

Metallo- β -Lactamase-Producing *Pseudomonas* spp. in Korea: High Prevalence of Isolates with VIM-2 Type and Emergence of Isolates with IMP-1 Type

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Purpose: Two Korean nationwide studies showed that metallo- β -lactamases (MBLs)-producing-*Pseudomonas* spp. are not rare. The aim of this study was to assess the trends of MBL-producing isolates among imipenem-resistant isolates of *Pseudomonas* spp. **Materials and Methods:** Imipenem-resistant clinical isolates were collected from 23 hospitals and one commercial laboratory participating in the KONSAR program in 2005. Polymerase chain reaction (PCR) was used to detect MBL genes. **Results:** Alleles of MBL genes were detected in 10.8% of 415 *Pseudomonas aeruginosa* and 66.7% of 12 *P. putida* isolates from 18 of 24 hospitals/laboratory. Among the 14 IMP-1-like and 39 VIM-2-like MBLs, emergence of IMP-6 was detected for the first time. **Conclusion:** Prevalence of MBL-producing *P. aeruginosa* has not significantly increased, but IMP-6 emerged in *P. aeruginosa*.

Key Words : IMP-1, IMP-6, VIM-2, metallo- β -lactamase, Korea

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INTRODUCTION

Pseudomonas aeruginosa is one of the most important nosocomial pathogens, while other *Pseudomonas* spp. are occasional causes of infections. Current isolates of *P. aeruginosa* are often multiresistant to many classes of potent antimicrobial agents, including β -lactams, aminoglycosides, and fluoroquinolones. Carbapenems are the most potent β -lactams, which are active even against extended-spectrum β -lactamase- and AmpC β -lactamase-producing gram-negative bacilli. Due to acquired metallo- β -lactamase (MBL) production, however, carbapenem resistance in *P. aeruginosa* and *Pseudomonas* spp., has increasingly been reported in some countries.^{1,2} Richet et al.³ initiated an international network and included carbapenemase-producing gram-negative bacilli in the early warning system list for emerging antimicrobial drug-resistant pathogens.

The Korean Nationwide Surveillance of Antimicrobial Resistance (KONSAR) program conducted in the years between 1998 and 2003 showed that *P. aeruginosa* ranked the 3rd by the number of isolates.^{4,5} The imipenem resistance rate of *P. aeruginosa* in 1999 was already relatively high (19%), but the rate rose further to reach 24% in 2004. Two previous studies with clinical isolates of *P. aeruginosa*, which were collected in 2000-2001 and in 2003 from hospitals participating in the program of KONSAR, showed that over 10% of all imipenem-resistant isolates were VIM-2 type MBL producers.^{6,7} Other studies also documented the presence of MBL-producing *P. aeruginosa* in Korean hospitals^{8,9} as well as the occasional

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presence of VIM-2-type MBL-producing isolates of *Enterobacteriaceae*.^{10,11} Moreover, the emergence of IMP-1-like-producing *P. aeruginosa* and finding of the fifth MBL, SIM-1-producing *Acinetobacter baumannii* isolates, were reported from a hospital in Seoul.¹²

The aim of this study was to determine any change in the prevalence of VIM-2- and IMP-1-like-producing clinical isolates of *Pseudomonas* spp. and possible emergence of new MBL variants two years after the second nationwide study. The possible presence of MBL-producing *Enterobacteriaceae* and spreading of *bla*_{SIM-1} type MBL gene to *Pseudomonas* spp. were also investigated.

MATERIALS AND METHODS

Bacterial strains and susceptibility testing

Non-duplicate, imipenem-resistant strains of *P. aeruginosa*, *Pseudomonas* spp., and *Enterobacteriaceae*, isolated during January to August 2005, were collected from 23 hospitals and one commercial laboratory participating in the KONSAR program. The number of isolates collected from any one hospital was limited to 25 for an individual species. Identification of the species was reconfirmed at the coordinating laboratory through the use of a conventional method^{13,14} or the ATB 32GN system (bioMerieux, Marcy l'Etoile, France). The MICs of antimicrobial agents for MBL-producing isolates were determined by the NCCLS agar dilution method.¹⁵

Screening MBL-producing isolates

Carbapenemase production was screened by the imipenem-disk Hodge (cloverleaf) test, using MacConkey agar instead of previously used Mueller-Hinton agar.¹⁶ MBL production was screened by the double-disk synergy test using an imipenem disk and an EDTA (750 µg) plus sodium mercaptoacetic acid (SMA, 2 mg) disk on Mueller-Hinton agar with 10 mm distance from the edge to the edge of the disk.¹⁶ Commercial imipenem disks and media (Becton-Dickinson, Sparks, MD, USA) were used for these tests, while the EDTA-SMA disks were prepared from commercially available chemicals (Sigma Chemical, St. Louis, MO, USA).

MBL gene detection by PCR

To detect alleles of *bla*_{IMP-1}, *bla*_{VIM-2}, and *bla*_{SIM-1}, DDS-positive isolates were tested by PCR as described previously.¹² Briefly, primers used were: IMP1-F 5'-CAT GGT TTG GTG GTT CTT GT-3' and IMP1-R 5'-ATA ATT TGG CGG ACT TTG GC-3'; VIM2-F 5'-ATG TTC AAA CTT TTG AGT AAG-3' and VIM2-R 5'-CTA CTC AAC GAC TGA GCG-3'; SIM1-F 5'-TAC AAG GGA TTC GGC ATC G-3' and SIM1-R 5'-TAA TGG CCT GTT CCC ATG TG-3'. PCR was performed with 1 µL of heat-extracted DNA template, 20 pmol of each primer, and PreMix (Bioneer, Cheongwon, Korea) containing 1 U of *Taq* DNA polymerase in a total volume of 20 µL. A Mastercycler instrument (Eppendorf, Hamburg, Germany) was used with the following reaction conditions: 94°C for 5 min, 25 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 45 s, and finally, 72°C for 7 min. Amplicons were visualized by electrophoresis and ethidium bromide staining.

Pulsed field gel electrophoresis and MBL gene sequencing

For PFGE, *xbaI*-digested genomic DNA was prepared according to the instruction of Bio-Rad (Hercules, Calif., USA), and fragments were separated using a CHEF-DR II system (Bio-Rad). We selected 17 isolates which represented PFGE types and performed the sequencing of PCR amplicons using IMP1-F or VIM2-F and Int2 (5'-AAG CAG ACT TGA CCT GA-3'), and IMP1-R or VIM2-R and Int1 (5'-GGC ATC CAA GCA GCA AG-3').

RESULTS

Among the 443 imipenem-resistant isolates collected, the majority were *P. aeruginosa* (93.7%), whereas 16 isolates were species of *Enterobacteriaceae* (9 *K. pneumoniae*, 2 each of *E. aerogenes*, *E. coli*, and *P. mirabilis*, and 1 *C. freundii*). Of these isolates, 127 were positive for the imipenem-disk Hodge test and 53 were positive for the DDS test (Table 1). Alleles of *bla*_{VIM-2} or *bla*_{IMP-1} were detected in all 53 DDS-positive isolates. Among all the isolates tested, the MBL-positive isolates comprised 10.8% of *P. aeruginosa* and 66.7% (8 of 12) of *P. putida*. MBL was not detected in

Table 1. Metallo-β-Lactamase Genes Detected in Imipenem-Resistant Isolates of *Pseudomonas* spp. and *Enterobacteriaceae* by PCR

Organism (No. of isolates tested)	No. of isolates Hodge test positive	No. of isolates DDS positive	No. (%) of isolates positive for					Total
			<i>bla</i> _{VIM-2}	<i>bla</i> _{VIM-2} - like	<i>bla</i> _{IMP-1}	<i>bla</i> _{IMP-1} - like	<i>bla</i> _{IMP-6}	
<i>P. aeruginosa</i> (415)	117	45	7	25	0	10	3	45 (10.8)
<i>P. putida</i> (12)	10	8	7	0	1	0	0	8 (66.7)
Total (427)	127	53	14	25	1	10	3	53 (12.0)

PCR, polymerase chain reaction; DDS, double disk synergy.

any of the 16 isolates of *Enterobacteriaceae*.

Overall, the proportion of *bla*_{VIM-2} allele-positive isolates was greater (73.6%) than *bla*_{IMP-1} allele-positive isolates (Table 1). *bla*_{SIM-1}-allele-positive isolate was not detected. All MBL-producing isolates of *Pseudomonas* spp. other than *P. aeruginosa* were identified as *P. fluorescens/P. putida* by the ATB 32 GN system. However, they were confirmed to have biochemical characteristics of *P. putida* by conventional tests. Nucleotide sequencing revealed that all the *bla*_{VIM-2}-like and *bla*_{IMP-1}-like in *P. aeruginosa* were VIM-2 and IMP-6. MBLs in *P. putida* were identified as VIM-2 and IMP-1. We found 13 different PFGE patterns in 23 *bla*_{VIM-2}-like *Pseudomonas* spp. and 4 patterns in 7 *bla*_{IMP-1}-like *Pseudomonas* spp., indicating that multiple MBL-producing *Pseudomonas* clones exist in Korea. The MIC_{50S} of

various β -lactams for MBL-producing *P. aeruginosa* were equal to over 64 μ g/mL except that of aztreonam (Table 2). The % resistances for aztreonam, piperacillin, amikacin, ciprofloxacin and other β -lactams were 67%, 83%, 87%, 96%, and 100%, respectively.

MBL-producing isolates were detected in 18 of 24 (75%) hospitals/laboratory, as well as in all four regions (Table 3). MBL-producing isolates were mostly derived from specimens of urine, sputum, and wounds (Table 4). All 8 MBL-producing *P. putida* isolates came either from urine or sputum. The MBL-producers were isolated mainly from inpatients (80: 63% isolates from general ward and 17% isolates from intensive-care unit); while 20% of isolates were from outpatient department.

Table 2. Antimicrobial Activities Against MBL-Producing *P. aeruginosa* and *P. putida* Strains

Antimicrobial agent	<i>P. aeruginosa</i> (n = 27)			<i>P. putida</i> (n = 7)	
	MIC (μ g/mL)		%R	MIC (μ g/mL) Range	%R
	50% of strains	90% of strains			
Piperacillin	> 128	> 128	83	128 - > 128	100
Ceftazidime	128	> 128	100	32 - > 128	100
Cefotaxime	> 128	> 128	100	> 128	100
Aztreonam	32	64	67	16 - 64	100
Imipenem	64	> 128	100	32 - > 128	100
Meropenem	> 128	> 128	100	64 - > 128	100
Gentamicin	> 128	> 128	100	128 - > 128	100
Amikacin	> 128	> 128	87	2 - > 128	83
Tobramycin	> 128	> 128	100	16 - 128	100
Ciprofloxacin	32	64	96	8 - > 128	100

MBL, metallo- β -lactamase.

Table 3. Metallo- β -Lactamase Genes Detected in Isolates of *Pseudomonas* spp. by the Location of Hospitals

No. of isolates tested / No. of hospitals participated*	No. of isolates/No. of hospitals positive for:		
	<i>bla</i> _{VIM-2} -like	<i>bla</i> _{IMP-1} -like	Total (% of hospitals) [†]
North (244 / 14)	27 / 10	4 / 3	31 / 10 (71.4)
Southeast (83 / 5)	4 / 3	8 / 3	12 / 4 (80.0)
Southwest (68 / 3)	5 / 1	1 / 1	6 / 2 (66.7)
Southwestern island (11 / 1)	1 / 1	0 / 0	1 / 1 (100)
Commercial Lab. (21 / 1)	2 / 1	1 / 1	3 / 1 (100)
Total (427 / 24)	39 / 16	14 / 8	53 / 18 (75.0)

*City or province: North, Seoul, Gyeonggi and Gangwon; Southeast, Busan, Daegu, Wulsan and Gyungsang; Southwest, Jeolla; Southwestern island, Jeju; Commercial Laboratory collected specimens from clinics located all over the country.

[†]Some hospitals had isolates with *bla*_{VIM-2} and with *bla*_{IMP-1}.

Table 4. Source of Isolation of Metallo- β -Lactamase Gene-Positive *Pseudomonas* spp.

MBL gene	No. (%) of isolates from:						
	Urine	Sputum	Wound	Blood	Peritoneal fluid	Others	Total
<i>bla</i> _{VIM-2} -like	27	3	6	1	1	1	39 (73.6)
<i>bla</i> _{IMP-1} -like	6	2	0	0	0	6	14 (26.4)
Total	33 (62.3)	5 (9.4)	6 (11.3)	1 (1.9)	1 (1.9)	7 (13.2)	53 (100)

DISCUSSION

Antimicrobial resistance surveillance has become increasingly important with the recent increase in the resistant organisms.¹⁷ Analysis of the data generated by the participating hospitals in 1998 and 2004 showed an alarming rise in the imipenem resistance rates of *P. aeruginosa*; from 19% to 24%.^{4,5} Decrease of carbapenem susceptible *P. aeruginosa* was also reported in Japan; from 80.7% to 62.0% over the period of 1998-2002.¹⁸

Among the β -lactamases, the genetically mobile MBL are among the most feared, because of their ability to hydrolyze virtually all β -lactams, except monobactams,¹⁹ and to spread horizontally. As a result of their geographic spread, these enzymes are among the greatest concerns to the medical communities.^{1,20} The rates of occurrence and number of enzyme types of MBL have escalated since 2000 in Asia, Europe, and Latin America.^{1,2,21} In the present study, 10.8% of imipenem-resistant isolates of *P. aeruginosa* were MBL producers. These proportions were similar to those in the previous study in 2000-2001 and in 2003.^{6,7} The rate was also similar to that in a Japanese study in 1998-2002.¹⁸

In this study, only 12 imipenem-resistant isolates of *Pseudomonas* spp. other than *P. aeruginosa* were collected. It is of an interest to note that all 8 MBL-producing isolates were identified as *P. putida*. *P. putida* is a species difficult to differentiate from *P. fluorescens* by commonly used biochemical tests. Although *P. putida* is an only organism occasionally isolated from clinical specimens,¹³ the detection of MBL gene in 8 of 12 imipenem-resistant isolates suggests that this organism may play a significant role as an important reservoir of MBL genes.

Only 16 imipenem-resistant *Enterobacteriaceae* isolates could be collected in this study, and MBL was not detected in any of these isolates. This indicates rarity of *Enterobacteriaceae* isolates not only producing MBL, but also resistant to imipenem. MBL has been detected only in a few isolates of *Enterobacteriaceae* in Korea.^{11,13,22}

It is interesting that the first acquired MBL discovered in the early 1990s was IMP-1 in nearby Japan,²³ whereas in Korea, VIM-2 was the first MBL type detected and IMP-1 was detected in *Acinetobacter* spp. in the early 2000s.⁶ In a study at a hospital in Seoul, IMP-1-like-producing *P. aeruginosa* was detected in strains isolated in 2003-2004, but the number was only two of 76 isolates of *Pseudomonas* spp.¹² In the present study, 14 of 53 MBL-producing isolates of *P. aeruginosa* and *P. putida* had *bla*_{IMP-1}, indicating spread of this MBL gene to these species (Table 1). However, *bla*_{SIM-1} which was discovered in *A. baumannii* isolates in Seoul has not yet spread to *Pseudomonas* spp.

Detection of MBL-producing *Pseudomonas* spp. in isolates collected from 18 of 24 (75.0%) hospitals/laboratory in

the present study (Table 3) is a great concern. As in previous studies,^{6,7} MBL-producing isolates were isolated mostly from urine and sputum specimens (Table 4), indicating that these specimens can serve as the important reservoirs for dissemination of the resistant organisms.

In conclusion, clinical isolates of *P. aeruginosa* in Korea are increasingly resistant to imipenem, and a significant proportion of the resistance is due to MBL production. *bla*_{VIM-2} remains the dominant type, however, IMP-1 variant, IMP-6-producing *P. aeruginosa* emerged. Extensive amount of effort is required to control further spread of MBL-producing *Pseudomonas* spp. in hospitals.

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