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Epidemiological Characterizations of Class 1 Integrons from Multidrug-Resistant *Acinetobacter* Isolates in Daejeon, Korea

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Background: Multidrug-resistant (MDR) *Acinetobacter* spp. acquire antimicrobial agent-resistance genes via class 1 integrons. In this study, integrons were characterized to investigate the antimicrobial resistance mechanisms of MDR *Acinetobacter* isolates. In addition, the relationship between the integron type and integron-harboring bacterial species was analyzed by using epidemiological typing methods.

Methods: Fifty-six MDR *Acinetobacter* spp.-*A. baumannii* (N=30), *A. bereziniae* (N=4), *A. nosocomialis* (N=5), and *A. pittii* (N=17)-were isolated. The minimum inhibitory concentrations (MICs) were determined on the basis of the results of the Epsilometer test (Etest). PCR and DNA sequencing was performed to characterize the gene cassette arrays of class 1 integrons. Multilocus sequence typing (MLST) and repetitive extragenic palindromic sequence (REP)-PCR were performed for epidemiological typing.

Results: Class 1 integrons were detected in 50 (89.3%) of the 56 isolates, but no class 2 or 3 integron was found within the cohorts. The class 1 integrons were classified into 4 types: 2.3-kb type A (*aacA4-catB8-aadA1*), 3.0-kb type B (*aacA4-blaIMP-1-blaOXA-2*), 3.0-kb type C (*blaVIM-2-aacA7-aadA1*), and 1.8-kb type D (*aac3-1-blaOXA-2-orfD*). Type A was most prevalent and was detected only in *A. baumannii* isolates, except for one *A. bereziniae* isolate; however, type B was amplified in all *Acinetobacter* isolates except for *A. baumannii* isolates, regardless of clone and separation time of the bacteria.

Conclusions: Although class 1 integron can be transferred horizontally between unrelated isolates belonging to different species, certain types of class 1 integrons tend to transfer horizontally and vertically among *A. baumannii* or non-*baumannii Acinetobacter* isolates.

Key Words: MDR *Acinetobacter* spp., Integron, MLST, Sequence types

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INTRODUCTION

Acinetobacter spp. are aerobic, glucose-nonfermenting gram-negative bacteria that are widely distributed in soil and water environments [1, 2]. Isolates belonging to the *A. calcoaceticus-A. baumannii* (Acb) complex of the genus *Acinetobacter* are important nosocomial pathogens. Finding an appropriate antimicrobial therapy against these pathogens is a serious concern. The prevalence of Acb complex-related infections has markedly increased in recent years [3-5]. The Acb complex is composed of *A. calcoaceticus*, *A. baumannii*, *A. pittii* (formerly known as *Acinetobacter* genospecies), and *A. nosocomialis* (formerly known as *Acinetobacter* genospecies), which are genetically and phenotypically related. With the exception of *A. calcoaceticus*, all members of the Acb complex are commonly associated with hospital-acquired infections, and they rapidly acquire di-

verse antimicrobial resistance determinants [6, 7].

In *Acinetobacter* spp., the acquisition and dissemination of an antimicrobial-resistant determinant is frequently facilitated by integrons [8]. Integrons contain integrase gene (*intI1*), the product of which facilitates site-specific acquisitions and removals of the gene cassettes contained in the integrons. Approximately 9-17% of the sequenced bacterial genomes harbor integrons, and integrons with the same organization and composition have been found in unrelated isolates in spatially and temporally distinct areas [9, 10]. In particular, class 1 integrons, which are characterized by two conserved segments, are the most widely disseminated isolate of *Acinetobacter* spp. Class 1 integrons harbor various antimicrobial resistance gene cassettes encoding broad-spectrum β-lactamase, dihydroflavonol-4-reductase (*dfr*), and aminoglycoside-modifying enzymes (AMEs), such as acetyltransferase (*aac*), adenylyltransferase (*aad*), and phosphotransferase (*aph*) [11-15]. Therefore, multidrug-resistant (MDR) *Acinetobacter* spp. harboring genetic elements are resistant to three or more classes of antimicrobial agents that contain quinolones, aminoglycosides, ampicillin-sulbactams, extended-spectrum cephalosporins, and carbapenems [16].

Genetic elements like integrons frequently transfer antimicrobial resistance determinants, and are widely disseminated among MDR *Acinetobacter* spp.; however, scarce data is available about the epidemiological characterization of integron-harboring bacteria. In this study, integrons were characterized to investigate the antimicrobial resistance mechanisms of MDR *Acinetobacter* spp. identified in a university hospital in Daejeon, Korea over a period of 7 yr. In addition, multilocus sequence typing (MLST) and repetitive extragenic palindromic sequence (REP)-PCR were performed to analyze the relationship between the integron types and MDR *Acinetobacter* isolates harboring integrons.

METHODS

1. Bacterial isolates and *rpoB* gene analysis

A total of 56 consecutive and MDR *Acinetobacter* isolates were collected from a university hospital laboratory in Daejeon, Korea between January 2006 and December 2012. *Acinetobacter* spp. were identified by using the Vitek 2 Automated Instrument ID System (bioMérieux; Marcy l'Etoile, France) and by sequencing the partial *rpoB* housekeeping gene as described previously [17].

2. Antimicrobial agents and minimum inhibitory concentration (MIC) determinations

The MICs of *Acinetobacter* isolates for amikacin, gentamicin, ceftazidime, cefepime, imipenem, meropenem, and ciprofloxacin were determined by the Epsiolon test (Etest; bioMérieux). The data were interpreted as per the criteria approved by CLSI [18]. *Escherichia coli* ATCC 25922 was used as a reference strain.

3. Characterization of integrons

Multiplex PCR was used to detect class 1, 2, and 3 integrons [19]. All of the integron-positive isolates were subjected to PCR and sequencing assays using specific primers for the analysis of gene cassette arrays. Class 1 integrons were amplified by using primers hep58 (5′-TCATGGCTTGTTATGACTGT-3′) and hep59 (5′-GTAGGGCTTATTATGCACGC-3′). Primers hep74 (5′-CGGGATCCCGGACGGCATGCACGATTTGTA-3′) and hep51 (5′- GATGCCATCGCAAGTACGAG-3′) were used to amplify class 2 integrons [20]. Whole-cell (genomic) DNA was obtained from each target strain by using a genomic DNA purification kit (SolGent; Daejeon, Korea) according to the manufacturer's instructions. For sequencing, PCR products were purified with a PCR purification kit (SolGent) according to the manufacturer's instructions. Sequencing was performed by using the BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems; Foster City, CA, USA) and the ABI PRISM 3730XL DNA Analyzer (PE Applied Biosystems). DNA fragments (up to 1 kb in size) were sequenced by using the overlapping PCR technique. The various DNA sequences were confirmed on the basis of the BLAST paired alignment facility (http://blast.ncbi.nlm.gov).

4. Molecular typing

For isolates of *A. pittii* spp., a MLST scheme with 7 housekeeping genes (*cpn60*, *fusA*, *gltA*, *pyrG*, *recA*, *rplB*, and *rpoB*) was used to determine the sequence types (STs) [21]. On the other hand, the Oxford MLST scheme with 7 housekeeping genes (*cpn60*, *gdhB*, *gltA*, *gpi*, *gyrB*, *recA*, and *rpoD24*) was used to determine the STs of *A. baumannii* isolates [22]. Each ST number was assigned by comparing the allele sequences to those in the MLST databases (http://www.pasteur.fr/mlst andhttp://pubmlst.org/abaumannii).

In addition, epidemiological typing of isolates was performed by REP-PCR [23]. In the REP-PCR method, the primer pair of REP1 (5′-III GCGCCGICATCAGGC-3′) and REP2 (5′-ACGTCTTATCAGGCCTAC-3′) was used to amplify putative REP-like elements in the genomic DNA. Amplification reactions were performed in a final volume of 50 μL, containing 100 ng of chromosomal DNA, 5 μL of 10× Taq buffer, 1.0 μL of 10 mM deoxyribonucleoside 5'-triphosphates (dNTPs) mix, 1.5 U of Taq DNA polymerase (SolGent), and 50 pmol of each primer. The

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cycling conditions were as follows: an initial denaturation step at 95℃ for 5 min followed by 30 cycles of 92℃ for 50 sec, 48℃ for 55 sec, and 70℃ for 5 min; and a final extension step at 70℃ for 10 min. The amplified products were separated via electrophoresis on 1.5% agarose gels containing ethidium bromide, and visualized by using the BioDoc-14TM Imaging system (UVP, Cambridge, UK).

RESULTS

1. Identification of *Acinetobacter* spp.

A total of 4 *Acinetobacter* spp. were identified: *A. baumannii* (N=30), *A. bereziniae* (N=4), *A. nosocomialis* (N=5), and *A. pittii* (N=17). The distribution of MICs for the 7 antimicrobial agents tested was similar among the 4 *Acinetobacter* isolates. Most of the *Acinetobacter* isolates showed high-level resistance to the antimicrobial agents, except for ciprofloxacin. MICs of ciprofloxacin were diverse, ranging from 0.19 mg/L to ≥32 mg/L (Table 1).

2. Characterization of class 1 integrons

Class 1 integrons were detected in 50 (89.3%) of the 56 isolates, but no class 2 or 3 integron was found within the cohorts. The class 1 integrons were classified into 4 types (types A, B, C, and D) on the basis of the gene cassette nucleotide sequence (Table 1). The 2.3-kb type A (*aacA4-catB8-aadA1*) was the most prevalent type but was detected only in *A. baumannii* isolates, except for one in an *A. bereziniae* isolate (Table 2). The 3.0-kb type B (*aacA4-bla*$_{IMP-1}$-*bla*$_{OXA-2}$) was detected in 3 *Acinetobacter* spp. (*A. bereziniae, A. nosocomialis*, and *A. pittii*). The 3.0-kb type C (*bla*$_{VIM-2}$-*aacA7-aadA1*) and 1.8-kb type D (*aac3-1-bla*$_{OXA-2}$-*orfD*) were detected only in *A. nosocomialis*.

3. Molecular typing

The *A. baumannii* isolates were grouped by MLST into 6 distinct STs, including ST69 (1-46-3-2-2-58-3), ST75 (1-3-3-2-2-11-3), ST92 (1-3-3-2-2-7-3), ST137 (1-3-3-2-2-12-3), ST138 (1-3-3-2-2-50-3), and ST358 (1-3-3-2-2-145-3). ST138 (n=11) was the most prevalent ST, followed by ST137 (n=10). In this study, the *A. pittii* isolates were grouped by MLST into 4 distinct STs, including ST63 (17-20-23-10-20-13-20), ST119 (36-20-38-16-38-18-20), and the novel STs N-1 (44-20-46-10-20-18-20) and N-2 (44-21-46-10-20-18-20). Two of the 4 STs corresponded to single isolates, whereas ST63 and N-2 corresponded to 8 and 7 strains, respectively.

All *A. baumannii* and *A. pittii* isolates were typed by REP-PCR. Although most *A. baumannii* isolates displayed the same REP-PCR type regardless of the STs, *A. pittii* isolates showed diverse patterns depending on the STs (Fig. 1).

DISCUSSION

In recent years, dissemination of the antimicrobial resistance genes through integrons in *Acinetobacter* spp. has become a major focus in the treatment of nosocomial infections by MDR bacteria [13]. Class 1 integrons have been found to be prevalent in *Acinetobacter* clinical isolates worldwide, which contain diverse gene cassettes that encode resistance factors to antimicrobial agents such as aminoglycosides, broad-spectrum β-lactams, and trimethoprims [14]. Therefore, our study was designed to determine the prevalence of various class 1 integrons of MDR *Acinetobacter* spp. In addition, we analyzed the relationship between the class 1 integron gene cassette array and *Acinetobacter* spp.

Most of the MDR *Acinetobacter* isolates (89.3%) analyzed in this study harbored class 1 integrons, and the frequency of these isolates in the study region was considerably higher than that in other geographical regions, including the United Kingdom (60%) and China (74.9%) [24]. However, the prevalence of class 1 integrons among MDR *Acinetobacter* spp. is similar to that documented previously showing the occurrence of class 1 integrase gene in only 15 of 123 *Acinetobacter* isolates (12%), with all of these 15 isolates showing MDR [25]. The higher detection rate of class 1 integrons was probably due to the pre-selection of clinical isolates showing the MDR phenotype. Our results indicate that class 1 integron is widely disseminated among MDR *Acinetobacter* spp. in the university hospital in Daejeon, Korea.

According to our results, most MDR *Acinetobacter* spp. carry class 1 integrons, but the specific integron type varies depending on the species. Type A is the most prevalent class 1 integron detected in *Acinetobacter* isolates. However, of the 4 *Acinetobacter* species tested, only an isolate belonging to *A. baumannii* harbored type A, except for one *A. bereziniae* isolate. Twenty-five *A. baumannii* strains harboring type A were collected between 2007 and 2012, and all of the strains belonged to 6 STs (ST69, ST75, ST92, ST137, ST138, and ST358). Our results suggest that type A is a dominant type of integron and that it has been conserved for many years in *A. baumannii* and other *Acinetobacter* species. The type A gene cassette array has been reported in *A. baumannii* as well as in other bacteria, including *Acinetobacter* spp. (92.0%; 126/137), *Burkholderia cepacia* (22.2%; 2/9), *Klebsiella pneumonia* (4.8%; 4/83), and *E. coli* (0.6%; 1/164) [24, 26]. Although the type A detection rate was highest in *Acinetobacter* isolates, there is a relative paucity of

Table 1. The MIC distribution of 7 antimicrobial agents for 56 MDR *Acinetobacter* spp. isolates

Species	Isolate code	Year of isolation	ST	Minimum inhibitory concentration (μg/mL)							Integron*
				AMK	GEM	CAZ	CFP	IMP	MEM	CIP	
A. baumannii (N=30)	C6-14	2006	69	>256	>1,024	>256	8	>32	12	>32	Type A
	C6-16	2006	69	>256	>1,024	>256	48	>32	>32	>32	Type A
	C6-22	2006	92	>256	>1,024	>256	32	>32	>32	>32	Type A
	C8-11	2008	137	>256	>1,024	>256	32	>32	>32	>32	Type A
	C8-13	2008	92	>256	>1,024	>256	32	8	6	>32	Type A
	C8-14	2008	137	>256	>1,024	>256	32	>32	>32	>32	Type A
	C8-16	2008	137	>256	>1,024	>256	32	>32	>32	>32	Type A
	C8-17	2008	92	>256	>1,024	>256	64	>16	>32	>32	Type A
	C8-18	2008	75	24	96	>256	32	8	6	>32	
	C8-21	2008	138	>256	>1,024	>256	32	>32	>32	>32	Type A
	C9-25	2009	137	>256	>1,024	>256	32	>32	>32	>32	Type A
	C9-26	2009	137	>256	>1,024	>256	64	>32	>32	>32	Type A
	C9-29	2009	138	>256	>1,024	>256	24	>32	>32	>32	Type A
	C9-30	2009	137	>256	128	128	8	>32	16	>32	Type A
	C9-32	2009	137	>256	512	>256	8	>32	12	>32	Type A
	C9-33	2009	137	>256	512	>256	8	>32	16	>32	Type A
	C10-06	2010	138	>256	>1,024	>256	24	>32	>32	>32	Type A
	C10-10	2010	137	4	8	>256	192	>32	12	>32	
	C10-12	2010	138	>256	>1,024	>256	48	>32	>32	>32	Type A
	C10-14	2010	137	>256	>1,024	>256	32	>32	>32	>32	Type A
	C10-16	2010	138	8	6	32	32	>32	>32	>32	
	C11-102	2011	138	8	12	>256	>256	>32	>32	>32	
	C11-103	2011	75	24	128	96	128	>32	>32	>32	
	C11-104	2011	138	>256	>1,024	>256	64	>32	>32	>32	Type A
	C11-106	2011	138	>256	>1,024	>256	128	>32	>32	>32	Type A
	C11-107	2011	138	>256	>1,024	>256	64	>32	>32	>32	Type A
	C11-112	2011	358	>256	>1,024	>256	64	>32	>32	>32	Type A
	C12-217	2012	358	>256	>1,024	96	64	>32	>32	>32	Type A
	C12-219	2012	138	>256	>1,024	256	>256	>32	>32	>32	Type A
	C12-221	2012	138	>256	>1,024	>256	96	>32	>32	>32	Type A
A. bereziniae (N=4)	C6-07	2006	-	>256	128	>256	>256	>32	>32	32	Type B
	C6-09	2006	-	64	16	128	64	>32	>32	32	Type B
	C7-14	2007	-	>256	>246	>256	>256	>32	>32	4	Type B
	C7-22	2007	-	>256	>256	>256	>256	>32	>32	32	Type A/Type B
A. nosocomialis (N=5)	C6-75	2006	-	64	>256	>256	>256	>32	16	32	Type B
	C6-814	2006	-	>256	>256	>256	>256	16	16	4	Type C
	C9-08	2009	-	>256	>256	64	64	8	4	4	Type C/Type D
	C10-09	2010	-	>256	>256	>256	64	8	6	4	Type C
	C12-280	2012	-	>256	128	128	64	16	16	4	

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Table 1. Continued

Species	Isolate code	Year of isolation	ST	Minimum inhibitory concentration (μg/mL)							Integron*
				AMK	GEM	CAZ	CFP	IMP	MEM	CIP	
A. pittii (N=17)	C6-01	2006	63	>256	>256	>256	>256	>32	>32	8	Type B
	C6-03	2006	63	>256	>256	>256	>256	>32	>32	4	Type B
	C6-05	2006	119	>256	>256	>256	128	>32	>32	0.38	Type B
	C6-06	2006	63	>256	>256	>256	24	>32	>32	0.25	Type B
	C6-08	2006	63	>256	>256	>256	48	>32	>32	0.25	Type B
	C6-10	2006	63	>256	>256	>256	48	>32	>32	0.25	Type B
	C6-24	2006	N-1	>256	>256	>256	32	>32	>32	0.25	Type B
	C9-09	2009	63	>256	>256	>256	32	>32	>32	0.19	Type B
	C9-31	2009	63	>256	32	>64	8	>32	>32	4	Type B
	C9-52	2009	63	>256	>256	>256	>256	>32	>32	0.25	Type B
	C11-14	2011	N-2	>256	>256	>256	>256	>32	>32	>32	Type B
	C11-15	2011	N-2	>256	>256	>256	>256	>32	>32	>32	Type B
	C11-47	2011	N-2	>256	>256	>256	>256	>32	>32	>32	Type B
	C11-48	2011	N-2	>256	>256	>256	>256	>32	>32	>32	Type B
	C12-200	2012	N-2	>256	>256	>256	>256	>32	>32	>32	Type B
	C12-247	2012	N-2	>256	>256	>256	>256	>32	>32	>32	Type B
	C12-273	2012	N-2	>256	>256	>256	>256	>32	>32	>32	Type B

*Type A, *aacA4-catB8-aadA1*; Type B, *aacA4-bla*$_{IMP-1}$-*bla*$_{OXA-2}$; Type C, *bla*$_{VIM-2}$-*aacA7-aadA1*; Type D, *aac3-1-bla*$_{OXA-2}$-*orfD*.
Abbreviations: MIC, minimum inhibitory concentration; ST, sequence type; AMK, amikacin; GEN, gentamicin; CAZ, ceftazidime; FEP, cefepime; IPM, imipenem; MEM, meropenem; CIP, ciprofloxacin; N, novelsequence type.

Table 2. Gene cassette arrays obtained from MDR *Acinetobacter* isolates–*A. baumannii*, *A. bereziniae*, *A. nosocomialis*, and *A. pittii*

Species	N of representative isolates (%)			
	Type A *aacA4-catB8-aadA1*	Type B *aacA4-bla*$_{IMP-1}$-*bla*$_{OXA-2}$	Type C *bla*$_{VIM-2}$-*aacA7-aadA1*	Type D *aac3-1-bla*$_{OXA-2}$-*orfD*
A. baumannii (N=30)	25 (83)			
A. bereziniae (N=4)	1 (25)	4 (100)		
A. nosocomialis (N=5)		1 (20)	3 (60)	1 (20)
A. pittii (N=17)		17 (100)		
Total (N=56)	26 (46)	22 (39)	3 (5)	1 (2)

Abbreviation: MDR, multidrug resistant.

data on the non-*A. baumannii Acinetobacter* isolates harboring type A. These earlier reports support our results that type A can usually be transmitted horizontally and vertically among *A. baumannii* isolates.

In contrast to type A, type B was amplified in *A. bereziniae, A. nosocomialis*, and *A. pittii* isolates, but not in *A. baumannii*. In particular, all *A. bereziniae* and *A. pittii* isolates contained type B, irrespective of the time sampled or the clone. This result was similar to that of previous studies that found type B in *A. pittii, A. bereziniae*, and *A. nosocomialis* strains isolated from Taiwan and South Korea [27, 28]. Our results suggest that type B can usually be transmitted across species among non-*A. baumannii Acinetobacter* isolates, and is conserved in the *Acinetobacter* isolates.

In this study, we performed MLST and REP-PCR for epidemiological typing of MDR *A. baumannii* and *A. pittii* isolates. The STs were not directly correlated with REP-PCR types in the *A. baumannii* isolates, but they were well correlated in *A. pittii* isolates. Previous studies have reported no correlation of STs with REP-PCR types, probably because of the REP-PCR methods used; however, the MLST results were correlated with the REP-PCR clusters [29, 30]. Further studies are required to confirm the relationship between REP-PCR and MLST types. On the other hand, we confirmed that *A. pittii* exhibited a diverse REP-PCR pattern, which was less clonal than that of *A. baumannii*

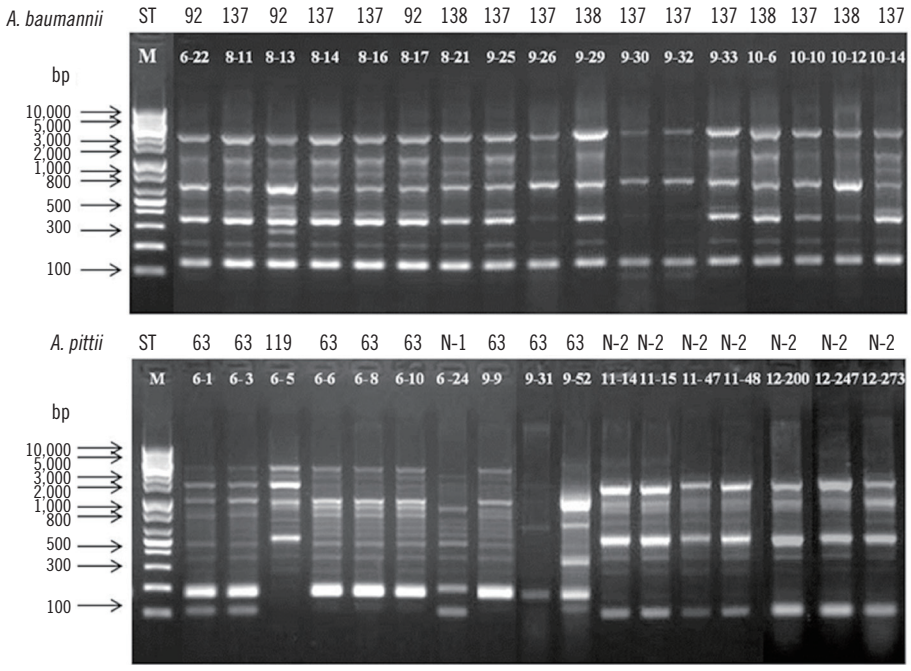

Fig. 1. Repetitive extragenic palindromic (REP)-PCR patterns of genomic DNA from MDR *Acinetobacter baumannii* (top panel) and *Acinetobacter pittii* (bottom panel) isolates. Lane M, 1-kb DNA size marker.
Abbreviations: MDR, multidrug resistant; ST, sequence type.

showing the same REP-PCR pattern.

All class 1 integrons detected in our study contained aminoglycoside-resistance genes such as acetyltransferase (*aac*) and adenylyltransferase (*aad*), which have been typically found within integron gene cassette arrays [13]. All isolates harboring class 1 integrons showed a high-level resistance rate to aminoglycoside compounds such as amikacin and gentamicin. In addition, the class 1 integrons harbored β-lactamase genes (bla_{OXA-2}, bla_{IMP-1}, and bla_{VIM-2}) and chloramphenicol-resistance gene (*catB8*). Consequently, the co-occurrence of several antimicrobial-resistance determinants via class 1 integrons can lead to the emergence of MDR *Acinetobacter* spp. In particular, type B and type C harboring metallo-β-lactamase (MBL) genes (bla_{IMP-1} and bla_{VIM-2}) were detected in non-*A. baumannii Acinetobacter* isolates. These results suggest that a major mechanism of carbapenem resistance in non-*A. baumannii Acinetobacter* isolates involves class 1 integrons containing MBL genes. However, in this study, integrons containing carbapenemase genes were not detected in the *A. baumannii* isolates. This result suggests that acquisitions of class 1 integrons were not considered as major factors of carbapenem resistance in *A. baumannii* isolates. It has been reported that the carbapenem resistance of *A. baumannii* isolates is often associated with the presence of carbapenem-hydrolyzing class D β-lactamase (CHDL) genes located in chromosomes, plasmids, or transposons [31].

Despite the increasingly frequent discovery of MDR *Acinetobacter* isolates in Korea, not much information is available regarding the distributions of integrons of MDR *Acinetobacter* strains isolated in Daejeon, Korea. We confirmed that class 1 integrons harboring diverse gene cassettes are widely disseminated among MDR *Acinetobacter* isolates. Although class 1 integrons can be transferred horizontally between unrelated isolates belonging to different species, each type of the 4 gene cassette arrays evaluated in this study was found in isolates belonging to *Acinetobacter* spp., irrespective of the time sampled and STs. Our findings suggest that certain class 1 integrons tend to transfer horizontally and vertically among *A. baumannii* or non-*A. baumannii Acinetobacter* isolates. Accordingly, the present study emphasizes that continuous analyses of gene cassette arrays of class 1 integrons will provide useful information regarding antimicrobial resistance mechanisms specific to different *Acinetobacter* species.

Authors' Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

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