# Genetic characteristics of and β-lactams effects on imipenem-resistant *Pseudomonas aeruginosa* clinical isolates

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# Genetic characteristics of and β-lactams effects on imipenem-resistant *Pseudomonas aeruginosa* clinical isolates

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ABSTRACT

Genetic characteristics of and β-lactams effects on imipenem-resistant *Pseudomonas aeruginosa* clinical isolates

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We conducted this study to investigate the characteristics and their resistant patterns of carbapenemase producing imipenem-resistant *Pseudomonas aeruginosa* (IRPA) clinical isolates. In addition, we evaluated the antimicrobial effects of β-lactams as detected by time-kill curve methods on CRPA isolates susceptible to ceftazidime. A total of 215 nonduplicate *P. aeruginosa* isolates were collected from hospitalized patients at 8 hospitals through a prospective surveillance study. Their susceptibility to 8 antibiotics and the existence of carbapenemase genes were investigated. Multi-locus sequence typing and sequencing of the *oprD* gene were performed to determine epidemiological characteristics of IRPA isolates. Time-kill curve methods in standard inoculum and high inoculum were performed on IRPA isolates susceptible to ceftazidime to evaluate the antimicrobial effects of β-lactams. Carbapenemase genes were identified in 34 (15.8%) *P. aeruginosa* isolates. Carbapenemase producers were significantly more resistant to all β-lactam and non-β-lactam antibiotics. Moreover, nine isolate (26.5%) were resistant to all drugs tested including colistin. Thirty-three isolates were positive for $bla_{\text{IMP-6}}$ genes, and one isolate was positive for $bla_{\text{VIM-2}}$ gene, which belonged to ST235. The *oprD* gene sequences showed the identical mutation in all ST235

isolates carrying *bla*$_{IMP-6}$, that is, a 1 base pair deletion at position 209. Mutational inactivation of *oprD* is the main mechanism of IRPA isolates susceptible to ceftazidime. Most of the examined clinical isolates had simultaneous downexpression of OprD and increased MexAB–OprM expression. A pronounced decrease in the rate of bacterial killing of IRPA isolates susceptible to ceftazidime by ceftazidime was shown at a high CFU, whereas piperacillin/tazobactam achieved similar killing activity at standard conditions and at a high inoculum. In conclusion, our study demonstrated that increasing IMP-6-harbouring ST235 *P. aeruginosa* isolates are considered a significant clinical threat in Korea because there remain few alternatives for the treatment of systemic infections. Moreover, we report the co-occurring *bla*$_{IMP-6}$ and a frameshift mutation of a 1-bp deletion of nt209ΔT in an IMP-6-producing *P. aeruginosa* ST235 strain. Our study also suggests the use of piperacillin/tazobactam rather than ceftazidime in patients with CRPA isolates susceptible to ceftazidime, especially in high-inoculum infections such as endocarditis and osteomyelitis.

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Key words : *Pseudomonas aeruginosa,* Carbapenemase OprD, IMP-6, Ceftazidime

# Genetic characteristics of and β-lactams effects on imipenem-resistant *Pseudomonas aeruginosa* clinical isolates

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## I. INTRODUCTION

*Pseudomonas aeruginosa* is a major nosocomial pathogen in healthcare-associated infections (HAIs), particularly in debilitated ill or immunocompromised patients.[1,2] The carbapenem-resistant *P. aeruginosa* (CRPA) in hospital environment is has now emerged and is disseminating worldwide. Infections caused by CRPA are a matter of concern in many hospitals worldwide, since they are associated with a three-fold higher rate of mortality, a nine-fold higher rate of secondary bacteremia, a two-fold increase in the length of hospital stay, and a considerable increase in healthcare costs.[3]

The increasing prevalence of production of carbapenemase, mainly Ambler class B metallo-β-lactamases (MBLs) is noteworthy in *P. aeruginosa* for acquiring carbapenem resistance. Diverse kinds of carbapenemases have been recognized in *P. aeruginosa*. Many class B metallo-β-lactamases (MBL), such as IMP-, VIM-, GIM-, SIM- and NDM-type, have been identified in *P. aeruginosa*. More recently KPC and GES class A serine carbapenemases have been also detected in this species.[4,5] MBL-producing *P. aeruginosa* (MPPA), particularly IMP and VIM-producing *P. aeruginosa*, has repeatedly been identified in Korea, since the first report for VIM-2 MPPA clinical isolates in 2002.[5-7] The clonal dissemination of MPPA ST235 is the principal cause for the diffusion of IMP-6 MBL genes in Korea.[5,7,8] MPPA ST235 isolates have also been found in Japan

and European countries.[9,10] Clonal spreading of ST235 isolates showing high resistance to meropenem due to IMP-6 would be a serious clinical concern.[11] However, the presence of intrinsic resistance mechanisms is far more commonly observed and carbapenem-resistant *P. aeruginosa* often have a combination of two or more of the following: hyperexpression of efflux pump systems, overexpression of chromosomal cephalosporinase and decreased expression or loss of the OprD outer membrane protein.[12-16] Carbapenemase producing CRPA clinical isolates usually exhibit co-resistance to other classes of antimicrobial agents. However, the isolates due to inactivation of o*prD* gene in conjunction with other mechanisms such as derepressed *ampC* or over-expression of efflux pumps sometimes showed susceptible to β-lactams.[17,18] Chromosomally encoded, large-spectrum AmpC β-lactamase contributes to the natural resistance of *P. aeruginosa* to many β-lactam antibiotics together with MexAB-OprM.[19] In addition, the well described inoculum effect, representing the requirement of higher β-lactam antibiotic concentrations to inhibit bacterial growth as the bacterial concentration increases, is related to the production of β-lactamase.[20]

Therefore, we conducted this study to investigate the characteristics and its resistant pattern of carbapenemase producing CRPA clinical isolates. In addition, we evaluated the antimicrobial effects of ceftazidime, cefepime, and piperacillin/tazobactam as detected by time-kill curve methods on imipenem-resistant and ceftazidime-susceptible *P. aeruginosa*.

## II. MATERIALS AND METHODS

### 1. Bacterial isolates

A total of 215 nonduplicate *P. aeruginosa* isolates were collected from hospitalized patients at 8 hospitals through a prospective surveillance study. The participating institutions included Kyunghee East-West Neo Medical Center, Samsung Medical Center, Kangbuk Samung Hospital, Kyungpook National University Hospital, Keimyung University Dongsan Medical Center, Chungnam National University Hospital, Chonnam National University Hospital, and Samsung Changwon Hospital. From November 2012 to August 2013, a total of

215 isolates (bacteremia, n = 138; UTI, n = 77) were included in this study. Species identification was performed with a VITEK-2 system (bioMérieux, Hazelwood, MO). Bacteremia was diagnosed if there were two or more of the following conditions of systemic inflammatory response syndrome: temperature >38 ◦C or <36 ◦C; heart rate >90 beats/min; respiratory rate >20 beats/min; and white blood cell count of >12 000 cells/μL or <4000 cells/μL and >10% bands. A bacterial isolate with a density $\geq 10^5$ CFU/mL in voided urine or $\geq 10^3$ CFU/mL in catheterized urine was identified as a causative pathogen of UTI.

2. Antimicrobial susceptibility testing

Antimicrobial susceptibility was tested by microdilution method following the Clinical and Laboratory Standards Institute (CLSI) guidelines.[21] Eight antimicrobial agents were tested, including imipenem, meropenem, piperacillin/tazobactam, cefepime, ceftazidime, ciprofloxacin, amikacin, and colistin. Interpretation of susceptibility was according to CLSI breakpoints. *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were employed as quality control strains.

3. Identification of carbapenemase gene

All of the carbapenem non-susceptible *P. aeruginosa* isolates were screened by PCR for $bla_{\text{IMP}}$, $bla_{\text{VIM}}$, $bla_{\text{SPM}}$, $bla_{\text{GIM}}$, $bla_{\text{SIM}}$, $bla_{\text{NDM}}$, and $bla_{\text{KPC}}$. PCR amplicons were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany). Presence of MBL genes was confirmed by sequencing of PCR products. Experimentally determined nucleotide sequences were compared with those in GenBank (http://www.ncbi.nlm.nih.gov/genbank) using the BLAST network service.

4. Multi-locus sequence typing (MLST)

PCR and sequencing for 7 housekeeping genes (*acsA, aroE, guaA, mutL*, *nuoD*, *ppsA*, and *trpE*) were performed as described previously.[22] Experimentally determined nucleotide nucleotide sequences of both strands were compared to pre-existing sequences in the MLST database to assign allelic numbers and STs (http://pubmlst.org/paeruginosa).

5. Sequencing of the *oprD* gene

The *oprD* gene sequence was investigated for all imipenem-resistant *P. aeruginosa* strains. Sequence was compared with that of the reference strain *P. aeruginosa* PAO1 (GenBank accession no. CAA78448).

6. Quantitative reverse transcription PCR (qRT-PCR)

mRNA transcription levels of *oprD, ampC, mexB, mexD, mexF* and *mexY* were determined by qRT-PCR in all imipenem-resistant and ceftazidime-susceptible *P. aeruginosa* as described previously.[23] Reverse transcription was performed in accordance with the protocol for the use of Omniscript® Reverse Transcriptase (QIAGEN GmbH, Hilden, Germany). Quantification of *oprD* transcripts was performed using SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA). The mRNA levels were normalized to that of housekeeping gene *rpsL* and expressed as a ratio to PAO1 (value of 1 by definition). Relative quantification was done by comparative Ct method ($2 - \Delta\Delta Ct$).[24] Reduced *oprD* expression was considered relevant when it was $\leq$50% compared with that of reference strains.[25] Isolates were considered to be AmpC, MexCD-OprJ, MexEF-OprN and MexXY-OprM hyperproducers when the levels of expression of *ampC, mexD, mexF* and *mexY* genes were $\geq$10-fold higher than that of the reference strains, negative if lower than 5-fold. Isolates were considered to be MexAB-OprM hyperproducers when the level of *mexB* expression was $\geq$3-fold higher than that of the reference strains, negative if lower than twofold.[26] Primers used for qRT-PCR are listed in Table 1.

Table 1. Primers used for Polymerase Chain Reaction and Reverse-Transcription Quantitative PCR

| | Primer | Oligonucleotide sequence |
|---|---|---|
| *IMP* | IMP-F | GAAGGYGTTTATGTTCATAC |
| | IMP-R | GTAMGTTTCAAGAGTGATGC |
| *VIM* | VIM-F | GTTTGGTCGCATATCGCAAC |
| | VIM-R | AATGCGCAGCACCAGGATAG |

| | | |
|---|---|---|
| *SPM* | SPM1-F | CTGCTTGGATTCATGGGCGC |
| | SPM1-R | CCTTTTCCGCGACCTTGATC |
| *GIM* | GIM-F | TCGACACACCTTGGTCTGAA |
| | GIM-R | AACTTCCAACTTTGCCATGC |
| *SIM* | SIM-F | TACAAGGGATTCGGCATCG |
| | SIM-R | TAATGGCCTGTTCCCATGTG |
| *NDM* | NDM-F | GCCCAATATTATGCACCCGG |
| | NDM-R | CTCATCACGATCATGCTGGC |
| *KPC* | KPC-F | GTATCGCCGTCTAGTTCTGC |
| | KPC-R | GGTCGTGTTTCCCTTTAGCC |
| Ribosomal protein S12 reference gene | rpsL-QF | GTGGTGAAGGTCACAACCTG |
| | rpsL-QR | CCTGCTTACGGTCTTTGACA |
| *oprD* | oprD-QF | ACGCAATCACCGATAACCTC |
| | oprD-QR | CATCGTTTGTGCGGTAGATG |
| *mexB* | mexB-F1 | TCGACAATCTGCGCTACATC |
| | mexB-R1 | CTTGTTCTGCACCTGGACCT |
| *mexD* | mexD-F1 | TCAACGGTCTGGGTAACTCC |
| | mexD-R1 | GTCTGGATCTCGCCAAGAAG |
| *mexF* | mexF-QF | TCTACGACCCGACCATCTTC |
| | mexF-QR | CAGGTCTGCAGGAACAGGAT |
| *mexY* | mexY-F | GAGCAATTGATGCAGGGACT |
| | mexY-R | GAACACGATCAACACCGAGA |

7. Antimicrobial effects using Time-kill assay

*P. aeruginosa* cells in a 0.1-ml volume containing approximately $10^5$ and $10^7$ colony forming unit (CFU)/ml were added into microtiter wells with ABTG medium.[27] Then, 0.1 ml of different concentrations of 0, 1, 4, 16, 128 μg/ml MIC of ceftazidime, cefepime, and piperacillin/tazobactam were mixed into different wells, and cultures were shaken and cultivated at 37°C for 0, 1, 4, and 8 hours. Then, 0.1-ml samples from wells were serially diluted and cultured on plates for overnight incubation, and CFU counts were determined for killing curves of antibiotics.

## III. RESULTS

During the study period, 215 *P. aeruginosa* clinical isolates were collected. The isolates were recovered from blood (n = 138), urine (n = 77). Resistance rates to imipenem, meropenem, ceftazidime and colistin were 35.8%, 33.5%, 44.2%, and 7.4%, respectively, higher than those reported previously.[23] Antimicrobial susceptibility rates, by specimen, are also described in Table 2. Compared with blood isolates, urine isolates exhibited higher resistance rates to all antibiotics.

Table 2. Antimicrobial resistance of *P. aeruginosa* clinical isolates

| Antimicrobial agent* | Number of resistant isolates (%) | | | | |
|---|---|---|---|---|---|
| | 2006-2007[23] (n=213) | 2012-2013 (n=215) | | | *p* |
| | | Total | Blood (n = 133) | Urine (n = 77) | |
| Imipenem | 57 (26.8) | 77 (35.8) | 45 (33.8) | 32 (41.6) | 0.043 |
| Meropenem | 47 (22.1) | 72 (33.5) | 39 (29.3) | 33 (42.9) | 0.008 |
| Piperacillin-Tazobactam | 76 (35.7) | 79 (36.7) | 39 (29.3) | 40 (51.9) | 0.819 |
| Cefepime | 71 (33.3) | 73 (34.0) | 37 (27.8) | 36 (46.8) | 0.892 |

| | | | | | |
|---|---|---|---|---|---|
| Ceftazidime | 70 (32.9) | 95 (44.2) | 56 (42.1) | 39 (50.6) | 0.016 |
| Ciprofloxacin | 64 (30.0) | 76 (35.3) | 35 (26.3) | 41 (53.2) | 0.242 |
| Amikacin | 48 (22.5) | 48 (22.3) | 14 (10.5) | 34 (44.2) | 0.999 |
| Colistin | 3 (1.4) | 16 (7.4) | 4 (3.0) | 12 (15.6) | 0.002 |

* MIC according to reference 21

1. The characteristics of carbapenemase producing imipenem-resistant *P. aeruginosa* clinical isolates

Out of the 215 *P. aeruginosa* clinical isolates, carbapenemase genes were identified in 34 (15.8%) *P. aeruginosa* isolates. The antimicrobial resistance of carbapenemase producers and carbapenemase non-producers among imipenem-resistant *P. aeruginosa* isolates was compared in Table 3. Carbapenemase producers were significantly more resistant to all β-lactam and non-β-lactam antibiotics. Moreover, nine isolate (26.5%) were resistant to all drugs tested including colistin. Carbapenemase producers showed extremely high rates of non-susceptibility to meropenem (100% vs. 86.0% in carbapenemase non-producers), piperacillin-tazobactam (91.2% vs. 62.8%), cefepime (94.1% vs. 51.2%) ceftazidime (100% vs. 58.1%), ciprofloxacin (100% vs. 53.5), amikacin (100% vs. 9.3%), and colistin (26.5% vs. 4.7%). All carbapenemase producing *P. aeruginosa* clinical isolates presented high MIC values (>64 mg/L) for meropenem.

Table 3. Antimicrobial resistance of imipenem-resistant *P. aeruginosa* clinical isolates

| Antimicrobial agent | Number of resistant isolates (%) | | *p* |
|---|---|---|---|
| | Carbapenemase producers (n=34) | Carbapenemase non-producers (n=43) | |
| Meropenem | 34 (100) | 37 (86.0) | 0.031 |
| Piperacillin-tazobactam | 31 (91.2) | 27 (62.8) | 0.004 |
| Cefepime | 32 (94.1) | 22 (51.2) | <0.001 |

| | | | |
|---|---|---|---|
| Ceftazidime | 34 (100) | 25 (58.1) | <0.001 |
| Ciprofloxacin | 34 (100) | 23 (53.5) | <0.001 |
| Amikacin | 34 (100) | 4 (9.3) | <0.001 |
| Colistin | 9 (26.5) | 2 (4.7) | 0.009 |

Thirty-three isolates were positive for $bla_{IMP}$ genes, and one isolate was positive for $bla_{VIM}$ gene. No $bla_{SPM}$, $bla_{GIM}$, $bla_{SIM}$, $bla_{NDM}$ genes were identified in the isolates. No $bla_{KPC}$ gene was identified in the isolates. The $bla_{IMP}$ and $bla_{VIM}$ genes identified were $bla_{IMP-6}$ and $bla_{VIM-2}$, respectively. All IMP-6-harbouring isolates and VIM-2-harbouring isolate belonged to ST235 (38-11-3-13-1-2-4). Sequence analysis of the entire *oprD* gene from all of the 34 carbapenemase producing *P. aeruginosa* clinical isolates showed a nearly identical mutation. The frameshift mutations of a 1-bp deletion of nt209ΔT, resulting in the formation of a premature stop codon, were found in all IMP-6-harbouring isolate, not a VIM-2-harbouring isolate. However, there is only a 1 bp difference at position 209 between 30 IMP-6-harbouring isolates and one VIM-2-harbouring isolate (Fig 1). The characteristics of *P. aeruginosa* isolates producing carbapenemase are described in Table 5.

Fig 1. Nucleotide sequence of *oprD* gene from PAO1 and carbapenemase-producing isolates. First line represents the *oprD* nucleotide sequence of *P. aeruginosa* PAO1 (GenBank accession no. CAA78448), second line represents the *oprD* nucleotide sequence of 30 IMP-6 harboring isolates († in Table 4), and third line represents the *oprD* nucleotide sequence of three IMP-6 harboring isolates (* in Table 4), and forth line represents the *oprD* nucleotide sequence of VIM-2 harboring isolates . Nucleotide sequence change was indicated by square.

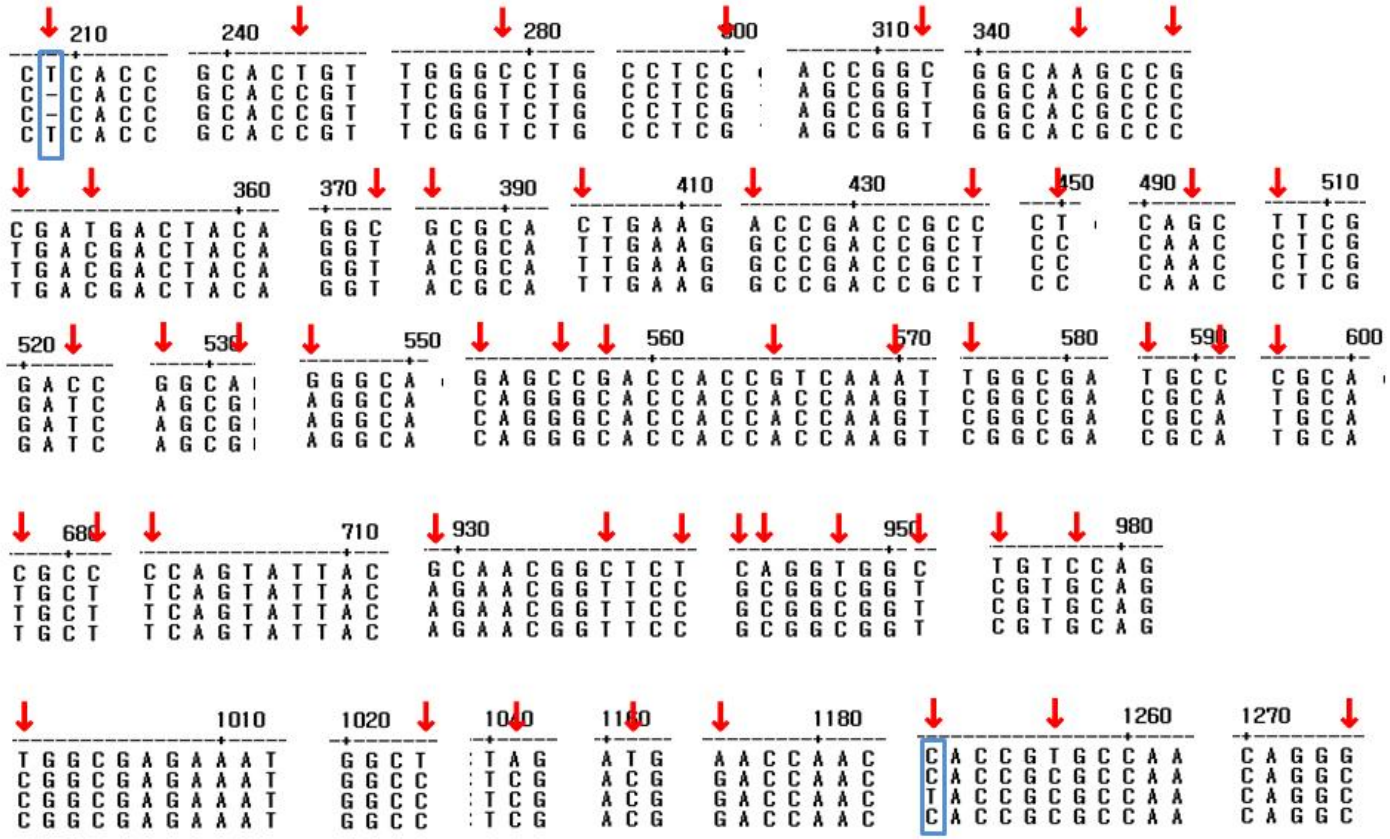


Table 4. Genotypes and antimicrobial resistance in 34 carbapenemase-producing *P. aeruginosa* clinical isolates

| Center | Source | ST | MBL enzyme | Mutation in the *oprD* gene | MIC (ug/ml) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | IMI | MRP | P/T | CPM | CAZ | CIP | AMI | CL |
| SMC | B | 235 | †IMP6 | Frameshift (1-bp deletion of nt209ΔT) | 32 | >64 | 128/4 | >64 | >64 | >64 | >128 | 1 |
| SMC | B | 235 | †IMP6 | Frameshift (1-bp deletion of nt209ΔT) | 16 | >64 | 64/4 | 1 | >64 | 64 | 128 | 0.5 |
| SMC | B | 235 | †IMP6 | Frameshift (1-bp deletion of nt209ΔT) | 16 | >64 | 128/4 | >64 | >64 | 64 | >128 | 8 |
| SMC | B | 235 | †IMP6 | Frameshift (1-bp deletion of nt209ΔT) | 32 | >64 | 128/4 | >64 | >64 | 64 | >128 | 8 |
| SMC | B | 235 | *IMP6 | Frameshift (1-bp deletion of nt209ΔT) | 16 | >64 | 128/4 | >64 | >64 | 64 | >128 | 4 |
| KUDMC | B | 235 | †IMP6 | Frameshift (1-bp deletion of nt209ΔT) | 32 | >64 | 256/4 | >64 | >64 | 64 | >128 | 2 |
| DFH | B | 235 | †IMP6 | Frameshift (1-bp deletion of nt209ΔT) | 16 | >64 | 256/4 | >64 | >64 | >64 | >128 | 1 |
| SCH | B | 235 | †IMP6 | Frameshift (1-bp deletion of nt209ΔT) | 32 | >64 | >256/4 | >64 | >64 | >64 | >128 | 1 |
| SCH | B | 235 | VIM2 | Substitution (Amino acid changes (T103S, K115T, F170L, E185Q, P186G, V189T, R310E, A315G, G425A)) | >64 | >64 | 32/4 | 32 | >64 | >64 | >128 | 1 |
| SCH | B | 235 | †IMP6 | Frameshift (1-bp deletion of nt209ΔT) | 32 | >64 | 128/4 | >64 | >64 | >64 | >128 | 1 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SMC | U | 235 | *IMP6 | Frameshift (1-bp deletion of nt209ΔT) | 16 | >64 | 128/4 | >64 | >64 | 32 | >128 | >64 |
| SMC | U | 235 | †IMP6 | Frameshift (1-bp deletion of nt209ΔT) | 16 | >64 | 256/4 | >64 | >64 | >64 | >128 | 8 |
| SMC | U | 235 | *IMP6 | Frameshift (1-bp deletion of nt209ΔT) | 16 | >64 | 256/4 | >64 | >64 | 32 | >128 | >64 |
| SMC | U | 235 | †IMP6 | Frameshift (1-bp deletion of nt209ΔT) | 16 | >64 | 256/4 | >64 | >64 | 32 | >128 | >64 |
| SMC | U | 235 | †IMP6 | Frameshift (1-bp deletion of nt209ΔT) | 16 | >64 | 128/4 | >64 | >64 | 32 | >128 | >64 |
| SMC | U | 235 | †IMP6 | Frameshift (1-bp deletion of nt209ΔT) | 16 | >64 | 128/4 | >64 | >64 | 32 | >128 | 4 |
| SMC | U | 235 | †IMP6 | Frameshift (1-bp deletion of nt209ΔT) | 16 | >64 | >256/4 | >64 | >64 | 32 | >128 | 4 |
| SMC | U | 235 | †IMP6 | Frameshift (1-bp deletion of nt209ΔT) | 32 | >64 | 128/4 | >64 | >64 | 32 | >128 | 8 |
| SMC | U | 235 | †IMP6 | Frameshift (1-bp deletion of nt209ΔT) | 32 | >64 | 64/4 | >64 | >64 | 32 | >128 | 8 |
| DFH | U | 235 | †IMP6 | Frameshift (1-bp deletion of nt209ΔT) | 32 | >64 | 128/4 | >64 | >64 | >64 | >128 | 2 |
| DFH | U | 235 | †IMP6 | Frameshift (1-bp deletion of nt209ΔT) | 64 | >64 | 128/4 | >64 | >64 | 16 | >128 | 4 |
| DFH | U | 235 | †IMP6 | Frameshift (1-bp deletion of nt209ΔT) | 32 | >64 | 128/4 | >64 | >64 | >64 | >128 | 2 |
| DFH | U | 235 | †IMP6 | Frameshift (1-bp deletion of nt209ΔT) | 32 | >64 | 128/4 | >64 | >64 | >64 | >128 | 4 |
| DFH | U | 235 | †IMP6 | Frameshift (1-bp deletion | 32 | >64 | 128/4 | >64 | >64 | >64 | >128 | 2 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | of nt209ΔT) | | | | | | | | |
| DFH | U | 235 | †IMP6 | Frameshift (1-bp deletion of nt209ΔT) | 32 | >64 | 128/4 | >64 | >64 | 64 | >128 | 4 |
| SCH | U | 235 | †IMP6 | Frameshift (1-bp deletion of nt209ΔT) | 32 | >64 | 256/4 | >64 | >64 | >64 | >128 | 2 |
| SCH | U | 235 | †IMP6 | Frameshift (1-bp deletion of nt209ΔT) | 32 | >64 | 128/4 | >64 | >64 | >64 | >128 | 4 |
| SCH | U | 235 | †IMP6 | Frameshift (1-bp deletion of nt209ΔT) | 16 | >64 | 128/4 | >64 | >64 | >64 | >128 | 2 |
| SCH | U | 235 | †IMP6 | Frameshift (1-bp deletion of nt209ΔT) | 32 | >64 | 128/4 | >64 | >64 | 32 | >128 | 4 |
| SCH | U | 235 | †IMP6 | Frameshift (1-bp deletion of nt209ΔT) | 32 | >64 | 256/4 | >64 | >64 | >64 | >128 | 2 |
| SCH | U | 235 | †IMP6 | Frameshift (1-bp deletion of nt209ΔT) | 32 | >64 | 256/4 | >64 | >64 | 64 | >128 | 4 |
| SCH | U | 235 | †IMP6 | Frameshift (1-bp deletion of nt209ΔT) | 32 | >64 | 256/4 | >64 | >64 | >64 | >128 | 2 |
| SCH | U | 235 | †IMP6 | Frameshift (1-bp deletion of nt209ΔT) | 32 | >64 | >256/4 | >64 | >64 | 32 | >128 | 2 |
| SCH | U | 235 | †IMP6 | Frameshift (1-bp deletion of nt209ΔT) | 32 | >64 | >256/4 | >64 | 64 | 32 | >128 | 1 |

MBL, metallo-β-lactamase; ST, sequence type; MIC, minimum inhibitory concentration; IMI, imipenem; MEM, meropenem; P/T, piperacillin-tazobactam; CPM, cefepime; CAZ, ceftazidime; CIP, ciprofloxacin; AMI, amikacin; CL, colistin. SMC, Samsung Medical Center (Seoul); KUDMC, Keimyung University Dongsan Medical Center (Daegu); DFH, Daegu Fatima Hospital (Daegu); SCMC, Samsung Changwon Hospital (Changwon). B, blood; U, urine.

2. The antimicrobial effects of ceftazidime, cefepime, and piperacillin/tazobactam as detected by time-kill curve methods on imipenem-resistant and ceftazidime-susceptible *P. aeruginosa*.

Eighteen isolates showed imipenem-resistant and ceftazidime-susceptible in imipenem-resistant *P. aeruginosa* isolates. None of the 18 clinical isolates produced carbapenemases. All isolates had a relevant decrease in *oprD* expression (≤50%) compared with that of PAO1. Decrease of *oprD* expression that appeared to be relevant was also detected in all five carbapenem-susceptible *P. aeruginosa* isolates studied. Whilst 15 (83.3%) overexpressed one or more genes of the efflux systems or *ampC*. Among the efflux systems, overexpression of the *mexB* gene was most prevalent (72.2%). *mexD*, *mexF*, and *mexY* overexpression was found in 2, 1 and 1 isolates, respectively. Overexpression of the *ampC* gene was observed in 2 isolates (11.1%).

Three (as described as * in Table 5) imipenem-resistant and ceftazidime-susceptible *P. aeruginosa* clinical isolates were selected for production of the time-kill curve.

A. Antimicrobial effects of ceftazidime in imipenem-resistant and ceftazidime-susceptible *P. aeruginosa* clinical isolates

Figure 2 shows the killing curves obtained with the three selected imipenem-resistant and ceftazidime-susceptible *P. aeruginosa* isolates at standard and high inocula at antibiotic concentrations of 0, 1, 4, 16, 128 μg/ml MIC. The MICs of ceftazidime against *P. aeruginosa* isolate 1, 2 and 3 were 4 μg/ml, 4 μg/ml, and 1 μg/ml, respectively. The killing activities of ceftazidime appeared to be time dependent for all three isolates. For the $10^5$ CFU, ceftazidime concentrations of X4 MIC and higher achieved a net bacterial killing of 1-2.5 log10 at 4 and 8 h. Concentrations of up to X32 MIC and higher achieved less than 1 log10 of net killing for the $10^7$ CFU. The onset of bacterial killing showed a lag time of approximately 1 h for the $10^5$ CFU in isolate 3.

Table 5. Resistance mechanism of imipenem-resistant *Pseudomonas aeruginosa* isolates susceptible to ceftazidime

| ST | MIC (ug/ml) | | | | | Gene expression[a] | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | IMP | MEM | FEP | CAZ | P/T | *oprD* | *mexB* | *mexD* | *mexF* | *mexY* | *ampC* |
| *1248 | 16 | 8 | 4 | 4 | 16/4 | **0.0036** | 1.4667 | 0.0675 | 2.1054 | 0.0066 | 0.0358 |
| 508 | 32 | 32 | 8 | 8 | 32/4 | **0.0002** | **5.0183** | 1.4400 | 7.5370 | 0.0690 | 0.1775 |
| 412 | 8 | 8 | 8 | 8 | 32/4 | **0.0001** | **5.4348** | 0.5630 | 1.4580 | 0.0867 | 0.0238 |
| *262 | 16 | 8 | 4 | 4 | 8/4 | **0.0009** | **4.5072** | 1.8481 | 1.6292 | 0.1226 | 0.0107 |
| 348 | 16 | 8 | 2 | 2 | 4/4 | **0.1760** | **3.6870** | 0.7149 | 1.6864 | 0.0330 | 0.0400 |
| 262 | 32 | 0.25 | 1 | 4 | 4/4 | **0.0001** | **8.9217** | 5.3937 | 1.0821 | 0.4419 | 0.1594 |
| 606 | 64 | 32 | 8 | 8 | 32/4 | **0.0026** | **12.4830** | 6.9456 | 0.6685 | 0.3809 | 0.1251 |
| 235 | 32 | 64 | 64 | 8 | 64/4 | **0.0181** | **3.4995** | 4.8098 | 1.2914 | 0.3316 | 0.1519 |
| *1154 | 8 | 0.25 | 2 | 1 | 1/4 | **0.2704** | **5.9681** | 5.8793 | 1.2825 | 0.0942 | 0.1268 |
| 298 | 64 | 8 | 2 | 2 | 16/4 | **0.1694** | **50.6143** | **678.478** | 0.8759 | **39.7410** | 0.0954 |
| 233 | 16 | 16 | 8 | 8 | 16/4 | **0.0015** | **19.6462** | 3.2918 | 0.1498 | 5.2202 | 0.4798 |
| 274 | 8 | 32 | 64 | 8 | 64/4 | **0.0009** | **11.5627** | 0.5830 | 0.1036 | 4.5985 | 0.1654 |
| 508 | 16 | 16 | 2 | 2 | 4/4 | **0.0016** | 2.1140 | 0.4180 | 0.1698 | 1.1847 | 0.1174 |
| 971 | 16 | 4 | 2 | 2 | 4/4 | **0.0015** | **4.0383** | 0.1337 | 0.5121 | 5.1170 | 1.1953 |
| 277 | 32 | 8 | 8 | 8 | 8/4 | **0.0618** | 0.7769 | 0.4164 | 0.3341 | 0.5608 | **61.3534** |
| 244 | 16 | 8 | 8 | 4 | 64/4 | **0.2484** | 0.1564 | 5.9590 | **46.943** | 0.7719 | 0.5415 |
| 235 | 16 | 8 | 16 | 4 | 16/4 | **0.0287** | 2.5572 | 0.5862 | 0.1074 | 5.6456 | 0.5649 |
| 641 | 16 | 4 | 32 | 2 | 128/4 | **0.0124** | **14.8698** | **12.7377** | 1.8272 | 0.7317 | **17.6862** |
| S1 | 0.5 | 0.25 | 2 | 2 | 4/4 | **0.0008** | 0.5111 | 0.1715 | 0.7191 | 0.0099 | 0.0099 |
| S2 | 1 | 0.12 | 1 | 2 | 4/4 | **0.0008** | 0.1692 | 0.2864 | 0.7016 | 0.0381 | 0.0067 |
| S3 | 1 | 1 | 2 | 2 | 8/4 | **0.0525** | 0.31791 | 0.2672 | 0.8638 | 0.0145 | 0.0175 |
| S4 | 1 | 2 | 4 | 8 | 8/4 | **0.0353** | 0.38601 | 0.1976 | 1.9848 | 0.0181 | 0.0075 |

ST, sequence type; MIC, minimum inhibitory concentration; IMI, imipenem; MEM, meropenem; P/T, piperacillin-tazobactam;

CPM, cefepime; CAZ, ceftazidime.

[a]Values in bold indicate a significant overexpression (or underexpression for oprD) of the corresponding gene according to the defined thresholds.

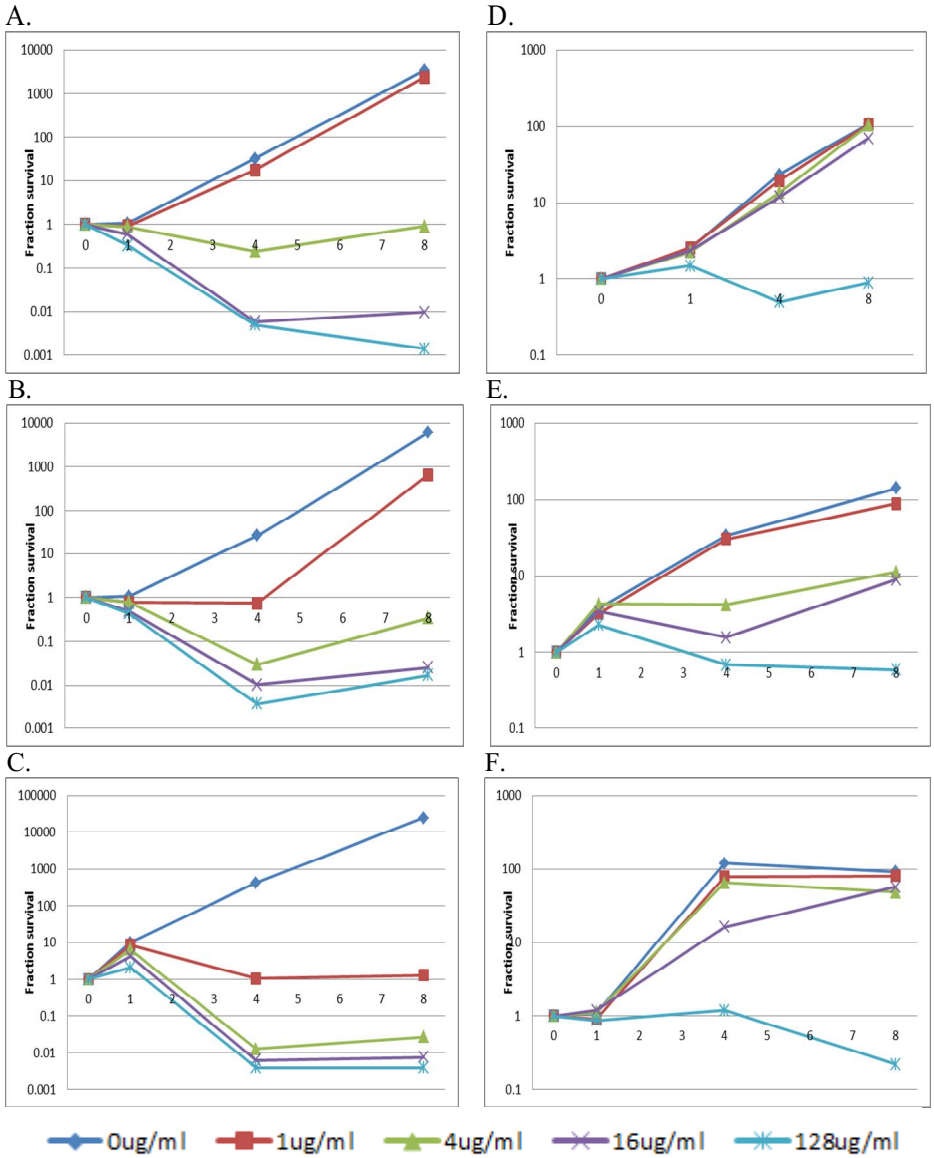
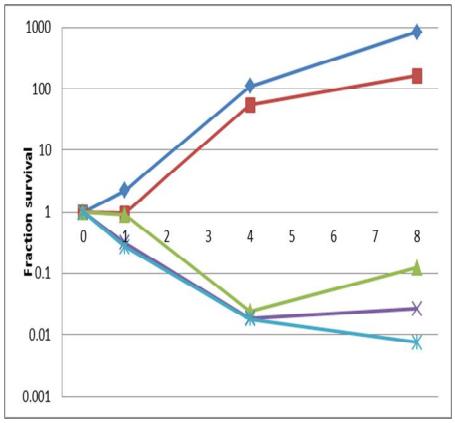



Fig 2. Time-kill curves of imipenem-resistant and ceftazidime-susceptible *P. aeruginosa* clinical isolates exposed to ceftazidime (A) Isolate 1, inoculum of 5 X$10^5$ CFU/ml; (B) Isolate 2, inoculum of 5 X $10^5$ CFU/ml; (C) Isolate 3, inoculum of 5 X$10^5$ CFU/ml; (D) Isolate 1, inoculum of 5 X$10^7$ CFU/ml; (E) Isolate 2, inoculum of 5 X $10^7$ CFU/ml; (F) Isolate 3, inoculum of 5 X$10^7$ CFU/ml. Portions of similar time-kill curves are not shown.

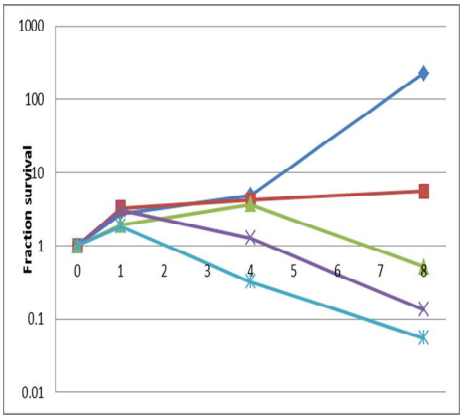
B. Antimicrobial effects of cefepime in imipenem-resistant and cefepime-susceptible *P. aeruginosa* clinical isolates

The killing profiles of cefepime at standard and high inocula against 3 isolates are shown in Fig 3. The MICs of cefepime against *P. aeruginosa* isolate 1, 2 and 3 were 4 μg/ml, 4 μg/ml, and 2 μg/ml, respectively. The killing activities of cefepime appeared to be time dependent for all three isolates. The onset of bacterial killing showed a lag time of approximately for 1 h in isolate 2 and 3 in standard inoculum. Bacterial growth was seen within 1 h when isolate 3 was exposed to X8 MIC of cefepime in standard inoculum and X64 MIC of cefepime in high inoculum. In isolate 1, the lag time of killing activities persisted 4 hours in high inoculum.

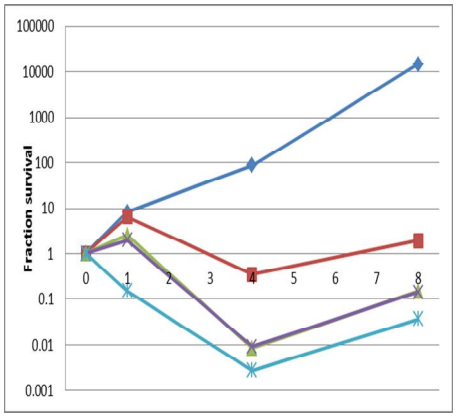
A.



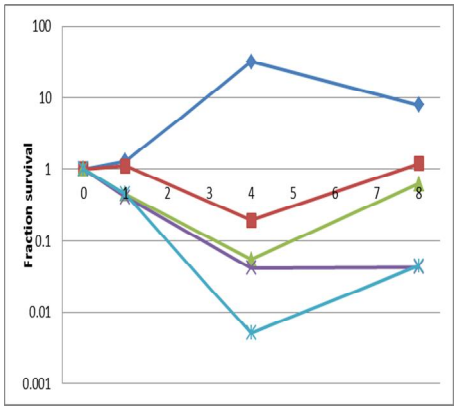

E.




B.




F.



C. G.

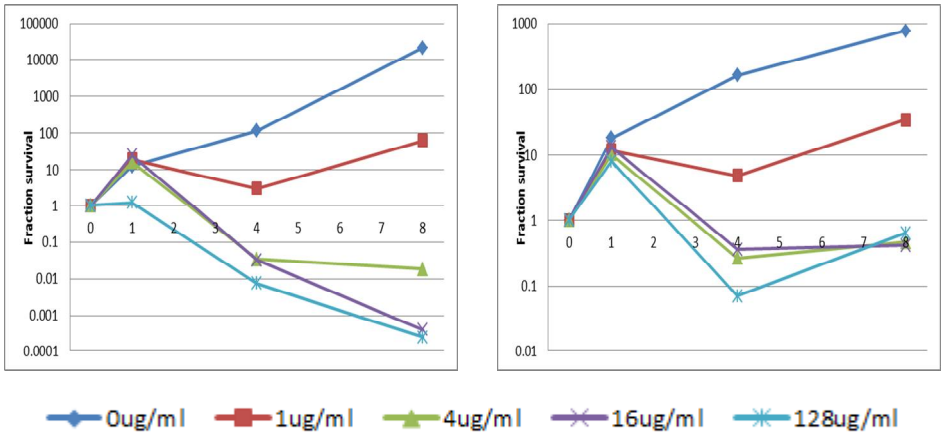



Fig 3. Time-kill curves of imipenem-resistant and ceftazidime-susceptible *P. aeruginosa* clinical isolates exposed to cefepime (A) Isolate 1, inoculum of 5 X$10^5$ CFU/ml; (B) Isolate 2, inoculum of 5 X $10^5$ CFU/ml; (C) Isolate 3, inoculum of 5 X$10^5$ CFU/ml; (D) Isolate 1, inoculum of 5 X$10^7$ CFU/ml; (E) Isolate 2, inoculum of 5 X $10^7$ CFU/ml; (F) Isolate 3, inoculum of 5 X$10^7$ CFU/ml. Portions of similar time-kill curves are not shown.

C. Antimicrobial effects of piperacillin/tazobactam in imipenem-resistant and piperacillin/tazobactam-susceptible *P. aeruginosa* clinical isolates

The MICs of piperacillin/tazobactam against *P. aeruginosa* isolate 1, 2 and 3 were 16/4 μg/ml, 8/4 μg/ml, and 1/4 μg/ml, respectively. Figure 4 shows the killing curves for standard and high inocula. The killing activities of piperacillin/tazobactam also appeared to be time dependent for all three isolates. At standard conditions and at a high inoculum, piperacillin/tazobactam achieved similar killing activity.

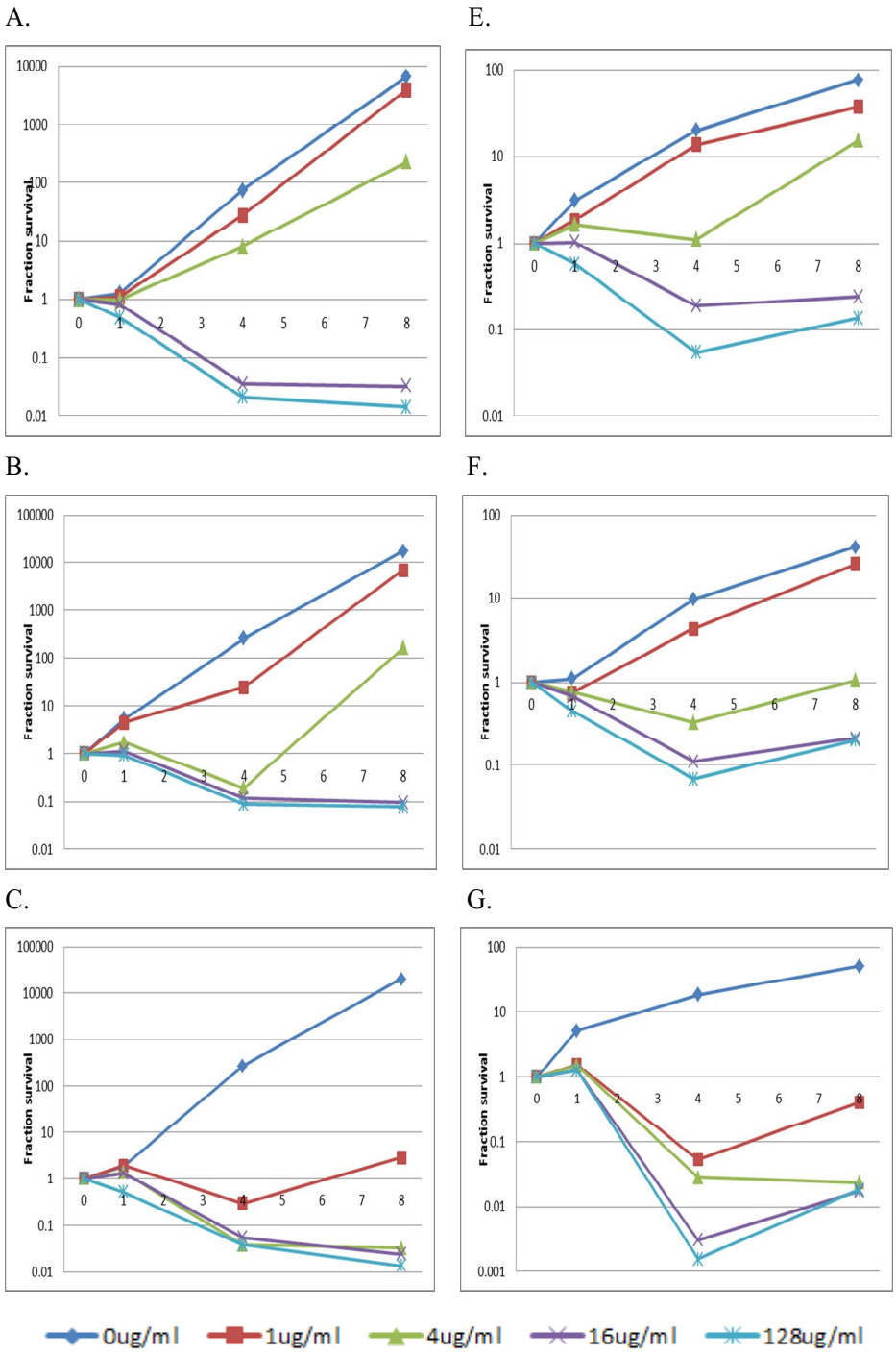



Fig 4. Time-kill curves of imipenem-resistant and ceftazidime-susceptible *P.*

*aeruginosa* clinical isolates exposed to piperacillin-tazobactam A) Isolate 1, inoculum of 5 X$10^5$ CFU/ml; (B) Isolate 2, inoculum of 5 X $10^5$ CFU/ml; (C) Isolate 3, inoculum of 5 X$10^5$ CFU/ml; (D) Isolate 1, inoculum of 5 X$10^7$ CFU/ml; (E) Isolate 2, inoculum of 5 X $10^7$ CFU/ml; (F) Isolate 3, inoculum of 5 X$10^7$ CFU/ml. Portions of similar time-kill curves are not shown.

## IV. DISCUSSION

In this work, out of the 215 *P. aeruginosa* clinical isolates, 35.8% (77/215) were found to be resistant to imipenem. MBL genes were identified in 15.8% (34/215). Compared with the earlier study,[23] the prevalence of MBL genes is higher in this study from 7.0% (15/213) to 15.8%. This is due to higher prevalence of IMP-6-harbouring isolates from 6.1% (13/213) to 15.3% (33/215). The increasing prevalence of production of carbapenemase is noteworthy in *P. aeruginosa* for acquiring carbapenem resistance. Carbapenemase producing *P. aeruginosa* isolates were significantly more resistant to all β-lactam and non-β-lactam antibiotics in the study. All carbapenemase producing *P. aeruginosa* clinical isolates presented higher MIC values (>64 mg/L) for meropenem than for imipenem. This observation is due to the Ser216-Gly substitution, which results in increased hydrolysis of meropenem compared with imipenem.[11] Intriguingly, nine isolate (26.5%) were resistant to all drugs tested including colistin. Therefore, carbapenemase producing *P. aeruginosa* isolates is considered a significant clinical threat because there remain few alternatives for the treatment of systemic infections.

Two international high-risk *P. aeruginosa* clones including sequence type 111 (ST111) and ST235 are responsible for the dissemination of carbapenemase genes worldwide.[28,29] Several typing studies have shown that the majority of multidrug-resistant isolates belong to ST235, ST111 and ST175, and are involved in the dissemination of genes encoding VIM-type MBLs in European countries.[9,10] In our study, the most prevalent gene, $bla_{\text{IMP-6}}$, was identified in 33 isolates and $bla_{\text{VIM2}}$ was identified in one isolate, which belonged to ST235.

Recent Korean studies[5,30] showed that clonal dissemination of MPPA ST235 is the principal cause for the diffusion of IMP-6 and VIM-2 MBL genes in Korea. Among 94 imipenem-resistant *P. aeruginosa* isolates collected from 23 Korean hospitals in 2014, IMP-6 and VIM-2 MBLs were identified in 21 (6.4%) isolates (n = 17 and 4, respectively).[30] Among 41 carbapenemase-producing *P. aeruginosa* isolates collected from 29 general hospitals in Korea in 2015, the clonal spread of an IMP-6-producing *P. aeruginosa* ST235 strain was demonstrated again.[5] All MBL-producing isolates in those studies showed multi-drug resistant phenotype, however, no isolate was resistant to colistin unlike our study.

Previous study showed that the *oprD* gene sequences showed the identical mutation in all ST235 isolates carrying $bla_{\text{IMP-6}}$, that is, a 1 base pair deletion at position 209.[7] Likewise, frameshift mutations of a 1-bp deletion of nt209ΔT resulting in truncated proteins were found in all IMP-6-harbouring isolates in our study. All thirty-seven isolates showing frameshift mutations of a 1-bp deletion of nt209ΔT belonged to ST235. However, only 33 isolates carry $bla_{\text{IMP-6.}}$ We confirmed that *oprD* mutational group sequences can be used as a surrogate to MLST for typing isolates. Moreover, we report here that co-occurring *bla*IMP-6 and a frameshift mutation of a 1-bp deletion of nt209ΔT in an IMP-6-producing *P. aeruginosa* ST235 strain.

Acquisition of a transferable resistance determinant such as metallo-β-lactamase could be more problematic in clinical settings. However, loss of OprD function is still the major determinant of non-carbapenemase -mediated resistance to carbapenems. Carbapenem resistance due to inactivation of OprD often occurs in conjunction with other mechanisms such as derepressed AmpC or MexAB–OprM.[12-16] Recently, several studies showed the increasing prevalence of the CRPA isolates susceptible to β-lactams.[17,18] Zeng AR et al. investigated 29 *P. aeruginosa* isolates, which are resistant to carbapenems but susceptible to ceftazidime or/and cefepime.[18] None of the 29 clinical isolates produced carbapenemases, extended-spectrum β-lactamases, or Ambler class C β-lactamases enzymes. On the other hands, Marina BS et al. examined 38 *P.*

*aeruginosa* isolates with reduced susceptibility to at least one of the carbapenems which were collected from hospitalized patients.[17] None of the collected isolates produced carbapenemases. Thirty-five patients (92.1%) showed susceptible to ceftazidime among imipenem-resistant isolates. In our study, 18 isolates showed imipenem-resistant and ceftazidime-susceptible *P. aeruginosa* clinical isolates. None of the 18 clinical isolates produced carbapenemases, which was consistent with the previous studies.[17,18] The MICs of imipenem indicated more moderate or low resistance than those of carbapenemase producing imipenem-resistant *P. aeruginosa* clinical isolates. Mutational inactivation of OprD is the main mechanism of imipenem-resistant and ceftazidime-susceptible *P. aeruginosa*. Most of the examined clinical isolates had simultaneous downexpression of OprD and increased efflux pumps expression, in addition to AmpC production. Intriguingly, overexpression of the *ampC* gene was observed in 2 isolates in our study. The overexpression of chromosomally encoded cephalosporinase, AmpC, is prevalent in *P. aerusinosa*.[31] Hyperproduction of chromosomally encoded AmpC β-lactamase had been known to be related to the resistance to ceftazidime in *P. aeruginosa*.[32] One among 2 isolates in our study showed deficient MexAB-OprM efflux systems, which would counterbalance the effect of AmpC production (TicHS phenotype).[33]

Our study also showed that antimicrobial effects of ceftazidime, cefepime, and piperacillin-tazobactam in imipenem-resistant and ceftazidime-susceptible *P. aeruginosa* clinical isolates using time-kill assay. To our knowledge, this is the first study analyzing the β-lactams' effects in imipenem-resistant and ceftazidime-susceptible *P. aeruginosa* clinical isolates. A pronounced decrease in the rate of bacterial killing of imipenem-resistant and ceftazidime-susceptible *P. aeruginosa* by ceftazidime was shown at a high CFU, whereas piperacillin/tazobactam achieved similar killing activity at standard conditions and at a high inoculum. The stability of piperacillin-tazobactam and the contrasting results obtained with ceftazidime against high inocula occurred in all three isolates. The well described inoculum effect, representing the requirement of higher β-lactam antibiotic concentration to inhibit bacterial growth as the

bacterial concentration increases, is related to the production of β-lactamase.[20] No isolate among three isolates showed the AmpC hyperproduction in our study. Therefore it is reasonable to assume that aspects other than the amount of β-lactamase, derived from a high inoculum or from a high level of production, are also related to the inoculum effect. Other possible reasons for the inoculum effect include factors related to quorum sensing,[34] decreasing expression of selected PBPs,[35] and expression of autolysins.[36] Although the clinical significance of the inoculum effect is unknown, animal infection models have shown for β-lactams and other anti-infectives that a high CFU or the delayed treatment of infections may greatly increase mortality or attenuate anti-infective effects.[20,37] According to this result, we recommend the use of piperacillin/tazobactam rather than ceftazidime in patients with imipenem-resistant and ceftazidime-susceptible *P. aeruginosa* isolate, especially in high-inoculum infections such as endocarditis and osteomyelitis. On the other hand, cefepime showed the lag time of bacterial killing in all three isolates. β-lactams were assumed to bind to penicillin binding proteins, which stimulate the autolysin effect, and the turnover of the autolysin effect caused the lag time of killing.[38,39] Therefore, cefepime should be used with caution in the early stage of severe infections.

## V. CONCLUSION

Our study demonstrated the increasing prevalence of carbapenemase producing CRPA, especially IMP-6-harbouring ST 235 isolates. Moreover, we report here the co-occurring $bla_{\text{IMP-6}}$ and a frameshift mutation of a 1-bp deletion of nt209ΔT in an IMP-6-producing *P. aeruginosa* ST235 strain. Increasing carbapenemase producing *P. aeruginosa* isolates are considered a significant clinical threat because there remain few alternatives for the treatment of systemic infections. Our study also suggests that the effects of ceftazidime monotherapy in imipenem-resistant and ceftazidime-susceptible *P. aeruginosa* isolates at high CFU are attenuated due to the inoculum effect. Therefore, we recommend the use of piperacillin/tazobactam rather than ceftazidime in patients with imipenem-resistant and ceftazidime-susceptible *P. aeruginosa* isolate, especially in high-inoculum infections such as endocarditis and osteomyelitis.

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ABSTRACT(IN KOREAN)

# Imipenem 내성 *Pseudomonas aeruginosa* 의 유전학적 특성과 β-lactams 항생제 효과

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## 위 유 미

본 연구는 carbapenemase를 분비하는 imipenem 내성 *Pseudomonas aeruginosa* (imipenem-resistant *P. aeruginosa,* IRPA) 임상균주의 특징과 그것의 항생제 내성패턴을 알고자 시행되었다. 더불어 ceftazidime에 감수성이 있는 IRPA에서의 β-lactams 항생제의 효과를 알고자 time-kill 연구를 진행하였다. 전향적 연구를 통해 8개의 병원에서 총 215개의 *P. aeruginosa* 균주가 수집되었다. 모든 균주에서 8개의 항생제에 대한 감수성과 carbapenemase 유전자의 유무를 확인하였다. IRPA의 역학적 특성을 확인하기 위해 Multi-locus sequence typing와 *oprD* 유전자분석을 시행하였다. ceftazidime에 감수성이 있는 IRPA에서의 β-lactams 항생제의 효과를 알고자 표준농도와 고농도의 균주에서 time-kill 연구를 진행하였다. Carbapenemase 유전자는 34 (15.8%) 균주에서 발견되었다. Carbapenemase 유전자를 가지고 있는 *P. aeruginosa* 균주가 그렇지 않은 균주에 비해 모든 항생제에 대해 내성률이 높았다. 더욱이 9균주 (26.5%) 에서는 colistin을 포함한 실험한 모든 항생제에 내성을 나타내고 있었다. 33균주에서 $bla_{\text{IMP-6}}$ 유전자를 한균주에서 $bla_{\text{VIM-2}}$ 유전자를 가지고 있었으며 모든 균주는 ST235로 분류되었다. *oprD* 유전자 분석상 ST235 모든 균주는 같은 변이를 보였는데 209번째 염기의 소실을 보이고 있었다. Ceftazidime에 감수성을 보이는 IRPA의 주된 내성기전은 OprD의

발현감소로 나타났다. Ceftazidime에 감수성을 보이는 대부분의 IRPA는 OprD의 발현감소 외에 MexAB–OprM의 과발현이 동시에 나타나고 있었다. Ceftazidime에 감수성을 보이는 IRPA 균주의 고농도에서 ceftazidime에 대한 살균효과 감소가 현격하게 나타났으나, piperacillin/tazobactam은 표준농도와 고농도에서 유사한 살균효과를 보였다. 결론적으로 본 연구에서는 IMP-6을 함유하는 ST235 *P. aeruginosa*의 증가가 국내에서 중요한 임상적 위협이 될 것이라는 것을 보여주었다. 더불어 IMP-6을 함유하는 ST235 *P. aeruginosa* 균주가 frameshift mutation of a 1-bp deletion of nt209ΔT 의 특성을 보임을 확인하였다. 본 연구는 또한 ceftazidime에 감수성을 보이는 IRPA에 의한 감염증에서는 ceftazidime 사용보다는 piperacillin/tazobactam 사용을 권하는 실험 결과를 보여주었다.

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핵심 되는 말: *Pseudomonas aeruginosa*, Carbapenemase, OprD, IMP-6, Ceftazidime