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Original Article

The Genetic Characteristics of Multidrug-resistant *Acinetobacter baumannii* Coproducing 16S rRNA Methylase *armA* and Carbapenemase OXA-23

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Acinetobacter baumannii is a gram-negative organism reported worldwide as a cause of health-care associated infections. Due to its increasing drug resistance, several studies on coproduction of *armA* and carbapenemase in South Korea and other parts of the world were reported, which can pose significant therapeutic threat. The aim of this study was to investigate genetic characteristics of multidrug-resistant *A. baumannii* coproducing *armA* and carbapenemase and its epidemiological relatedness. Forty-five multidrug resistant (MDR) *A. baumannii* clinical isolates were collected. Antimicrobial susceptibility was determined by agar dilution, Etest and VITEK 2 system. The presence of 16S rRNA methylase and carbapenemase were analyzed by polymerase chain reaction (PCR) and sequencing. Repetitive element palindromic (REP)-PCR was also performed for epidemiologic investigation. All of *A. baumannii* isolates harbored bla_{OXA-51}-like gene and 10 isolates showed an upstream ISA*ba1*. 36 isolates (80%) showed amplification of OXA-23, all of which except one had an upstream ISA*ba1*. 16S rRNA methylase *armA* was found in 44 isolates with high level resistance to aminoglycosides. The rate of coproduction was found in 36 isolates (80%). All isolates showed dominant two patterns in REP-PCR profile. The prevalence of MDR *A. baumannii* coproducing OXA-23 and *armA* was high, which the rate of bla_{OXA-23} coproduction was also high.

Key Words: *Acinetobacter baumannii*, 16S rRNA methylase, Aminoglycoside resistance

INTRODUCTION

Acinetobacter baumannii is a gram-negative organism reported worldwide as a cause of health-care associated infections, particularly in intensive care units (ICUs) (1). It is responsible for pneumonia, urinary tract infections, skin and soft tissue infections, and bloodstream infections (2). Despite intensive efforts, nosocomial acquisition of multidrug resistant (MDR) *A. baumannii* is still a problem due to its great ability to disseminate from and colonize human and environmental reservoir (3, 4).

For a long time, imipenem and meropenem have been the drugs of choice for the treatment of infections due to MDR *A. baumannii*. Currently, however, their efficacy has been compromised by increased dissemination of isolates showing resistance to these antibiotics (5). Resistance to carbapenem in *A. baumannii* is mainly mediated by the acquisition of class D and class B carbapenemase-encoding genes, with bla_{OXA-23}-like being the most frequently identified

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carbapenemase-encoding gene (6).

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 synergistic effects (7). Resistance to amino glycosides is most commonly encountered by amino glycoside-modifying enzymes, including acetylaminotransferases, nucleotidyltransferase, and phosphotransferases (8). But recently the production of 16S rRNA methylases has been implicated in aminoglycoside resistance among the gram-negative pathogens (9). They were found to confer extraordinarily high levels of resistance to clinically useful aminoglycosides, such as amikacin, tobramycin and gentamicin, which thus effectively eliminating the entire class as a therapeutic option (9, 10). The dissemination of different types of 16S rRNA methylase were found in different bacterial species or regions (11~14) but *armA* has been the only 16S rRNA methylase found in *A. baumannii* (14~16).

Recently in several studies of *A. baumannii* coproducing *armA* and carbapenemase in South Korea (16, 17) and other parts of the world were reported (10, 18, 19), which can pose significant therapeutic threat.

The aim of this study was to describe the genetic characteristics of MDR *A. baumannii* coproducing *armA* and carbapenemase and to investigate the genetic relatedness through epidemiologic study.

MATERIAL AND METHODS

Identification of *A. baumannnii*

Isolates identified as *A. baumannnii* or *Acinetobacter* spp. by the VITEK2 (bioMérieux, Marcy l'Etoile, France) automated microbiology system were collected between January 2012 and November 2012 from Chungnam National University Hospital. Identification of *A. baumannnii* was confirmed by *rpoB* gene analysis (20). Genomic DNA was obtained from each target strain by using the genomic DNA purification kit (Solgent, Daejeon, South Korea) according to the standard protocols.

Determination of minimal inhibitory concentrations (MICs)

The MICs of different antimicrobials (amikacin, gentamicin, tobramycin, imipenem, meropenem, cefepime, aztreonam, pipercillin/tazobactam, ciprofloxacin, colistin and minocycline) were determined by agar dilution or Etest (bioMérieux, Marcy l'Etoile, France) according to Clinical and laboratory standards institute (21). *Escherichia coli* ATCC 25922 was used as quality control strain. For 11 isolates, the MICs of cefepime, ciprofloxacin, piperacillin/tazobactam, aztreonam, colistin and minocycline were determined by VITEK 2 system.

The MDR phenotype was defined as resistance to representative antimicrobial agents of at least 3 different classes of drugs: aminoglycosides (gentamicin, amikacin), antipseudomonal penicillins (ticarcillin, piperacillin, piperacillin/tazobactam), carbapenems (imipenem, meropenem), antipseudomonal cephalosporins (ceftazidime, cefepime), and fluoroquinolone (ciprofloxacin) (22).

Detection of 16S rRNA methylase genes and carbapenemase genes $bla_{\text{OXA-23}}$, $bla_{\text{OXA-24}}$, $bla_{\text{OXA-58}}$, $bla_{\text{VIM-1}}$, and $bla_{\text{IMP-1}}$

All MDR *A. baumannii* isolates were subjected to PCR and sequencing assay for the detection of 16S rRNA methylase genes (*armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD* and *npmA*) (10, 13) and carbapenemase genes ($bla_{\text{OXA-51}}$, $bla_{\text{OXA-23}}$, $bla_{\text{OXA-24}}$, $bla_{\text{OXA-58}}$, $bla_{\text{VIM-1}}$, and $bla_{\text{IMP-1}}$) (23, 24) (Table 1). Upstream ISA*ba1* was investigated using the primers ISA*ba1*/OXA-23 and ISA*ba1*/OXA-51 (23). Chromosomal DNA was obtained from each target strains as mentioned previously. PCR was performed using 50 ng of genomic DNA, 2.5 μl of 10 X *Taq* buffer, 0.5 μl of 10 mM dNTP mix, 20 pmol of each primer, and 0.7 U of *Taq* DNA polymerase (Bioneer, Daejeon, South Korea), in a total volume of 25 μl. Each gene was amplified in a Gene Amp PCR system 9600 thermal cycler (Perkin-Elmer Cetus Corp., Norwalk, CT, USA) by pre-denaturation of the reaction mixture at 95℃ for 5 min, followed by 35 cycles of 95℃ for 30 sec, 52℃ for 40 sec, and 72℃ for 30 sec,

Table 1. Primers used in this study

Primer	Nucleotide sequence (5' → 3')	Reference
armA F	AGGTTGTTTCCATTTCTGAG	14
armA R	TCTCTTCCATTCCCTTCTCC	
rmtA F	CTA GCG TCC ATC CTT TCC TC	14
rmtA R	TTT GCT TCC ATG CCC TTG CC	
rmtB F	CCC AAA CAG ACC GTA GAG GC	14
rmtB R	CTC AAA CTC GGC GGG CAA GC	
rmtC F	CGA AGA AGT AAC AGC CAA AG	14
rmtC R	ATC CCA ACA TCT CTC CCA CT	
rmtD F	ATG AGC GAA CTG AAG GAA AAA CTG C	14
rmtD R	GCT CCA AAA GCG GCA GCA CCT TA	
npmA F	GGAGGGCTATCTAATGTGGT	11
npmA R	GCCCAAAGAGAATTAAACTG	
OXA-23-like F	CTTGCTATGTGGTTGCTTCTC	23
OXA-23-like R	ATCCATTGCCCAACCAGTC	
OXA-51-like F	ATGAACATTAAAGCACTC	23
OXA-51-like R	CTATAAAATACCTAATTGTTC	
OXA-24-like F	GTACTAATCAAAGTTGTGAA	24
OXA-24-like R	TTCCCCTAACATGAATTTGT	
OXA-58-like F	CGATCAGAATGTTCAAGCGC	24
OXA-58-like R	ACGATTCTCCCTCTGCGC	
IMP F	CATGGTTTGGTGGTTCTTGT	24
IMF R	ATAATTTGGCGGACTTTGGC	
VIM F	ATTGGTCTATTTGACCGCGTC	24
VIM R	TGCTACTCAACGACTGAGCG	
PW 166 (ISA*ba1*)	CCTATCAGGGTTCTGCCTTCT	23
REP 1	IIIGCGCCGICATCAGGC	25
REP 2	ACGTCTTATCAGGCCTAC	

with a final extension at 72℃ for 5 min.

Repetitive element palindromic (REP)-PCR typing for assessing clonality

DNA fingerprinting was performed by REP-PCR using primers REP1 and REP2 (25) to amplify putative REP-like elements. The reaction 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-14TM Imaging system (UVP, Cambridge, UK).

RESULTS

Identification of bacterial strains and antibiotic susceptibility testing

A total of 45 isolates were confirmed as *A. baumannii* by

Table 2. Minimal inhibitory concentrations of different antimicrobial agents

Isolates	MIC (μg/ml)										
	CIP	MN	CS	AN	GEN	TOB	IMP	MEM	FEP	P/T	AZ
171	>64	≤1	1	≥1024	≥1024	≥1024	≥32	≥32	32	≥256	64
173	64	≤1	1	≥1024	≥1024	≥1024	≥32	≥32	16	≥256	64
177	>64	≤1	1	≥1024	≥1024	≥1024	≥32	≥32	16	≥256	64
183	64	≤1	1	≥1024	≥1024	≥1024	≥32	≥32	48	≥256	32
191	>64	≤1	1	≥1024	≥1024	≥1024	≥32	≥32	32	≥256	64
193	64	2	1	≥1024	≥1024	≥1024	≥32	≥32	8	≥256	16
196	64	2	2	≥1024	≥1024	≥1024	≥32	≥32	24	≥256	16
197	>64	≤1	1	≥1024	≥1024	≥1024	≥32	≥32	32	≥256	≥256
202	64	2	1	≥1024	≥1024	≥1024	≥32	≥32	12	≥256	16
207	64	≤1	1	≥1024	≥1024	≥1024	≥32	≥32	32	≥256	16
208	>64	≤1	1	≥1024	≥1024	≥1024	≥32	≥32	≥256	≥256	48
211	>64	≤1	1	≥1024	≥1024	≥1024	≥32	≥32	≥256	≥256	48
216	64	4	1	≥1024	≥1024	≥1024	≥32	≥32	24	≥256	48
217	64	2	2	6	24	<256	1	1	24	≥256	32
218	64	2	1	≥1024	≥1024	≥1024	1	0.5	12	≥256	16
219	64	≤1	1	≥1024	≥1024	≥1024	≥32	≥32	24	<0.16	16
223	>64	≤1	1	≥1024	≥1024	≥1024	≥32	≥32	24	≥256	≥256
225	64	2	1	≥1024	≥1024	≥1024	≥32	≥32	≥256	≥256	64
226	64	≤1	1	≥1024	≥1024	≥1024	≥32	≥32	24	≥256	≥256
227	64	2	0.5	≥1024	≥1024	≥1024	≥32	≥32	8	≥256	16
228	>64	≤1	1	≥1024	≥1024	≥1024	≥32	≥32	32	≥256	≥256
229	>64	≤1	1	≥1024	≥1024	≥1024	≥32	≥32	≥256	≥256	≥256
230	>64	≤1	1	≥1024	≥1024	≥1024	≥32	≥32	48	≥256	≥256
231	64	≤1	1	≥1024	≥1024	≥1024	≥32	≥32	≥256	≥256	≥256
232	64	≤1	1	≥1024	≥1024	≥1024	≥32	≥32	32	≥256	48
233	32	≤1	1	≥1024	≥1024	≥1024	≥32	≥32	≥256	≥256	≥256
234	32	≤1	1	≥1024	≥1024	≥1024	2	0.75	8	≥256	16
235	64	≤1	1	≥1024	≥1024	≥1024	≥32	≥32	32	≥256	≥256
236	>64	≤1	1	≥1024	≥1024	≥1024	≥32	≥32	32	≥256	64
237	>64	≤1	1	≥1024	≥1024	≥1024	≥32	≥32	32	≥256	64
238	64	≤1	1	≥1024	≥1024	≥1024	≥32	≥32	32	≥256	≥256
241	>64	≤1	2	≥1024	≥1024	≥1024	≥32	≥32	≥256	≥256	16
242	64	2	1	≥1024	≥1024	≥1024	4	8	16	≥256	24
243	16	≤1	2	512	≥1024	<265	1	0.75	≥64	≥128	≥64
61	≥4	≤1	≤0.5	≥1024	≥1024	≥1024	≥32	≥32	32	≥128	32
68	≥4	≤1	≤0.5	≥1024	≥1024	≥1024	≥32	≥32	≥64	≥128	≥64

Table 2. Continued

Isolates	MIC (μg/ml)										
	CIP	MN	CS	AN	GEN	TOB	IMP	MEM	FEP	P/T	AZ
90	≥4	8	1	≥1024	≥1024	≥1024	≥32	≥32	≥64	≥128	≥64
93	≥4	8	2	≥1024	≥1024	≥1024	≥32	≥32	≥64	≥128	≥64
94	≥4	16	1	≥1024	≥1024	≥1024	≥32	≥32	≥64	≥128	≥64
96	≥4	16	2	≥1024	≥1024	≥1024	≥32	≥32	≥64	≥128	≥64
104	≥4	8	≤0.5	≥1024	≥1024	≥1024	≥32	≥32	≥64	≥128	≥64
108	≥4	4	≤0.5	512	128	128	≥32	≥32	≥64	≥128	≥64
146	≥4	16	≤0.5	128	1024	128	≥32	≥32	≥64	≥128	≥64
154	≤0.25	≤1	2	≥1024	≥1024	≥1024	≥32	≥32	4	≤4	32
159	≥4	≤1	2	≥1024	≥1024	≥1024	≥32	≥32	≥64	≥128	≥64

MICs were determined by Etest (bioMérieux, Marcy l'Etoile, France). But MICs of antimicrobials except Amikacin, gentamicin, tobramycin, imipenem and meropenem were determined by VITEK 2 system (bioMérieux, Marcy l'Etoile, France) in isolates in red color.
Abbreviations: MIC, minimal inhibitory concentration; CIP, ciprofloxacin; MN, minocycline; CS, colistin; AN, amikacin; GEN, gentamicin; TOB, tobramycin; IMP, imipenem, MEM, meropenem, FEP, cefepime, P/T, piperacillin/tazobactam; AZ, azteronam.

rpoB gene sequencing. The isolates were highly resistant to carbapenems and aminoglycosides except a few isolates. The MICs of other antimicrobials were listed in Table 2.

Genetic characterization of MDR *A. baumannii*

All *A. baumannii* harbored bla_{OXA-51}-like gene which is intrinsic beta-lactamase to *A. baumannii* (Table 3). The bla_{OXA-23} was amplified in 36 isolates (80%), all of which except one showed upstream ISA*ba1*. These 36 isolates showed high level of resistance to carbapenems including imipenem and meropem. In 7 among 9 isolates without bla_{OXA-23}, ISA*ba1* was present upstream of bla_{OXA-51}-like gene. The bla_{OXA-24}, bla_{OXA-58}, bla_{VIM}, and bla_{IMP} were not detected in any isolate.

16S rRNA methylase *armA* was found almost all isolates except one and they were highly resistant to all classes of aminoglycosides. Other types of 16S rRNA methylase, *rmtA*, *rmtB*, *rmtC*, *rmtD* and *npmA*, were not detected in any isolate.

The isolates coproducing bla_{OXA-23} and *armA* were 36 isolates (82.2%) and they were highly resistant to carbapenems and aminoglycosides. And besides they were also resistant to ciprofloxacin, cefepime, azteronam and piperacillin/tazobactam while susceptible to minocycline and colistin.

REP-PCR patterns

The 45 isolates showed dominantly 2 types (A and B) of REP-PCR patterns, while a few showed C types (Fig. 1).

DISCUSSION

MDR *A. baumannii* has been recognized as an increasing threat in hospitals and as a global challenge (8). Carbapenems are stable against most beta-lactamases and are often used as a last resort to treat cases of MDR *A. baumannii* (17). Aminoglycoside continue to play an important role in the management of serious infections caused by gram-negative pathogens, often in combination with broad-spectrum beta-lactams (9), but the activity of aminoglycosides is lower for MDR isolates of *A. baumannii* compared with non-multiresistant ones (26). Incidence of infection by *A. baumannii* with *armA* 16S rRNA methylase has increased, leading to reports of high-level resistance to most aminoglycosides (27). Recently several countries have documented the occurrence of co-production of $bla_{\text{OXA-23}}$ and *armA* in *A. baumannii*, which can pose therapeutic challenge (10, 16~19).

Table 3. Genetic characteristics of MDR *A. bauamannii*

Isolates	Antimicrobial resistance determinants					
	OXA-51	ISA*ba1*/OXA-51	OXA-23	ISA*ba1*/OXA-23	*armA*	REP-PCR
171	+	−	+	+	+	A
173	+	−	+	+	+	A
177	+	−	+	+	+	A
183	+	−	+	+	+	A
191	+	−	+	+	+	A
193	+	+	−	−	+	A
196	+	−	+	+	+	A
197	+	−	+	+	+	A
202	+	+	−	−	+	A
207	+	−	+	+	+	A
208	+	−	+	+	+	B
211	+	−	+	+	+	A
216	+	−	+	+	+	A
217	+	−	−	−	−	A
218	+	+	−	−	+	B
219	+	−	+	+	+	A
223	+	−	+	+	+	A
225	+	+	+	+	+	B
226	+	−	+	+	+	A
227	+	+	−	−	+	A
228	+	−	+	+	+	B
229	+	−	+	+	+	A
230	+	+	+	+	+	B
231	+	−	+	+	+	A
232	+	−	+	+	+	A
233	+	+	−	−	+	A
234	+	+	−	−	+	B
235	+	−	+	+	+	A
236	+	−	+	+	+	A
237	+	−	+	+	+	A
238	+	−	+	+	+	C
241	+	−	+	+	+	A
242	+	+	−	−	+	A
243	+	−	−	−	+	C
61	+	−	+	+	+	A
68	+	−	+	+	+	A

Table 3. Continued

Isolates	Antimicrobial resistance determinants					
	OXA-51	ISA*ba1*/OXA-51	OXA-23	ISA*ba1*/OXA-23	*armA*	REP-PCR
90	+	−	+	+	+	A
93	+	−	+	−	+	A
94	+	−	+	+	+	A
96	+	−	+	+	+	A
104	+	+	+	+	+	A
108	+	−	+	+	+	A
146	+	−	+	+	+	A
154	+	−	+	+	+	A
159	+	−	+	+	+	A

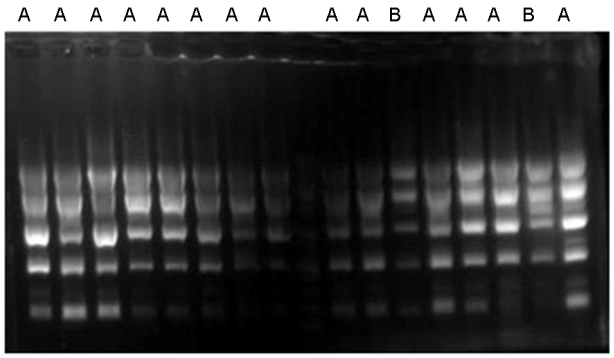

Figure 1. Repetitive extragenic palindromic (REP)-PCR of genomic DNA from MDR *A. baumannii* clinical isolates. Most isolates showed A pattern, while some isolate 208 and 218 showed B type.

In this study, the genetic characterisctics MDR *A. baumannii* and its coproduction of carbapenemase and 16S rRNA methylase were investigated. Only *armA* among different kinds of 16S rRNA methylase was detected like previous studies (27). The prevalence rate of *armA* among MDR *A. baumannii* was 97.8% and the isolates with *armA* in our study were highly resistant to all available aminoglycosides (MIC over 1024). The rate of coproduction of $bla_{\text{OXA-23}}$ was 100% among isolates with *armA* and they were multidrug resistant to aminoglycoside, carbapenems, ciprofloxacin, ampicillin/sulbactam, piperacillin, and aztreonam while susceptible to colistin and minocycline. These phenotypic profiles in these isolates were very similar to the previous studies (10, 16~19). Confounding evidence regarding *A. baumannii* isolates with *armA* were present in our country. Lee *et al*. reported that most of *A. baumannii* with *armA* were carbapenem nonsusceptible (28), while in a study in 2010, 11 isolates (10%) with *armA* among 112 *A. baumannii* were all carbapenem susceptible (14). In our study, almost all MDR *A. baumannii* isolates harbored both *armA* and carbapenemase OXA-23, which seems to be endemic in our hospital. The prevalence of *armA* in *A. baumannii* slightly increased from previously reported data (29) and showed similar result with recent report in China (12). Phenotypic characteristics of isolates with *armA* and its high prevalence in MDR *A. baumannii* make aminoglycoside less effective agent in the treatment of MDR *A. buamannii* infections.

Carbapenem resistance in MDR *A. baumannii* was mostly attributable to carbapenemase OXA-23 in 36 isolates (80%) and partially to OXA-51 with upstream ISA*ba1* in 5 isolates. The presence of ISA*ba1* upstream of bla_{OXA} genes provides a promoter sequence enhancing their expression (30). In our study most isolates with OXA-23 harbored upstream of ISA*ba1* and they were all related to resistance to carbapenems. In the 9 isolates without OXA-23, 5 isolates were carbapenem non-susceptible presumably due to upreatream ISA*ba1* of OXA-51 but 4 isolates remained

susceptible to carbepenem. Since carbapenem resistance is known to be significant risk factor for morbidity and mortality in *A. baumannii* infection, these MDR carbapenem resistant isolates are prevalent in tertiary care hospital is quite alarming.

The rate of coproduction of OXA-23 and *armA* in MDR *A. baumannii* was 80%. This high rate of coproduction of major resistant determents significantly narrows therapeutic options for MDR *A. baumannii* infection.

Dominant two patterns seen in REP-PCR profiles suggest that there are both clonal and horizontal spreads of resistance genes in MDR *A. baumannii* isolates. This emphasizes the necessity of a screening program and strict infection control.

Additionally, what was interesting in our study was amikacin susceptibility error in VITEK2 system. Several studies have reported aminogylcoside susceptibility error in *A. baumannii* (31, 32). Manufacturer recommends manual testing such as disk diffusion or Etest for *A. baumannii* showing susceptibility to amikacin. In our study, the rate of very major error (false susceptibility) was 77.3% (34 out of 44 isolates), which was much higher than previous report (36.4%) (31). Jung *et al*. (32) reported false susceptibility to amikacin by VITEK2 in *A. baumannii* was related to harboring *armA* but in our study almost all isolates harbored *armA* and some of them showed concordant result with reference method. Thus susceptibility testing error can occur regardless of harboring *armA* but tend to occur more often in MDR *A. buamannii*. So additional testing for susceptibility to amikacin in MDR *A. baumannii* may not be necessary for confirmation.

In conclusion, our study showed 16S rRNA methylase *armA* and carbapenemase OXA-23 was highly prevalent in MDR *A. baumannii*. Due to its patient to patient transfer in the spread of antimicrobial resistance as shown in REP-PCR, the needs for hospitals to isolate and screen for MDR pathogens and more strict infection control are pivotal for preventing further dissemination. The high prevalence of MDR isolates coproducing *armA* and $bla_{\text{OXA-23}}$ can also threaten therapeutic options for these infections.

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