Distribution and characterization of carbapenemase-producing *Pseudomonas aeruginosa* in Korea

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Directed by Professor Kyungwon Lee

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Distribution and characterization of carbapenemase-producing *Pseudomonas aeruginosa* in Korea

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A total of 431 *Pseudomonas aeruginosa* (PA) clinical isolates were collected from 29 general hospitals in Korea in 2015. Antimicrobial susceptibility was tested by the disk diffusion method and MICs of carbapenems were determined by agar dilution method. Carbapenemase genes were amplified by PCR and sequenced and the structures of class 1 integrons surrounding the carbapenemase gene cassettes were analyzed by PCR mapping. Multi-locus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) were performed for strain typing. Whole-genome sequencing was carried out to analyze PA genomic islands (PAGIs) carrying the $bla_{\text{IMP-6}}$, $bla_{\text{IMP-10}}$, and $bla_{\text{GES-24}}$ genes. The rates of carbapenem-non-susceptible and carbapenemase-producing PA isolates were 34.3% (148/431) and 9.5% (41/431), respectively. IMP-6

was the most prevalent carbapenemase type, followed by VIM-2, IMP-10, and GES-24. All carbapenemase genes were located on class 1 integrons showing 6 different types in chromosome. All isolates harboring carbapenemase genes exhibited genetic relatedness by PFGE (similarity >80%); moreover, all isolates were identified as sequence type 235 (ST235), with the exception of two ST244 isolates by MLST. The $bla_{\text{IMP-6}}$, $bla_{\text{IMP-10}}$, and $bla_{\text{GES-24}}$ genes were found to be located on two novel PAGIs, designated PAGI-15 and PAGI-16. Our data support the exclusive clonal spread of an IMP-6-producing PA ST235 strain, and the emergence of IMP-10 and GES-24 demonstrates the diversification of carbapenemases in *P. aeruginosa* from Korea.

Key words: IMP-6, VIM-2, IMP-10, GES-24, ST235, class I integron, *Pseudomonas aeruginosa*, genomic island

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I. INTRODUCTION

Pseudomonas aeruginosa (PA) is an opportunistic pathogen that causes various nosocomial infections including sepsis, pneumonia, and urinary tract infections[1]. Treatment of PA infections is often difficult because of the intrinsic drug resistance of PA and the ability of this pathogen to acquire genes for antimicrobial resistance determinants[2]. Carbapenems are used as the last resort drugs for the treatment of infections caused by multidrug-resistant PA, due to their high affinity to penicillin-binding proteins, stability to various β-lactamases, and ability to easily pass through the bacterial outer membrane. However, the increasing use of carbapenems

has resulted in the emerging phenomenon of carbapenem resistance[3].

PA can become resistant to carbapenems by reduced permeability of the outer membrane due to loss of substrate-specific outer membrane porin OprD, frequently accompanied by AmpC hyperproduction and overexpression of efflux systems[4], along with acquisition of genes encoding carbapenemases[5]. While molecular mechanism of carbapenem resistance is geographically variable, diverse classes of carbapenemase have been increasingly identified in PA including class A [KPC and GES variants], class B [IMP, VIM, and NDM metallo-β-lactamases (MBLs)], and class D [OXA variants][6]. The MBLs, of particular IMP and VIM enzymes, are the most widespread carbapenemases in PA[7], exclusively IMP-6 in South Korea[8].

Carbapenemase genes are found often on integrons in PA clinical isolates. Integrons are prevalent in Gram-negative clinical isolates and offer the ability to capture and excise gene cassettes, frequently including antibiotic resistance determinants, to the bacterial host[9]. However, integrons accomplish mobility only when they are associated with a specific mobile genetic element such as a transposon, conjugative plasmid, or genomic island[10]. The genomic islands in PA are mostly integrative and conjugative elements (ICEs) and are named according to their characteristics, i.e. PA pathogenicity island; the name of their host, i.e. LES genomic islands; or more broadly, i.e. PA genomic island (PAGI)[11]. Thus far, 14 PAGIs have been identified[12]. These genomic islands typically carry genes for integration, transfer, and maintenance, which confer self-transferability to the composite ICE, along with

genes conferring metabolic, virulence, and heavy-metal-resistance capability, which offer extra benefit to the bacterial host. Although PAGIs associated with antimicrobial resistance are rare, some examples have been identified, e.g. derivatives of PAGI-1 and PAGI-2[13].

Two nucleotide sequence-based bacterial typing techniques, pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST), are considered to be the gold standards for use in epidemiological studies. The PFGE provides fingerprints of entire bacterial genome by restriction enzyme mapping to determine epidemiologic relationships of an outbreak in detail, while the MLST method gives strain lineages based on DNA fragments of seven house-keeping genes which allow fewer and larger groups feasible for global comparison[14]. Two international high-risk PA clones including sequence type 111 (ST111) and ST235 are responsible for the dissemination of carbapenemase genes worldwide[15]: GES-type carbapenemases-producing PA ST235 in Spain[16] and IMP-6-producing PA ST235 in South Korea[8]. In the meanwhile, ST175 and ST244 carrying genes that confer resistance to carbapenems have also been reported to be responsible for regional dissemination, as a result, it could be considered that they were associated with concerning high-risk clone[17]. Together with these two, relatively high-cost whole genome sequencing (WGS) is now commonly used for epidemiologic studies expecting incomparable power of discrimination[14].

The aims of this study were to investigate the prevalence of carbapenemase genes in PA clinical isolates recovered from general hospitals in Korea in 2015 and to

determine the genetic environments surrounding the carbapenemase genes and epidemiological characteristics of carbapenemase-producing PA (CP-PA) clinical isolates.

II. MATERIALS AND METHODS

1. Bacterial strains

A total of 431 non-duplicated PA clinical isolates were collected from 29 general hospitals located in 11 cities and provinces in South Korea between June and August 2015. The isolates were recovered from blood (n = 33), respiratory specimens (n = 180), urine (n = 93), pus (n = 97), and other specimens (n = 28). Bacterial species were identified by matrix-assisted laser desorption ionization-time of flight (MALDI) using a Bruker MALDI mass spectrometry instrument. Species identification was made only when the log score of the species was above 2.0 and by using partial sequences of the 16S rRNA gene.

2. Antimicrobial susceptibility testing

Antimicrobial susceptibilities were tested by the disk diffusion method following the Clinical and Laboratory Standards Institute guidelines[18]. Briefly, disks (Oxoid Ltd., Basingstoke, United Kingdom) containing the following antimicrobial agents were used: piperacillin, ceftazidime, cefepime, aztreonam, gentamicin, tobramycin, amikacin, ciprofloxacin, meropenem, imipenem, and colistin. MICs of meropenem and imipenem were determined by the agar dilution method on Mueller-Hinton agar (Becton, Dickinson and Company, Sparks, MD, USA). PA ATCC 27853 and *Escherichia coli* ATCC 25922 were used as control strains.

3. Identification of carbapenem resistance determinants

The genes encoding IMP-, VIM-, and NDM-type MBLs, KPC- and GES-like serine β-lactamases[19], and OprD outer membrane porin were amplified by PCR in carbapenem-non-susceptible PA clinical isolates. PCR amplicons were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced using an Applied Biosystems 3730xl DNA analyzer (Applied Biosystems, Foster City, CA, USA) to identify the variant types of each carbapenemase family. Sequences were compared with those in GenBank (http://www.ncbi.nlm.nih.gov/genbank) using the BLAST network service.

4. Integron mapping and sequencing

The structures of the class 1 integrons carrying the carbapenemase gene cassettes were determined by PCR mapping and sequencing using the primers (**Table 1**).

Table 1. Primers used in this study for carbapenemase genes and integron characterization

Target	Primer	Nucleotide sequence (5' to 3')	Position[a]	Reference
IntI	INT1-mF	GCCTGTTCGGTTCGTAAGCT	908/927	This study
	INT1-F	CCAAGCTCTCGGGTAACATC	462/481	
	INT1-R	CATGAAAACCGCCACTGC	997/1014	
qacEΔ1	qacEΔ1-F	GAAAGGCTGGCTTTTTCTTG	3/22	This study
	qacEΔ1-R	GCAGCGACTTCCACGATG	311/328	
ereA	ereA2-F	TTGAGCGATTTTCGGATACC	269/288	This study
	ereA2-R	GGCATGAATCCTCCTTACCA	1071/1090	
qac	qac-F	CAATCTTTGGCGAGGTCATC	29/48	8
	qac-R	CGCTGACCTTGGATAGCAG	307/325	
catB3	catB3-F	AAGGCAAGCTGCTTTCTGAG	29/48	21
	catB3-R	AACGATAGCGTAAGGCTCCA	440/459	
aadA1	aadA1-F	ACATCATTCCGTGGCGTTAT	284/303	This study
	aadA1-R	AGGTTTCATTTAGCGCCTCA	489/508	
aacA4	aacA4-F	TGACCTTGCGATGCTCTATG	12/31	This study
	aacA4-R	CTGGCGTGTTTGAACCATGT	470/489	
aacA7	aacA7-F	CAGGCCTGTTGAAACTACCG	21/40	22
	aacA7-R	CTTGAGCAACCTCCGTGAAT	414/433	
bla_{OXA}	OXA-1F	TATCTACAGCAGCGCCAGTG	54/73	21
	OXA-1R	TGCACCAGTTTTCCCATACA	635/654	
	OXA-2F	CGATAGTTGTGGCAGACGAA	128/147	
	OXA-2R	CTTGACCAAGCGCTGATGT	564/582	
tniC	TniC-R	TTTCCGAGCGAACAGTCGCT	229/248	22
bla_{IMP}	IMP-1F	AAGGCGTTTATGTTCATACTTCG	95/117	19
	IMP-1R	TTTAACCGCCTGCTCTAATGTAA	677/699	
bla_{VIM}	VIM-2F	ATCATGGCTATTGCGAGTCC	46/65	19
	VIM-2R	ACGACTGAGCGATTTGTGTG	775/794	
bla_{KPC}	KPC-F	GTCACTGTATCGCCGTCTAGTTC	3/25	19
	KPC-R	TGGTGGGCCAATAGATGATT	919/938	
bla_{GES}	GES-F	ATGCGCTTCATTCACGCAC	1/19	This study
	GES-R	CTATTTGTCCGTGCTCAGGA	864/845	

bla$_{NDM}$	NDM-F	GCCCAATATTATGCACCCGG	9/28	19
	NDM-R	CTCATCACGATCATGCTGGC	649/668	
oprD	oprD-F	GGAACCTCAACTATCGCCAAG	-120/-99	This study
	oprD-R	GTTGCCTGTCGGTCGATTAC	+17/1328	

[a]Coordinates refer to the first base of each gene.

5. Multilocus sequence typing

PCR amplification and sequencing of partial fragments of seven house-keeping genes (*acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA*, and *trpE*) were performed and the experimentally determined nucleotide sequences of both strands were compared to pre-existing sequences in the MLST database to assign allele numbers and STs (http://pubmlst.org/paeruginosa).

6. Pulsed-field gel electrophoresis

Agarose plugs containing *Xba*I-digested genomic DNA from PA clinical isolates were prepared and DNA fragments were separated for 20 h at 6 V/cm at 11℃ using a CHEF-DRII System (Bio-Rad, Hercules, CA, USA). Pulsing was carried out with initial and final pulse times of 0.5 s and 30 s, respectively. A lambda ladder (Bio-Rad) was used as a DNA size marker. Band patterns were analyzed with UVIband/Map software (UVItech Ltd, Cambridge, UK) and used to generate dendrograms based on the unweighted pair group method using arithmetic averages from the Dice coefficient.

7. Southern blot and hybridization

Southern blotting was performed to determine the locations of the carbapenemase genes. Briefly, I-*Ceu*I or S1 nuclease-digested DNA was blotted onto nylon membranes (Zeta-Probe Blotting Membranes, Bio-Rad) and hybridized with probes

specific for the carbapenemase genes or 16S rRNA. The probes were obtained via PCR amplification as described above. Probe labeling, hybridization, and detection were performed with a DIG DNA Labeling and Detection Kit (Roche Diagnostics, Indianapolis, IN, USA).

8. Whole genome sequencing (WGS)

For bacterial whole genome sequencing (WGS), Single-Molecule Real Time (SMRT) sequencing was carried out on a PacBio RSII instrument (Pacific Biosciences, Menlo Park, CA, USA). Genomic DNA was extracted from the PA strains using a Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA). After extraction, DNA shearing was performed using a g-TUBE™ apparatus (Covaris, Inc., Moburn, MA, USA) and the fragments were purified by using 0.45x of the final volume of washed Agencourt AMPure XP magnetic beads (Beckman Coulter Inc., Brea, CA, USA). SMRTbell template libraries were subsequently prepared and adapter ligation was performed, followed by exonuclease digestion of incompletely ligated products. Reads with lengths that were less than 50 bp were filtered out after acquisition of the sequencing data; the minimum polymerase read quality was set to 0.75. *De novo* genome assembly of PacBio SMRT reads was performed with the PacBio SMRT analysis software suite (version 2.3.0)[20]. Briefly, the hierarchical genome assembly process was used with default parameters and a seed read length cut-off of 6 kb. Following assembly, individual contigs with duplicate sequences on

their 5’ and 3’ ends were manually trimmed. Similarly, overlapping sequences were manually trimmed and joined based on sequence similarity to form circular fragments. Following chromosome and plasmid circularization, the sequences were polished using Quiver. In this program, the raw reads are mapped back to the chromosome and plasmid sequences to validate the assembly and resolve any remaining sequence errors. The annotations of coding sequences, tRNA sequences, and rRNA sequences were performed using the NCBI Prokaryotic Genome Annotation Pipeline (http://www.ncbi.nlm.nih.gov/books/NBK174280).

9. Nucleotide sequence accession numbers

The nucleotide sequence data reported in this paper are available in the GenBank nucleotide database under accession numbers KX196167 (PAGI-16 carrying the $bla_{\text{IMP-6}}$ gene), KX196168 (PAGI-15 carrying the $bla_{\text{GES-24}}$ gene), and KX196169 (PAGI-16 carrying the $bla_{\text{IMP-10}}$ gene).

III. RESULTS

1. Antimicrobial resistance profiles of the PA isolates

Of the 431 PA clinical isolates examined, 283 (65.7%) were found to be susceptible to carbapenems. The remaining 148 isolates (34.3%) that were non-susceptible to imipenem and/or meropenem were classified by PCR into two groups: carbapenemase-producing *P. aeruginosa* (CP-PA) (n = 41), whose members carried the bla_{IMP}, bla_{VIM}, or bla_{GES} gene, and carbapenemase-non-producing PA (CNP-PA; n = 107), whose members did not. The 20 isolates (18.7%) among the CNP-PA group had a OprD porin loss. The vast majority (40/41) of the clinical isolates in the CP-PA group exhibited an extensively drug-resistant phenotype and were resistant to all drugs tested except colistin (**Figure 1**). Moreover, the CP-PA group showed extremely high rates of non-susceptibility to piperacillin (97.6% compared to 62.6% in the CNP-PA group and 10.2% in the carbapenem-susceptible group), ceftazidime (97.6% compared to 29.9% and 7.1%, respectively), ciprofloxacin (100% compared to 70.1% and 13.8%, respectively), and amikacin (100% compared to 23.4% and 3.5%, respectively).

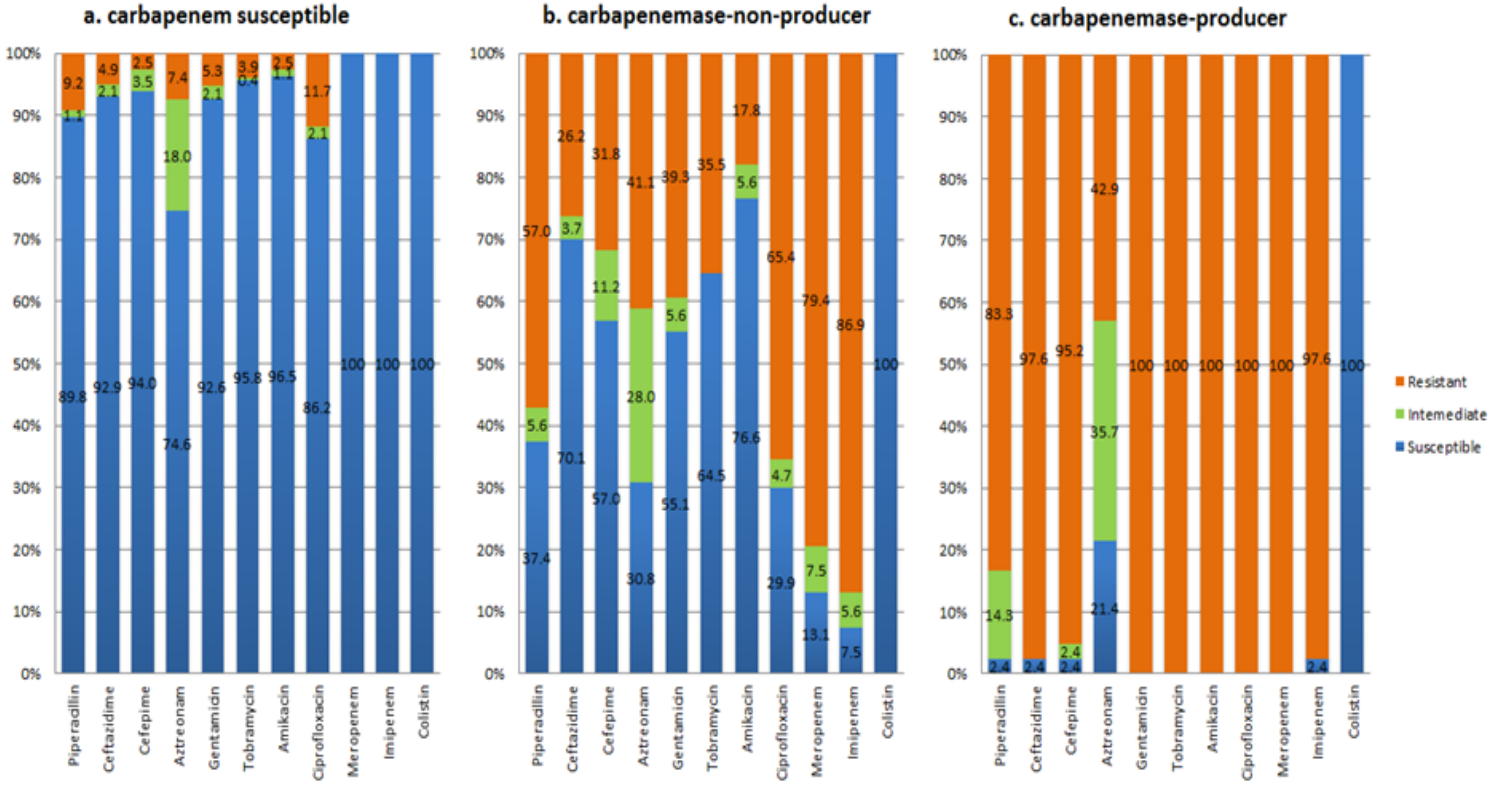


Figure 1. Antimicrobial susceptibilities of *P. aeruginosa* clinical isolates. a) carbapenem susceptible (n=238), b) carbapenem-non-susceptible carbapenemase-non-producers (n=107), and c) carbapenem-non-susceptible carbpanemase-producers (n=41).

2. Presence of the carbapenemase gene and its correlation with carbapenem susceptibility

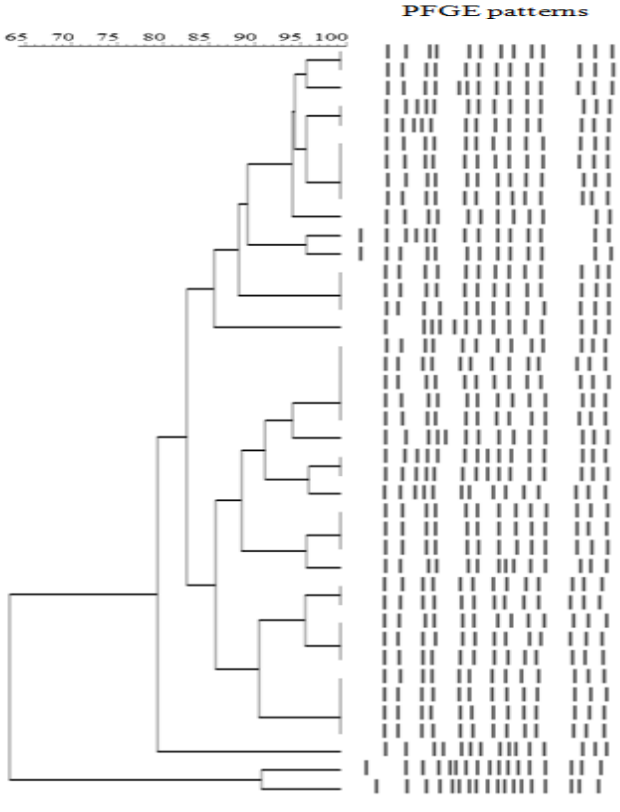
The most prevalent gene, bla_{IMP-6}, was identified in 36 isolates; moreover, another subtype bla_{IMP-10} was identified in one isolate. PA clinical isolates harboring bla_{IMP-6} exhibited higher resistance to meropenem (MICs ranged from 64 to >256 μg/ml): MIC_{50} >256 μg/ml and MIC_{90} >256 μg/ml than to imipenem (MICs ranged from 16 to 256 μg/ml): MIC_{50} 16 μg/ml and MIC_{90} 32 μg/ml. The isolate BP14 carrying bla_{IMP-10} had similar carbapenem MICs: meropenem (MIC, >256 μg/ml) and imipenem (MIC, 16 μg/ml). The products encoded by bla_{IMP-6} and bla_{IMP-1} differ by one amino acid, with the former having a Ser216-Gly substitution compared with the latter. Similarly, the product encoded by bla_{IMP-10} exhibits a single Val49-Phe substitution compared with the product encoded by bla_{IMP-1}.

The bla_{VIM-2} gene was identified in three isolates. These three isolates had meropenem MICs ranging from 16 to 128 μg/ml and imipenem MICs ranging from 16 to 256 μg/ml. The bla_{GES-24} gene was identified in one isolate and the isolate presented resistance to meropenem (MIC, 128 μg/ml) and imipenem (MIC, 64 μg/ml). Of note, this strain had a truncated OprD by a novel insertion sequence IS*PA67*. Considering relatively low carbapenem MICs for 20 PA strains having only the porin loss, of meropenem 1 to 32 μg/ml (MIC50 8 μg/ml and MIC90 16 μg/ml) and of imipenem 4 to 32 μg/ml (MIC50 16 μg/ml and MIC90 32 μg/ml), the elevated carbapenem MICs were mostly by GES-24. The GES-24 differs from GES-1 by two substitutions, Met62-Thr and Gly170-Ser, and the latter change is the same as the

carbapenem-hydrolyzing GES-5.

3. Strain typing for the CP-PA isolates

All CP-PA isolates were identified as ST235 (allele profile, 38-11-3-13-1-2-4), with the exception of two isolates, isolate JNH6, which carried $bla_{\text{VIM-2}}$, and isolate IH2, which carried $bla_{\text{GES-24}}$. These two isolates were identified as ST244 (17-5-12-3-14-4-7) very different from ST235 (**Table 2**). The ST235 CP-PA isolates shared more than 80% similarity by PFGE analysis, and the remaining two ST244 isolates also exhibited close relatedness (similarity, >90%). The ST235 and ST244 isolates did not show significant relatedness (similarity, <70%) to each other (**Figure 2**).



Isolate	Carbapenemase gene	MIC (μg/ml)		Sequence type
		Meropenem	Imipenem	
CMDAE1	*bla*IMP-6	>256	32	ST235
SCHGM14	*bla*IMP-6	>256	16	ST235
CMDAE3	*bla*IMP-6	>256	16	ST235
CMDAE6	*bla*IMP-6	>256	16	ST235
CMDAE7	*bla*IMP-6	>256	16	ST235
CBU1	*bla*IMP-6	>256	16	ST235
CBU14	*bla*IMP-6	>256	16	ST235
CMDAE11	*bla*IMP-6	>256	16	ST235
CMDAE12	*bla*IMP-6	>256	16	ST235
CMDAE4	*bla*IMP-6	>256	16	ST235
CMDAE5	*bla*IMP-6	>256	16	ST235
SCHGM15	*bla*IMP-6	64	16	ST235
GS4	*bla*VIM-2	128	256	ST235
KMU11	***bla*IMP-6**	**>256**	**32**	**ST235**
IH6	*bla*IMP-6	>256	16	ST235
KMU3	*bla*IMP-6	>256	32	ST235
BP13	*bla*IMP-6	>256	256	ST235
CSU11	*bla*IMP-6	>256	16	ST235
KMU4	*bla*IMP-6	>256	32	ST235
SCHGM1	*bla*IMP-6	>256	16	ST235
SCHBU12	*bla*IMP-6	>256	32	ST235
JNH1	*bla*IMP-6	>256	32	ST235
BP14	***bla*IMP-10**	**>256**	**16**	**ST235**
BP7	*bla*IMP-6	>256	16	ST235
CMDAE15	*bla*IMP-6	>256	16	ST235
SCHGM10	*bla*IMP-6	>256	16	ST235
SCHGM13	*bla*IMP-6	>256	16	ST235
SCHGM12	*bla*IMP-6	>256	16	ST235
CAU11	*bla*IMP-6	>256	16	ST235
GNU11	*bla*IMP-6	>256	16	ST235
GNU14	*bla*IMP-6	>256	16	ST235
CMDAE14	*bla*IMP-6	>256	16	ST235
SCHGM4	*bla*IMP-6	>256	32	ST235
WCH1	*bla*IMP-6	>256	16	ST235
CSU6	*bla*IMP-6	>256	32	ST235
JN5	*bla*IMP-6	>256	64	ST235
EUMC2	*bla*IMP-6	>256	32	ST235
HP6	*bla*IMP-6	>256	32	ST235
JNH5	*bla*VIM-2	32	16	ST235
IH2	***bla*GES-24**	**128**	**64**	**ST244**
JNH6	*bla*VIM-2	16	128	ST244

Figure 2. Characteristics of chromosome-encoded carbapenem-producing *P. aeruginosa* clinical isolates. Strains which were performed whole genome sequencing are shown in boldface.

Table 2. SNP differences of the allelic sequence for MLST between ST235 and ST244

Sequence type	Allelic profile (size of the template)						
	acsA (390 bp)	*aroE* (498 bp)	*guaA* (373bp)	*mutL* (442bp)	*nuoD* (366bp)	*ppsA* (370bp)	*trpE* (443bp)
ST235	38	11	3	13	1	2	4
ST244	17	5	12	3	14	4	7
No. of SNPs	4	10	2	3	2	3	6

4. Genetic contexts of the class 1 integrons

The *bla*$_{IMP-6}$, *bla*I$_{MP-10}$, *bla*$_{VIM-2}$, and *bla*$_{GES-24}$ genes were located on class 1 integrons as gene cassettes and the structures determined by PCR mapping are indicated in **Figure 3**. The *bla*$_{IMP-6}$ gene in all 36 isolates was carried by the InIMP-6D *intI1-bla*$_{IMP-6}$*-qac-aacA4-catB3-aacA4-bla*$_{OXA-1}$*-aadA1*. This class 1 integron has been identified in 2008 in one PA ST235 clinical isolate from Korea[21]. The *bla*$_{IMP-10}$ gene in isolate BP14 composed a new class 1 integron InIMP-10, *intI1-bla*$_{IMP-10}$*-qac-aacA4-catB3-aacA4-bla*$_{OXA-1}$*-aadA1*. Between the integron integrase coding gene and the gene cassettes, an integron-associated 136-bp *attI* recombination site was identified.

The *bla*$_{VIM-2}$ gene cassette was found in three different integrons: In559 *intI1-aacA7-bla*$_{VIM-2}$*-dhfa-aadA5*, InVIM-2$_{JH5}$ *intI1-aadB-bla*$_{VIM-2}$*-aacA4-orf-ereA*, and InVIM-2$_{JH6}$ *intI1-aacA4-bla*$_{VIM-2}$*-aacA4-fosC-bla*$_{OXA-2}$*-qac*. The In559 was first identified in PA ST235 clinical isolates from Russia in 2002[22] and the InVIM-2$_{JH5}$ and InVIM-2$_{JH6}$ class 1 integrons were first found in PA and *Pseudomonas putida* isolates, respectively, from South Korea (unpublished data; GenBank accession: EF207719 for InVIM-2$_{JH5}$ and AY907717 for InVIM-2$_{JH6}$). Notably, the In559 was distinct from the other class 1 integrons that culminate with *qacEΔ1* in that the Tn5090 *tniC* gene (also called *tniR* of Tn402) for the 3'CS indicates a distinct evolutionary path driving the excision and addition of gene cassettes[23]. The isolate IH2 was found to harbor a novel integron, InGES-24 *intI1-aacA4-aadB-bla*$_{GES-24}$*-aacA4-bla*$_{OXA-2}$.

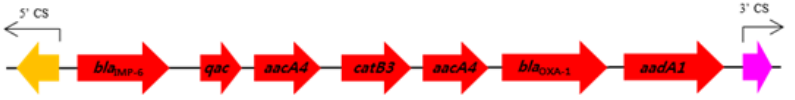
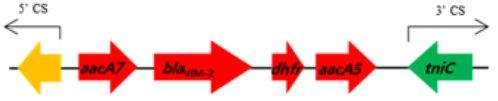
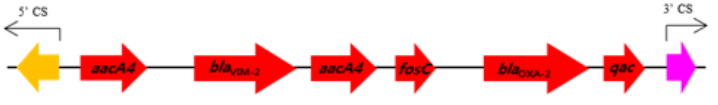
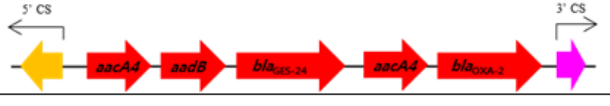
	Antimicrobial resistance gene cassette array[a]	No. of isolates	Reference	GenBank accession no.
InIMP-6D		36	21	KC960557
In559		1	22	DQ522233
InVIM-2$_{JH5}$		1	unpublished	EF207719
InVIM-2$_{JH6}$		1	unpublished	AY907717
InIMP-10		1	This study	KX196169
InGES-24		1	This study	KX196168

Figure 3. Schematic representation of class 1 integrons carrying the carbapenemase gene cassettes. Orange arrows indicate integrase; red arrows, genes for resistance; pink arrows, *qacEΔ1*; blue arrow, a gene for tyrosine recombinase XerD; dotted line, unknown nucleotide sequences.

5. Two novel genomic islands PAGI-15 and PAGI-16

By Southern blot analysis using I-*Ceu*I-macrorestriction digested fragments, the $bla_{\text{IMP-6}}$, $bla_{\text{IMP-10}}$, $bla_{\text{VIM-2}}$, and $bla_{\text{GES-24}}$ were verified to be chromosome-encoded in all CP-PA isolates (data not shown). Probes specific for the carbapenemase genes did not bind to any S1 nuclease-treated plasmids. To further characterize the genomic environments of the class 1 integrons, WGS was conducted using the PacBio RSII platform for three PA isolates: KMU11 of $bla_{\text{IMP-6}}$, BP14 of $bla_{\text{IMP-10}}$, and IH2 of $bla_{\text{GES-24}}$.

The draft genome sequences of KMU11 were composed of nine contigs and three (a total of 6.9 Mb in size) of those carried the genes for ribosomal proteins indicating chromosome. The class 1 integron InIMP-6D was found on a chromosome in a 1.6-Mb contig possessing together the genes encoding ribosomal proteins S6, S9, S15, S18, S20, L9, L13, L21, L25, L27, and L31. Moreover, the integron was in a novel genomic island, PAGI-16, being 95,029-bp in size. The PAGI-16 was integrated at the end of the $tRNA^{gly}$ gene, as the other genomic islands found in PA[11], and flanked at extremity by 20-bp direct repeats (DR) GATTCCCTTCACCCGCTCCA (**Figure 4A**). The PAGI-16 in the isolate KMU11 had a G+C content of 61.4% and it encoded 99 ORFs including a cluster of nine genes originating from the *clc*-element which is also found in PFGI-1 of *Pseudomonas fluorescens*[26]. The *clc*-element integrates through the *attB* site using a bacteriophage P4-like integrase; this site is situated in between two tandem $tRNA^{Gly}$ genes in the 3'-end[11]. The cluster also contains ten genes associated with genome instability such as integrases, transposases, and

insertion sequences; eight genes for antimicrobial resistance, i.e., two copies of *aacA4*, *aadA1*, *catB3*, *cmx*, *sul1*, bla_{OXA-1}, together with bla_{IMP-6} which are mostly found in the class 1 integron between bps 57,464 and 77,106; and genes encoding hypothetical proteins, which comprise the rest of the sequences.

The isolate BP14 had 7.1-Mb circularized chromosome without any plasmid and the class 1 integron InIMP-10 was found within a 69,208-bp 5'-portion of genomic island sharing 99.99% nucleic acid identity with PAGI-16 in the isolate KMU11. This portion was integrated at the end of $tRNA^{gly}$ gene and the 20-bp DR GATTCCCTTCACCCGCTCCA was present in the 5'-end as that in KMU11. The PAGI-16 was ended by IS*6100* and a truncated *wspC*, encoding a biofilm formation methyltransferase WspC, was found in the downstream (**Figure 4A**). The 1,269-bp *wspC* gene encodes the biofilm formation methyltransferase WspC and the left 619-bp was found 3.3-Mb away from the portion together with the rest part of the PAGI-16 in an opposite direction. The 26,701-bp 3'-portion of the PAGI-16 in the isolate BP16 was identical to that in KMU11 and the 20-bp DR sequence was found indicating chromosomal inversion by a duplication-insertion of IS*6100*[25]. The entire PAGI-16 in the isolate BP14 had the same G+C content of 61.4%

The IH2 had 7.3-Mb circularized chromosome devoid of any plasmid. In the chromosome, the class I integron InGES-24 was located within an 118,715-bp genomic island designated as PAGI-15. The location of the PAGI-15 was the same as PAGI-16, next to the $tRNA^{gly}$ gene, but the DR was 27-bp in size, AGGGTTCGATTCCCTTCGCCCGCTCCA (**Figure 4A**). The G+C content of the

PAGI-16 was 61.3%, slightly smaller than that of the PAGI-15. This genomic island shared the backbone with PAGI-16, presenting 99.99% nucleic acid identity excluding the class 1 integron and a short putative prophage possessing nine ORFs located in the 3'-end of the genomic island (**Figure 4A**). The PAGI-16 encoded 116 putative ORFs: mostly shared with PAGI-15 except 11 antimicrobial resistance genes, two copies of *aacA4*, *aadB*, *strA*, *strB*, *tet(G)*, *cmx*, two copies of *sul1*, $bla_{\text{OXA-2}}$, and $bla_{\text{GES-24}}$.

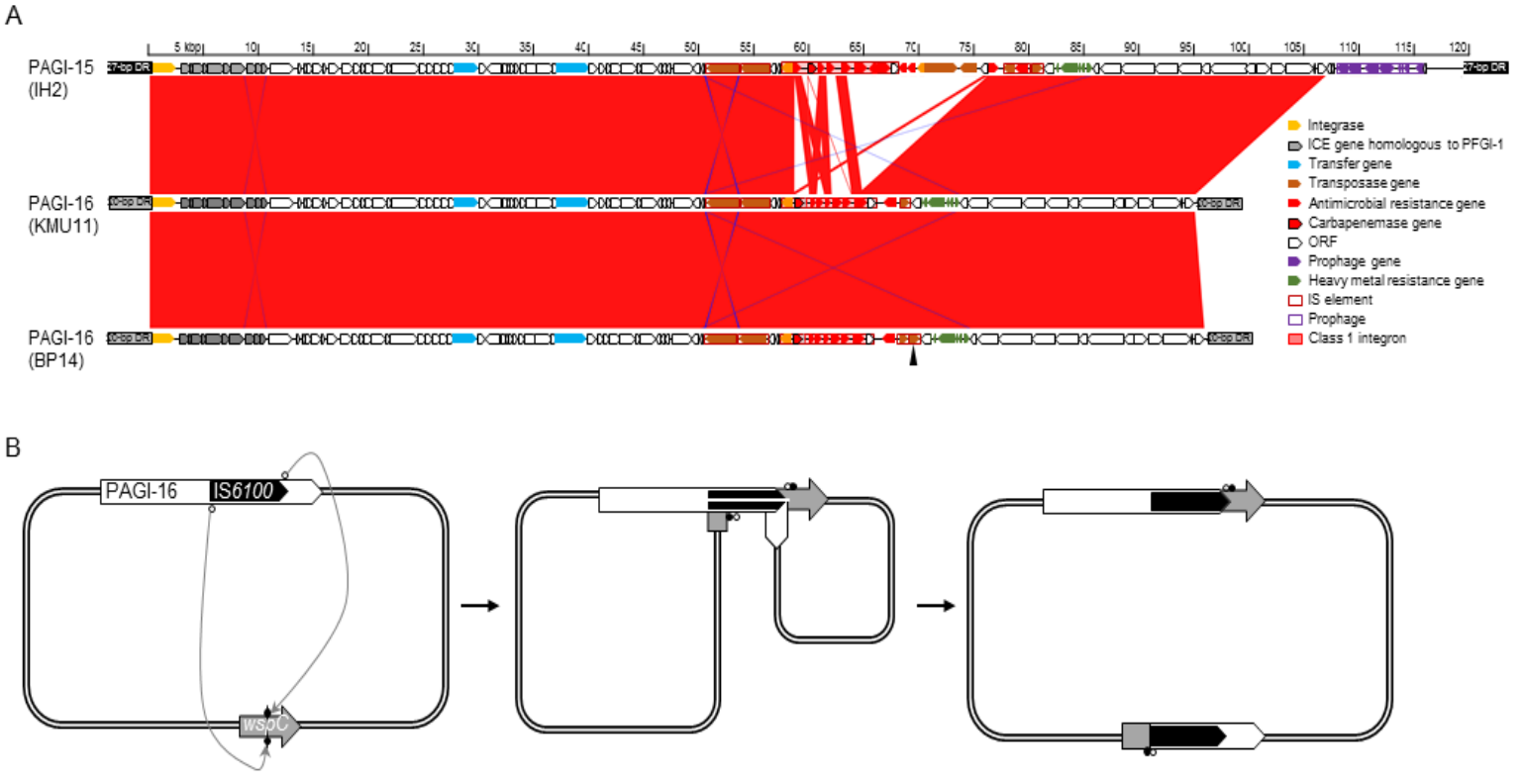


Figure 4. Schematic representation of genomic islands in IH2, KMU11, and BP14 strains. A The sequences of genomic islands of PA isolates were aligned using BlastN, and compared using Artemis Comparison Tool[24]. The conserved regions (> 95% identity) are indicated in red. Arrow head, location of chromosomal inversion due to duplication-insertion of IS*6100*.

B Schematic mechanism for the large chromosomal inversion mediated by duplication of IS*6100* adapted from[25]. In a chromosome carrying PAGI-16, catalyzed 3'-OH groups of the IS*6100* (white circle) targeted the 5' phosphate in the *wspC* gene (black circle) located elsewhere. The replication broke the cointegration leading the duplication in the opposite direction of IS*6100* and a chromosomal inversion. And the final chromosomal structure became 5'PAGI-16-IS*6100*-5'Δ*wspC* and 3'PAGI-16-IS*6100* (opposite direction)-3'Δ*wspC*. Open arrow, PAGI-16; black arrow, IS*6100*; gray arrow, *wspC*.

IV. DISCUSSION

A nationwide survey in 2009 reported the carbapenem resistance rate in PA was 35.8% (138/386) and 8.0% (31/386) of the strain produced carbapenemase IMP-6 (n=30) and VIM-2 (n=1)[8]. In this surveillance, similar rate of CP-PA was found, 9.5% (41/431). Of CP-PA, prevalence of *bla*$_{IMP-6}$-producing PA has been detected exclusively in South Korea. The IMP-6 production by the bacterial host results in a higher MIC for meropenem than for imipenem. This observation is due to the Ser216-Gly substitution, which results in increased hydrolysis of meropenem compared with imipenem[27]. Interestingly, meropenem is highly recommended for treating Gram-negative bacterial infections rather than imipenem[28] and meropenem usage is increasing faster than that of imipenem[29]. It is likely that antibiotic selective pressure leads to the predominance of IMP-6 in PA.

Clonal diversity of the epidemic isolates, which consisted mostly of ST235 isolates presenting indistinguishable PFGE patterns, allowed us to understand the dissemination of IMP-6 was mostly by clonal dissemination of the PA ST235 isolates possessing the gene. The gene cassette array added another evidence for that showing all IMP-6-positive strains possessed an identical class 1 integron, InIMP-6D *intI1-bla*$_{IMP-6}$*-qac-aacA-catB3-aacA4-bla*$_{OXA-1}$*-aadA1*. Interestingly, the gene cassette array of this class 1 integron resembled that of the integron, *intI1-bla*$_{IMP-6}$*-qac-aacA4-bla*$_{OXA-1}$*-aadA1,* in epidemic strains of PA isolated in 2009[8] (**Figure 3**).

The second most prevalent carbapenemase was VIM-2, although it was only

identified in three isolates. The bla_{VIM-2} gene was sporadically identified to be associated with unrelated integrons in PA of two different STs, ST235 and ST244, each with different PFGE patterns (**Figure 2**). These results suggest that bla_{VIM-2} dissemination was not due to clonal spread, but rather to an occasional occurrence. Interestingly, In559, one of the three class 1 integrons carrying the bla_{VIM-2} gene cassette, had a distinct 3'-end, matured by *tniC* of Tn5090 instead of the usual 3'CS with *qacEΔ1*. This finding likely indicates that the In559 has been developed through a different evolutionary scheme used by Tn5090. A similar bla_{VIM-2} gene cassette-carrying integron, which was identified as an ancestral class 1 integron 3'-ended by the *tniC* gene, was identified in a PA strain isolated in India in 2003[23]. The precise epidemiological relationship of the unusual integrons in our strain and in the Indian strain is unclear, since no ST information could be obtained for the case.

This surveillance study enabled the first observation of IMP-10 and GES-24, indicating diversification of carbapenemases in PA in South Korea. The bla_{IMP-10}-associated integron has previously been identified in PA in Japan[30]. We initially suspected that the first identification of bla_{IMP-10} reflected a traveler carrier; however, after inspecting the genomic environment of this gene, we concluded that this gene appearance was a result of spontaneous nucleotide substitutions independently from bla_{IMP-1}, a G640A substitution resulted in Gly216-Ser mutation for IMP-6 in PAGI-16 of KMU11 and G145T leading to Val49-Phe mutation in IMP-10 in PAGI-16 of BP14. Indeed, this conclusion is supported by two additional pieces of evidence. First, the InIMP-6D and InIMP-10 have the same genetic context, with the exception of the

MBL gene cassettes (bla_{IMP-6} and bla_{IMP-10}) between the 5'CS and the 3'CS (**Figure 3**). Second, the PAGI-16, which was found to carry the bla_{IMP-6} gene (KX196167), and the PAGI-16, which was found to carry the bla_{IMP-10} gene (KX196169), exhibited a high degree of homology based on WGS results and shared most of the conserved regions (95% identity) (**Figure 4A**).

So far, six GES β-lactamases, namely GES-2, -4, -5, -6, -14 and -18, have been shown to present detectable carbapenemase activity[6]. This activity is predominantly a factor of amino acid substitutions of residues 104 and 170. The GES-24 enzyme has Gly170 as GES-5, which results in high affinity for carbapenems and high turnover rates due to the low rate constants for acylation and deacylation[31]. The GES-5 producers, *Klebsiella pneumoniae* and *E. coli*, have been found in South Korea[32], but the gene was on very different integrons. The gene for GES-24 in *Enterobacter clocae* and *Acinetobacter baumannii* from Japan are available in GenBank (last updated on 05/13/2016) but, to the best of our knowledge, this is the first description of GES-24 in PA.

Interestingly, PAGI-15 and PAGI-16 were found to differ only by their integrons. It is likely that both genomic islands derived from a common origin. They once possessed a backbone possessing a *clc*-like element, into which two unrelated integrons were later integrated. Moreover, one of the PAGI-16s was found to have split into two parts by large chromosomal inversion that resulted in duplication-insertion of the IS*6100* gene in the opposite direction (**Figure 4B**). Similar IS*6100*-mediated large chromosomal inversions have been found in PA clinical isolates and

are likely to be involved in phenotypic adaptations of the different strains to the environment[25].

The PAGI-15 and -16 of this study were found in PA belonging to ST244 and ST235. These genomic islands have shared origin evidenced by the same backbone in spite of the distinct STs of the host PA. Interestingly, a genomic island having a same backbone is found also in chromosome of PA ST395 isolated in France in 1997 (available in GenBank accession CP013993). The genomic island has a class 1 integron possessing one gene cassette, *aadB*. This highly mobile genomic island is likely having a key role for capturing and disseminating the multiple antibiotic resistance genes in PA belonging to various STs similarly to the PAGI-1 and -2[13].

V. CONCLUSION

In conclusion, our data indicate that IMP-6 is highly prevalent in CP-PA ST235 isolates. Moreover, our results clearly demonstrate that WGS methodology can be used to identify the genomic environments associated with resistance determinants, which promises to shed light on many epidemiologic questions regarding the mechanism of dissemination of resistance determinants.

REFERENCES

1. Dortet L, Poirel L, Nordmann P. 2012. Rapid identification of carbapenemase types in *Enterobacteriaceae* and *Pseudomonas* spp. by using a biochemical test. Antimicrob Agents Chemother 56: 6437-40.

2. Morita Y, Kimura N, Mima T, Mizushima T, Tsuchiya T. 2001. Roles of MexXY- and MexAB-multidrug efflux pumps in intrinsic multidrug resistance of *Pseudomonas aeruginosa* PA01. J Gen Appl Microbiol 47: 27-32.

3. Zavascki AP, Carvalhaes CG, Picão RC, Gales AC. 2010. Multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii*: resistance mechanisms and implications for therapy. Expert Rev Anti Infect Ther 8: 71-93.

4. Jacoby GA. 2009. AmpC beta-lactamases. Clin Microbiol Rev 22: 161-82.

5. Crespo MP, Woodford N, Sinclair A, Kaufmann ME, Turton J, Glover J et al. 2004. Outbreak of carbapenem-resistant *Pseudomonas aeruginosa* producing VIM-8, a novel metallo-beta-lactamase, in a tertiary care center in Cali, Colombia. J Clin Microbiol 42: 5094-101.

6. Diene SM, Rolain JM. 2014. Carbapenemase genes and genetic platforms in Gram-negative bacilli: *Enterobacteriaceae, Pseudomonas* and *Acinetobacter* species. Clin Microbiol Infect 20: 831-8.

7. Pasteran F, Faccone D, Gomez S, De Bunder S, Spinelli F, Rapoport M et al; *Pseudomonas aeruginosa* KPC Group. 2012. Detection of an international

multiresistant clone belonging to sequence type 654 involved in the dissemination of KPC-producing *Pseudomonas aeruginosa* in Argentina. J Antimicrob Chemother 67: 1291-3.

8. Seok Y, Bae IK, Jeong SH, Kim SH, Lee H, Lee K. 2011. Dissemination of IMP-6 metallo-β-lactamses-producing *Pseudomonas aeruginosa* sequence type 235 in Korea. J Antimicrob Chemother 66: 2791-6.

9. Boucher Y, Labbate M, Koenig JE, Stokes HW. 2007. Integrons: mobilizable platforms that promote genetic diversity in bacteria. Trends Microbiol 15: 301-9.

10. Hall RM. 2012. Integrons and gene cassettes: hotspots of diversity in bacterial genomes. Ann N Y Acad Sci 1267: 71-8.

11. Kung VL, Ozer EA, Hauser AR. 2010. The accessory genome of *Pseudomonas aeruginosa*. Microbiol Mol Biol Rev 74: 621-41.

12. Silveira MC, Albano RM, Asensi MD, Carvalho-Assef AP. 2016. Description of genomic islands associated to the multidrug-resistant *Pseudomonas aeruginosa* clone ST277. Infect Genet Evol 42: 60-65.

13. Roy Chowdhury P, Scott M, Worden P, Huntington P, Hudson B, Karagiannis T et al. 2016. Genomic islands 1 and 2 play key roles in the evolution of extensively drug-resistant ST235 isolates of *Pseudomonas aeruginosa*. Open Biol 6: pii=150175.

14. Foxman B, Zhang L, Koopman JS, Manning SD, Marrs CF. 2005. Choosing an

appropriate bacterial typing technique for epidemiologic studies. Epidemiol Perspect Innov 2: 10.

15. Castanheira M, Deshpande LM, Costello A, Davies TA, Jones RN. 2014. Epidemiology and carbapenem resistance mechanisms of carbapenem-non-susceptible *Pseuomonas aeruginosa* collected during 2009-11 in 14 European and Mediterranean countries. J Antimicrob Chemother 69: 1804-14.

16. Viedma E, Juan C, Acosta J, Zamorano L, Otero JR, Sanz F et al. 2009. Nosocomial spread of colistin-only-sensitive sequence type 235 *Pseudomonas aeruginosa* isolates producing the extended-spectrum beta-lactamases GES-1 and GES-5 in Spain. Antimicrob Agents Chemother 53: 4930-3.

17. Oliver A, Mulet X, López-Causapé C, Juan C. 2015. The increasing threat of *Pseudomonas aeruginosa* high-risk clones. Drug Resist Updat 21-22: 41-59.

18. CLSI. 2016. Performance standards for antimicrobial susceptibility testing; twenty-fifth informational supplement. Document M100-S25. CLSI, Wayne, PA.

19. Hong JS, Kim JO, Lee H, Bae IK, Jeong SH, Lee K. 2015. Characteristics of Metallo-β-Lactamase-Producing *Pseudomonas aeruginosa* in Korea. Infect Chemother 47: 33-40.

20. Chin CS, Alexander DH, Marks P, Klammer AA, Drake J, Heiner C P et al. 2013. Nonhybrid, finished microbial genome assemblies from long-read SMRT sequencing data. Nat Methods 10: 563–569.

21. Bae IK, Suh B, Jeong SH, Wang KK, Kim YR, Yong D et al. 2014. Molecular epidemiology of *Pseudomonas aeruginosa* clinical isolates from Korea producing β-lactamases with extended-spectrum activity. Diagn Microbiol Infect Dis 79: 373-7.

22. Edelstein MV, Skleenova EN, Shevchenko OV, D'souza JW, Tapalski DV, Azizov IS et al. 2013. Spread of extensively resistant VIM-2-positive ST235 *Pseudomonas aeruginosa* in Belarus, Kazakhstan, and Russia: a longitudinal epidemiological and clinical study. Lancet Infect Dis 13: 867-76.

23. Toleman MA, Vinodh H, Sekar U, Kamat V, Walsh TR. 2007. *bla*$_{\text{VIM-2}}$-harbouring integrons isolated in India, Russia, and the United States arise from an ancestral class 1 integron predating the formation of the 3' conserved sequence. Antimicrob Agents Chemother 51: 2636-8.

24. Guindon S, Gascuel O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol 52: 696-704.

25. Coyne S, Courvalin P, Galimand M. 2010. Acquisition of multidrug resistance transposon Tn6061 and IS*6100*-mediated large chromosomal inversions in *Pseudomonas aeruginosa* clinical isolates. Microbiology 156: 1448-58.

26. Mavrodi DV, Loper JE, Paulsen IT, Thomashow LS. 2009. Mobile genetic elements in the genome of the beneficial rhizobacterium *Pseudomonas fluorescens* Pf-5. BMC Microbiol 9: 8.

27. Yano H, Kuga A, Okamoto R, Kitasato H, Kobayashi T, Inoue M. 2001. Plasmid-encoded metallo-beta-lactamase (IMP-6) conferring resistance to carbapenems, especially meropenem. Antimicrob Agents Chemother 45: 1343-8.

28. Joly-Guillou ML, Kempf M, Cavallo JD, Chomarat M, Dubreuil L, Maugein J et al. 2010. Comparative in vitro activity of Meropenem, Imipenem and Piperacillin/tazobactam against 1071 clinical isolates using 2 different methods: a French multicentre study. BMC Infect Dis 10: 72.

29. Kuo RN, Dong YH, Liu JP, Chang CH, Shau WY, Lai MS. 2011. Predicting healthcare utilization using a pharmacy-based metric with the WHO's Anatomic Therapeutic Chemical algorithm. Med Care 49: 1031-9.

30. Iyobe S, Kusadokoro H, Takahashi A, Yomoda S, Okubo T, Nakamura A, O'Hara K. 2001. Detection of a variant metallo-beta-lactamase, IMP-10, from two unrelated strains of *Pseudomonas aeruginosa* and an *Alcaligenes xylosoxidans* strain. Antimicrob Agents Chemother 46: 2014-6.

31. Frase H, Shi Q, Testero SA, Mobashery S, Vakulenko SB. 2009. Mechanistic basis for the emergence of catalytic competence against carbapenem antibiotics by the GES family of β-lactamases. J Biol Chem 284: 29509-13.

32. Kim J, Hong SG, Bae IK, Kang JR, Jeong SH, Lee W et al. 2011. Emergence of *Escherichia coli* sequence type ST131 carrying both the $bla_{\text{GES-5}}$ and $bla_{\text{CTX-M-15}}$ genes. Antimicrob Agents Chemother 55: 2974-5.

Abstract (in Korean)

카바페네메이즈를 생성하는 국내 녹농균의 분포와 특성규명

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2015년 국내의 29개 종합병원에서 총 431주의 임상 분리 녹농균을 수집하였다. 항균제 감수성 시험은 디스크 확산법을 사용하였고 카바페넴의 최소 억제 농도는 한천희석법으로 시험하였다. 카바페네메이즈 유전자들은 중합연쇄반응과 시퀀싱을 통해 증폭 및 확인하였고, 카바페네메이즈 유전자 카세트들 주변의 class I 인테그론 구조는 중합연쇄반응 지도화로 분석하였다. 시험 균주의 역학적 분류는 다좌위 서열 형별분석과 유전자 지문 분석법을 시행하였다. 그리고 bla_{IMP-6}, bla_{IMP-10}, 그리고 bla_{GES-24}의 베타 락타메이즈 유전자를 가지고 있는 녹농균 내성섬을 분석하기 위해 전장 유전체 분석을 실시하였다. 카바페넴 비감수성 균주와 카바페네메이즈를 생성하는 녹농균 균주는 총 431주 중에서 각각 34.3% (148주) 와 9.5% (41주) 이었다. IMP-6는 가장 흔한 카바페네메이즈 유형이였고, 그 다음으로 VIM-2,

IMP-10, 그리고 GES-24가 있었다. 모든 카바페네메이즈 유전자들은 염색체상에 6개의 다른 유형을 가지는 class I 인테그론에 위치해 있었다. 또한 모든 카바페네메이즈 유전자를 가지는 균주들은 80% 이상의 유전자 지문 연관성을 가지고 있었다. 더욱이, 2균주의 244시퀀스 타입을 제외하고는 모두 235시퀀스 타입으로 확인되었다. bla_{IMP-6}, bla_{IMP-10}, 그리고 bla_{GES-24}의 베타 락타메이즈 유전자들은 새롭게 발견된 녹농균 내성섬에 위치해 있었고, 본 연구에서 내성섬 -15와 -16이라고 명명하였다. 본 연구의 결과는 국내에서 IMP-6를 생산하는 235시퀀스 타입 녹농균의 분명한 클론 확산과 IMP-10과 GES-24의 출현으로 인한 국내 카바페네메이즈의 다양화를 나타내고 있다.

핵심되는 말: IMP-6, VIM-2, IMP-10, GES-24, ST235, class I 인테그론, 녹농균, 내성섬