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Characterization of *Acinetobacter baumannii* Co-producing Carbapenemases OXA-23 and OXA-66, and *armA* 16S Ribosomal RNA Methylase at a University Hospital in South Korea

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Background: In the present study, the resistance mechanisms against carbapenems and aminoglycosides for 23 strains of multi-drug-resistant *Acinetobacter baumannii* isolated at a university hospital were investigated.
Methods: The minimal inhibitory concentrations (MICs) were determined via broth microdilution or Etest. The genes encoding OXA-type carbapenemases and 16S rRNA methylase were identified using multiplex PCR, and the amplified products were sequenced. Conjugation experiments were conducted, and an epidemiologic study was performed using enterobacterial repetitive intergenic consensus (ERIC)-PCR.
Results: In the isolates, the MICs of the tested aminoglycosides, including arbekacin, were >1024 μg/mL; the MICs of aztreonam, cefepime, ceftazidime, and ciprofloxacin ranged from 64 to 128 μg/mL; and the MICs of carbapenem ranged from 32 to 64 μg/mL, as determined through the broth microdilution test. According to the E-test, the MICs of ampicillin/sulbactam and colistin were 8 and 0.25 to 0.38 μg/mL, respectively. Sequence analysis confirmed that all of the isolates expressed carbapenemases OXA-23 and OXA-66, as well as *armA* 16S rRNA methylase. In addition, ISAba1 was identified upstream of the gene encoding OXA-23. OXA-23 and *armA* were not transferred to *Escherichia coli* J53 cells in the transconjugation experiments. ERIC-PCR molecular fingerprinting produced a single pattern in all cases.
Conclusion: The co-production of OXA-23 and armA 16S rRNA methylase may be attributed to the multidrug resistance of the *A. baumannii* isolates in the present study. Stricter surveillance and more rapid detection are necessary to prevent the spread of this type of resistance in the future. **(Korean J Clin Microbiol 2011;14:67-73)**

Key Words: *Acinetobacter baumannii, armA*, Outbreak, OXA-23

INTRODUCTION

Infections caused by multi-drug resistant *Acinetobacter baumannii* (MDRAB) have been reported in many countries [1,2]. These infections are most common in intensive care units (ICUs), where they occur mainly in conjunction with ventilator-associated pneumonia, urinary tract infections, or bacteremia. Carbapenem-resistant *Acinetobacter* spp. are increasing due to the emergence of carbapenem-hydrolyzing β-lactamases belonging to molecular classes B and D [3,4]. Whereas class B carbapenemase, metallo-β-lactamase (MBL), have been frequently founded in non-*baumannii* members of this genus, an increase in class D carbapenemases has been reported in *A. baumannii* in South Korea [4,5]. The OXA-type class D carbapenemases of *A. baumannii* can be divided into the following subgroups: OXA-23, -24, -51, and -58. Of these, OXA-23 and -51 are the most common and frequently occur simultaneously in a single isolate; however, OXA-23 is acquired enzyme that frequently leads to an outbreak at ICUs around the world [4,6,7], whereas OXA-51 is an intrinsic enzyme. Recently, a 16S rRNA methylase that confers high-level resistance to 4,6-substituted deoxystreptamines, including arbekacin, amikacin, tobramycin, and gentamicin, through the methylation of 16S rRNA leading to a loss of affinity for aminoglycosides was identified in vari-

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ous Gram-negative bacilli, including *Acinetobacter* spp., *Pseudomonas* spp., and various Enterobacteriaceae [8,9]. Additionally, the dissemination of various 16S rRNA methylase genes, including *armA*, *rtmB*, and *rtmA*, has been reported in Japan [10], Europe [11], North America [12], Brazil [13], Taiwan [14], and South Korea [9].

Carbapenems and aminoglycosides can produce a synergistic effect and thus are often used together to treat MDRAB infections [15]. Therefore, strains bearing resistance to both carbapenems and aminoglycosides would have considerable clinical impact.

Twenty-three strains of MDRAB were isolated from an ICU and a few wards of a university hospital in South Korea, over a 5-month period in 2007. The strains were resistant to all of the β-lactam drugs tested, and they yielded a positive modified Hodge test result, but a negative MBL disk test result. In addition, the strains did not produce an inhibitory zone around a Kirby-Bauer disk impregnated with 30 μg of arbekacin, suggesting the production of 16S rRNA methylase.

Here, we investigated the mechanism of resistance to carbapenems and aminoglycosides in the isolates, using phenotypic and molecular methods in addition to molecular epidemiologic experiments.

MATERIALS AND METHODS

1. Bacterial strains identification, antimicrobial susceptibility testing and carbapenemase screening

The bacterial strains identification was firstly carried out by the Vitek system with a GNI+ card (bioMérieux, Hazelwood, MO, USA), and subsequently were confirmed by determination of the partial *rpoB* gene sequence [16].

The minimal inhibitory concentrations (MICs) of imipenem (Merk & Co., Inc., Elkton, MD, USA), meropenem (Yuhanyanghang Co, Seoul, Korea), ceftazidime (CJ Jeiljedang Co, Seoul, Korea), cefepime (Boryung Pharmaceutical, Seoul, Korea), arbekacin (Joongwae Phamaceutical Co., Seoul, Korea), amikacin (Boryung Pharmaceutical Co., Seoul, Korea), gentamicin (Dongshin Pharmaceutical, Seoul, Korea), tobramycin (Daewoong Pharmaceutical Co, Seoul, Korea), and ciprofloxacin (Bayer Korea Co, Seoul, Korea) were determined for the isolates by the CLSI broth microdilution method [17]. The MICs of ampicillin/sulbactam, piperacillin/tazobactam, and colistin were determined by Etest (AB BIODISK, Solna, Sweden). *Pseudomonas aeruginosa* ATCC 27853 was used as a reference strain. A modified Hodge test [18] and MBL disk test [19] were performed for carbapenemase and MBL screening, respectively.

Table 1. Primers used in this study

Primer	Sequence 5′→3′	Target	Reference
Ac1055 F	GTG ATA ARA TGG CBG GTC GT	*rpoB*	[16]
Ac1598 R	CGB GCR TGC ATY TTG TCR T		
OXA-23-like F	GAT CGG ATT GGA GAA CCA GA	$bla_{\text{OXA-23}}$-like	[23]
OXA-23-like R	ATT TCT GAC CGC ATT TCC AT		
OXA-24-like F	GGT TAG TTG GCC CCC TTA AA	$bla_{\text{OXA-24}}$-like	[23]
OXA-24-like R	AGT TGA GCG AAA AGG GGA TT		
OXA-51-like F	TAA TGC TTT GAT CGG CCT TG	$bla_{\text{OXA-51}}$-like	[23]
OXA-51-like R	TGG ATT GCA CTT CAT CTT GG		
OXA-58-like F	AAG TAT TGG GGC TTG TGC TG	$bla_{\text{OXA-58}}$-like	[23]
OXA-58-like R	CCC CTC TGC GCT CTA CAT AC		
IS*Aba1*F	CAC GAA TGC AGA AGT TG	IS*Aba1*/$bla_{\text{OXA-23}}$-like	[24]
OXA-23-like R	ATT TCT GAC CGC ATT TCC AT		
OXA-51-like R	TGG ATT GCA CTT CAT CTT GG	IS*Aba1*/$bla_{\text{OXA-51}}$-like	[24]
IMP-1 F	CAT GGT TTG GTG GTT CTT GT	$bla_{\text{IMP-1}}$	[20]
IMP-1 R	ATA ATT TGG CGG ACT TTG GC		
VIM-2 F	ATG TTC AAA CTT TTG AGT AAG	$bla_{\text{VIM-2}}$	[21]
VIM-2 R	CTA CTC AAC GAC TGA GCG		
SIM-1 F	TAC AAG GGA TTC GGC ATCG	$bla_{\text{SIM-1}}$	[22]
SIM-2 R	TAA TGG CCT GTT CCC ATG TG		
ArmA F	CAA ATG GAT AAG AAT GAT GTT	*armA*	[8]
ArmA R	TTA TTT CTG AAA TCC ACT		
RtmB F	ATG AAC ATC AAC GAT GCC CT	*rtmB*	[14]
RtmB R	CCT TCT GAT TGG CTT ATC CA		
ERIC1 R	ATGTAAGCTCCTGGGGATTCAC	ERIC sequences	[26]
ERIC2	AAGTAAGTGACTGGGGTGAGCG		

Abbreviation: ERIC, enterobacterial repetitive intergenic consensus.

2. PCR and sequencing

The amplification of bla_{IMP-1}, bla_{VIM-2}, and bla_{SIM-1} was done as described previously [20-22]. Screening for OXA-type carbapenemases in the isolates was carried out using a multiplex PCR assay with the primers and reaction conditions described by Woodford et al. [23]. IS*Aba1* was amplified using the primers IS*Aba1*/OXA-23 and IS*Aba1*/OXA-51 like [24]. The amplification of *armA* and *rtmB* was conducted by duplex PCR for 16S rRNA methylase using previously described primers [8,14]. For each reaction, a fresh bacterial colony was suspended in 500 μL of sterile distilled water and boiled at 100°C for 10 min. After centrifugation, 2 μL of the supernatant were used for PCR with the primers described in Table 1. Amplification of the template DNA was performed using a GeneAmp 9700 PCR system (Applied Biosystems, Foster City, CA, USA). The reaction conditions were: 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 7 min.

For eight arbitrarily selected isolates, the amplified products were purified using a QIAquick PCR purification kit (Qiagen, Inc., Chatsworth, CA, USA) and sequenced on both strands using an ABI PRISM 3100 genetic analyzer (Applied Biosystems). The sequences were compared with those in the GenBank nucleotide database (www. ncbi.nlm.nih.gov/blast/).

3. Transconjugation

Conjugation experiments were conducted by broth mating, using *Escherichia coli* J53 as the recipient [25]. Transconjugants were selected on MacConkey agar supplemented with 100 μg/mL sodium azide and 2 μg/mL ceftazidime (for bla_{OXA-23}) or 30 μg/mL amikacin (for *armA* 16S rRNA methylase).

4. Enterobacterial repetitive intergenic consensus (ERIC)-PCR typing

DNA fingerprinting was performed by ERIC-PCR using the primers ERIC1R and ERIC2 with genomic DNA as the template, as described previously [26]. The reaction conditions were: 91°C for 5 min, followed by 35 cycles of 94°C for 1 min, 51°C for 1 min, and 72°C for 5 min, with a final extension at 72°C for 10 min. The products were electrophoresed in 2% agarose gels.

Table 2. Minimal inhibitory concentration (MIC) of *Acinetobacter baumannii* clinical isolates

No.	Minimal inhibitory concentration (μg/mL)								
	CTZ	FEP	ATM	A/S*	P/T*	IMP	MEM	CIP	CS*
88	128	64	128	8	>256	32	64	64	0.25
89	64	64	128	8	>256	32	64	64	0.25
91	128	64	128	8	>256	32	64	64	0.25
92	128	64	128	8	>256	32	64	64	0.38
94	128	64	128	8	>256	32	32	64	0.25
95	64	64	128	8	>256	32	64	64	0.25
96	64	64	128	8	>256	32	32	64	0.25
98	64	64	128	8	>256	32	64	64	0.25
103	128	64	128	8	>256	32	64	64	0.25
104	128	64	128	8	>256	32	64	64	0.25
105	128	64	128	8	>256	64	64	64	0.25
106	64	64	128	8	>256	32	64	64	0.25
201	128	64	128	8	>256	32	64	64	0.25
202	128	64	128	8	>256	64	64	64	0.25
203	64	64	128	8	>256	32	32	64	0.25
205	64	64	128	8	>256	32	64	64	0.25
207	128	64	128	8	>256	32	64	64	0.38
208	128	64	128	8	>256	32	64	64	0.25
209	128	128	128	8	>256	32	32	64	0.25
210	128	128	128	8	>256	32	64	64	0.25
211	128	128	128	8	>256	32	64	64	0.25
212	64	64	128	8	>256	32	64	64	0.25
213	128	64	128	8	>256	32	32	64	0.25

MIC of four aminoglycoside antimicrobial agents including amikacin, gentamicin, tobramycin and arbekacin was >1,024 μg/mL in all of the isolates.

*MIC was determined by Etest (AB BIODISK, Solna, Sweden).

Abbreviations: CTZ, ceftazidime; FEP, cefepime; ATM, aztreonam; A/S, ampicillin/sulbactam; P/T, piperacillin/tazobactam; IMP, imepenem; MEM, meropenem; CIP, ciprofloxacin; CS, colistin.

RESULTS

1. Bacterial strains identification and characterization

All of the isolates were identified as *A. baumannii* using a Vitek 2 system and *rpoB* gene sequencing. The isolates exhibited resistance to all of the antimicrobial agents tested, including imipenem and meropenem (except colistin and ampicillin/sulbactam), and they showed high-level resistance (> 1,024 μg/mL) to aminoglycosides, including amikacin, gentamicin, tobramycin, and arbekacin (Table 2). Positive results were obtained using a modified Hodge test, and negative results were obtained with MBL disk tests, suggesting the production of carbapenemase, rather than MBLs. In addition, the lack of a zone of inhibition for arbekacin suggests the existence of 16S rRNA methylase.

2. Detection of OXA-type carbapenemase and 16S ribosomal RNA genes

None of the isolates produced products for bla_{IMP-1}, bla_{VIM-2}, or bla_{SIM-1}. All of the isolates harbored bla_{OXA-23}- and bla_{OXA-51}-like carbapenemase genes, and the amplified products from eight arbitrarily selected isolates were confirmed to be bla_{OXA-23} and bla_{OXA-66} by sequence analysis. Furthermore, IS*Aba1* was

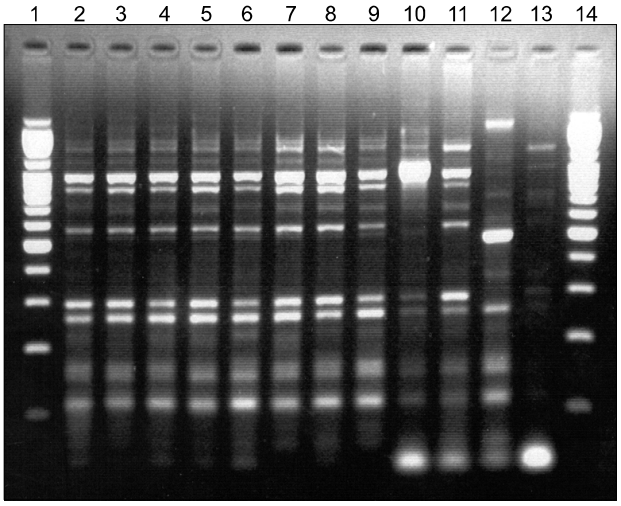

Fig. 1. The finding of DNA fingerprints by ERIC-PCR of *Acinetobacter baumannii* clinical isolates. Clinical isolates with outbreak (lanes 2~9) exhibits single DNA fingerprinting pattern but non-outbreak strains (lanes 10~13) showed different patterns. Lane 1, molecular size marker (100 bp ladder); lanes 2~9, clinical isolates of *A. baumannii* with outbreak; lanes 10~13, clinical isolates of *A. baumannii* with non-outbreak strain, including cabapenem resistant *A. baumannii* isolated from other two University hospital; lane 14, molecular size marker (100 bp ladder).

Table 3. Clinical information and genetic characteristics of *Acinetobacter baumannii* clinical isolates

No	Isolation date	Isolation ward	Specimen	OXA-like*		IS*Aba1*/ OXA-23†	*armA*‡	ERIC-PCR
				23	51			
88	07-05-07	ICU	Sputum	+	+	+	+	A
89	07-05-08	ICU	Sputum	+	+	+	+	A
91	07-05-11	ICU	Ascitic F	+	+	+	+	A
92	07-05-15	ICU	Sputum	+	+	+	+	A
94	07-05-28	ICU	Ascitic F	+	+	+	+	A
95	07-05-25	ICU	Sputum	+	+	+	+	A
96	07-05-30	ICU	Sputum	+	+	+	+	A
98	07-06-20	ICU	Sputum	+	+	+	+	A
103	07-07-05	A-W	Sputum	+	+	+	+	A
104	07-07-09	B-W	Ascitic F	+	+	+	+	A
105	07-07-10	C-W	BA	+	+	+	+	A
106	07-07-10	C-W	BA	+	+	+	+	A
201	07-07-19	ICU	BA	+	+	+	+	A
202	07-07-26	D-W	BA	+	+	+	+	A
203	07-07-24	E-W	Urine	+	+	+	+	A
205	07-07-24	B-W	BA	+	+	+	+	A
207	07-09-27	ICU	Sputum	+	+	+	+	A
208	07-09-27	ICU	Blood	+	+	+	+	A
209	07-10-03	ICU	Sputum	+	+	+	+	A
210	07-10-11	ICU	Sputum	+	+	+	+	A
211	07-10-11	ICU	Blood	+	+	+	+	A
212	07-10-13	E-W	Other	+	+	+	+	A
213	07-10-29	ICU	Sputum	+	+	+	+	A

*bla_{OXA-23} like and bla_{OXA-51} like was positive by multiplex PCR, and the amplified products were respectively confirmed to OXA-23 and OXA-66 type by sequencing analysis in eight isolates selected arbitrarily. †IS*Ab1* was located to upstream of bla_{OXA-23} (separated to 27 bp) but was not presented at upstream of bla_{OXA-66}. ‡The amplified products were confirmed to be *armA* gene by the sequencing analysis.
Abbreviations: ICU, intensive care unit; W, ward; F, fluid; BA, bronchial aspirate.

positively identified by IS*Aba1*/OXA-23 PCR and was located 27 bp upstream of bla_{OXA-23} based on sequence analysis. In addition, the isolates were found to possess *armA*, a 16S rRNA methylase gene, by PCR and sequencing (Table 3).

3. Transconjugation and strain typing

The genes encoding OXA-23 carbapenemase and armA 16S rRNA methylase were not transferred to *E. coli* J53 cells in repeated transconjugation experiments. Moreover, the isolates exhibited single DNA fingerprinting patterns by ERIC-PCR (Fig. 1).

DISCUSSION

Carbapenems are stable against most β-lactamases and are often used as a last resort to treat cases of MDRAB. However, since OXA-23 carbapenemase was first described from *A. baumannii* detected in Scotland in 1985 [27], the global incidence of OXA-23 carbapenemase-producing *A. baumannii* has increased, creating difficulty in treating infected patients, especially those admitted to ICUs [6,28]. Aminoglycoside antibiotics are frequently ineffective against strains of *A. baumannii*, but are nevertheless used together with carbapenems to treat infected patients because the two agents have a synergistic effect [15]. The incidence of infection by *A. baumannii* with armA 16S rRNA methylase has increased, leading to reports of high-level resistance to most aminoglycosides [29]. Moreover, North American [12], Chinese [30,31], and South Korean [32] reports have documented the co-production of bla_{OXA-23} and *armA*, which can pose therapeutic challenges.

During a 5-month period in 2007, 23 strains of imipenem-resistant *A. baumannii* were isolated from a university hospital, beginning with an isolate obtained from a patient in an ICU. All of the isolates co-expressed bla_{OXA-23} and *armA* 16S rRNA methylase and showed pan-drug resistance, except to colistin and ampicillin/sulbactam. Moreover, the isolates showed a single DNA fingerprinting pattern by ERIC-PCR, suggesting an outbreak caused by single clonal isolates. Six of the 23 isolates might have caused the infection and the other 17 patients had colonized forms of these isolates. Interestingly, five of the 17 colonized isolates were obtained from bronchial aspirates, suggesting that contaminated bronchoscopy equipment was the source of infection.

The isolates had bla_{OXA-66} in addition to bla_{OXA-23}, as reported previously in China [31]. The isolates also possessed IS*Aba1* upstream of bla_{OXA-23}, which plays an important role in resistance to carbapenems, including imipenem. There was a 27-bp sequence between IS*Aba1* and bla_{OXA-23}, which was identical to ARI-1 [27]. At upstream of bla_{OXA-66}, IS*Aba1* was not amplified by IS*Aba1*/OXA-51-like PCR. Phenotypic and genetic analyses for MBLs produced negative results for all of the isolates. Therefore, the resistance to imipenem and meropenem (MIC, 32 ~64 μg/mL) may be caused by the presence of carbapenemases OXA-23 (mainly) and -66.

On the one other hand, no zone of inhibition was produced with any of the tested aminoglycoside antibiotic disks, including disks with amikacin, gentamicin, tobramycin, and arbekacin, suggesting the production of 16S rRNA methylase. Among the known 16S rRNA methylases, armA was identified in all of the isolates, which is similar to isolates of *A. baumannii* in Korea [9]. Consequently, the multidrug resistance of *A. baumannii* strains in this study may be attributable to the co-production of carbapenemase OXA-23 and armA 16S rRNA methylase. Interestingly, the isolates in this study were similar to those that caused an outbreak of *A. baumannii* infections, as described by Kim et al. [32], in that they simultaneously carried bla_{OXA-23}, bla_{OXA-51}, bla_{PER-1}, and *armA*. The isolates also showed similar antibiotic resistance patterns, except for ampicillin-sulbactam (>32 μg/mL), which might have been caused by the acquisition of bla_{PER-1}. In addition, the infections occurred during the same time period, i.e., May to October 2007 in this study and May to July 2007 in Gyungnam province [32], in different regions with interrelated life zones, suggesting the dissemination in South Korea of *A. baumannii* isolates co-producing OXA-type carbapenemases and 16S rRNA methylase.

In conclusion, infections of MDRAB co-producing OXA-23 carbapenemase and armA 16S rRNA methylase occurred in a university hospital in South Korea. The resistance mechanism identified in this study could threaten existing therapeutic modalities for such infections. Stricter surveillance and more rapid detection are essential to reduce the spread of MDRAB, including OXA-type carbapenemases and 16S rRNA methylase co-producing microorganism.

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=국문초록=

한 대학병원에서 발생한 OXA-23, OXA-66형 Carbapenemase와 armA 형 16S Ribosomal RNA Methylase를 동시 생성하는 *Acinetobacter baumanni*의 특성분석

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배경: 저자들은 한 대학병원에서 분리된 imipenem 및 amikacin 내성 *Acinetobacter baumannii* 23균주의 carbapenem과 aminoglycoside에 대한 내성기전의 특성을 분석하였다.

방법: 최소억제농도(MIC)는 미량액체희석법과 E-test에 의해 결정되었다. Multiplex PCR에 의해 OXA형 carbapenemase와 16S rRNA methylase에 대한 유전자의 검출을 시도하였고, 이어 증폭 산물의 염기서열을 분석하였다. 접합실험을 시행하였으며, enterobacterial repetitive intergenic consensus (ERIC)-PCR을 이용하여 역학조사를 시행하였다.

결과: 미량액체 희석법에 의해 arbekacin을 포함한 시험한 aminoglycoside의 MIC는 1,024 μg/mL 이상이었으며, aztreonam, cefepime, ceftazidime 및 ciprofloxacin의 MIC는 64～128 μg/mL의 범위였다. Carbapenem MIC는 32～64 μg/mL이었다. Ampicillin/sulbactam과 colistin의 MIC는 E-test에 의해 각각 8과 0.25～0.38 μg/mL이었다. 모든 균주는 OXA-23, OXA-66형 carbapenemase와 armA형 16S rRNA methylase를 생성하고 있었으며 IS*Aba1*이 bla_{OXA-23} 유전자 전방에 위치하고 있었다. 접합실험에서 OXA-23과 armA는 *Escherichia coli* J53에 전달되지 않았으며, ERIC-PCR에 의해 모두 동일한 유전형을 보였다.

결론: OXA-23과 armA 16S rRNA methyasle의 동시 생성이 이 연구에 포함된 *A. baumannii*의 내성에 기여하였을 것이다. 앞으로 이들 내성의 전파를 막기 위해 좀 더 철저한 감시와 조기 검출이 필요할 것이다. **[대한임상미생물학회지 2011; 14:67-73]**

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