoroquinolone drugs – ciprofloxacin (CIP) and levofloxacin (LEV) – have several targets such as DNA gyrase (type II topoisomerase), topoisomerase IV, and the efflux pump regulatory protein encoded by *mexR*.^{5,10–12} Fluoroquinolone resistance is presented mainly by mutations in the fluoroquinolone resistance-determining region (FRDR) of *gyrA*, which codes for DNA gyrase subunits, and *parC*, which codes for topoisomerase IV subunits. Consequently, mutations within the topoisomerase II (*gyrA*), topoisomerase IV (*parC*), and the efflux regulatory (*mexR*) genes are the main mechanisms of fluoroquinolone resistance in gram-negative bacteria.^{13–15} Amino acid alterations found in *gyrA*, *parC*, and *mexR* are associated with high-level fluoroquinolone resistance in gram-negative bacteria, including *P. aeruginosa*.^{5,11,15–17}

Several studies on fluoroquinolone resistance have revealed that the prevalence of mutations in *gyrA*, *parC*, and *mexR* for *P. aeruginosa* is significantly localized to countries such as South Korea, Taiwan, People's Republic of China, and Japan.^{5,11,16,17} However, data on fluoroquinolone-resistant clinical isolates of *P. aeruginosa* in Vietnam are not well established. In this study, mutations of *gyrA*, *parC*, and *mexR* in fluoroquinolone-resistant clinical isolates of *P. aeruginosa* from Vietnam are investigated, and the correlation between fluoroquinolone minimum inhibitory concentrations (MICs) and point mutations in *gyrA*, *parC*, and *mexR* are compared.

Materials and methods

Clinical isolates of *P. aeruginosa*

We collected 141 clinical isolates of *P. aeruginosa* from 13 national hospitals located throughout Vietnam during 2013–2016. Of these, 21 isolates were susceptible to both CIP and LEV (MIC of CIP ≤ 1 µg/mL, MIC of LEV ≤ 2 µg/mL), 10 isolates had intermediate resistance to both CIP and LEV (MIC of CIP = 2 µg/mL, MIC of LEV = 4 µg/mL), and 110 isolates were highly resistant to both CIP and LEV (MIC of CIP ≥ 4 µg/mL, MIC of LEV ≥ 8 µg/mL). To test the antibiotic susceptibility of all clinical isolates of *P. aeruginosa*, MICs of fluoroquinolone antibiotics were determined at the Bacterial Laboratory (National Hospital for Tropical Diseases, Vietnam) using the VITEK 2 Compact System (BioMerieux, Marcy l'Etoile, France).

DNA extraction and PCR amplification of partial sequences of *gyrA*, *parC*, and *mexR*

Qiagen kits (QIAamp DNA Mini Kit, Qiagen Sciences, Germantown, MD, USA) were used for extraction of total DNA from *P. aeruginosa* isolates in accordance with the manufacturer's protocol. Briefly, cell pellets were resuspended in 200 µL of the given lysis buffer (Buffer AL [>8 mM EDTA and $>0.5\%$ SDS]) containing 20 µL proteinase K, and incubated at 56°C for 30 minutes. Thereafter, 4 µL RNase was added and the sample was treated in accordance with the manufacturer's protocol (for microfuge-scale preparations).

The PCR amplification of *gyrA*, *parC*, and *mexR* of *P. aeruginosa* was done in a 50-µL reaction volume. The PCR mixture was prepared in a 0.2 mL thin-walled tube, containing 25 ng template DNA (total DNA isolated from *P. aeruginosa* isolates), 30 pmol primers, and 0.2 mM dNTP mix (containing equimolar quantities of dATP, dCTP, dGTP, and dTTP). The primers *gyrA*-A1 (5'-GTGTGCTTTATGCCATGAG-3') and *gyrA*-A2 (5'-GGTTTCCTTTTCCAGGTC-3') were used to amplify 287 bp of the FRDR of the *gyrA* gene. The primers *parC*-C1 (5'-CATCGTCTACGCCATGAG-3') and *parC*-C2 (5'-AGCAGCACCTCGGAATAG-3') were used to amplify 267 bp of the FRDR of the *parC* gene. The primers *mexR*-R1 (5'-CTGGATCAACCACATTTACA-3') and *mexR*-R2 (5'-CTTCGAAAAGAATGTTCTTAAA-3') were used to amplify 503 bp of the FRDR of the *mexR* gene.¹⁰ The final volume was made up with 10X *Pyrococcus furiosus* (Pfu) buffer and distilled water. In addition, 1 µL Pfu DNA polymerase was added to the reaction mixture after denaturation at 100°C for 2 minutes. The PCR was carried out in a Genius PCR Thermal Cycler (Techne) with a 105°C heated lid. The amplification parameters included 35 cycles of initial heat activation at 95°C for 45 seconds, annealing at 51°C for 30 seconds, elongation at 72°C for 30 seconds, and a final 72°C elongation step for 10 minutes.

Sanger sequencing and partial sequence analysis of *gyrA*, *parC*, and *mexR*

Dideoxy sequencing of PCR products was undertaken using BigDye™ Terminator Chemistry v. 3.1 (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions. As described previously, the forward and reverse primers for *gyrA*, *parC*, and *mexR* were used as sequencing primers using the ABI 3130 Bio-analyzer (Applied Biosystems).

Original nucleotide sequences of *gyrA*, *parC*, and *mexR* were obtained from the GenBank nucleotide sequence database with accession numbers L29147, AB003428, and U23763, respectively. Target regions of these three genes were analyzed for all 141 clinical isolates of *P. aeruginosa*, using ATGC 7.2 (supported by the Japanese ACC program). The DNA sequences were then compared with original nucleotide sequences of *gyrA* (accession number L29147), *parC* (accession number AB003428), and *mexR* (accession number U23763) genes in the GenBank data for wild-type *P. aeruginosa*.

Results

Antibiotic resistance of *P. aeruginosa* to CIP and LEV

In total, 141 clinical isolates of *P. aeruginosa* were collected from different specimens and all of these isolates were then tested for antimicrobial susceptibility to both CIP and LEV. Considering the susceptibility breakpoints suggested by the Clinical and Laboratory Standard Institute in 2015 (CLSI 2015), the majority of isolates (134/141 strains) were mainly collected from pus, sputum, urine, and blood – of which 21 strains were found to be susceptible, 10 strains were intermediate, and 102 strains were highly resistant to both CIP and LEV. In addition, the minority of clinical isolates (7/141 strains) were collected from peritoneal fluid, bronchial lavage, and cerebrospinal fluid, with no strain found to be susceptible to CIP and LEV – all of these isolates were revealed to be highly resistant to both CIP and LEV (Table 1).

Amino acid alterations in *gyrA*, *parC*, and *mexR*

Partial DNA fragments of *gyrA*, *parC*, and *mexR* were amplified using PCR, and nucleotide sequences of all the

PCR-amplified fragments were determined using Sanger sequencing. Alterations found in 141 clinical isolates of *P. aeruginosa* were classified into eight groups according to the pattern of amino acid changes detected in *gyrA*, *parC*, and *mexR* as follows (Table 2): Group I had no mutations in all three genes; Group II mutations only occurred in *gyrA*; Group III mutations only occurred in *parC*; Group IV mutations only occurred in *mexR*; Group V mutations occurred in both *gyrA* and *parC*; Group VI mutations appeared in all three genes; Group VII mutations occurred in *gyrA* and *mexR*; and, finally, Group VIII mutations appear in both *parC* and *mexR*.

Mutations found in the topoisomerase II, topoisomerase IV, and the efflux regulatory *mexR* genes of *P. aeruginosa* isolates were then compared with the corresponding nucleotide sequences of the *gyrA*, *parC*, and *mexR* genes of *P. aeruginosa* ATCC 27896. Amino acid replacements found in *gyrA*, *parC*, and *mexR* are listed in Table 2. Of these eight mutation groups, the majority of the *P. aeruginosa* clinical isolates, resistant to CIP and LEV, were found in groups V (48 isolates), II (28 isolates), VII (10 isolates), and III (09 isolates). The results showed that some of the *P. aeruginosa* clinical isolates possessing a single amino acid substitution were either susceptible or intermediately resistant to CIP and LEV. Susceptible *P. aeruginosa* isolates were found only in groups I (11 isolates) and IV (10 isolates), whereas intermediately resistant isolates were found in groups I (five isolates), II (four isolates), and IV (one isolate). In addition, the results showed that mutations – either only in *gyrA* or in both *gyrA* and *parC* – were most frequently found in the *P. aeruginosa* isolates resistant to CIP and LEV. In contrast, mutations found in *mexR* were consistent with only a small proportion of the *P. aeruginosa* isolates resistant to CIP and LEV.

Clinical isolates of *P. aeruginosa*, sorted by amino acid alterations found in the target genes (*gyrA*, *parC*, and *mexR*),

Table 1 Results of a screen for fluoroquinolone susceptibility from 141 clinical isolates of *P. aeruginosa*

Types of specimen	Total isolates	No. of isolates with fluoroquinolone susceptibility		
		Susceptible (CIP≤1, LEV≤2)	Intermediate (CIP=2, LEV=4)	Resistant (CIP≥4, LEV≥8)
		No.	No.	No.
Pus	48	6	1	41
Sputum	59	12	6	41
Urine	17	2	1	14
Blood	10	1	2	7
Peritoneal fluid	3	0	0	3
Bronchial lavage	2	0	0	2
Cerebrospinal fluid	2	0	0	2
Total	141	21	10	110

Abbreviations: CIP, ciprofloxacin; LEV, levofloxacin.

and the fluoroquinolone susceptibility pattern of clinical isolates of *P. aeruginosa* are shown in Tables 2 and 3. The comparison of the DNA sequences of *P. aeruginosa* ATCC 27853 with the *gyrA* DNA sequences in 141 *P. aeruginosa* isolates showed that an alteration in the *gyrA* gene at codon 83 (Thr83→Ile) was found in 76 clinical isolates of *P. aeruginosa*, harboring *gyrA* mutations (groups II, V, VI, and VII), as shown in several studies. However, a novel single-nucleotide mutation was also found in *gyrA* at codon 133 (Thr133→Met) in a total of 51 clinical isolates of *P. aeruginosa*. Further analysis of the data revealed that a total of 93 fluoroquinolone-resistant isolates had mutations in *gyrA* – of which, 47 isolates possessed only a single-nucleotide mutation at codon 83 (Thr83→Ile), 19 isolates were found to have a novel single-nucleotide alteration at codon 133 (Thr133→Met), and the remaining isolates possessed double mutations in *gyrA* (Thr83→Ile and Thr133→Met).

The main amino acid alterations found in *parC* mainly occurred at codon 87 (Ser87→Leu), as reported in previous studies. Moreover, other minor novel mutations were determined in *parC* at codon 141 (Ser141→His) in one isolate and (Ser141→Thr) in another isolate (Table 2). Furthermore, results revealed that the single amino acid alteration in *parC* at codon 87 (Ser87→Leu) was observed in 66 fluoroquinolone-resistant clinical isolates, and a single-nucleotide mutation was found at codon 141 (Ser141→Thr), whereas double mutations at codons 87 and 141 (Ser87→Leu and Ser141→His) were detected in another clinical isolate of *P. aeruginosa* (Table 3).

Common mutations in the efflux regulatory *mexR* gene were mainly detected at codon 126 (Glu126→Val). Other alterations in *mexR* for fluoroquinolone resistance were observed only in the fluoroquinolone-resistant isolates at codon 25 (Ile25→Thr and Ile25→Asn); moreover, these mutations were mentioned in previous studies. None of the mutations found in these clinical isolates correlated with low and intermediate MIC values of the fluoroquinolones tested. However, the single amino acid substitution occurring at codon 126 (Glu126→Val) of *mexR* was found in 16 fluoroquinolone-resistant isolates, 10 susceptible isolates, and only one intermediately resistant isolate of *P. aeruginosa* (Tables 2 and 3).

Drug resistance of *P. aeruginosa* clinical isolates to CIP and LEV in relation to *gyrA*, *parC*, and *mexR* mutations and distribution of specimen types

Results obtained from mutation data of *gyrA*, *parC*, and *mexR* were compared with the antibiotic susceptibility data of *P. aeruginosa* clinical isolates and distribution of specimen types among these isolates in order to elucidate the correlation between amino acid alterations and phenotypic resistance to the fluoroquinolone drugs (CIP and LEV), and original specimens of isolations are shown in Table 3. In the 141 *P. aeruginosa* isolates, no amino acid alteration in *gyrA* was identified in susceptible isolates. Mutations of *gyrA* were

Table 2 Comparison of amino acid alterations in *gyrA*, *parC*, and *mexR* with fluoroquinolone susceptibility of *P. aeruginosa* isolates

Group of mutation	Amino acid alteration of						No. isolates with fluoroquinolone susceptibility		
	<i>gyrA</i>		<i>parC</i>		<i>mexR</i>		Susceptible	Intermediate	Resistant
	83	133	87	141	25	126			
ATCC 27853	Thr (ACC)	Thr (ACG)	Ser (TCG)	Ser (AGC)	Ile (ATC)	Glu (GAG)	—	—	—
I	—	—	—	—	—	—	11	05	02
II	Ile (ATC)	Met (ATG)	—	—	—	—	0	04	28
III	—	—	Leu (TTG)	His (CAC) Thr (ACC)	—	—	0	0	09
IV	—	—	—	—	Thr (ACC) Asn (AAC)	Val (GTG)	10	01	02
V	Ile (ATC)	Met (ATG)	Leu (TTG)	His (CAC) Thr (ACC)	—	—	0	0	48
VI	Ile (ATC)	Met (ATG)	Leu (TTG)	His (CAC) Thr (ACC)	Thr (ACC) Asn (AAC)	Val (GTG)	0	0	05
VII	Ile (ATC)	Met (ATG)	—	—	Thr (ACC) Asn (AAC)	Val (GTG)	0	0	10
VIII	—	—	Leu (TTG)	His (CAC) Thr (ACC)	Thr (ACC) Asn (AAC)	Val (GTG)	0	0	06
Total							21	10	110

found only in the resistant isolates. Moreover, the comparison indicated that mutations occurring in *gyrA* were consistent with the phenotypic resistance of most *P. aeruginosa* isolates to fluoroquinolone drugs. In addition, mutations occurring in *parC* were reported to correlate well with fluoroquinolone-resistant clinical isolates. However, none of the mutations in *parC* was detected in susceptible isolates of *P. aeruginosa*. In contrast, amino acid alterations of *mexR* were found in both susceptible and resistant isolates. Furthermore, the results indicated that differences in the relative resistance of *P. aeruginosa* clinical isolates to fluoroquinolone drugs were indistinguishable.

Of the 141 clinical isolates of *P. aeruginosa* collected during 2013–2016 from 13 national hospitals across nine cities in Vietnam, most of the *P. aeruginosa* strains were isolated from different types of specimen (Table 3), and the highest numbers of isolates (59 and 48) were obtained from sputum and pus, respectively, whereas only 2–17 isolates were collected from cerebrospinal fluid, bronchial lavage, peritoneal fluid, urine, and blood. Data showed that amino acid substitutions at codons 83 and 133 of *gyrA* and at codons 87 and 141 of *parC* were most frequently identified in all types of specimen – excepting mutations in *parC* of the resistant clinical isolates collected from cerebrospinal fluid and bronchial lavage. In addition, amino acid substitutions at codon 25 of *mexR* were mainly detected in highly resistant isolates of *P. aeruginosa*, whereas the mutation at codon 126 of *mexR* was detected in both susceptible and resistant isolates, and it was not detected in all specimen types. The correlation between mutations occurring in *mexR* and the antibiotic resistance of clinical isolates remained inconsistent.

Discussion

Single amino acid alteration in target genes is one of the common pathways for antibiotic resistance in bacteria.¹⁸ Previous studies have indicated that molecular resistance mechanisms of *P. aeruginosa* to fluoroquinolone drugs are mainly due to mutations in the FRDR of *gyrA*, *parC*, and *mexR*.^{5,10–12} Several known mutations in the FRDR of these genes have been described in previous studies such as a single-nucleotide mutation at codon 83 (Thr83→Ile) of *gyrA*, codon 87 (Ser87→Leu) of *parC*, and codon 126 (Glu126→Val) of *mexR*, in which most studies demonstrated that clinical isolates of *P. aeruginosa* comprising mutations at codon 83 (*gyrA*) and 87 (*parC*) are highly associated with fluoroquinolone resistance.^{5,11,12} In contrast, other studies that reported mutations occur at codon 126 of *mexR* did not show correlation with the resistance to fluoroquinolone drugs.¹⁰

In this study, the FRDR of these genes were amplified by PCR, and the full nucleotide sequences of these PCR-amplified fragments demonstrated that the nucleotide sequences are intact. Our results showed several point mutations in all three genes and that most of these mutations were found in fluoroquinolone-resistant clinical isolates of *P. aeruginosa*. In contrast, fewer mutations were detected in the susceptible clinical isolates, including a mutation at codon 126 of *mexR*. These results indicated that amino acid alterations within the DNA gyrase (*gyrA*) and topoisomerase IV (*parC*) genes are highly associated with resistance to CIP and LEV in the isolates, whereas alterations in the efflux regulatory *mexR* gene are not consistent with resistance to CIP and LEV in these isolates. In addition, this study found that mutations occurring in *gyrA* are consistent with highly fluoroquinolone-resistant *P. aeruginosa* isolates. Moreover, amino acid alterations, frequently found in *parC*, show a high correlation with fluoroquinolone resistance in most of the resistant *P. aeruginosa* isolates. These findings indicate that DNA gyrase and type II topoisomerase enzymes may be primary physiological targets of fluoroquinolone drugs, thereby establishing alterations in *gyrA*, *parC*, and other target enzymes. Although mutations are observed in *gyrA*, *parC*, and *mexR*, the accumulation of mutations in the *mexR* gene does not correlate with the resistance phenotype of *P. aeruginosa* isolates. In addition, our data revealed that the accumulation of mutations in the *gyrA* gene accompanied by the simultaneous presence of mutations in the *parC* gene correlate with the development of high-level fluoroquinolone resistance in clinical isolates of *P. aeruginosa* (Table 2). Moreover, *P. aeruginosa* possesses a low-permeability outer membrane, leading to very slow antibiotic diffusion.¹⁹ Therefore, active efflux systems play an important role in decreasing intracellular concentrations of antimicrobial agents.^{20,21} Previous studies indicated there are several repressors involved in controlling expression of *mexAP-oprM* operon in *P. aeruginosa*, including *mexR*, *nalD*, *nalC*, and *cpxR*.²² Mutations in these genes code for repressor proteins in order to generate defective form, typically leading to enhanced multidrug resistance in *P. aeruginosa* due to overexpression of the *mexAB-oprM* operon. In this study, mutations in the *mexR* gene are not only found in resistant isolates but also appear in susceptible strains, indicating that single or double alterations occurring in the *mexR* gene may not change the antibiotic susceptibility of *P. aeruginosa* clinical isolates; moreover, this suggests that these mutations may not alter the *mexR* binding site in the promoter region but can introduce resistance when co-occurring with mutations in other promoters such as *nalD*, *nalC*, or *cpxR*.

Table 3 Source of isolation and amino acid mutations of *gyrA*, *parC*, and *mexR* in correlation with fluoroquinolone susceptibility of clinical isolates of *P. aeruginosa*

Type of specimen	No. of isolates	No. of isolates carrying mutations (fluoroquinolone susceptibility of both ciprofloxacin and levofloxacin)							
		Susceptible						Intermediate	
		<i>gyrA</i>		<i>parC</i>		<i>mexR</i>		<i>gyrA</i>	
		83	133	87	141	25	126	83	133
		Thr (ACC)	Thr (ACG)	Ser (TCG)	Ser (AGC)	Ile (ATC)	Glu (GAG)	Thr (ACC)	Thr (ACG)
Pus	48	—	—	—	—	—	Val (2)	—	—
Sputum	59	—	—	—	—	—	Val (7)	Ile (2)	Met (3)
Urine	17	—	—	—	—	—	Val (1)	—	—
Blood	10	—	—	—	—	—	—	Ile (1)	—
Peritoneal fluid	3	—	—	—	—	—	—	—	—
Bronchial lavage	2	—	—	—	—	—	—	—	—
Cerebrospinal fluid	2	—	—	—	—	—	—	—	—
Total	141						10	3	3

Previous studies have reported that major amino acid alterations in *gyrA* frequently occur at codon 83 (Thr83→Ile).^{5,10,11,23} However, novel mutations in *gyrA* at codon 133 (Thr133→Met) were first reported in this study. In addition, minor mutations are found in *gyrA* at codon 87 (Asp87→Asn, Asp87→Gly, Asp87→His, and Asp87→Tyr) and at codon 106 (Glu106→Leu) as described in a previous study.¹¹ Our data indicate that amino acid substitutions are frequently detected in *gyrA* at codons 83 and 133 (Thr83→Ile and Thr133→Met), and most of the *P. aeruginosa* clinical isolates possessing these mutations are consistent with fluoroquinolone resistance. Furthermore, the results showed that mutations in *gyrA* occurring either at codon 87 or 133 were both associated with highly resistant isolates. The finding of a novel mutation at codon 133 in *gyrA* suggests that genetic modification of antibiotic resistant gene of clinical strains of *P. aeruginosa* isolated in Vietnam was considerably different from clinical isolates in other countries. Similar amino acid substitutions frequently observed in *parC* at codon 87 (Ser87→Leu) are always present only in the resistant isolates, as reported in several studies,^{5,11} and other minor novel mutations detected at codon 141 (Ser141→His and Ser141→Thr). Although mutations at codon 141 in *parC* were rare (presented in a few clinical isolates), the present data show that *P. aeruginosa* clinical isolates possessing these mutations are consistent with fluoroquinolone resistance.

Interestingly, results of this study showed that the number of clinical isolates of *P. aeruginosa* with amino acid alterations in both *gyrA* and *parC* was higher than that of strains carrying single-nucleotide mutations in *gyrA*, as well as that

amino acid alterations in *parC* account for a small proportion of clinical isolates. This finding indicates that *gyrA* is the primary target for fluoroquinolones, and that point mutations occurring in *parC* have been shown to enhance the level of drug resistance of the isolates.

Although the number of clinical isolates carrying mutations in *gyrA* is higher than that in *parC*, the relevant correlation between amino acid mutations in the *gyrA* and the *parC* genes and fluoroquinolone resistance in the *P. aeruginosa* isolates cannot be demonstrated. Major amino acid alterations in the *mexR* gene are mainly identified at codon 126 (Glu126→Val), and other minor mutations are detected at codon 25 (Ile25→Thr and Ile25→Asn); these mutations were reported in previous investigations. Earlier studies have shown that mutations in *mexR* do not show any drug resistance. Thus, it maybe speculated that mutations at codons 25 and 126 in *mexR* alone may not change the antibiotic susceptibility of fluoroquinolone-resistant clinical isolates of *P. aeruginosa*, but may promote the levels of resistance if accompanied by mutations that occur in other repressors such as nalA, nalD, or CpxR. Moreover, a combination of alterations in *mexR* and *gyrA* or in *mexR* and *parC* does not improve fluoroquinolone resistance, suggesting that mutations co-occurring in *mexR* and *gyrA* or in *mexR* and *parC* do not always indicate resistance to fluoroquinolones. The study, while trying to identify novel mutations consistent with fluoroquinolone resistance, recognizes that the alteration frequently found at codon 133 of *gyrA* (Thr133→Met) is highly correlated with fluoroquinolone resistance. Further, minor novel alterations were found in *parC* and *mexR*, but

				Resistant					
<i>parC</i>		<i>mexR</i>		<i>gyrA</i>		<i>parC</i>		<i>mexR</i>	
87	141	25	126	83	133	87	141	25	126
Ser (TCG)	Ser (AGC)	Ile (ATC)	Glu (GAG)	Thr (ACC)	Thr (ACG)	Ser (TCG)	Ser (AGC)	Ile (ATC)	Glu (GAG)
—	—	—	Val (1)	Ile (28)	Met (13)	Leu (21)	His (1)	Asn (4)	Val (6)
—	—	—	—	Ile (26)	Met (23)	Leu (29)	Thr (1)	Thr (2)	Val (3)
—	—	—	—	Ile (11)	Met (6)	Leu (9)	—	Asn (2)	Val (3)
—	—	—	—	Ile (3)	Met (2)	Leu (5)	—	Lys (1)	Val (1)
—	—	—	—	Ile (3)	Met (2)	Leu (3)	—	Leu (1)	Val (1)
—	—	—	—	—	Met (2)	—	—	Pro (1)	Val (1)
—	—	—	—	Ile (2)	—	—	—	—	Val (1)
—	—	—	1	73	48	67	2	11	16

they do not support any evidence consistent with fluoroquinolone resistance.

A total of 141 clinical isolates of *P. aeruginosa* were collected from 13 independent hospitals across nine cities in Vietnam; most of these strains were mainly isolated from pus and sputum specimens, but only a few isolates were collected from cerebrospinal fluid, bronchial lavage, peritoneal fluid, urine, and blood, with mutations found most frequently at codons 83 and 133 in *gyrA*, at codon 87 in *parC*, and at codons 25 and 126 in *mexR*. It is now known that *P. aeruginosa* is an opportunistic bacterium and is found everywhere in the soil, water, and the air or on the human body, especially in the hospital environment (nosocomial infection). Combined with favorable conditions such as wound, incision, or penetration into the respiratory system in patients, they then have a favorable environment to develop and cause disease. In addition, *P. aeruginosa* strains were mainly isolated from patients in the Emergency Department and General Surgery Department. The reason for this is that most patients in the departments normally have very serious medical conditions, high mortality risk, and have to face treatment interventions such as surgery, mechanical ventilation, endotracheal intubation, surgical drainage, and nasogastric intubation – all with a very high risk of infection. Therefore, the rate of resistance clinical isolates of *P. aeruginosa* collected from pus and sputum is highest in clinical specimens.

In conclusion, most of the clinical isolates of *P. aeruginosa* collected in Vietnam were mainly isolated from pus and sputum specimens, with amino acid alterations frequently occurring at codon 83 (Thr83→Ile and Thr133→Met) in

gyrA and at codon 87 (Ser87→Leu) in *parC* consistent with high-level resistance to CIP and LEV in clinical isolates of *P. aeruginosa* isolated from Vietnam, whereas the mutation detected at codon 126 (Glu126→Val) in *mexR* does not correlate with the antibiotic resistance of the bacterium. Substitution mutations co-occurring in *gyrA* and *parC* show higher-level fluoroquinolone resistance compared with single-nucleotide mutations occurring in *gyrA*.

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Disclosure

The authors report no conflicts of interest in this work.

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