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Genetic Basis of Multidrug-resistant *Acinetobacter baumannii* Clinical Isolates from Three University Hospitals in Chungcheong Province, Korea

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Background: The emergence of multidrug-resistant (MDR) *Acinetobacter baumannii* as an important opportunistic pathogen has given rise to significant therapeutic challenges in the treatment of nosocomial infections. In the present study, we assess the antibiotic resistance mechanisms of MDR *A. baumannii* strains by estimating the prevalence of antibiotic resistance determinants, including integrons, β-lactamases, *str* genes, and *gyrA* and *parC* mutations.

Methods: Thirty-five MDR *A. baumannii* clinical isolates were collected from 3 Korean university hospitals over a 2-yr period. *A. baumannii* was confirmed by *rpoB* gene analysis. For each isolate, the minimal inhibitory concentrations (MICs) of 9 antibiotics were determined by the agar dilution method. PCR and DNA sequencing were used to identify the genes that potentially contribute to each resistance phenotype.

Results: Of the 35 MDR *A. baumannii* isolates examined, 7 antibiotic resistance gene determinants were detected. These resistance gene determinants included the gene bla_{OXA-23}, with an upstream element IS*Aba1* to promote increased gene expression and subsequent resistance to carbapenems, in 8 isolates (22.9%); *aacA4*, located within class 1 integrons, in 7 isolates (20.0%); and fluoroquinolone resistance conferred by *gyrA* and *parC* sense mutations in 31 isolates.

Conclusions: Of the 35 MDR *A. baumannii* isolates, 26 (74.3%) from both outbreak and sporadic cases possessed at least 4 of the 7 antibiotic resistance gene determinants that give rise to the MDR phenotype. The co-occurrence of several resistance determinants may present a significant threat.
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Key Words : *MDR A. baumannii, Antibiotic resistance gene determinants, ISAba1*

INTRODUCTION

Acinetobacter baumannii is an aerobic, gram-negative, non-glucose fermenting bacterium, which has recently emerged as an important opportunistic pathogen causing nosocomial infections, including pneumonia, septicemia, and urinary tract and wound infections, and is also frequently involved in outbreaks. The organism is characterized by its tendency to acquire resistance to multiple classes of antimicrobials [1].

Multidrug-resistant (MDR) *A. baumannii* strains have been known to acquire their antibiotic-resistant genes via class 1 integrons that carry single or multiple gene cassettes [2]. Integrons are genetic elements that mostly encode antibiotic resistance and have the ability to integrate or mobilize their inherent gene cassettes [3]. Aminoglycoside resistance genes, such as acetyltransferase

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(*aac*), phosphotransferase (*aph*), and adenylyltransferase (*aad*), contribute to the enzymatic inactivation of aminoglycoside antibiotics and are the genes typically found within the gene cassettes. In some instances, carbapenemase genes, such as bla_{IMP}, bla_{VIM}, bla_{GIM}, bla_{SIM}, or $bla_{OXA-like}$, are located within class 1 integrons and lead to carbapenem resistance [4-6].

Although carbapenems have become the drug of choice for treating *Acinetobacter* infections, their efficacy is now compromised by the emergence of carbapenem resistance. Carbapenemase production is the most well-described resistance mechanism to carbapenems [7]. Ambler class B enzymes (also referred to as metallo-β-lactamases, or MBLs) and carbapenem hydrolyzing class D-β-lactamases have been isolated from carbapenem-resistant *A. baumannii* strains worldwide [8, 9]. In particular, the region upstream of OXA-type class D carbapenemase in *Acinetobacter* frequently includes the insertion sequence (IS) IS*Aba1*, which can modulate the expression and transfer of OXA-type carbapenemase genes [4]. An IS is a mobile genetic element known to influence the evolutionary pattern of bacterial genomes. Upon integration, the IS elements may cause DNA insertion/deletion, chromosomal rearrangement, and modulation of neighboring gene expression, thereby influencing the bacterial phenotype [10].

In addition, increases in the number fluoroquinolone-resistant *A. baumannii* strains have resulted in a limited choice of efficacious antimicrobial agents against *A. baumannii*. Fluoroquinolone resistance is mediated primarily by spontaneous mutations in the quinolone resistance-determining regions (QRDRs) of either the DNA gyrase (*gyrA*) or topoisomerase IV (*parC*) genes or both [11].

The emergence of *A. baumannii* isolates resistant to multiple classes of antimicrobials, including carbapenems, aminoglycosides, and fluoroquinolones, was observed in Korea [12]. However, there is a relative paucity of data on the number and type of resistance genes in MDR *A. baumannii* strains.

In the present study, we aimed to determine the genetic basis for MDR, as well as the clonal relationship between clinical isolates obtained from the Chungcheong province of Korea. We investigated the molecular determinants known to enable MDR strains to exhibit co-resistance to carbapenems, aminoglycosides, and/or fluoroquinolones, with particular focus on integrons, carbapenemases, *str* genes (aminoglycoside resistance), and *gyrA* and *parC* gene mutations.

MATERIALS AND METHODS

1. Bacterial isolates and *rpoB* gene analysis

A total of 35 consecutive and non-duplicated MDR *A. baumannii* isolates were obtained between January 2007 and December 2008 from 3 university hospital laboratories in Chungcheong province of Korea. *A. baumannii* was confirmed by determination of the partial *rpoB* gene sequence using primers Ac1055F (5′-GTGATAARATG-GCBGGTCGT-3′) and Ac1598R (5′-CGBGCRTGCATYT-TGTCRT-3′) [13]. Chromosomal DNA was obtained from each target strain by using the genomic DNA purification kit (Promega, Madison, WI, USA) in accordance with standard protocols. PCR was performed using 50 ng template DNA (genomic DNA), 2.5 μL 10×Taq buffer, 0.5 μL 10 mM dNTP mix, 20 pmol of each primer, and 0.7 U Taq DNA polymerase (SolGent, Daejeon, Korea), in a total volume of 25 μL. The partial *rpoB* gene was amplified in a GeneAmp PCR System 9600 (Perkin-Elmer Cetus Corp., Norwalk, CT, USA) by pre-denaturation of the reaction mixture for 5 min at 95℃, followed by 35 cycles consisting of 30 sec at 95℃, 40 sec at 52℃, and 30 sec at 72℃, and finally elongated for 5 min at 72℃. The amplicons were purified with a PCR purification kit (SolGent) and were sequenced using a BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 3730XL DNA analyzer (PE Applied Biosystems).

2. Antimicrobial agents and MIC determinations

The CLSI agar dilution method was used to determine

the MIC [14]. The following antibiotics were obtained from the indicated providers: amikacin, gentamicin, streptomycin, and ampicillin from Sigma-Aldrich (St. Louis, MO, USA); cefotaxime from Handok (Seoul, Korea); ceftazidime from Hanmi (Seoul, Korea); cefepime from Boryung (Seoul, Korea); imipenem from MSD (Westpoint, PA, USA); and ciprofloxacin from ICN Biomedicals (Aurora, OH, USA). *E. coli* ATCC 25922 was used as a reference strain. MDR was defined as resistance to 3 or more of the following antibiotic classes: quinolones (ciprofloxacin), extended-spectrum cephalosporins (ceftazidime), aminoglycosides (amikacin, streptomycin, gentamicin, kanamycin), and carbapenems (imipenem) [4].

3. Characterization of antimicrobial resistance determinants

All MDR *A. baumannii* isolates were sequenced by PCR to detect antibiotic resistance determinants and to identify mutations associated with fluoroquinolone resistance. The specific primers used in detecting antibiotic resistance determinants are described in Table 1.

PCR and sequencing analysis of antibiotic resistance determinants, excluding integron, was performed by the same method as *rpoB* gene analysis with the exception of primer annealing (for 40 sec at 59℃ instead of 52℃). Integron detection, characterization, and gene cassette amplification were carried out using previously described

Table 1. Primers used in this study

Primer	Target gene/region	Sequence (5′→3′)	Reference
PW166	IS*Aba1*	CCTATCAGGGTTCTGCCTTCT	15
PW200	*strA*	ATGATGTCTAACAGCAAACTG	15
PW201		TCAACCCCAAGTAAGAGG	
PW202	*strB*	ATGGGGTTGATGTTCATGCCGC	15
PW203		CTAGTATGACGTCTGTCGCAC	
TEM F	bla_{TEM-1} and derivative	ATGAGTATTCAACATTTCCGT	5
TEM R		TTACCAATGCTTAATCAGTGA	
SHV F	bla_{SHV-1} and derivative	CCGGGTTATTCTTATTTGTCGCT	5
SHV R		TAGCGTTGCCAGTGCTCG	
CTX-M F	$bla_{CTX-M-1,2,9}$ group	GATTGACCGTATTGGGAGTTT	5
CTX-M R		CGGCTGGGTAAAATAGGTCA	
PER F	bla_{PER-1}	GTTAATTTGGGCTTAGGGCAGA	5
PER R		CAGCGCAATCCCCACTGT	
VEB F	bla_{VEB-1}	CGACTTCCATTTCCCGATGC	5
VEB R		GGACTCTGCAACAAATACGC	
GES F	$bla_{GES-1,-2,-3,-4}$, bla_{IBC-1}	GTTAGACGGGCGTACAAAGATAAT	5
GES R		TGTCCGTGCTCAGGATGAGT	
PSE F	bla_{PSE-1}	AATGGCAATCAGCGCTTC	5
PSE R		GCGCGACTGTGATGTATA	
IMP F	bla_{IMP}	CATGGTTTGGTGGTTCTTGT	5
IMP R		ATAATTTGGCGGACTTTGGC	
VIM F	bla_{VIM}	ATTGGTCTATTTGACCGCGTC	5
VIM R		TGCTACTCAACGACTGAGCG	
OXA-23-like F	$bla_{OXA-23-like}$	CTTGCTATGTGGTTGCTTCTC	15
OXA-23-like R		ATCCATTGCCCAACCAGTC	
OXA-24-like F	$bla_{OXA-24-like}$	GTACTAATCAAAGTTGTGAA	5
OXA-24-like R		TTCCCCTAACATGAATTTGT	
OXA-51-like F	$bla_{OXA-51-like}$	ATGAACATTAAAGCACTC	16
OXA-51-like R		CTATAAAATACCTAATTGTTC	
OXA-58-like F	$bla_{OXA-58-like}$	CGATCAGAATGTTCAAGCGC	5
OXA-58-like R		ACGATTCTCCCCTCTGCGC	
GyrA F	*gyrA*	AAATCTGCCCGTGTCGTTGGT	17
GyrA R		GCCATACCTACGGCGATACC	
ParC F	*parC*	AAGCCCGTACAGCGCCGTATT-	18
ParC R		AAAGTTATCTTGCCATTCGCT	

PCR methods [19].

4. Repetitive extragenic palindromic-PCR (REP-PCR) for clonality assessment

REP-PCR was conducted in a 50 μL reaction mixture containing 100 ng chromosomal DNA, 5 μL 10×Taq buffer, 1.0 μL 10 mM dNTP mix, 1.5 U Taq DNA polymerase (SolGent), and 50 pmol each of the primers REP1 (5′-IIIGCGCCGICATCAGGC-3′) and REP2 (5′-ACGTCT-TATCAGGCCTAC-3′) [20]. The cycling conditions were as follows: initial denaturation for 5 min at 95℃; 30 amplification cycles consisting of 50 sec at 92℃, 55 sec at 48℃, and 5 min at 70℃; and final elongation for 10 min at 70℃. The amplified products were separated via electrophoresis on a 1.5% agarose gel containing ethidium bromide, and visualized using a BioDoc-14™ Imaging system (UVP, Cambridge, UK).

RESULTS

1. Antibiotic susceptibility testing

Of the 35 MDR *A. baumannii* strains, strains 11, 18, and 6 were isolated in hospitals A, B, and C, respectively, and were tested for susceptibility to 9 antibiotics by MIC determination (Table 2). Twenty-seven isolates demonstrated high-level resistance to gentamicin, streptomycin, ampicillin, and ciprofloxacin (Table 2). Of the 9 antimicrobials tested, the most efficacious was amikacin.

2. Detection and characterization of integrons

Class 1 integrons were detected in 7 of the 35 isolates (20.0%), however, no class 2 or class 3 integrons were found within the cohorts (Table 3). Class 1 integron gene cassettes can be classified into 2 types according to the cassette composition (Fig. 1). The class 1 integrons detected in 3 of the isolates were type 1 (3.3 kb) carrying *aacA4*, bla_{OXA-2} and bla_{IMP-1} genes. The type 2 (3.2 kb) amplicon obtained in 4 of the clinical isolates contained *aacA4* (coding for amikacin, netilmicin, and tobramycin resistance), *aadA1* (coding for spectinomycin and streptomycin resistance), and *catB8* genes.

3. Detection of antibiotic resistance determinants

The *aadA1* gene cassette was present in 4 out of 7 integron-positive isolates (A14, B6, B9, B13), yet all 35 isolates were resistant to streptomycin. Hence, we investigated the determinants *strA* and *strB* for their ability to confer resistance to streptomycin. Twenty-seven of the 35 isolates contained the single *strB* gene, while the single *strA* gene was not found in any isolate (Table 3). The isolates containing *strB* were highly resistant to streptomycin (MIC≥512).

The gene, bla_{OXA-23}, was amplified from 8 out of the 35 isolates (22.9%), all of which harbored IS*Aba1* upstream. These 8 isolates were highly resistant to imipenem (MIC ≥64). Although the intrinsic β-lactamase gene, $bla_{OXA-51-like}$, was amplified in all 35 MDR *A. baumannii* isolates, the upstream IS*Aba1* was identified in 21 of the isolates. Three of the 35 MDR *A. baumannii* strains harbored the MBL gene, bla_{IMP-1}, and these 3 strains were only isolated from hospital C. However, we were unable to detect Ambler class A β-lactamases (bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{PER}, bla_{VEB}, bla_{GES}, and bla_{PSE}) or OXA-type carbapenemases ($bla_{OXA-24-like}$ and $bla_{OXA-58-like}$).

In addition, to determine whether ciprofloxacin resistance was due to structural changes in fluoroquinolone protein targets, the *gyrA* and *parC* genes were PCR amplified and sequenced. In 31 of the 35 isolates (88.6%), sequencing results revealed a point mutation on the *gyrA* gene that converted serine at position 83 (Ser-83) to leucine (Leu) in GyrA. No additional amino acid sequence changes were observed for the GyrA polypeptide in these clinical isolates. Sequencing also indicated that in 31 of the 35 isolates (88.6%), mutations in *parC*, triggered amino acid changes either at Ser-80 or glutamate (Glu)-84 of ParC, but not in both. Among these 31 isolates, 21 showed Leu to Ser conversions at position 80, and 10 lysine to Glu conversions at position 84. All 31 isolates

Table 2. MICs of the 9 antibiotics for the 35 *Acinetobacter baumannii* isolates as determined by agar dilution

Isolates	MIC (mg/L)								
	AMK	GEN	STR	AMP	CAZ	CTX	FEP	IPM	CIP
A4	64	<16	32	128	128	128	64	16	4
A14	64	>128	>512	>512	512	>512	64	64	64
A15	<2	<16	64	128	128	128	64	16	4
A16	16	>128	>512	>512	>512	>512	256	16	>64
A20	<2	>128	>512	>512	>512	>512	256	64	>64
A22	6	>128	>512	>512	128	512	32	8	>64
A23	64	>128	256	>512	128	256	64	16	>64
A31	4	>128	>512	>512	512	>512	32	16	>64
A32	4	>128	>512	>512	256	512	32	16	>64
A34	4	>128	>512	>512	256	512	32	16	>64
A36	4	>128	>512	>512	512	512	32	16	>64
B1	64	>128	>512	>512	512	>512	64	128	>64
B3	64	>128	>512	>512	>512	>512	256	16	>64
B4	64	>128	>512	>512	512	512	64	64	>64
B6	64	>128	>512	>512	>512	>512	256	64	>64
B9	64	>128	>512	>512	>512	>512	256	64	>64
B13	64	>128	>512	>512	512	>512	64	64	>64
B21	64	>128	>512	>512	128	512	64	16	>64
B22	64	>128	>512	>512	128	512	64	16	>64
B23	64	>128	>512	>512	128	512	64	16	>64
B24	64	>128	>512	>512	>512	>512	>512	16	>64
B25	64	>128	>512	>512	>512	512	512	16	>64
B27	64	>128	>512	>512	128	512	64	16	>64
B28	64	>128	>512	>512	128	512	64	16	>64
B29	64	>128	>512	>512	128	512	64	16	>64
B30	64	>128	>512	>512	128	512	64	16	>64
B33	64	>128	>512	>512	128	256	64	16	>64
B36	64	>128	>512	>512	128	512	64	16	>64
B37	64	>128	>512	>512	128	512	64	16	>64
C1	64	64	64	>512	>512	256	64	64	4
C4	64	64	64	>512	>512	128	64	64	64
C6	64	>128	>512	>512	128	512	64	64	>64
C8	64	>128	>512	>512	>512	>512	512	64	>64
C11	64	>128	>512	>512	512	512	64	64	>64
C25	4	<16	32	128	128	128	64	16	4

Abbreviations: MIC, minimum inhibitory concentration; AMK, amikacin; GEN, gentamicin; STR, streptomycin; AMP, ampicillin; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; IPM, imipenem; CIP, ciprofloxacin.

that harbored amino acid substitutions in GyrA and ParC polypeptides exhibited high-level fluoroquinolone resistance (MIC≥64 mg/mL).

4. REP-PCR patterns

In order to determine the clonality of all the 35 MDR *A. baumannii* isolates, REP-PCR was carried out on genomic DNA. The 17 strains isolated from both hospital A and hospital C displayed diverse band patterns. However, 17 of the 18 strains isolated from hospital B displayed 2 REP-PCR types: type a and type b. (Fig. 2).

DISCUSSION

A. baumannii is an increasingly important nosocomial pathogen, frequently causing nosocomial outbreaks in intensive care units. Most outbreak strains are highly resistant to antibiotics, and therefore therapeutic options are becoming increasingly limited [21]. The present study aimed to define the genetic basis of MDR in *A. baumannii* by detecting antibiotic resistance gene determinants.

Table 3. Profiles of antibiotic resistance determinants in multidrug-resistant *Acinetobacter baumannii* Isolates

Isolates	Antibiotic resistance determinants in MDR *A. baumannii*						
	strB	IS*Aba1*/bla_{oxa-23}	IS*Aba1*/$bla_{oxa-51-like}$	bla_{IMP-1}	*gyrA* mutation	*parC* mutation	Integron
A4	-	-	-	-	-	-	-
A14	+	+	-	-	+	+	Type 2
A15	-	-	-	-	-	-	-
A16	+	-	+	-	+	+	-
A20	+	+	-	-	+	+	-
A22	+	-	+	-	+	+	-
A23	-	-	-	-	+	+	-
A31	+	-	+	-	+	+	-
A32	+	-	+	-	+	+	-
A34	+	-	+	-	+	+	-
A36	+	-	+	-	+	+	-
B1	+	+	-	-	+	+	-
B3	+	-	-	-	+	+	-
B4	+	+	-	-	+	+	-
B6	+	+	+	-	+	+	Type 2
B9	+	+	+	-	+	+	Type 2
B13	+	+	-	-	+	+	Type 2
B21	+	-	+	-	+	+	-
B22	+	-	+	-	+	+	-
B23	+	-	+	-	+	+	-
B24	+	-	-	-	+	+	-
B25	+	-	+	-	+	+	-
B27	+	-	+	-	+	+	-
B28	+	-	+	-	+	+	-
B29	+	-	+	-	+	+	-
B30	+	-	+	-	+	+	-
B33	-	-	+	-	+	+	-
B36	+	-	+	-	+	+	-
B37	-	-	+	-	+	+	-
C1	-	-	-	+	-	-	Type 1
C4	-	-	-	+	+	+	Type 1
C6	+	-	+	-	+	+	-
C8	+	-	+	-	+	+	-
C11	+	+	-	-	+	+	-
C25	-	-	-	+	-	-	Type 1

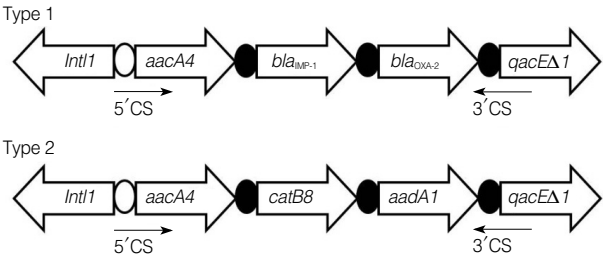

Fig. 1. Schematic representation of the gene cassette structure located in the class 1 integron isolated from multidrug-resistant *Acinetobacter baumannii*. The horizontal arrows indicate the translation orientation of the genes.

Integron classes have been identified by sequencing the integrase genes, with class 1 being the most prevalent in *Acinetobacter* spp. [22, 23]. In this study, only the class 1 integron was identified in MDR *A. baumannii*. Class 1 integrons harboring bla_{IMP-1} belong to the type 1 gene cassette array and have been identified in the *A. baumannii* (EF375699) and *A. junii* (EU014166) isolates recovered from Korea in 2007. The type 2 gene cassette array has been reported in many bacterial species including *Klebsiella pneumoniae* (AY748453), *Citrobacter freundii* (AF550415), *Salmonella enterica* (DQ238105), and *A. baumannii* (AY922989) [24].

Various gram-negative bacteria, including *A. baumannii*, have been shown to contain insertion sequences with promoters that alter the levels of gene expression [25, 26]. The IS*Aba1* element provides promoter sequences

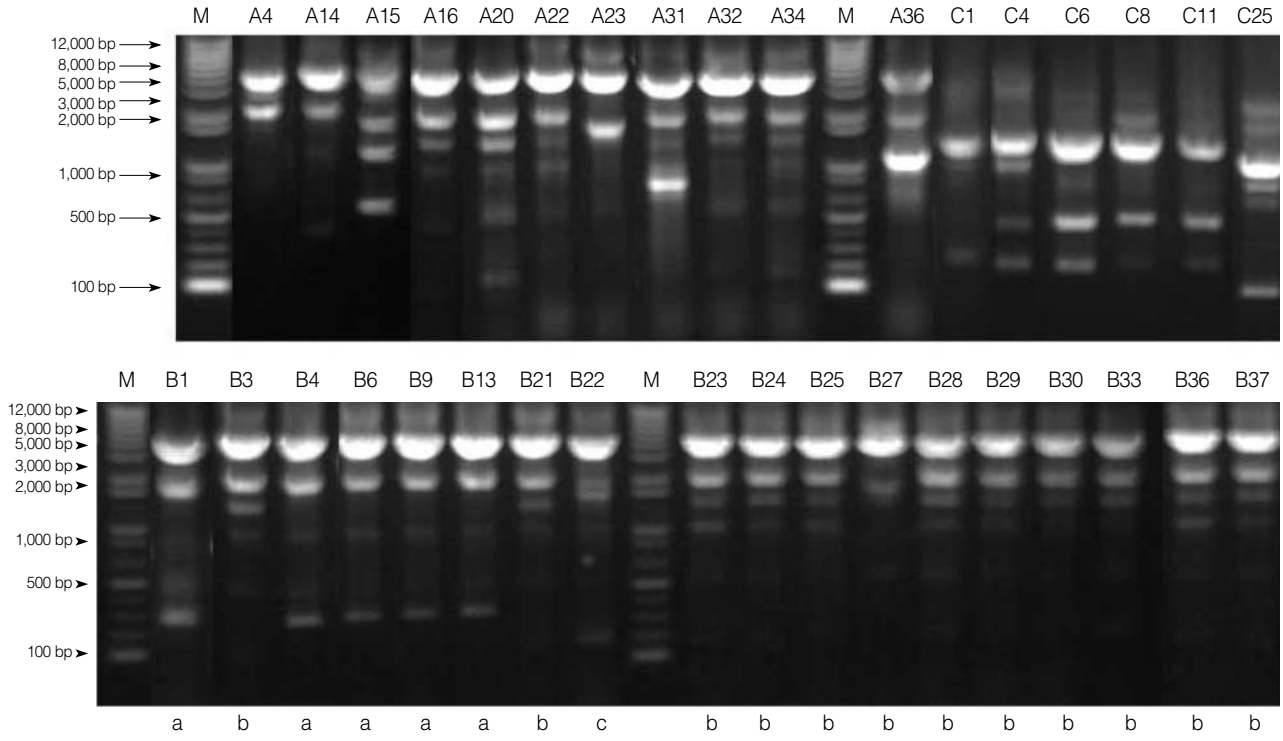

Fig. 2. Repetitive element sequence-based (REP)-PCR patterns of genomic DNA from 35 multidrug resistant *A. baumannii*. Lane M contains a 1 kb DNA size marker.

upstream of bla_{OXA} genes, thereby enhancing the expression of these genes [21]. In this study, IS*Aba1* conferred imipenem resistance by virtue of its location upstream of bla_{OXA-23} in 8 isolates (22.9%) and of $bla_{OXA-51-like}$ in 21 isolates (60.0%). We also observed a correlation between the presence of the IS*Aba1* element upstream of bla_{OXA-23} and the high-level antibiotic resistance in the 8 isolates (22.9%) that harbored the bla_{OXA-23} gene. In addition, the 5 strains which harbored the bla_{OXA-23} gene exhibited identical banding patterns on REP-PCR profiles and were isolated from hospital B. Our results suggest that the clonal relationship and horizontal spread of OXA-23 effected the development of MDR *A. baumannii* in hospital B. The dissemination of *Acinetobacter* harboring the bla_{OXA-23} gene has been previously reported in Korea [5].

A major mechanism of fluoroquinolone resistance in gram-negative bacteria involves structural changes of the drug targets DNA gyrase and DNA topoisomerase IV [27]. In *A. baumannii*, the most frequent amino acid substitutions occur at position 83 (Ser-83) of GyrA and at position 80 (Ser-80) of ParC [28]. Our sequencing results revealed concurrent mutations in *gyrA* (Ser-83) and *parC* (Ser-80 or Glu-84) in 31 of the 35 isolates.

Despite the increased frequency of MDR *A. baumannii* strains isolated in Korea, there exists a relative paucity of information regarding the antimicrobial resistance of this gram-negative bacillus in the Chungcheong province of Korea. In this study, 26 of the 35 MDR *A. baumannii* strains possessed at least 4 antibiotic resistance determinants, including *strB*, IS*Aba1*/bla_{oxa-23}, and/or IS*Aba1*/$bla_{oxa-51-like}$, and mutations in *gyrA* and *parC* (Table 3). These molecular determinants enable co-resistance to carbapenems, aminoglycosides, and/or fluoroquinolones in the *A. baumannii* strains. This is the first study that attempted to determine the resistance gene profile of MDR *A. baumannii* isolates in Korea. We found that the MDR *A. baumannii* isolates that have the IS*Aba1* element upstream of bla_{OXA-23} show a high level of antibiotic resistance. These antibiotic resistance gene determinants were present in isolates obtained from both outbreak and sporadic cases; this finding emphasizes that *A. baumannii* can potentially possess a multitude of

resistance genes.

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