Molecular Characterization and Antimicrobial Susceptibility of Biofilm-forming *Acinetobacter baumannii* Clinical Isolates from Daejeon, Korea

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대전지역에서 분리된 생물막 형성 *Acinetobacter baumannii* 임상분리주의 분자유전학적 특성과 항균제 감수성양상

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The emergence and dissemination of multidrug-resistant (MDR) *Acinetobacter baumannii* isolates have been reported worldwide, with most of these possessing the ability to form biofilms. Biofilm formation is an important virulence factor associated with the resistance to disinfection and desiccation. This study examined the genetic basis of antimicrobial resistance mechanisms of biofilm-forming *A. baumannii* clinical isolates. Imaging and quantification of biofilms were performed by a crystal violet assay and 46 biofilm-forming *A. baumannii* isolates were selected. Subsequently, 16 isolates belonging to different clones were identified using REP-PCR, and detection of the antimicrobial determinants in the isolates was carried out. The 16 isolates included 9 non-MDR and 7 MDR isolates. The mean biomass OD₅₆₀ values of the non-MDR (0.96) and MDR (1.05) isolates differed but this difference was not significant. In this study, most biofilm-forming MDR *A. baumannii* isolates contained various antimicrobial resistance determinants (*bla*_{OXA-23}, *armA*, and mutations of *gyrA* and *parC*). On the other hand, most biofilm-forming non-MDR *A. baumannii* isolates did not contain antimicrobial resistance determinants. These results suggest that there is little correlation between the biofilm-forming ability and antimicrobial susceptibility in *A. baumannii* isolates. In addition, the emergence of MDR *A. baumannii* clinical isolates is generally caused by mutations of the genes associated with antimicrobial resistance and/or the acquisition of various antimicrobial resistance determinants.

Key words: *Acinetobacter baumannii*, Antimicrobial resistance, Biofilm, Crystal violet assay

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INTRODUCTION

Acinetobacter baumannii is an important opportunistic and hospital-acquired pathogen responsible for various types of serious infections, such as epidemic pneumonia,

secondary meningitis, urinary tract infections, and bacteremia [1]. Many *A. baumannii* clinical isolates causing nosocomial infections exhibit antimicrobial resistance to most major classes of antimicrobial agents, including aminoglycosides, β -lactams, and fluoro-

quinolones. Accordingly, infectious diseases caused by *A. baumannii* are often difficult to treat and have been cured with carbapenems, which have been the drugs of choice until recently [2]. However, the emergence of carbapenem-resistant *A. baumannii* isolates has been reported worldwide, with most of these isolates possessing carbapenem-hydrolyzing β -lactamase genes. Among carbapenemases spread throughout *A. baumannii* isolates, carbapenem-hydrolyzing class D β -lactamases (CHDLs) are the most common and have become a current threat, while metallo- β -lactamase (MBL)-producing *A. baumannii* isolates have also continued to be identified [3]. Antimicrobial agents belonging to the aminoglycosides and quinolones have also long been used to treat *Acinetobacter* infections. Subsequently, the emergence of *A. baumannii* isolates resistant to these agents is not new, and many studies have reported on the resistance mechanisms of these isolates. Resistance to aminoglycosides is most commonly mediated by aminoglycoside-modifying enzymes, but high-level resistance to clinically important aminoglycosides, including amikacin, gentamicin, and tobramycin, results from the acquisition of 16S rRNA methyltransferase genes. More recently, a total of 8 16S rRNA methyltransferase enzymes (ArmA, RmtA, RmtB, RmtC, RmtD, RmtE, RmtF, and NpmA) have been identified among pathogenic bacteria, including *A. baumannii*, worldwide [4, 5]. In addition, quinolone resistance among *A. baumannii* isolates is due to chromosomal mutations in genes located in the quinolone resistance-determining region (QRDR) and/or acquisition of plasmid-mediated quinolone resistance (PMQR) genes. In particular, high-level resistance to quinolones among *A. baumannii* isolates is caused by mutations in the *gyrA* gene, encoding DNA gyrase A, and/or the *parC* gene, encoding the topoisomerase IV A subunit, located in the QRDR [6,7]. The development of multidrug-resistant (MDR) *A. baumannii* isolates has occurred by chromosomal mutation and/or acquisition of antimicrobial resistance genes. However, some recent studies have suggested that biofilms may play a significant role in the antimicrobial resistance of MDR *A. baumannii* isolates [8]. Biofilms allow microorganisms to

persist within unfavorable environments and to disseminate under favorable conditions. In addition, biofilms also function as a site for the exchange and spread of antimicrobial resistance determinants. Therefore, the biofilm formation ability of *A. baumannii* isolates enables the colonization of surfaces such as those of hospital instruments, indwelling catheters, and endotracheal tubes [9, 10]. According to previous studies, the efflux pumps involved in the MDR phenotype play an important role in transporting quorum sensing (QS) molecules out of the cells, and QS molecules are associated with biofilm formation in *A. baumannii* isolates [11]. Bacterial components playing a significant role in the maturation and maintenance of biofilms have been previously characterized in *A. baumannii* isolates by researchers. Biofilm-related virulence determinants include outer membrane protein A (OmpA), the chaperone-usher assembly system of pili (CsuA BABCDE), the *A. baumannii* biofilm-associated protein (Bap), and PER-1 belonging to the β -lactamase family [10].

Previous studies have reported that biofilms are significantly involved in chronic *A. baumannii* infections as well as colonization and persistence of the isolates. In addition, the biofilm formation rate among *A. baumannii* isolates is higher (80~91%) than the rate among other *Acinetobacter* species (5~24%) [11]. However, few studies of biofilm-forming *A. baumannii* isolates have been reported from Korea. In this study, we investigated the genetic basis of antimicrobial resistance mechanisms among biofilm-forming *A. baumannii* isolates obtained from Chungcheong, Korea. For selection of genetically diverse strains, clonal selection was performed by repetitive extragenic palindromic (REP)-PCR. We also investigated biofilm-associated genes and assessed the correlation between antimicrobial resistance and biofilm formation among *A. baumannii* isolates.

MATERIALS AND METHODS

1. Bacterial isolation and identification

A total of 58 consecutive and non-duplicate *A.*

baumannii clinical isolates were collected from a tertiary hospital located in Daejeon, Korea from 2012 to 2016. Until to investigate, the isolates had been stored long term in brain heart infusion broth (Difco Laboratories, Detroit, MI, USA) containing 20% glycerol at -70°C . All *A. baumannii* isolates were identified using biochemical tests with the VITEK 2 Automated Instrument ID System (bioMérieux, Marcy l'Etoile, France) and analysis of partial *rpoB* house-keeping gene sequences, as described previously [12].

2. Detection of biofilm formation

Biofilm imaging and quantification were performed using the crystal violet assay as described by Martí et al. with some modifications [13]. Each well of a polystyrene-bottomed 96-well plate was filled with 100 μL of brain heart infusion broth, in which *A. baumannii* strains were inoculated at approximately 1×10^7 CFU/mL. Each of the isolates was inoculated in triplicate. After incubation at 37°C for 18 hr, planktonic cells from each well were aspirated and well-bound cells were washed three times with sterile deionized water. The biofilm remaining in each well was stained using 1% crystal violet solution at room temperature for 10 min and washed three times with sterile deionized water. After the last wash, the biofilm was air-dried at 55°C for 30 min. For imaging, biofilms stained with crystal violet were observed by a phase contrast microscope (Meiji Techno, San Jose, CA, USA). Subsequently, stained biofilm cells were dissolved with 100 μL of 95% ethanol, and the optical density (OD) of each well at a wavelength of 560 nm was measured by a Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific, Langenselbold, Germany). Each assay for isolates involved in this study was performed in triplicate, and averages of triplicate values were calculated. Three *A. baumannii* isolates obtained and confirmed to be non-biofilm-forming in our previous study were used as negative control strains for biofilm formation [14].

3. REP-PCR for clonal assessment

Clonal selection of biofilm-forming *A. baumannii* isolates was performed using REP-PCR with primers REP1

(5'-IIIGCGCCGICATCAGGC-3') and REP2 (5'-ACGTCT-TATCAGGCCTAC-3') [15]. The cycling conditions were as follows: initial denaturation at 95°C for 5 min; amplification for 35 cycles of 95°C for 1 min, 48°C for 1 min, and 70°C for 6 min; and a final extension at 70°C for 10 min. Amplified products were electrophoresed on 2% agarose gels containing ethidium bromide and visualized with a BioDoc-14 imaging system (UVP, Cambridge, UK). Strains belonging to the same clonal clusters displayed identical REP-PCR profiles with the same band sizes and densities.

4. Determination of antimicrobial susceptibilities and minimum inhibitory concentrations (MICs)

The antimicrobial susceptibility of each isolate against 12 antimicrobial agents was analyzed on Müller-Hinton agar (Difco) by the Kirby-Bauer disk diffusion method, according to the CLSI M100-S21 guidelines [3]. The following antimicrobial disks (BBL, Cockeysville, MI, USA) were used: amikacin (30 μg), ampicillin/sulbactam (10/10 μg), aztreonam (30 μg), cefepime (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), ciprofloxacin (5 μg), gentamicin (10 μg), imipenem (10 μg), meropenem (10 μg), piperacillin/tazobactam (100/10 μg), and ticarcillin/clavulanic acid (75/10 μg). *Escherichia coli* strain ATCC 25922 was used as a reference strain. Multidrug resistance was defined as resistance to three or more representatives of the following five drug classes: aminoglycosides, carbapenems, cephalosporins, fluoroquinolones, and β -lactam/ β -lactam inhibitor [16]. MIC determination against imipenem, meropenem, amikacin, gentamicin, ciprofloxacin and ceftazidime of clonally selected *A. baumannii* isolates was performed with the Epsilon test (Etest; bioMérieux), according to the guidelines recommended by CLSI M100-S21 [17].

5. Characterization of biofilm-associated genes and antimicrobial resistance determinants

Clonally selected *A. baumannii* isolates were subjected to PCR and sequencing assays for detection of biofilm-associated genes and antimicrobial resistance deter-

minants. Total DNA was extracted from each target strain showing different banding patterns from REP-PCR using a DNA purification kit (SolGent, Daejeon, Korea) according to standardized protocols. PCR amplification was performed in separate 25 µL reactions containing 0.7 U Taq DNA polymerase MasterMix (SolGent), 50 ng total

DNA, and 20 pmol of each specific forward and reverse primer (Table 1) [5-7, 18-20]. PCR amplification was performed in a GeneAmp PCR System 9600 thermal cycler (Perkin-Elmer Cetus Corp., Norwalk, CT, USA) with an initial melting of the reaction mixture at 95°C for 5 min; followed by 30 cycles of 95°C for 30 s, 52°C for 30 s, and

Table 1. Oligonucleotides used in this study for the detection of biofilm-associated genes and antimicrobial resistance determinants

Target genes	Primer	Sequence (5'-3')	Gene	Reference
Aminoglycoside resistance genes	armA-F	CAATGGATAAGAAATGATGTT	<i>armA</i>	[5]
	armA-R	TTATTCTGAAATCCACT		
	rmtA-F	ATGAGCTTTGACGATGCCCTA	<i>rmtA</i>	[5]
	rmtA-R	TCATTATTCCTTTTATCATG		
	rmtB-F	ATGAACATCAACGATGCCCT	<i>rmtB</i>	[5]
	rmtB-R	CCTTCTGATTGGCTTATCCA		
	rmtC-F	CGAAGAAGTAACAGCCAAAG	<i>rmtC</i>	[5]
	rmtC-R	ATCCCAACATCTCTCCACT		
	rmtD-F	CGGCACGCGATTGGGAAGC	<i>rmtD</i>	[5]
	rmtD-R	CGGAAACGATGCGACGAT		
	rmtE-F	ATGAATATTGATGAAATGGTTGC	<i>rmtE</i>	[5]
	rmtE-R	TGATTGATTTCTCCGTTTTTG		
	rmtF-F	GCGATACAGAAAACCGAAGG	<i>rmtF</i>	[5]
	rmtF-R	ACCAGTCGGCATAGTGCTTT		
Quinolone resistance genes	QnrA-F	AGAGGATTTCTCACGCCAGG	<i>qnrA</i>	[6]
	QnrA-R	TGCCAGGCACAGATCTTGAC		
	QnrB-F	GGMATHGAAATTCGCCACTG	<i>qnrB</i>	[6]
	QnrB-R	TTTGCYGYCGCCAGTCGAA		
	QnrS-F	GCAAGTTCATTGAACAGGGT	<i>qnrS</i>	[6]
	QnrS-R	TCTAAACGTCGAGTTCGGCG		
	gyrAaF	ATGAGCGTATCGGAAATCC	<i>gyrA</i>	[7]
	gyrAaR	ATCAATCCTTCAATCGAGATATTC		
	parCaF	ATGGAAGATAAGCTGACTATG	<i>parC</i>	[7]
	parCaR	GTTGGTAAATCCGGAGC		
Biofilm associated genes	bapF	ATGCCTGAGATACAAATTAT	<i>bap</i>	[19]
	bapR	GTCAATCGTAAAGGTAACG		
	ompAF	GTAAAGGCGACGTAGACG	<i>ompA</i>	[19]
	ompAR	CCAGTGTTATCTGTGTGACC		
	per-1F	ATGAATGTCATTATAAAAGC	<i>bla_{PER-1}</i>	[19]
	per1R	AATTTGGGCTTAGGCAGAA		
	csuEF	CATCTTCTATTTCCGTCCC	<i>csuE</i>	[19]
Carbapenemase genes	csuER	CGGTCTGAGCATTGGTAA		
	ISAba1	CCTATCAGGGTTCTGCCTTCT	<i>ISAba1</i>	[18]
	OXA-23-F	GATCGGATTGGAGAACCAGA	<i>bla_{OXA-23-like}</i>	[20]
	OXA-23-R	ATTTCTGACCGCATTTCCAT		
	OXA-24-F	GGTTAGTTGGCCCCCTTAAA	<i>bla_{OXA-24-like}</i>	[20]
	OXA-24-R	AGTTGAGCGAAAAGGGGATT		
	OXA-51-F	TAATGCTTTGATCGGCCTTG	<i>bla_{OXA-51-like}</i>	[20]
	OXA-51-R	TGGATTGCACTTCATCTTGG		
	OXA-58-F	AAGTATTGGGGCTTGTGCTG	<i>bla_{OXA-58-like}</i>	[20]
	OXA-58-R	CCCCTCTGCGCTCTACATAC		
	IMP-F	GGAATAGAGTGGCTTAAYTCTC	<i>bla_{IMP}</i>	[20]
	IMP-R	GGTTTAAAYAAAACAACCACC		
	VIM-F	GATGGTGTGTTGTCGCATA	<i>bla_{VIM}</i>	[20]
	VIM-R	CGAATGCGCAGCACCAG		

Abbreviations: F, sense primer; R, antisense primer; H, A/C/T; M, A/C; Y, C/T.

72°C for 30 s; and a final extension at 72°C for 3 min. After confirmation of the presence of appropriate amplified products on 1.5% (w/v) agarose gels, the amplicons were purified according to the manufacturer’s instructions using a PCR purification kit (SolGent), and these purified amplicons 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). The DNA sequences of biofilm-associated genes and antimicrobial resistance determinants were confirmed using the NCBI BLAST paired alignment function.

RESULTS

1. Biofilm imaging and quantification

Fifty-eight *A. baumannii* isolates belonging to diverse strains were obtained from various clinical specimens over 5 years. Biofilm imaging and formation assays of all isolates involved in this study and negative control strains were carried out. The mean OD₅₆₀ value of the negative control

strains was 0.27, and this value was used as a cut-off OD value for biofilm formation ability. Isolates were classified as follows: OD₅₆₀ ≤ 0.27, non-biofilm former; 0.27 < OD₅₆₀ ≤ 0.54, weak biofilm former; 0.54 < OD₅₆₀ ≤ 1.08, moderate biofilm former; OD₅₆₀ > 1.08, strong biofilm former. As the OD value of the biofilm increased, the abundance of matrix-like structures; thick, contained cells; and biofilm-covered surface areas increased (Figure 1). Among the 58 isolates, 12 isolates were non-biofilm formers and 46 (79.3%) were biofilm formers, including 11 (19.0%), 32 (55.2%), and 3 (5.2%) isolates categorized as strong, moderate, and weak biofilm formers, respectively.

2. Association of MDR phenotype with biofilm-forming capability

Epidemiological typing of the 46 *A. baumannii* isolates with confirmed biofilm formation capacity was performed by REP-PCR, and a total of 16 REP genotypes were identified (Figure 2). Subsequently, antimicrobial susceptibility test was performed to determine MDR and non-MDR status of 16 randomly selected *A. baumannii*

Figure 1. Imaging and quantification of biofilm formation by *A. baumannii* isolates using a phase contrast microscope and a 96-well microplate spectrophotometer, after crystal violet staining.

Figure 2. Repetitive extragenic palindromic (REP)-PCR banding pattern of clonal selected nonmultidrug-resistance and multidrug-resistance *A. baumannii* strains, with 1-kb DNA size markers in M lanes.

Table 2. Antimicrobial susceptibilities of biofilm-forming *A. baumannii* strains

Isolates	Specimen	Antimicrobial susceptibility												Biomass OD ₅₆₀ *	Biofilm-associated genes			
		AN	SAM	ATM	FEP	CTX	CAZ	CIP	GM	IPM	MEM	TZP	TIM		<i>bla</i> _{PER-1}	<i>ompA</i>	<i>csuE</i>	<i>bap</i>
Non-MDR	12-1	Sputum	S	S	R	S	S	S	S	S	S	S	S	1.09	-	-	+	+
	12-17	Sputum	S	S	R	S	I	S	S	S	S	S	S	0.93	-	+	+	+
	14-125	Sputum	S	S	R	S	S	S	S	S	S	S	S	0.95	-	+	+	-
	15-35	Bronchial	S	S	R	S	I	S	S	S	S	S	S	0.89	-	+	+	-
	15-58	Sputum	S	S	R	S	R	S	S	S	S	S	S	0.97	-	+	+	-
	16-2	Sputum	S	S	R	S	S	S	S	S	S	S	S	0.71	-	-	+	+
	16-7	Sputum	S	S	R	S	S	S	S	S	S	S	S	0.89	-	-	+	+
	16-29	Bronchial	S	S	R	S	I	S	S	S	S	S	S	0.89	-	-	+	+
	16-36	Sputum	S	S	R	R	R	R	R	S	S	R	I	1.33	-	+	+	+
MDR	12-4	Sputum	R	R	R	R	R	R	R	R	R	R	R	1.00	-	-	-	+
	12-16	Sputum	R	R	R	R	R	R	R	R	R	R	R	0.92	+	+	+	+
	14-143	Sputum	R	R	R	R	R	R	R	R	R	R	R	0.91	-	+	+	-
	14-146	Sputum	R	R	R	R	R	R	R	R	R	R	R	0.96	-	+	+	+
	15-27	Sputum	R	I	R	R	R	R	R	R	R	R	R	1.70	-	+	+	+
	16-8	Sputum	R	R	R	R	R	R	R	R	R	R	R	1.04	-	+	+	+
	16-25	Urine	R	R	R	R	R	R	R	R	R	R	R	0.80	-	+	+	+

Abbreviations: MDR, multidrug-resistance; AN, amikacin; SAM, ampicillin/sulbactam; ATM, aztreonam; FEP, cefepime; CTX, cefotaxime; CAZ, ceftazidime; CIP, ciprofloxacin; GM, gentamicin; IPM, imipenem; MEM, meropenem; TZP, piperacillin/tazobactam; TIM, ticarcillin/clavulanic acid; S, susceptible; I, intermediate resistant; R, resistant; OD, optical density.

*OD₅₆₀: Biofilm formation was quantified by measuring optical absorbance (560 nm) using crystal violet.

isolates, each belonging to a different REP genotype. Among the 16 isolates, 9 and 7 strains were identified as non-MDR and MDR strains, respectively (Table 2). Only one MDR *A. baumannii* isolate was resistant to 11 antimicrobial agents tested in this study (all agents except ampicillin/sulbactam), while the remaining 6 MDR isolates were resistant to all 12 agents in the panel. In contrast, 8 of the 9 non-MDR *A. baumannii* isolates were susceptible to 10 antimicrobial agents tested in this study. PCR and sequencing analysis were performed to detect 4 genes associated with biofilm formation in the 16 biofilm-forming isolates. Among the 4 genes, *csuE* (93.8%) was the most prevalent, followed by *bap* (75.0%) and *ompA*

(68.8%), while the *bla*_{PER-1} gene (6.3%) was rare among the isolates (Table 2). Among MDR isolates, the detection rates of *ompA* (85.7%) and *bap* (85.7%) were higher than the rates of *ompA* (55.6%) and *bap* (66.7%) among non-MDR isolates. However, the prevalence of the *csuE* gene was higher among non-MDR isolates (100.0%) than among MDR isolates (85.7%). Despite this, there were no significant differences among the detection rates of biofilm-associated genes between MDR and non-MDR isolates. In addition, the mean OD₅₆₀ values of non-MDR and MDR isolates were 0.96 and 1.05, respectively, but this difference was not significant (Table 2).

3. Correlation between MICs and antimicrobial resistance determinants

Detection of antimicrobial resistance determinants and MIC determinations for 6 antimicrobial agents were performed to evaluate the association between the presence of these determinants and MDR status (Table 3). All 16 biofilm-forming isolates possessed *bla*_{OXA-51}-like genes, which are ubiquitous among *A. baumannii* isolates. Among carbapenemase genes, *bla*_{OXA-23} and *bla*_{OXA-51} were detected in 5 MDR isolates, while only *bla*_{OXA-51} was detected in non-MDR isolates. All 5 isolates harboring *bla*_{OXA-23} possessed an insertion of *ISAbal1* upstream of the gene and were resistant to imipenem and meropenem. Among the 16S rRNA methyltransferase genes (*armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD*, *rmtE*, and *rmtF*), only *armA* was observed in this study. Among the 5 isolates harboring the *armA* gene, 1 isolate was a non-MDR strain and 4 isolates were MDR strains with high-level resistance to gentamicin

(>256 µg/mL). Seven of the 16 isolates possessed point mutations in both *gyrA* (Ser83Leu) and *parC* (Ser80Leu or Glu84Lys), and all were resistant to ciprofloxacin with high MICs (>32 µg/mL). However, no isolates involved in this study possessed any *qnr* genes (*qnrA*, *qnrB*, or *qnrS*).

DISCUSSION

Among recent studies, many researchers have reported that the ability to form biofilms is an important factor involved in resistance to disinfection and desiccation, as well as antimicrobial agents. In addition, they have reported that biofilms act as a virulence factor, facilitating the extended survival of *A. baumannii* isolates in a hospital environment. The aim of the present study was to analyze the correlation between biofilm-forming ability and antimicrobial resistance in *A. baumannii* isolates. In addition, we evaluated the association between high-level resistance to antimicrobial agents and antimicrobial

Table 3. Characterization of biofilm-forming *A. baumannii* strains

Isolates		Specimen	Minimum inhibitory concentrations (MICs)						Antimicrobial resistance determinants					
			MEM	IPM	AMK	CM	CIP	CAZ	Carbapenemase	<i>abaI</i> ~ OXA23	<i>abaI</i> ~ OXA51	<i>armA</i>	<i>gyrA</i>	<i>parC</i>
Non-MDR	12-1	Sputum	0.25	0.38	3.00	1.50	0.13	1.50	OXA-51	-	+	-	-	-
	12-17	Sputum	2.00	1.00	8.00	4.00	0.25	2.00	OXA-51	-	+	+	-	80 Ser →Leu
	14-125	Sputum	0.25	0.38	2.00	0.50	0.13	2.00	OXA-51	-	-	-	-	-
	15-35	Bronchial	0.38	0.38	4.00	0.75	0.19	2.00	OXA-51	-	-	-	-	-
	15-58	Sputum	1.00	0.38	3.00	1.00	0.50	3.00	OXA-51	-	-	-	-	-
	16-2	Sputum	0.64	0.94	3.00	1.00	0.25	1.50	OXA-51	-	-	-	-	-
	16-7	Sputum	0.23	0.47	0.75	0.25	0.25	1.00	OXA-51	-	-	-	-	-
	16-29	Bronchial	0.47	0.64	2.00	0.25	0.38	1.50	OXA-51	-	-	-	-	-
	16-36	Sputum	0.75	1.00	3.00	6.00	>32	48	OXA-51	-	-	-	83 Ser → Leu	80 Ser →Leu
MDR	12-4	Sputum	16	16	64	8	4	128	OXA-51	-	-	-	-	-
	12-16	Sputum	>32	>32	64	>256	>32	>256	OXA-51	-	+	-	83 Ser → Leu	84 Glu →Lys
	14-143	Sputum	>32	>32	>256	>256	>32	64	OXA-51, OXA-23	+	-	+	83 Ser → Leu	80 Ser →Leu
	14-146	Sputum	16	16	>256	>256	>32	128	OXA-51, OXA-23	+	-	+	83 Ser → Leu	80 Ser →Leu
	15-27	Sputum	>32	>32	64	>256	>32	128	OXA-51, OXA-23	+	-	-	83 Ser → Leu	80 Ser →Leu
	16-8	Sputum	>32	>32	64	>256	>32	64	OXA-51, OXA-23	+	-	+	83 Ser → Leu	80 Ser →Leu
	16-25	Urine	16	>32	64	>256	>32	>256	OXA-51, OXA-23	+	-	+	83 Ser → Leu	80 Ser →Leu

Abbreviations: MDR, multidrug-resistance; MEM, meropenem; IPM, imipenem; AMK, amikacin; CM, gentamicin; CIP, ciprofloxacin; CAZ, ceftazidime.

resistance determinants among biofilm-forming *A. baumannii* isolates. For these purposes, we identified a total of 46 (79.3%) biofilm-forming *A. baumannii* isolates, including 11 strong (19.0%), 32 moderate (55.2%), and 3 weak (5.2%) biofilm formers. Our results were similar to previous reports, which showed that more than 75% of clinical *A. baumannii* isolates form biofilms [10, 21]. Subsequently, biofilm-forming *A. baumannii* isolates exhibited 16 different REP genotypes and were clonally selected. Biomass analysis results of the 16 isolates, including 9 non-MDR and 7 MDR isolates, were compared. There was little difference between the mean biomass OD560 values of the non-MDR (0.96) and MDR (1.05) isolates. These results suggest that the MDR phenotype is not associated with biofilm-producing ability. Previous researchers have also reported that antimicrobial resistance is similar between biofilm-forming and biofilm non-forming isolates [21]. Next, biofilm-associated genes were investigated in the 16 isolates. We found that MDR isolates had a higher prevalence of biofilm-associated genes, with the exception of *csuE*, but no significant differences in their biofilm-forming abilities and MDR phenotype were observed based on the presence of biofilm-associated genes. However, one biofilm-associated gene, *ompA*, was associated with an antimicrobial-resistant phenotype. In our study, only one (9.1%) of the 11 isolates harboring the *ompA* gene was susceptible to cefotaxime, while 3 (60.0%) of the 5 isolates not harboring the *ompA* gene were susceptible to this antibiotic (Table 2). Our results are consistent with previous studies, which reported that OmpA is involved in the attachment and development of biofilms as well as resistance to antimicrobial agents such as cefotaxime, ciprofloxacin, and imipenem [10, 21]. In this study, we investigated correlations between antimicrobial resistance determinants and the MICs of 6 antimicrobial agents. Five (71.4%) of 7 imipenem- and meropenem-resistant isolates contained the *bla*_{OXA-23} gene and the upstream *ISAbal* insertion sequence and exhibited the MDR phenotype. These results agree with the worldwide dissemination of the *bla*_{OXA-23} gene, which is more prevalent than other

carbapenemase genes among MDR *A. baumannii* isolates [16]. Four (66.7%) of the 6 amikacin- and gentamicin-resistant isolates harbored *armA* genes and exhibited high-level resistance to gentamicin (>256 µg/mL). *ArmA* is widely known as a 16S rRNA methyltransferase causing high-level resistance to various antimicrobial agents belonging to the aminoglycosides, and it is most frequently detected among *A. baumannii* isolates [4]. Quinolone resistance among gram-negative bacteria usually occurs due to chromosomal mutations in QRDRs or the acquisition of PMQR determinants. Seven isolates showed resistance to ciprofloxacin with high MICs (>32 µg/mL) and possessed point mutations in both *gyrA* and *parC*. Similarly, other investigators have reported that *A. baumannii* isolates possessing mutations in both *gyrA* and *parC* exhibit high-level resistance to ciprofloxacin [7].

In this study, we showed that most biofilm-forming MDR *A. baumannii* clinical isolates contained various antimicrobial resistance determinants to combat aminoglycosides, carbapenems, cephalosporins, and fluoroquinolones. In addition, most MDR isolates showed high-level resistance to various antimicrobial agents. In contrast, we found that most biofilm-forming non-MDR *A. baumannii* clinical isolates did not contain antimicrobial resistance determinants and were susceptible to various antimicrobial agents. Therefore, our results suggest that there is little correlation between biofilm-forming ability and antimicrobial susceptibility in MDR *A. baumannii* isolates. However, in this study, there were limitations that the number of clinical isolates was small and the isolates were separated from limited areas. So, further study with a large number of isolates from wide areas might be necessary in future. Correlations between antimicrobial resistance and biofilm-forming ability have been reported in *A. baumannii* and *P. aeruginosa* isolates [22–24]. Previous researchers demonstrated that *A. baumannii* isolates had achieved high levels of resistance to antimicrobial agents despite producing weak biofilms [22]. They reported that biofilm-forming ability acts as a strategy for *A. baumannii* isolates to get a better survival, particularly in isolates with low levels of resistance to

antimicrobial agents. However, previous study showed that MDR *P. aeruginosa* isolates produced biofilms at a significantly higher rate than non-MDR isolates [23]. In addition, other study also reported that positive correlations between antimicrobial resistance and biofilm-forming ability in imipenem resistant *A. baumannii* isolates [24]. Therefore, the correlation between biofilm formation and antimicrobial resistance phenotypes remains unclear, and further studies are needed to explore this issue more fully. The present study investigated the genetic basis of antimicrobial resistance mechanisms of biofilm-forming *A. baumannii* clinical isolates. We confirmed that the emergence of MDR *A. baumannii* clinical isolates appears to be caused by mutations of genes associated with antimicrobial resistance and/or acquisition of various antimicrobial resistance determinants.

요 약

다제내성(multidrug-resistant, MDR) *Acinetobacter baumannii* 균주의 출현 및 확산이 전 세계적으로 보고되어 왔는데, 이들 대부분은 생물막 형성능력을 가지고 있다. 생물막 형성은 소독 및 건조에 대해 세균이 저항할 수 있도록 해주는 중요한 병독성 인자이다. 본 연구에서는 생물막을 형성하는 *A. baumannii* 임상 분리주를 대상으로 항균제 내성 기전에 대한 유전적 기초를 조사하였다. 크리스탈 바이올렛 분석법을 통해 생물막 형성능력을 확인한 결과 46균주가 생물막을 형성하는 것으로 나타났다. 이어서 REP-PCR을 수행하여 서로 다른 클론에 속하는 16 균주를 선택한 후 이 균주들을 대상으로 항균제 내성 인자를 검출하였다. 본 연구에서 분리된 9개 non-MDR (0.96) 균주와 7개의 MDR (1.05) 균주의 평균 생물량은 서로 달랐지만, 이 차이는 유의하지 않았다. 대부분의 생물막 형성 MDR 균주는 다양한 항균제 내성인자 (*bla*_{OXA-23}, *armA* 및 *gyrA* 및 *parC*의 돌연변이)를 가지고 있는 것으로 나타났다. 그러나 대부분의 non-MDR 균주는 항균제 내성인자를 포함하지 않는 것으로 나타났다. 우리의 결과는 *A. baumannii* 균주의 생물막 형성능력이 항균제 감수성과 상관관계가 적음을 시사한다. 또한 MDR *A. baumannii* 임상 분리주의 출현은 대개 항균제 내성과 관련된 유전자의 돌연변이나 또는 다양한 항균제 내성인자의 획득에 의한 것으로 사료된다.

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