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Dissemination of IMP-6 metallo-β-lactamase-producing *Pseudomonas aeruginosa* sequence type 235 in Korea

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Objectives: To investigate the epidemiological traits of *Pseudomonas aeruginosa* clinical isolates producing metallo-β-lactamases (MBLs) in Korea.

Methods: A total of 386 non-duplicate *P. aeruginosa* clinical isolates were collected from Korea in 2009. Detection of MBL genes was performed by PCR. The genetic organization of class 1 integrons carrying the MBL gene cassette was investigated by PCR mapping and sequencing. The epidemiological relationships of the isolates were investigated by multilocus sequence typing and PFGE.

Results: Of 386 *P. aeruginosa* isolates, 30 (7.8%) isolates carried the bla_{IMP-6} gene and 1 (0.3%) isolate carried the bla_{VIM-2} gene. A probe specific for the bla_{IMP-6} gene was hybridized to an ∼950 kbp I-CeuI-macrorestriction fragment from all 30 isolates and a probe specific for the bla_{VIM-2} gene also hybridized to an ∼500 kbp I-CeuI-macrorestriction fragment from 1 isolate (BDC10). All 31 MBL-producing isolates shared an identical sequence type (ST), ST235, and they carried the same bla_{OXA-50} allelic type, $bla_{OXA-50g}$. All MBL-producing isolates showed similar XbaI-macrorestriction patterns (similarity >85%), irrespective of MBL genotype.

Conclusions: *P. aeruginosa* ST235 carrying the chromosomally located bla_{IMP-6} gene is widely disseminated in Korea.

Keywords: MBLs, meropenem, clonal complex 235

Introduction

Metallo-β-lactamases (MBLs) have a wide hydrolytic spectrum against β-lactams, including penicillins, cephalosporins, and carbapenems, but not monobactams.[1] The dissemination of MBL-producing *Pseudomonas aeruginosa* (MPPA) is considered an important clinical threat with very limited antibacterial treatment options. In addition, MPPA strains are thought to cause life-threatening infections more frequently than MBL-negative *P. aeruginosa* strains.[2]

The development of a *P. aeruginosa* multilocus sequence typing (MLST) scheme by Curran *et al*. in 2004 has allowed comparisons of epidemiological data from different countries.[3] MLST experiments have shown that *P. aeruginosa* strains of two international clonal complexes (CCs), CC111 (CC4 was moved to CC111 after an update of allelic profiles at the web site http://pubmlst.org) and CC235 (previously CC11), are responsible for the dissemination of MBL genes in European countries.[4–6] However, reports of epidemiological characteristics of MPPA isolates from Asian countries are rare, with the exception of the description of IMP-1-producing *P. aeruginosa* isolates of sequence type (ST) 357 and ST235 in Japan.[7]

The aim of this study was to investigate the epidemiological traits of MPPA clinical isolates collected in a nationwide surveillance programme in Korea.

Materials and methods

Bacterial strains and susceptibility testing

A total of 386 non-duplicate *P. aeruginosa* clinical isolates were collected from 21 hospitals in Korea between June and August 2009. Species identification was performed using the ATB 32GN system (bioMérieux, Marcy l'Étoile, France) and/or 16S rRNA gene sequencing. Susceptibilities to piperacillin, piperacillin/tazobactam, ceftazidime, cefepime, aztreonam, imipenem, amikacin, gentamicin, tobramycin, ciprofloxacin and colistin were determined by disc diffusion assays or the agar dilution method for routine antibiograms at each participating hospital. Breakpoints were applied following CLSI recommendations.[8] MICs of imipenem and meropenem were determined using the agar dilution method according to CLSI guidelines. *P. aeruginosa* ATCC 27853 was used as a

control strain for susceptibility testing. The presence of MBLs was screened for by a modified Hodge test and an EDTA-based double-disc synergy (DDS) test, as previously described.[9]

Characterization of genes encoding MBLs

Genes coding for MBLs were detected by PCR, as previously described.[10] Templates for PCR amplification from clinical isolates were whole cell lysates, and amplified products were subjected to direct sequencing. Both strands of the PCR products were sequenced twice with an automatic sequencer (model 3730*xl*; Applied Biosystems, Weiterstadt, Germany).

Analyses of genetic environments surrounding the MBL genes

The genetic organization of class 1 integrons carrying the MBL gene cassettes was investigated by PCR mapping and sequencing the regions surrounding the genes using the primers described in Table 1.

Determination of bla_{OXA-50} allelic types

The bla_{OXA-50} gene was amplified using newly designed primers (Table 1). Experimentally determined nucleotide sequences were compared with pre-existing sequence databases using BLAST (http://www.ncbi.nlm.nih.gov/blast).

Table 1. Nucleotide sequences of oligonucleotides used for analysis of MBL genetic environments and bla_{OXA-50} alleles

Name	Target gene	Primer sequence (5′–3′)
VIM-2F	VIM-2 cluster	ATC ATG GCT ATT GCG AGT CC
VIM-2R		ACG ACT GAG CGA TTT GTG TG
IMP-1F	IMP-1 cluster	AAG GCG TTT ATG TTC ATA CTT CG
IMP-1R		TTT AAC CGC CTG CTC TAA TGT AA
INT1-F	class 1 integron	GGC ATC CAA GCA GCA AG
INT1-R		AAG CAG ACT TGA CCT GA
N3CS		ATC AAG CTT TTG CCC ATG AA
aacA4-F	*aacA4* gene	TGA CCT TGC GAT GCT CTA TG
aacA4-mF		GGT TCG AGC TCT GGT TGA GT
aacA4-R		CTG GCG TGT TTG AAC CAT GT
aadA1-mF	*aadA1* gene	ACA TCA TTC CGT GGC GTT AT
aadA1-mR		AGG TTT CAT TTA GCG CCT CA
orfII-mR	group IIC-*attC* integron	GCC GTT TGA AGT TAG TGG TCA
orfII-F		CCG CGT AAA TAT TGG CTG AT
orfIII-R		CGA CCT TGG AGA GCG AAT TA
orfIII-F		ATG GGC GTT TGG CTA CTA TG
qac-F	*qac* gene	CAA TCT TTG GCG AGG TCA TC
qac-R		CGC TGA CCT TGG ATA GCA G
OXA-1F	bla_{OXA-1} gene	TAT CTA CAG CAG CGC CAG TG
OXA-1R		TGC ACC AGT TTT CCC ATA CA
OXA-50F	bla_{OXA-50} gene	GCC GTG GAC AAG CTA TTC G
OXA-50R		CAG TAT CCC GAG AGC CTT GA
OXA-50F-mF		GTG ACC GCA CCT ACC CTT T
OXA 50 -mR		GCC ATA GGG AAG AAC CTC GT
OXA-50FF		GAT TTC GAC CTC TGG CTG TG

PFGE

XbaI-digested genomic DNA was prepared and DNA fragments were separated for 20 h at 6 V/cm at 11°C using a CHEF-DR II System (Bio-Rad, Hercules, CA, USA) 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. Banding patterns were analysed with UVIband/Map software (UVItech Ltd, Cambridge, UK) to generate a dendrogram based on the unweighted pair group method using arithmetic averages from the Dice coefficient.

MLST

MLST experiments were performed as previously described.[3] PCR and sequencing experiments for seven housekeeping genes (*acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA* and *trpE*) were performed. Nucleotide sequences were determined for both strands and compared with existing sequences in the MLST database (http://pubmlst.org/paeruginosa/) for assignment of allelic numbers and STs.

Southern blotting

Plugs containing whole genomic DNA of the *P. aeruginosa* isolates were treated with I-CeuI or S1 nuclease. DNA fragments were separated by PFGE using a CHEF-DRII device (Bio-Rad), as previously described.[11] I-CeuI-digested chromosomal DNA and S1 nuclease-digested linearized plasmids were blotted onto nylon membranes (Zeta-Probe Blotting Membranes, Bio-Rad) and hybridized with probes specific for the bla_{IMP-6} gene, bla_{VIM-2} gene or 16S rRNA. The probes were obtained via PCR experiments as described above. Probe labelling, hybridization and detection were performed with the DIG DNA Labeling and Detection Kit (Roche Diagnostics, Indianapolis, IN, USA).

Nucleotide sequence accession numbers

Nucleotide sequence data reported in this paper are available in the GenBank nucleotide database under accession numbers JF429900 (class 1 integron carrying the bla_{VIM-2} gene cassette), HQ833036 ($bla_{OXA-50i}$), HQ833037 ($bla_{OXA-50j}$) and HQ833038 ($bla_{OXA-50i}$).

Results

Identification of MBL-producing isolates

Of 386 *P. aeruginosa* isolates, 138 (35.8%) isolates showed resistance or intermediate susceptibility to imipenem and/or meropenem. Among them, 31 (8.0%) isolates showed positive results in both the modified Hodge and the DDS tests, suggesting MBL production. PCR and sequencing experiments detected the bla_{IMP-6} gene in all DDS-positive isolates, except for one isolate carrying the bla_{VIM-2} gene. MBL-producing isolates were detected in 14 of 21 (67%) participating hospitals. Figure 1 shows the distribution of the MBL-producing *P. aeruginosa* isolates in Korea.

MICs of carbapenems

The MICs of meropenem (MIC range 128–>256 mg/L; MIC_{50} >256 mg/L; MIC_{90} >256 mg/L) for 30 *P. aeruginosa* isolates were higher than those of imipenem (MIC range 8–>256 mg/L; MIC_{50} 128 mg/L; MIC_{90} 256 mg/L) (Figure 2). The MICs of imipenem and meropenem for an isolate carrying the bla_{VIM-2} gene were 128 mg/L and 64 mg/L, respectively.

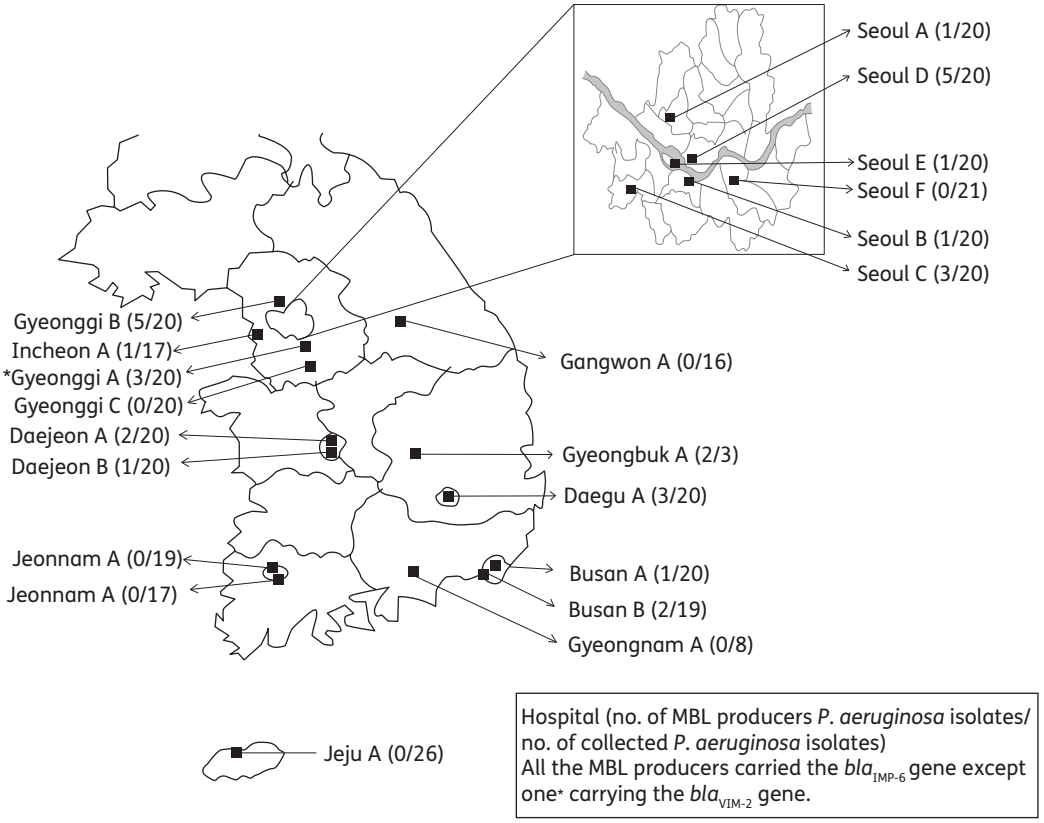


Figure 1. Map showing the locations of the hospitals participating in this study.

Genetic contexts of the MBL genes

The bla_{IMP-6} gene was located in a class 1 integron of an identical structure as a gene cassette in all 30 isolates. The variable region between the 5′ conserved segment (5′-CS) and the 3′-CS elements in the class 1 integron contained five insert gene cassettes (bla_{IMP-6}, *qac*, *aacA4*, bla_{OXA-1} and *aadA1*), as previously reported.[12] In one isolate, the bla_{VIM-2} gene was also located in a class 1 integron, but showed a novel gene cassette array. The variable region between the 5′-CS and 3′-CS elements contained three insert gene cassettes (*aacA4*, bla_{VIM-2} and *aadA1*) followed by a group IIC-*attC* intron, which encodes a putative reverse transcriptase that plays a role in the formation of gene cassettes.[13]

Locations of the MBL genes

The probe specific for the bla_{IMP-6} gene hybridized to an ~950 kbp I-CeuI-macrorestriction fragment from all 30 isolates. The probe specific for the bla_{VIM-2} gene also hybridized to an ~950 kbp I-CeuI-macrorestriction fragment from one isolate (BDC10). Probes specific for the MBL genes did not bind to any plasmids linearized by S1 nuclease treatment. The hybridization of I-CeuI-macrorestriction fragments with the probe specific for 16s rRNA confirmed the chromosomal location of the MBL genes (Figure 3).

Clonal relationships

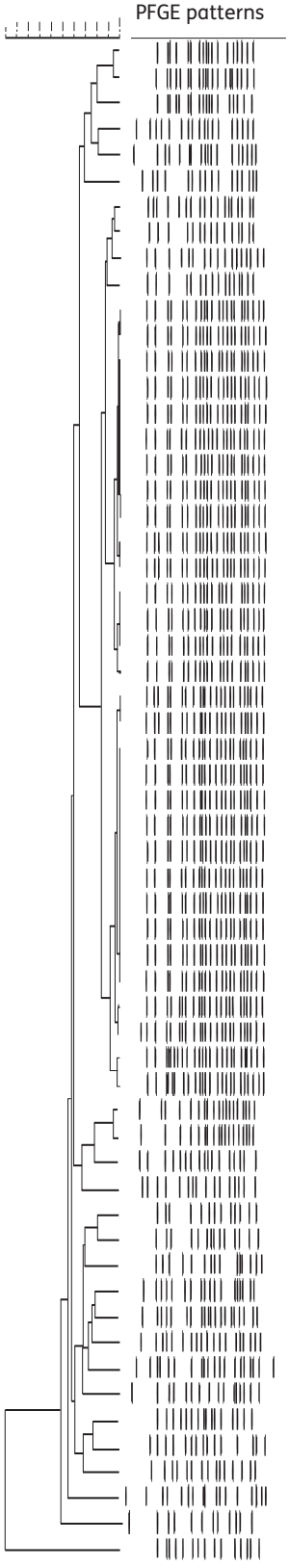
All 31 MPPA isolates showed similar XbaI-macrorestriction patterns (similarity >85%), irrespective of MBL genotype (Figure 2). MLST experiments showed that all the isolates shared an identical ST, ST235. Twenty-eight MBL-negative isolates were included in this study for comparison by random selection. Twelve of them were selected among those that were carbapenem resistant and 16 were selected among those that were carbapenem susceptible. MBL-negative isolates showed great diversity ($n=22$) of STs. International STs, ST111 and ST235, were identified in two and four MBL-negative isolates, respectively (Figure 2). Four MBL-negative *P. aeruginosa* isolates of ST235 were closely related to MPPA isolates by PFGE. Five new STs (STn1–STn5) were identified in this study.

bla_{OXA-50} allelic type

All MPPA isolates of ST235 shared an identical bla_{OXA-50} allele, $bla_{OXA-50g}$. Four MBL-negative isolates of ST235 also carried the $bla_{OXA-50g}$ allele, suggesting that $bla_{OXA-50g}$ is a strain-specific allele of *P. aeruginosa* ST235. The remaining 24 MBL-negative isolates carried one of eight bla_{OXA-50} alleles ($bla_{OXA-50a}$, $bla_{OXA-50b}$, $bla_{OXA-50d}$–$bla_{OXA-50g}$, $bla_{OXA-50i}$, and $bla_{OXA-50j}$) (Figure 2). Both MBL-negative isolates of ST111 carried the $bla_{OXA-50d}$ allele. Two novel bla_{OXA-50} alleles, $bla_{OXA-50i}$ and $bla_{OXA-50j}$, were identified in this study.

Discussion

VIM-2 has been described as the most prevalent MBL variant worldwide;[4,14,15] however, IMP-6 appears to be the dominant



PFGE patterns	Isolate	Hospital	MBL genotype	bla_{OXA-50} allele	ST	*P. aeruginosa* MLST alleles							MIC (mg/L)	
						acs	*aro*	*gua*	*mut*	*nuo*	*pps*	*trp*	Imipenem	Meropenem
	BSP13	Busan B		f	274	23	5	11	7	1	12	7	256	128
	KSS12	Seoul B		f	274	23	5	11	7	1	12	7	256	64
	YSM20	Seoul E		d	532	5	4	5	5	5	20	4	8	1
	BSN11	Busan A		e	n1	17	5	26	31	4	4	7	256	32
	KSM07	Seoul F		a	357	2	4	5	3	1	6	11	256	128
	KSM22	Seoul F		i	882	17	5	11	3	4	6	37	256	128
	JJH03	Jeju A		g	235	38	11	3	13	1	2	4	64	64
	WK10	Gangwon A		g	235	38	11	3	13	1	2	4	128	128
	WK03	Gangwon A		g	235	38	11	3	13	1	2	4	4	4
	GS02	Gyeongnam A		g	235	38	11	3	13	1	2	4	128	64
	JAY05	Seoul D	bla_{VIM-2}	g	235	38	11	3	13	1	2	4	128	64
	JAY15	Seoul D	bla_{IMP-6}	g	235	38	11	3	13	1	2	4	8	256
	BDC08	Gyeonggi A	bla_{IMP-6}	g	235	38	11	3	13	1	2	4	128	>256
	ESM01	Gyeonggi B	bla_{IMP-6}	g	235	38	11	3	13	1	2	4	128	>256
	ESM04	Gyeonggi B	bla_{IMP-6}	g	235	38	11	3	13	1	2	4	128	>256
	ESM20	Gyeonggi B	bla_{IMP-6}	g	235	38	11	3	13	1	2	4	128	>256
	JAY-8	Seoul D	bla_{IMP-6}	g	235	38	11	3	13	1	2	4	128	>256
	JAY09	Seoul D	bla_{IMP-6}	g	235	38	11	3	13	1	2	4	8	256
	JAY10	Seoul D	bla_{IMP-6}	g	235	38	11	3	13	1	2	4	8	256
	ESM14	Gyeonggi B	bla_{IMP-6}	g	235	38	11	3	13	1	2	4	64	>256
	ESM15	Gyeonggi B	bla_{IMP-6}	g	235	38	11	3	13	1	2	4	64	>256
	BDC05	Gyeonggi B	bla_{IMP-6}	g	235	38	11	3	13	1	2	4	128	>256
	DSM20	Daejeon B	bla_{IMP-6}	g	235	38	11	3	13	1	2	4	128	>256
	KY01	Daejeon A	bla_{IMP-6}	g	235	38	11	3	13	1	2	4	64	>256
	KY07	Daejeon A	bla_{IMP-6}	g	235	38	11	3	13	1	2	4	16	256
	JAH02	Seoul C	bla_{IMP-6}	g	235	38	11	3	13	1	2	4	64	>256
	KSS02	Seoul B	bla_{IMP-6}	g	235	38	11	3	13	1	2	4	128	>256
	BSN20	Busan A	bla_{IMP-6}	g	235	38	11	3	13	1	2	4	256	>256
	BSP01	Busan B	bla_{IMP-6}	g	235	38	11	3	13	1	2	4	128	>256
	BSP05	Busan B	bla_{IMP-6}	g	235	38	11	3	13	1	2	4	128	>256
	ISM02	Incheon A	bla_{IMP-6}	g	235	38	11	3	13	1	2	4	128	>256
	JAH14	Seoul C	bla_{IMP-6}	g	235	38	11	3	13	1	2	4	128	>256
	JAH16	Seoul C	bla_{IMP-6}	g	235	38	11	3	13	1	2	4	128	>256
	KSC03	Gyeongbuk A	bla_{IMP-6}	g	235	83	11	3	13	1	2	4	128	>256
	SS02	Seoul A	bla_{IMP-6}	g	235	38	11	3	13	1	2	4	128	>256
	YN18	Daegu A	bla_{IMP-6}	g	235	38	11	3	13	1	2	4	>256	>256
	YSM10	Seoul E	bla_{IMP-6}	g	235	38	11	3	13	1	2	4	128	>256
	BDC10	Gyeonggi A	bla_{VIM-2}	g	235	38	11	3	13	1	2	4	>256	>256
	KSC01	Gyeongbuk A	bla_{IMP-6}	g	235	38	11	3	13	1	2	4	128	128
	YN19	Daegu A	bla_{IMP-6}	g	235	38	11	3	13	1	2	4	128	128
	YN20	Daegu A	bla_{IMP-6}	g	235	38	11	3	13	1	2	4	64	>256
	KY10	Daejeon A		d	111	17	5	5	4	4	4	3	256	256
	SS01	Seoul A		d	111	17	5	5	4	4	4	3	256	256
	DSM10	Daejeon B		f	244	17	5	12	3	14	4	7	128	64
	KSS03	Seoul B		i	233	16	5	30	11	4	31	41	8	1
	BSN02	Busan A		a	n2	22	5	91	33	4	4	1	8	128
	DSM02	Daejeon B		a	381	11	20	1	65	4	4	10	4	2
	KY11	Daejeon A		e	179	36	27	28	3	4	13	7	8	2
	ISM06	Incheon A		a	151	19	5	11	7	4	15	26	8	1
	JJH01	Jeju A		a	563	6	75	26	5	1	4	26	8	1
	SS05	Seoul A		b	131	47	8	7	6	8	11	40	4	1
	YN01	Daegu A		g	395	6	5	1	1	3	12	1	4	1
	BDC04	Gyeonggi A		b	588	5	5	95	3	1	7	47	8	0.5
	BSP02	Busan B		a	n3	17	5	5	31	4	42	3	8	0.5
	JJH06	Jeju A		a	n4	8	n1	6	28	2	20	23	8	4
	KSC02	Gyeongbuk A		d	532	5	4	5	5	5	20	4	4	2
	ESM03	Gyeonggi B		j	n5	87	34	114	123	n1	n1	n1	8	0.5
	VIN01	Gyeonggi C		a	2	6	130	1	3	2	4	17	8	4
	CS02	Jeonnam A		e	250	28	5	36	3	1	13	7	8	1

Figure 2. Dendrogram based on XbaI-macrorestriction patterns of the 31 MBL-producing *P. aeruginosa* isolates and the 28 MBL-negative *P. aeruginosa* isolates. All MBL-producing isolates showed >80% similarity.

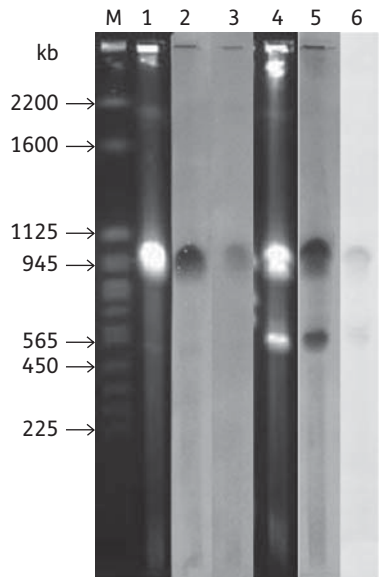


Figure 3. Southern blot analysis of I-CeuI-digested genomic DNA of *P. aeruginosa* isolates carrying the bla_{IMP-6} gene (lanes 1–3) or the bla_{VIM-2} gene (lanes 4–6). Lanes: M, DNA ladder; 1, I-CeuI-macrorestriction banding pattern of genomic DNA from a *P. aeruginosa* isolate carrying the bla_{IMP-6} gene; 2, Southern blot analysis of genomic DNA with a probe specific to the 16S rRNA gene; 3, Southern blot analysis of genomic DNA with a probe specific to the bla_{IMP-6} gene; 4, I-CeuI-macrorestriction banding pattern of genomic DNA from a *P. aeruginosa* isolate carrying the bla_{VIM-2} gene; 5, Southern blot analysis of genomic DNA with a probe specific to the 16S rRNA gene; lane 6, Southern blot analysis of genomic DNA with a probe specific to the bla_{VIM-2} gene.

MBL variant in *P. aeruginosa* isolates from Korea.[12,16] IMP-6 differs from IMP-1 MBL only by the substitution Ser196Gly, resulting in increased hydrolytic activity for meropenem.[17] Our *P. aeruginosa* isolates harbouring IMP-6 also exhibited a higher level of resistance to meropenem than imipenem. The bla_{IMP} alleles have been found to be located on plasmids; however, the bla_{IMP-6} gene was located in chromosomes in all 30 isolates in this study.[18] The mechanism and significance of this phenomenon need to be elucidated.

P. aeruginosa ST235 is the founder strain of an international CC, CC235.[19,20] The strain has been involved in the dissemination of genes encoding VIM-type MBLs and class A extended-spectrum β-lactamases, such as PER-1, BEL-1, GES-1, and GES-5, in European countries.[19–22] In this study, MBL genes bla_{IMP-6} and bla_{VIM-2} were exclusively detected in *P. aeruginosa* isolates of ST235, which showed similar XbaI-macrorestriction banding patterns by PFGE. Furthermore, the bla_{IMP-6} gene was located in a class 1 integron in the same genetic context in all isolates. The results indicate that clonal spreading of the strain ST235 played a key role in dissemination of the bla_{IMP-6} gene in *P. aeruginosa* in Korea.

Interestingly, an isolate carrying the chromosomally located bla_{VIM-2} gene was also identified as ST235, and showed similar PFGE fingerprinting patterns to the isolates carrying the bla_{IMP-6} gene. The results support the concept that *P. aeruginosa* ST235, which carry the strain-specific $bla_{OXA-50g}$, may be prone to the acquisition of MBL genes resulting in carbapenem resistance. Another international ST, ST111, was identified in two MBL-negative isolates, but not in MBL-producing isolates, suggesting that *P. aeruginosa* ST111 may not play a role in the dissemination of MBL genes in Korea.

In conclusion, our data showed that *P. aeruginosa* ST235 carrying the chromosomally located bla_{IMP-6} gene is widely disseminated in Korea. Further dissemination of the strain could pose a serious clinical threat.

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Transparency declarations

None to declare.

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