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# Correlation Between Virulence Genotype and Fluoroquinolone Resistance in Carbapenem-Resistant *Pseudomonas aeruginosa*

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**Background:** *Pseudomonas aeruginosa* is a clinically important pathogen that causes opportunistic infections and nosocomial outbreaks. Recently, the type III secretion system (TTSS) has been shown to play an important role in the virulence of *P. aeruginosa*. ExoU, in particular, has the greatest impact on disease severity. We examined the relationship among the TTSS effector genotype (*exoS* and *exoU*), fluoroquinolone resistance, and target site mutations in 66 carbapenem-resistant *P. aeruginosa* strains.

**Methods:** Sixty-six carbapenem-resistant *P. aeruginosa* strains were collected from patients in a university hospital in Daejeon, Korea, from January 2008 to May 2012. Minimum inhibitory concentrations (MICs) of fluoroquinolones (ciprofloxacin and levofloxacin) were determined by using the agar dilution method. We used PCR and sequencing to determine the TTSS effector genotype and quinolone resistance-determining regions (QRDRs) of the respective target genes *gyrA, gyrB, parC*, and *parE*.

**Results:** A higher proportion of exoU+ strains were fluoroquinolone-resistant than exoS+ strains (93.2%, 41/44 vs. 45.0%, 9/20; $P \leq 0.0001$). Additionally, *exoU*+ strains were more likely to carry combined mutations than *exoS*+ strains (97.6%, 40/41 vs. 70%, 7/10; $P = 0.021$), and MIC increased as the number of active mutations increased.

**Conclusions:** The recent overuse of fluoroquinolone has led to both increased resistance and enhanced virulence of carbapenem-resistant *P. aeruginosa*. These data indicate a specific relationship among *exoU* genotype, fluoroquinolone resistance, and resistance-conferring mutations.

**Key Words:** TTSS effector genotype, *exoS*, *exoU*, Fluoroquinolone resistance

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## INTRODUCTION

*Pseudomonas aeruginosa* is an opportunistic pathogen that can cause a wide spectrum of acute infections in hospitalized and immunocompromised patients as well as chronic lung infections in patients with cystic fibrosis. The pathogenicity of *P. aeruginosa* is the result of numerous secreted virulence factors. Among the multitude of virulence determinants of *Pseudomonas*, the type III secretion system (TTSS) is considered an important contributor to the cytotoxicity and the invasion process [1-3]. This system allows these bacteria to directly inject effector proteins into the host cell, where they subvert host cell defense and signaling systems [4]. Four effector proteins have been identified: ExoU, a phospholipase; ExoY, an adenylate cyclase; and ExoS and ExoT, which are bifunctional proteins. ExoT and ExoY are encoded by almost all strains, though not all strains

produce functional ExoY because of the presence of frameshift mutations. ExoS and ExoU are variably encoded genes depending on the disease site or background. In particular, ExoT and ExoY have a minor effect on virulence, whereas ExoS and ExoU contribute greatly to the pathogenesis [5-8]. In a mouse model of acute pneumonia, secretion of ExoU has the greatest impact on mortality, bacterial persistence, and dissemination in the lungs, followed by secretion of ExoS and ExoT [1, 9-11].

In recent years, the incidence of carbapenem-resistant *P. aeruginosa* strains has increased worldwide. The fluoroquinolone antimicrobials are the most potent agents for treatment of carbapenem-resistant *P. aeruginosa* infections. Nonetheless, a number of carbapenem-resistant *P. aeruginosa* strains have exhibited increased resistance to fluoroquinolone, and these strains have emerged rapidly in South Korea [12-15]. According to the study by Lee *et al*. [16], 57 carbapenem-resistant *P. aeruginosa* strains isolated in 10 South Korean hospitals showed strong resistance to ciprofloxacin (59.6%). On the other hand, there are few studies on the resistance to fluoroquinolones and on the prevalence of alterations in topoisomerase II and IV in carbapenem-resistant *P. aeruginosa* strains collected in South Korea.

Alterations in the quinolone resistance-determining regions (QRDRs) within topoisomerase II (GyrA and GyrB subunits) and topoisomerase IV (ParC and ParE subunits) are the major contributors to fluoroquinolone resistance in gram-negative bacteria [17, 18]. In particular, amino acid alterations in the GyrA and ParC subunits play a major role in conferring strong fluoroquinolone resistance to gram-negative bacteria such as *Escherichia coli, Acinetobacter baumannii*, and *P. aeruginosa* [19-21].

The aim of this study was to assess a possible correlation between the TTSS effector genotype (*exoS* vs. *exoU*) and fluoroquinolone resistance in carbapenem-resistant *P. aeruginosa* strains. In addition, we compared the prevalence and degree of fluoroquinolone resistance with respect to the alterations in topoisomerases II and IV.

## METHODS

### 1. Isolation and identification of bacteria

In total, 66 consecutive and nonduplicated carbapenem-resistant *P. aeruginosa* strains were obtained from patients in a university hospital in Daejeon, Korea, from January 2008 to May 2012. The strains were identified with the Vitek 2 automated ID system (BioMérieux, Hazelwood, MO, USA), and carbapenem-resistant *P. aeruginosa* strains were selected on the basis of resistance to imipenem and meropenem.

### 2. Testing of susceptibility to antimicrobials

Using the agar dilution method and CLSI guidelines, antimicrobial susceptibility tests were performed by determining the minimum inhibitory concentration (MIC) [22]. The susceptibility of *P. aeruginosa* to the following antimicrobial agents was tested: ciprofloxacin and levofloxacin (Sigma Chemical Co., St. Louis, MO, USA). The interpretation of susceptibility was performed according to the CLSI breakpoints. *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as quality controls.

### 3. Multilocus sequence typing (MLST)

MLST was carried out according to the methods described on the website of the *P. aeruginosa* MLST database (http://pubmlst.org/paeruginosa/). Genomic DNA was extracted from the clinical strains by using the Genomic DNA Prep Kit (SolGent, Daejeon, Korea). PCR was performed by using 50 ng of template DNA (genomic DNA) using 2.5 μL 10× *Taq* buffer, 0.5 μL of 10 mM dNTP mix, 20 pmol of each primer, and 0.7 U Taq DNA polymerase (SolGent) in a total volume of 25 μL. Internal fragments of 7 housekeeping genes (*acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA*, and *trpE*) were amplified by using a GeneAmp PCR System 9600 (Perkin-Elmer Cetus Corp., Norwalk, CT, USA). The reaction mixture was denatured for 1 min at 96℃, and then subjected to 30 cycles of 1 min at 96℃, 1 min at 55℃, and 1 min at 72℃, with a final elongation step at 72℃ for 10 min. The amplicons were purified by using a PCR purification kit (SolGent) and were then sequenced by using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 3730XL DNA analyzer (Applied Biosystems). The nucleotide sequences of each of the 7 housekeeping genes were compared with the sequences stored in the MLST database to determine the allelic numbers and sequence types (STs).

### 4. Analysis of TTSS effector genotypes (*exoS* and *exoU*) and sequencing of target site mutations

To detect the TTSS effector genotype (*exoS* and *exoU*) and the QRDRs of the *gyrA*, *gyrB*, *parC*, and *parE* genes, genomic DNA of all carbapenem-resistant *P. aeruginosa* strains was amplified by means of PCR as described previously [23, 24]. The resulting DNA sequences were compared with published nucleotide sequences from the GenBank database (Accession numbers: L29417 [*gyrA*], AE004440 [*gyrB*], AB003428 [*parC*], and AB003429 [*parE*]) by using the BLAST search available on the website of the National Center for Biotechnology Information (Bethesda, MD, USA).

### 5. Statistical analysis

All 66 strains were grouped into specific TTSS effector genotypes on the basis of their degree of resistance to fluoroquinolones, as indicated by MIC and by the number and type of target site mutations. Chi-square and Fisher's exact tests were used where appropriate. A difference with a *P* value of ≤0.05 was considered statistically significant.

## RESULTS

### 1. TTSS effector genotypes and results of MLST

Among the 66 carbapenem-resistant *P. aeruginosa* strains, the site of isolation was known for 65 strains except 1 strain, with most being obtained from sputum (64.6%), followed by urine (26.2%), wounds (4.6%), pus (3.1%), and bile (1.5%; Table 1).

A TTSS effector genotype, according to the PCR results, for the *exoS* and *exoU* genes revealed that all 66 clinical strains carried *exoS* and/or *exoU* (Table 1). Overall, more than a half of the 66 strains (66.7%, 44/66) were *exoU*+, while 30.3% (20/66) were *exoS*+; 2 strains were positive for both effector genes (3.0%). The 44 *exoU*+ strains tested were classified into 3 STs (ST235, 34 strains; ST357, 9 strains; and ST313, 1 strain), and the 20 *exoS*+ strains analyzed were assigned to 10 STs (ST245, 6 strains; ST244, 5 strains; ST139, 2 strains; ST155, ST260, ST274, ST612, ST645, ST654, and ST1154: 1 strain each).

### 2. The prevalence and degree of fluoroquinolone resistance

All 66 carbapenem-resistant *P. aeruginosa* strains were tested for susceptibility to 2 fluoroquinolone agents. Fifty-two strains (78.8%) exhibited nonsusceptibility to fluoroquinolones, with a ciprofloxacin $MIC_{50}$ of 64 μg/mL and levofloxacin $MIC_{50}$ of 32 μg/mL, whereas only 21.2% (14 strains) showed susceptibility (ciprofloxacin: MIC range, <0.25-1.00 μg/mL; levofloxacin: MIC range, <0.25-1.00 μg/mL). The 52 fluoroquinolone nonsusceptible strains were grouped into 6 STs (ST235, 34 strains; ST357, 7 strains; ST245, 6 strains; ST244, 2 strains; ST654, 1 strain; and ST111, 2 strains). Of these, the 41 *exoU*+ strains were grouped into ST235 and ST357 (Table 2). Notably, *exoU*+ strains were significantly associated with fluoroquinolone nonsusceptibility compared with *exoS*+ strains (93.2%, 41/44 vs. 45.0%,

**Table 1.** Distribution of the sequence types among 66 carbapenem-resistant *Pseudomonas aeruginosa* strains according to disease sites and TTSS genes

| Source | *exoU* (n=44) | | *exoS* (n=20) | | *exoU* and *exoS* (n=2) | |
|---|---|---|---|---|---|---|
| | N (%) | ST (N) | N (%) | ST (N) | N (%) | ST (N) |
| Sputum | 25 (56.8) | 235 (15), 357 (9)<br>313 (1) | 16 (80.0) | 245 (6), 244 (4)<br>139 (1), 155 (1)<br>260 (1), 274 (1)<br>612 (1), 654 (1) | 1 (50.0) | 111 (1) |
| Urine | 15 (34.1) | 235 (15) | 2 (10.0) | 139 (1), 244 (1) | | |
| Wounds | 3 (6.8) | 235 (3) | | | | |
| Pus | | | 2 (10.0) | 645 (1), 1154 (1) | | |
| Bile | | | | | 1 (50.0) | 111 (1) |
| Unknown | 1 (2.3) | 235 (1) | | | | |

Abbreviations: TTSS, the type III secretion system; N, number of strains; ST, sequence type.

**Table 2.** Prevalence of fluoroquinolone (FQ) resistance and multilocus sequence typing analysis in *exoU*(+) and *exoS*(+) *Pseudomonas aeruginosa* strains

| | *exoU* (n=44) | | *exoS* (n=20) | | *exoU* and *exoS* (n=2) | |
|---|---|---|---|---|---|---|
| | N (%) | ST (N) | N (%) | ST (N) | N (%) | ST (N) |
| FQ nonsusceptible | 41 (93.2) | 235 (34), 357 (7) | 9 (45.0) | 245 (6), 244 (2), 654 (1) | 2 (100) | 111 (2) |
| FQ susceptible | 3 (6.8) | 357 (2), 313 (1) | 11 (55.0) | 244 (3), 139 (2), 155 (1),<br>260 (1), 274 (1), 612 (1),<br>645 (1), 1154 (1) | | |

Abbreviations: N, number of strains; ST, sequence type.


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9/20; $P \leq 0.0001$).

### 3. Target site mutations

Of the 66 carbapenem-resistant strains, 53 (80.3%) displayed QRDR mutations in 1 or more of the genes analyzed (Table 3). Fifty-two (98.1%) of the 53 mutants possessed a substitution Ile83→Thr in *gyrA*. This amino acid change was the most frequently identified substitution among strains with active mutations. Of the 53 strains, 4 strains (ciprofloxacin: MIC range, 1-8 μg/mL; levofloxacin: MIC range, 2-32 μg/mL) had only a single mutation in *gyrA*. The remaining strains (ciprofloxacin MIC range 16-256 μg/mL, levofloxacin MIC range 32-256 μg/mL) had additional substitutions at other positions in *gyrB*, *parC*, and *parE*.

Six strains had alterations in *gyrB* as follows: Glu 468→Asp (5 strains), Ser 466→Phe (1 strain). Glu 468→Asp was a dominant alteration in *gyrB*, and strains showing 3 alterations, including Glu 468→Asp, had a high level of resistance to ciprofloxacin (MICs, 64-128 μg/mL) and levofloxacin (MICs, 64-128 μg/mL). Furthermore, the change Ser 466→Phe conferred even stronger resistance to ciprofloxacin and levofloxacin (MIC, 256 μg/

**Table 3.** Amino acid substitutions in quinolone resistance-determining regions of GyrA, GyrB, ParC, and ParE subunits in 52 fluoroquinolone-nonsusceptible *Pseudomonas aeruginosa* strains

| ST (N) | N of isolates | MIC range | | Amino acid substitution | | | |
|---|---|---|---|---|---|---|---|
| | | CIP | LEV | GyrA | GyrB | ParC | ParE |
| 235 (34) | 28 | 64-128 | 32-128 | Thr83Ile | | Ser87Leu | |
| | 3 | 64-128 | 64-128 | Thr83Ile | Glu468Asp | Ser87Leu | |
| | 1 | 8 | 32 | Thr83Ile | | | |
| | 1 | 64 | 32 | Thr83Ile | | | Ala503Val |
| | 1 | 256 | 256 | Thr83Ile | Ser466Phe | Ser87Leu | |
| 357 (7) | 7 | 64 | 32 | Thr83Ile | | Ser87Trp | |
| 245 (6) | 3 | 16-64 | 32 | Thr83Ile | | Ser87Leu | |
| | 2 | 2 | 8 | Thr83Ile | | | |
| | 1 | 16 | 32 | Thr83Ile | | | Asp419Asn |
| 244 (2) | 1 | 64 | 32 | Thr83Ile | | Ser87Leu | |
| | 1 | 128 | 128 | Thr83Ile | Glu468Asp | Ser87Leu | |
| 654 (1) | 1 | 64 | 32 | Thr83Ile | | Ser87Leu | |
| 111 (2) | 1 | 64 | 32 | Thr83Ile | | Ser87Leu | |
| | 1 | 128 | 64 | Thr83Ile | Glu468Asp | Ser87Leu | |

Abbreviation: MIC, minimum inhibitory concentration.

**Table 4.** Comparison of target site mutations (TSMs) in *exoU*(+) and *exoS*(+) *Pseudomonas aeruginosa* strains

| Target protein | Amino acid substitution | *exoU* (n=44) (%) | *exoS* (n=20) (%) | *exoU* & *exoS* (n=2) (%) |
|---|---|---|---|---|
| No TSM | | 3/44 (6.8) | 10/20 (50.0) | |
| Single TSM | | 1/44 (2.2) | 3/20 (15.0) | |
| GyrA | Thr83Ile | 1 | 2 | |
| | Thr83Ala | | 1 | |
| Combined TSMs | | 40/44 (90.9) | 7/20 (35.0) | 2/2 (100.0) |
| GyrA+ParC | Thr83Ile+Ser87Leu | 28 | 5 | 1 |
| | Thr83Ile+Ser87Trp | 7 | | |
| GyrA+ParE | Thr83Ile+Asp419Asn | | 1 | |
| | Thr83Ile+Ala503Val | 1 | | |
| GyrA+GyrB+ParC | Thr83Ile+Glu468Asp+Ser87Leu | 3 | 1 | 1 |
| | Thr83Ile+Ser466Phe+Ser87Leu | 1 | | |

mL) than did the change at position 468.

Substitution of Leu (40 strains) or Trp (7 strains) for Ser 87 in *parC* was found in 47 strains, and these strains also displayed an additional substitution, Thr 83→Ile, in *gyrA* and/or Glu 468→Asp or Ser 466→Phe in *gyrB*. Characteristically, substitution of Trp for Ser at position 87 in *parC* was detected only in ST357 strains. Alterations at Asp 419 and Ala 503 in *parE* were identified in 2 strains with an additional substitution in *gyrA*.

Additionally, single or combined target site mutations (TSMs) showed a significant difference between *exoU*+ and *exoS*+ strains (Table 4). Of the 66 strains, 41 *exoU*+ strains (41/44, 93.2%) had either single or combined TSMs, whereas 10 *exoS*+ strains (10/20, 50.0%) had no TSMs ($P \leq 0.0001$). Moreover, among the 53 strains with active mutations, the *exoS*+ strains showed an increased number of single TSMs (30.0%, 3/10 vs. 2.4% 1/41; $P=0.021$), whereas the *exoU*+ strains had a greater number of combined TSMs (97.6%, 40/41 vs. 70%, 7/10; $P=0.021$).

## DISCUSSION

Fluoroquinolone resistance among carbapenem-resistant *P. aeruginosa* strains has increased at an alarming rate because of the extensive use of these agents, thereby severely limiting their usefulness [16, 25, 26]. Fluoroquinolone resistance can lead to treatment failure in infections caused by carbapenem-resistant *P. aeruginosa*. In particular, fluoroquinolone resistance and expression of TTSS effector proteins are independently associated with poor outcomes in *P. aeruginosa* infections [3]. TTSS is considered an important determinant of virulence of *P. aeruginosa* [9, 27]. Using TTSS, *P. aeruginosa* transports ExoS and ExoU inside a eukaryotic cell. ExoS, a major cytotoxin involved in colonization, invasion, and dissemination of bacteria during infection, is regarded as the most prevalent TTSS effector protein [28]. ExoU has been found to be associated with diverse infections and to have the greatest impact on disease severity [1, 29]. Compared to *exoS*+ strains, *exoU*+ strains have been shown to have higher cytotoxicity, which correlates with increased fluoroquinolone resistance [9, 30]. Previous studies found that in the *exoU*+ genotype, multidrug resistance (MDR) and ciprofloxacin resistance are linked [3, 23]. In addition, our present results show that 93.2% of *exoU*+ strains are associated significantly with fluoroquinolone nonsusceptibility compared to only 45.0% of the *exoS*+ strains. The 41 fluoroquinolone nonsusceptible *exoU*+ strains were grouped into 2 STs (ST235 and ST357). ST235, which has recently been found in a MDR clone, is the founder strain of an international clonal complex, CC235. In addition, Cho *et al*. [31] reported that IMP-6-producing strains belong to MDR *P. aeruginosa* ST235. In the present study, all ST235 strains were MDR and featured mainly the virulence gene *exoU* in contrast to ST357 strains (82.9% vs. 17.1%). Similarly, ST235 carrying the *exoU* gene was identified in 21 (36.8%) of the 57 carbapenem-resistant *P. aeruginosa* strains collected in 7 Korean hospitals [32].

In particular, only ST357 strains had the substitution Trp→Ser at position 87 in *parC*, and they showed strong resistance (ciprofloxacin $MIC_{50}$ of 64 μg/mL, levofloxacin $MIC_{50}$ of 32 μg/mL). Recently, Matsumoto *et al*. [33] reported that 2 (9.1%) of 22 levofloxacin-resistant *P. aeruginosa* strains isolated in Japan have this mutation in *parC*. In addition, Hrabák *et al*. [34] showed a wider spread of the IMP-7-producing *P. aeruginosa* ST357 in Central Europe. As a result, it appears that *exoU*+ and increased resistance to fluoroquinolones may be coselected traits.

The most prevalent mechanisms of fluoroquinolone resistance in *P. aeruginosa* involve mutations in QRDRs. Resistance to fluoroquinolones has been shown to be associated with alterations in the GyrA subunit of DNA gyrase and in the ParC subunit of DNA topoisomerase IV [19, 35, 36].

Of the 53 mutants, 52 (98.1%) possessed a single substitution, Thr to Ile, at position 83 in *gyrA*. This change was the most common substitution and is known as the principal mutation for the fluoroquinolone resistance of *P. aeruginosa* strains [37, 38]. The combined alterations, Thr 83→Ile in *gyrA* and Ser 87→Leu in *parC* (75.5%, 40 strains), were the second most common mutations in the 53 strains. In *P. aeruginosa*, like in *Neisseria gonorrhoeae* or *Escherichia coli*, amino acid substitutions in *parC* rarely occurred alone and were usually accompanied by *gyrA* mutations [39].

Alterations in the 3 QRDRs—*gyrA*, *gyrB*, and *parC*—increased fluoroquinolone resistance further compared to a single mutation in *gyrA* (MIC range for ciprofloxacin, 64-256 μg/mL vs. 1-8 μg/mL; levofloxacin, 64-256 μg/mL vs. 2-32 μg/mL). Similarly, Parry *et al*. [40] reported that double mutants show greater MICs than do single mutants, whereas the triple mutant exhibits the highest MIC in *Salmonella typhi*. In general, the increased number of active mutations is linked to the development of stronger fluoroquinolone resistance.

According to our present study, *exoU*+ strains were more likely to acquire 2 or more TSMs than *exoS*+ strains. Furthermore, 10 *exoS*+ strains had no TSMs (50.0%, 10/20) and showed susceptibility to ciprofloxacin (MIC range, <0.25-1 μg/mL) and levofloxacin (MIC range, <0.25-1 μg/mL). In these data, we ob-


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served a robust correlation between TTSS effector proteins and the number of resistance-associated alterations in GyrA, GyrB, ParC, and ParE (QRDR mutations).

In summary, the recent overuse of fluoroquinolones has led to both increased resistance and enhanced virulence in carbapenem-resistant *P. aeruginosa*. The present results indicate a specific relationship among the *exoU* genotype, fluoroquinolone resistance, and differences in active target site mutations conferring resistance. To prevent the spread of fluoroquinolone-resistant *exoU+ P. aeruginosa* strains, the current widespread use of fluoroquinolones needs to be curtailed, and novel antivirulence therapeutic strategies should be developed as soon as possible.

## Authors' Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

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