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Increasing Carbapenem-Resistant Gram-Negative Bacilli and Decreasing Metallo-β-Lactamase Producers over Eight Years from Korea

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The trends and types of carbapenemase-producing Gram-negative bacilli were analyzed from clinical specimens collected between 2005 and 2012 at a Korean teaching hospital. The proportions of carbapenem-resistant *Acinetobacter* spp. increased markedly to 66%. Metallo-β-lactamase producers significantly decreased and the majority shifted from the *bla*$_{VIM-2}$ type to the *bla*$_{IMP-1}$ type.

Key Words: Gram-negative bacilli, carbapenem, metallo-β-lactamase

Carbapenems such as imipenem and meropenem are first-line drugs in the treatment of serious infections caused by multidrug-resistant *Pseudomonas aeruginosa*, *Acinetobacter* spp., and *Enterobacteriaceae*. However, in recent years, imipenem resistance in *P. aeruginosa* and *Acinetobacter* spp. has been increasing steadily around the world, and in Korea, these resistance rates reached 22% and 64%, respectively, in 2011.[1] Among several mechanisms of carbapenem resistance, acquired metallo-β-lactamases (MBLs) have a more serious impact as the enzymes confer a high level of resistance and the genes can be transferred horizontally.[2,3] Among acquired MBLs, VIM-type and IMP-type enzymes are the most common types of MBLs with worldwide distribution,[4] and the VIM-2 type has been highly prevalent in Korea.[5,6] In our previous study in 2003–2004, imipenem-nonsusceptible *P. aeruginosa* isolates carrying the *bla*$_{VIM-2}$ allele were highly prevalent, and the incidence of *Acinetobacter* spp. carrying the *bla*$_{VIM-2}$ allele had increased compared to those carrying the *bla*$_{IMP-1}$ allele.[5] The aim of the present study was to determine the trends of carbapenem-resistant and MBL-producing Gram-negative bacilli over the past 8 years in a Korean teaching hospital with more than 2000 beds. This is a unique report on the long term trend of carbapenem-resistant and MBL-producing Gram-negative bacilli isolated in a single hospital.

In total, non-duplicated clinical isolates of 12650 *P. aeruginosa*, 1096 other *Pseudomonas* spp., and 7650 *Acinetobacter* spp. in addition to 14026 *Klebsiella pneumoniae*, 6110 *Enterobacter cloacae*, and 3162 *Serratia marcescens* isolates among *Enterobacteriaceae* were recovered from patients at the hospital from 2005 to 2012 (Table 1). The isolates were identified by conventional methods using ATB 32 GN or VITEK-2 systems (bioMerieux, Marcy-l'E´toile, France). Antimicrobial susceptibilities were determined using the disk-diffusion method or the VITEK-2 system (bioMerieux). The Clinical and Laboratory Standards Institute 2010 breakpoints were used after January 2011.[7] The modified Hodge test (MHT) and the imipenem and ethylenediaminetetraacetic acid sodium mercaptoacetic acid double disk potentiation (IEDDP) test were conducted to screen for MBL producers in imipenem- or meropenem-nonsusceptible isolates.[8] Polymerase chain reaction (PCR) was performed to detect and sequence $bla_{\text{VIM-2}}$-like, $bla_{\text{IMP-1}}$-like and $bla_{\text{SIM-1}}$-like genes.[9,10] In carbapenem-nonsusceptible strains showing MHT-positive yet the aforementioned PCR negative results, bla_{NDM} and bla_{KPC} genes were also screened by PCR.[11,12] The nucleotide sequences of PCR-generated amplicons were analyzed for the representative strains. XbaI- or SmaI-digested genomic DNAs from 75 *P. aeruginosa*, 8 *P.*

Table 1. Annual Imipenem Resistance Rates and MBL-Producing Clinical Isolates over 8 Years

Species	Yr	No. of isolates (% imipenem resistance)	No. (%) of carbapenem-nonsusceptible isolates with positive results					
			Tested	Modified Hodge test	Double disk potentiation test	$bla_{\text{VIM-2}}$ -like gene	$bla_{\text{IMP-1}}$ -like gene	$bla_{\text{SIM-1}}$ -like gene
P. aeruginosa	2005	1409 (21)	474	102 (22)	39 (8)	37 (95)	2 (5)	0
	2006	1635 (20)	454	92 (20)	39 (9)	26 (67)	13 (33)	0
	2007	1675 (24)	513	36 (7)	20 (4)	16 (80)	4 (20)	0
	2008	1777 (22)	465	66 (14)	41 (9)	14 (34)	27 (66)	0
	2009	1721 (29)	631	63 (10)	50 (8)	28 (56)	22 (44)	0
	2010	1383 (25)	604	86 (14)	29 (5)*	10 (34)	20 (69)	0
	2011	1510 (19)	361	23 (6)	10 (3)	5 (50)	5 (50)	0
	2012	1540 (23)	575	77 (13)	15 (3)	10 (67)	5 (33)	0
	Subtotal	12650 (23)	4077	545 (13)	243 (6)	146 (61)	98 (40)	0
Other *Pseudomonas* spp.	2005	137 (34)	6	3 (50)	3 (50)	3 (100)	0	0
	2006	157 (35)	34	29 (85)	25 (74)	24 (96)	1 (4)	0
	2007	142 (32)	27	20 (74)	20 (74)	17 (85)	3 (15)	0
	2008	126 (34)	26	19 (73)	18 (69)	18 (100)	0	0
	2009	102 (31)	26	19 (73)	14 (54)	13 (93)	1 (7)	0
	2010	137 (27)	35	27 (77)	27 (77)	26 (96)	1 (4)	0
	2011	166 (27)	56	28 (50)	26 (46)	25 (96)	1 (4)	0
	2012	129 (33)	37	25 (68)	24 (65)	23 (96)	1 (4)	0
	Subtotal	1096 (32)	247	170 (69)	157 (64)	149 (95)	8 (5)	0
Acinetobacter spp.	2005	793 (29)	358	332 (93)	72 (20)†	21 (29)	38 (53)	14 (19)
	2006	847 (15)	302	245 (81)	42 (14)*	12 (29)	26 (62)	6 (14)
	2007	721 (16)	159	115 (72)	33 (21)*	17 (52)	15 (45)	2 (6)
	2008	1158 (39)	447	399 (89)	41 (9)	13 (32)	28 (68)	0
	2009	1141 (58)	668	660 (99)	32 (5)	8 (25)	19 (59)	5 (16)
	2010	1100 (64)	828	799 (97)	20 (2)	7 (35)	12 (60)	1 (5)
	2011	885 (61)	510	485 (95)	9 (2)‡	5 (56)	2 (22)	0
	2012	1005 (66)	726	723 (99)	13 (2)*	2 (15)	11 (85)	1 (8)
	Subtotal	7650 (46)	3998	3758 (94)	262 (7)	85 (37)	151 (58)	29 (11)
K. pneumoniae	2005–2012	14026 (<1)	205	25 (12)	2 (1)	2 (100)	0	0
E. cloacae	2005–2012	6110 (<1)	36	20 (56)	3 (8)	2 (67)	1 (33)	0
S. marcescens	2005–2012	3162 (<1)	13	6 (46)	1 (8)	1 (100)	0	0

MBL, metallo-β-lactamase.

*Both $bla_{\text{VIM-2}}$-like and $bla_{\text{IMP-1}}$-like genes were detected in two isolates in 2006 and one isolate per year in 2007, 2010, and 2012.

†Both a $bla_{\text{VIM-2}}$-like gene and a $bla_{\text{SIM-1}}$-like gene were detected in one isolate.

‡An NDM-1 gene was detected in two isolates.

putida, and 109 *Acinetobacter* spp., all of which were randomly selected MBL-producing spp. isolated in 2005–2006, were separated by pulsed field gel electrophoresis (PFGE) using a CHEF-DR II system (Bio-Rad, Hercules, CA, USA) and BioNumerics software v. 5.10 (Applied Maths, Sint-Martens-Latem, Belgium). PFGE banding pattern clustering with an 80% similarity threshold was determined using the Dice coefficients and the unweighted pair group method using arithmetic averages using Molecular Analyst Fingerprinting Software (Bio-Rad). Related clones with one or two independent genetic events were designated as subtype numbers in Table 2. The S1-digested DNA of randomly selected *P. aeruginosa*, *Pseudomonas putida*, and *Acinetobacter* spp. strains carrying the *bla*$_{VIM-2}$ allele were blotted onto nylon membranes (Bio-Rad) and hybridized with *bla*$_{VIM-2}$ gene probes to observe the differences in the plasmids carrying the gene.

While annual imipenem resistance rates in *P. aeruginosa* increased from 14% in 2003 to 29% in 2009, they decreased from 58% in 2003 to 33% in 2012 in other *Pseudomonas* spp.; in *Acinetobacter* spp., resistance rates increased steeply from 13% in 2003 to 66% in 2012.[5] However, the imipenem resistance rates in *K. pneumoniae*, *E. cloacae*, and *S. marcescens* isolates remained at less than 1%. The positive rates of MHT were highly variable depending on the species, being only 13% in *P. aeruginosa* isolates, yet reaching 69% in other *Pseudomonas* spp. and 94% in *Acinetobacter* spp. These findings suggest that the majority of other *Pseudomonas* and *Acinetobacter* spp. isolates clearly produced the carbapenem-hydrolyzing enzymes to gain their resistance to carbapenem, while *P. aeruginosa* isolates obtained other resistance mechanisms such as AmpC or extended spectrum β-lactamase and porin loss, as previously reported.[13,14]

Among the carbapenem-nonsusceptible isolates, MBL producers included only 6% (243 of 4077) of *P. aeruginosa* spp. and only 7% (262 of 3998) of *Acinetobacter* spp. Interestingly, 157 of 247 (64%) of the other *Pseudomonas* spp. produced MBL, and almost all of them (152 of 157) were *P. putida*, which was a much higher incidence than those of the other species. These findings suggest that *P. putida* can be a reservoir for MBL, as previously described.[15] *Acinetobacter* spp. with different carbapenem-resistance mechanisms, such as OXA-type β-lactamases, have become prevalent.[16]

In *P. aeruginosa*, the incidence of MBL producers increased until the mid-2000s in this study, as shown in Japan,[17] while in more recent years, these isolates gradually decreased in this study, as described in a previous report.[18] Likewise, Cavalcanti, et al.[19] reported that a higher prevalence of MBL-producing *P. aeruginosa* was observed in 2002–2003 in Brazil, while the level decreased significantly in 2008–2009, suggesting that the resistance to carbapenems by these recent *P. aeruginosa* isolates was not due to the spread of MBL-positive clones. In this study, *P. aeruginosa* isolates carrying a *bla*$_{VIM-2}$-like gene were highly prevalent, comprising 90% to 100% of the *P. aeruginosa* strains in 2003 to 2004,[5] although they were reduced to 34% while those with *bla*$_{IMP-1}$-like genes increased to 69% in 2010.

Among MBL-producing *Acinetobacter* spp. isolates, the prevalence of *bla*$_{IMP-1}$-like genes also increased to 85% in 2012. The range of strains carrying *bla*$_{SIM-1}$-like genes remained low. Two isolates carrying *bla*$_{NDM-1}$-like genes that were isolated in 2011 were identified as *A. pitti* and *A. guillouiae*. To our knowledge, this is the first report of a clinical isolate of *A. guillouiae* carrying the *bla*$_{NDM-1}$-like gene. Most of the other *Pseudomonas* spp. isolates carrying MBL genes were identified as *P. putida*, and their MBL genes were *bla*$_{VIM-2}$-like.

Among the 254 carbapenem-nonsusceptible *Enterobacteriaceae*, only five isolates, two *K. pneumoniae*, two *E. cloacae*, and one *S. marcescens*, produced MBL, suggesting that the major carbapenem resistance mechanism in *Enterobacteriaceae* was not MBL. Our results support previous reports that suggested that carbapenem resistance in *Enterobacteriaceae* was comediated with AmpC beta-lactamase and outer membrane protein loss in *K. pneumoniae*, *E. cloacae*, and *S. marcescens*.[20-22]

PFGE analysis revealed that the pulsotypes of IMP-6- and VIM-2-producing *P. aeruginosa* strains were clearly separated. The major pulsotypes in the IMP-6-producing *P. aeruginosa* were A2 and A3, while those in the VIM-2-producing *P. aeruginosa* were A1 and C1 types (Table 2). Likewise, the pulsotypes of *Acinetobacter* spp. isolates obviously differed according to MBL type (Table 2). These findings suggest that the plasmids carrying the MBL gene are not promiscuous, although they do have clone preference. Interestingly, *Acinetobacter* spp. isolates in the E1, H1, I4, J3, N1, and Q subgroups showed identical PFGE patterns, despite the differences in species. This suggests that the identification of *Acinetobacter* species is important for evaluating clonal outbreaks in hospital settings, as the misinterpretation of a clonal outbreak occurred among the different species.

Other mechanisms may block the cross-over of resistance plasmids between clones. Further study is warranted to elucidate this supposition. The hybridization of S1-digested DNA

Table 2. Pulsotypes of 109 MBL-Producing *Acinetobacter* spp. and 75 *P. aeruginosa* Isolates

Species with MBL genes	*A. pitti*			*A. bereziniae*			*A. nosocomialis*			*A. junii*			*A. genomosp* 14TU			*Acinetobacter* spp.*			*P. aeruginosa*		
	IMP -1	VIM -2	SIM -1	IMP -1	VIM -2	SIM -1	IMP -1	VIM -2	SIM -1	IMP -1	VIM -2	SIM -1	IMP -1	VIM -2	SIM -1	IMP -1	VIM -2	SIM -1	IMP -1	IMP -6	VIM -2
A																					
1																				2	41
2																				7	
3																			1	5	
4-7																					1e
B																					
1																					2
2-3																					1e
C																					
1																					2
D																					
1																					1
E																					
1					1			4	1												
2		1																			
3									4												
4								1													
5								2													
6				1	2																
7																	1				
F																					
1	7																				
2	1																				
3-5			1e																		
G																					
1		1																			
2-4		2e																			
5-6													1e								
7														1							
H																					
1				5			1														
2				1																	
3				2																	
4				1																	
I																					
1			2																		
2			1																		
3			2																		
4			1															1			
5			1																		
J																					
1	3																				
2					1																
3	1															1					
K																					
1				3																	
2						1															
3-4										1e											

Table 2. Pulsotypes of 109 MBL-Producing *Acinetobacter* spp. and 75 *P. aeruginosa* Isolates (Continued)

Species with MBL genes	*A. pitti*			*A. bereziniae*			*A. nosocomialis*			*A. junii*			*A. genomosp* 14TU			*Acinetobacter* spp.*			*P. aeruginosa*		
	IMP -1	VIM -2	SIM -1	IMP -1	VIM -2	SIM -1	IMP -1	VIM -2	SIM -1	IMP -1	VIM -2	SIM -1	IMP -1	VIM -2	SIM -1	IMP -1	VIM -2	SIM -1	IMP -1	IMP -6	VIM -2
L																					
1	2																				
2	1																				
3			1																		
4	1																				
M																					
1										3											
2-3										1e											
N																					
1		1						1													
2-3								1e													
O																					
1	2																				
2	1																				
P																					
1-2	1e																				
3														1							
Q				1			1														
R	2																				
Miscel-laneous	8	3			1								1			1	2				8
Total	31	12	11	14	5	1	2	10	5	7	0	0	3	2	0	2	3	1	1	14	60

e, each; MBL, metallo-β-lactamase.
**A. johnsonii* (n=2), *A. baumannii* (n=1), *A. baylyi* (n=1), *A. soli* (n=1), *A. ursingii* (n=1).

showed that the sizes of bla_{VIM-2} gene-carrying plasmids in *P. aeruginosa*, *P. putida*, and *Acinetobacter* spp. isolates were diverse (Supplementary Fig. 1, only online). It is noteworthy that the bla_{VIM-2} gene-carrying plasmids in *Acinetobacter* spp. were in multimer forms, indicating that the plasmids did not replicate themselves in the same way as with *P. aeruginosa*. Further plasmid sequence analysis using massive parallel sequencing technology has been undertaken.

In conclusion, MBL-producing clinical isolates of *P. aeruginosa* and *Acinetobacter* spp. were reduced, and carbapenemase-producing *Enterobacteriaceae* were found to be rare in Korea. Continuous surveillance studies and further deep sequencing are necessary to understand the dissemination mechanism of the carbapenem-nonsusceptible Gram-negative bacilli isolates in order to control their spread.

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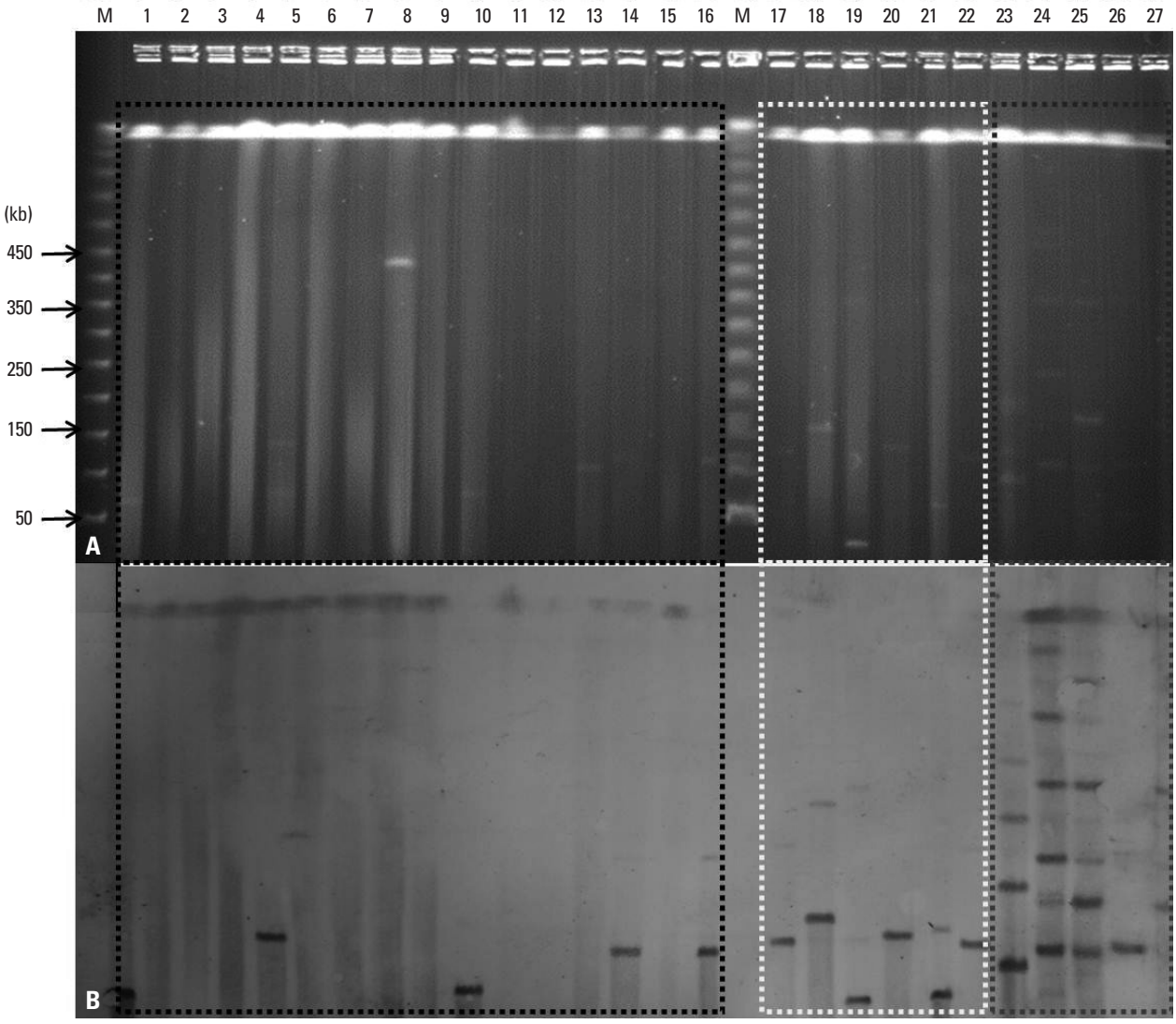

Supplementary Fig. 1. (A) Pulsed field gel electrophoresis of whole genomic DNA of VIM-2-producing *P. aeruginosa* (lanes 2 to 16), *P. putida* (lanes 17 to 22), and *Acinetobacter* spp. isolates (lanes 23 to 27) digested with S1 nuclease. (B) Southern blot hybridization with bla_{VIM-2} gene probe. Lane M, lambda ladder (Bio-Rad) as a marker (kb). The genomic DNA of *P. aeruginosa* stains with high endogenous DNase activities were degraded not to show positive bands (lanes 2, 3, 4, 6, 7, 8, 9, 11, 12, 13, and 15).