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In Vivo Selection of Pan-Drug Resistant *Acinetobacter baumannii* during Antibiotic Treatment

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Purpose: Colistin resistance in *Acinetobacter baumannii* (*A. baumannii*) is mediated by a complete loss of lipopolysaccharide production via mutations in *lpxA*, *lpxC,* and *lpxD* gene or lipid A modifications via mutations in the *pmrA* and *pmrB* genes. However, the exact mechanism of therapy-induced colistin resistance in *A. baumannii* is not well understood. **Materials and Methods:** We investigated the genotypic and phenotypic changes that underlie pan-drug resistance mechanisms by determining differences between the alterations in extensively drug-resistant (XDR) *A. baumannii* (AB001 and AB002) isolates and a pan-drug resistant (PDR) counterpart (AB003) recovered from one patient before and after antibiotic treatment, respectively. **Results:** All three clinical isolates shared an identical sequence type (ST138), belonging to the global epidemic clone, clonal complex 92, and all produced OXA-23 carbapenemase. The PDR AB003 showed two genetic differences, acquisition of *armA* gene and an amino acid substitution (Glu229Asp) in *pmrB* gene, relative to XDR isolates. No mutations were detected in the *pmrA*, *pmrC*, *lpxA*, *lpxC*, or *lpxD* genes in all three isolates. In matrix-assisted laser desorption ionization-time of flight analysis, the three isolates commonly showed two major peaks at 1728 m/z and 1912 m/z, but peaks at 2034 m/z, 2157 m/z, 2261 m/z, and 2384 m/z were detected only in the PDR *A. baumannii* AB003 isolate. **Conclusion:** Our results show that changes in lipid A structure via a mutation in the *pmrB* gene and acquisition of *armA* gene might confer resistance to colistin and aminoglycosides to XDR *A. baumannii* strains, resulting in appearance of a PDR *A. baumannii* strain of ST138.

Key Words: *pmrB* gene, *armA* gene, colistin, lipid A, *Acinetobacter baumannii*

INTRODUCTION

The extensive use of antimicrobial agents and the propensity of pathogenic bacteria that accumulate antimicrobial resistance have led to a considerable increase in pathogenic bacteria acquiring resistance to multiple categories of antimicrobial agents. As this problem continues to grow, the definition of terms such as multidrug resistant (MDR), extensively drug-resistant (XDR), and pan-drug resistant (PDR) strains were standardized in order to more specifically define those microorganisms. MDR strains are defined as non-susceptible to at least one agent in three

or more antimicrobial categories, XDR strains are defined as non-susceptible to at least one agent in all but two or fewer antimicrobial categories, and PDR strains are defined as non-susceptible to all agents in all antimicrobial categories.[1]

Acinetobacter baumannii (A. baumannii) is associated with infections including bacteremia, pneumonia, meningitis, and urinary tract infections especially in patients hospitalized in intensive care units (ICUs).[2,3] The prevalence of XDR *A. baumannii* isolates with carbapenem resistance has persistently increased, and most of XDR *A. baumannii* isolates in Asia belong to the globally-distributed clone, clonal complex 92.[4-7] After the reintroduction of colistin in clinical practice to treat infections caused by XDR *A. baumannii* due to the lack of applicable antibiotics, the emergence of PDR *A. baumannii* strains with colistin resistance has repeatedly been reported in many parts of the world.[8-11]

According to a few *in vitro* studies regarding colistin resistance mechanisms in *A. baumannii*, colistin resistance is mediated by a complete loss of lipopolysaccharide (LPS) production via mutations in LPS producing genes (*lpxA*, *lpxC,* and *lpxD*) or by mutations in the *pmrA* and *pmrB* genes that encode a two-component signal transduction system.[12,13] However, the exact mechanism of therapy-induced colistin resistance in *A. baumannii* is not well understood.

Here, we studied colistin resistance mechanisms by evaluating the genetic alterations between two colistin-susceptible XDR *A. baumannii* clinical isolates and a colistin-resistant counterpart isolated from one patient before and after colistin therapy, respectively. Furthermore, we investigated the genetic determinants of antimicrobial resistance to various antibiotics and the molecular epidemiology of the clinical isolates.

MATERIALS AND METHODS

Patient characteristics and bacterial strains

A 72-year-old man with a history of hypertension, atrial fibrillation, and diabetes mellitus was admitted to the emergency department of a tertiary-care hospital in Seoul, Korea, on August 29th, 2011 due to abdominal pain. Abdominal computed tomography revealed an impending thoracoabdominal aortic aneurysm, and a graft replacement of the abdominal aorta was performed. After emergency surgery, he was admitted to the ICU and was mechanically ventilated from day one. He was treated with ciprofloxacin (400 mg/day) and piperacillin+tazobactam (6.75 g/day). An XDR *A. baumannii* isolate (AB001) was first recovered from a respiratory tract specimen on day five, and it was then serially recovered from respiratory tract specimens. And an XDR *A. baumannii* isolate (AB002) was recovered from a urinary specimen on day six. Intravenous administration of colistimethate sodium (150 mg/day) was started on day six. After five days of colistin therapy, a colistin-resistant *A. baumannii* isolate (AB003) was recovered from a blood specimen, which prompted the physicians to change antibiotics to tigecycline. However, urinary tract infection, acute renal failure, atelectasis at both lungs, and sepsis were not controlled. On day 17, the patient was transferred to a local hospital near the patient's home for continuation of treatment. In this study, we included the two colistin-susceptible XDR *A. baumannii* isolates (AB001 and AB002), which were recovered from the respiratory tract and urinary specimens, respectively, and their colistin-resistant counterpart (AB003). The isolates were identified as *A. baumannii* using the Vitek GNI system (bioMérieux, Marcy l'Etoile, France) and sequence analysis of the RNA polymerase β-subunit (*rpoB*) gene.[14] *A. baumannii* ATCC 19606 was used as the reference strain.

Antimicrobial susceptibility testing

Antimicrobial susceptibilities were determined using antibiotic-containing disks (Becton Dickinson, Sparks, MD, USA) on Mueller-Hinton agar (Difco Laboratories, Detroit, MI, USA). The minimum inhibitory concentrations (MICs) of meropenem and imipenem were determined using the agar dilution method, and the MIC of colistin was determined by the E-test (bioMérieux) according to the Clinical and Laboratory Standards Institute guidelines.[15]

Multi-locus sequence typing (MLST)

PCR and sequencing experiments were performed on seven housekeeping genes encoding citrate synthase (*gltA*), DNA gyrase subunit B (*gyrB*), glucose dehydrogenase B (*gdhB*), homologous recombination factor (*recA*), 60-kDa chaperonin (*cpn60*), glucose-6-phosphate isomerase (*gpi*), and RNA polymerase sigma factor (*rpoD*), as described previously.[6,16] Nucleotide sequences of both strands were determined. Allelic profiles of the seven housekeeping genes and STs of the strains were analyzed using the *Acinetobacter* MLST database (http://www.pasteur.fr/recherche/genopole/PF8/ mlst/).

Pulsed-field gel electrophoresis (PFGE)

Pulsed-field gel electrophoresis (PFGE) was performed with *Sma*I-digested genomic DNA extracted from the three iso-

Table 1. Characteristics of *A. baumannii* Clinical Isolates Included in This Study

Isolate*	Antimicrobial susceptibilities (inhibition zone diameter, mm)						MIC (mg/mL)			MLST
	CAZ	CTX	FEP	AN	GM	CIP	CL	MEM	IPM	ST
AB001	R (9)	R (9)	R (10)	S (24)	S (18)	R (9)	0.25	256	64	138
AB002	R (9)	R (9)	R (10)	S (23)	S (17)	R (9)	0.25	256	64	138
AB003	R (9)	R (9)	R (11)	R (9)	R (9)	R (9)	16	256	64	138

AN, amikacin; CAZ, ceftazidime; CIP, ciprofloxacin; CL, colistin; CTX, cefotaxime; FEP, cefepime; GM, gentamicin; IPM, imipenem; MEM, meropenem; MLST, multi-locus sequence typing; R, resistance; S, susceptible; ST, sequence type.
*AB001 and AB002 are colistin-susceptible isolates and AB003 is the colistin-resistant counterpart.

Table 2. Antibiotic Resistance Genes of *Acinetobacter baumannii* Clinical Isolates

Strain†	Detected antimicrobial resistance determinants				Detected mutations				
	bla_{OXA-51}-like*	IS*Aba1*-bla_{OXA-23}	IS*Aba1*-bla_{AmpC}	*armA*	*gyrA*	*parC*	*pmrB*	*pmrA* and *C*	*lpxA*, *C*, and *D*
AB001	+	+	+	ND	Ser83Leu	Ser80Leu	WT	WT	WT
AB002	+	+	+	ND	Ser83Leu	Ser80Leu	WT	WT	WT
AB003	+	+	+	+	Ser83Leu	Ser80Leu	Glu229Asp (A→T substitution at nt687)	WT	WT

ND, not detected; WT, wild type.
*IS*Aba1* upstream of bla_{OXA-51}-like was not detected in all isolates.
†Genes detected by PCR and sequencing.

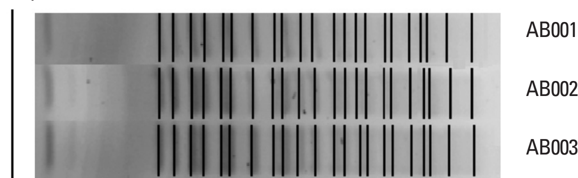

Fig. 1. *Sma*I-macroresticion patterns of two colistin-susceptible isolates (AB001 and AB002) and a colistin-resistant isolate (AB003). All three isolates showed 100% of similarity.

lates using a CHEF-DRII device (Bio-Rad, Hercules, CA, USA). The conditions of PFGE were 6 V/cm for 20 h with pulse times of 3–10 seconds at a temperature of 11ºC. *Sma*I-digested band patterns were analyzed using Molecular Analyst Fingerprinting Software Ver. 3.2 (Bio-Rad). The PFGE patterns were interpreted using the criteria of Tenover, et al.[17]

Characterization of antimicrobial resistance determinants

PCR and sequence analysis of antimicrobial resistance determinants including bla_{OXA51}-like, bla_{OXA-23}, bla_{AmpC}, *gyrA*, *parC*, and *armA* genes were performed according to methods described in previous studies.[18-21] The presence of IS *Aba1* upstream of the bla_{OXA51}-like, bla_{OXA-23}, and bla_{AmpC} genes was also evaluated.[22] LPS-producing genes (*lpxA*, *lpxC*, and *lpxD*) and *pmrA* and *pmrB* genes encoding a two-component signal transduction system were sought by PCR and sequencing.[13,23] Briefly, genomic DNA was extracted using a QIAamp DNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. After PCR, amplicons were purified using a QIAquick Gel Extraction Kit (Qiagen) and sequenced using an ABI 3500dx system (Applied Biosystems, Foster City, CA, USA). Sequences for all antimicrobial resistance determinant loci in *A. baumannii* isolates were compared using the GenBank database (http://www.ncbi.nlm.nih.gov) with reference sequences of *A. baumannii* ATCC 17978 (GenBank accession number CP000 521).

Analysis of lipid A structure

LPS and lipid A were extracted from whole bacterial cells using Tri-Reagent extraction and mild acid hydrolysis methods, and were then subjected to negative-ion matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometer (Bruker Daltonik GmbH, Leipzig, Germany) analysis in the negative reflective mode.[24]

RESULTS

Antimicrobial susceptibilities and antimicrobial resistance determinants

The antimicrobial resistance determinants of the two colistin-susceptible XDR *A. baumannii* isolates (AB001 and

AB002) and a PDR *A. baumannii* isolate (AB003) were evaluated. All three isolates showed resistance to ciprofloxacin, and they contained the *gyrA* gene with an amino acid substitution (Ser83Leu). While isolates AB001 and AB002 showed susceptibility to amikacin and gentamicin, the AB003 isolate showed resistance to these aminoglycosides and harbored the *armA* gene. All three isolates showed resistance to ceftazidime and cefepime, and they carried and the IS*Aba1*-*bla*$_{AmpC}$ structure. MICs of imipenem and meropenem for all three isolates were greater than 32 mg/L, and they carried the *bla*$_{OXA-23}$ gene with an insertion sequence IS*Aba1* upstream of the gene. The MIC of colistin for both AB001 and AB002 isolates was 0.25 mg/L, while that for AB003 isolate was 16 mg/L (Table 1 and 2).

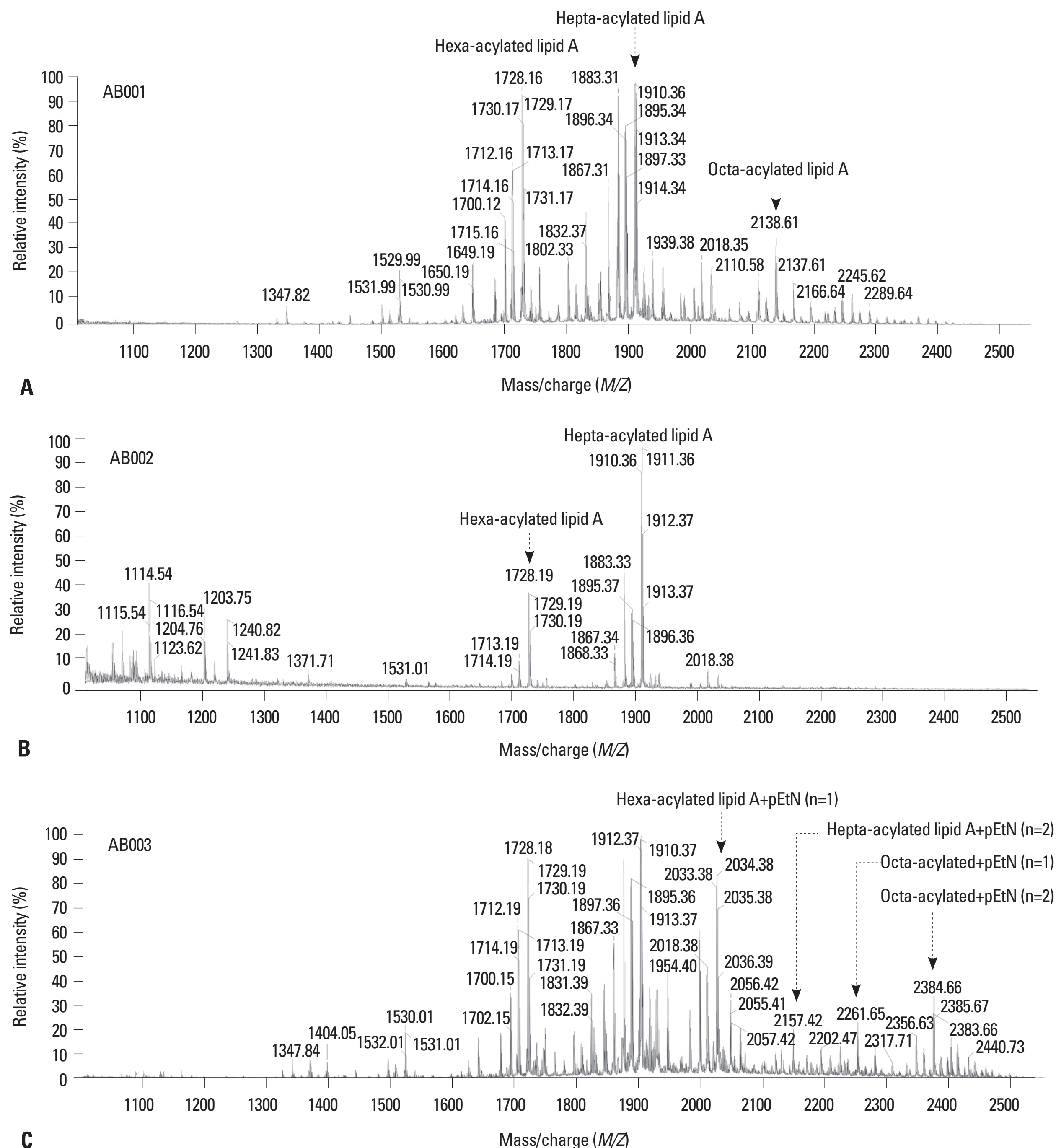

Fig. 2. Mass spectrometry of lipid A extracted from the colistin-susceptible isolates (A) AB001 and (B) AB002 and their colistin-resistant counterpart (C) AB003 with Glu229Asp in *pmrB* gene. In MALDI-TOF analysis, AB001, AB002, and AB003 isolates all showed two major peaks at 1728 m/z and 1912 m/z, but peaks at 2034 m/z, 2157 m/z, 2261 m/z, and 2384 m/z were detected only in the PDR *A. baumannii* AB003 isolate. MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; PDR, pan-drug resistant.

Strain type

All three isolates were identified as an identical ST, ST 138 (1-3-3-2-2-50-3), and they showed identical *Sma*I-macrorestriction patterns by PFGE (Fig. 1).

Molecular analysis of the colistin-susceptible/resistant isogenic clinical isolates

The sequences of the *pmrB* gene in the two colistin-susceptible AB001 and AB002 isolates were identical to those of the reference *A. baumannii* ATCC 17978, whereas the PDR *A. baumannii* AB003 isolate harbored a novel mutation (encoding a Glu229Asp change) in the *pmrB* gene (Table 2). No mutations were detected in the *pmrA*, *pmrC*, *lpxA*, *lpxC*, and *lpxD* genes in all three isolates.

Analysis of lipid A structure

The two colistin-susceptible AB001 and AB002 isolates and the PDR *A. baumannii* AB003 isolate all showed two major peaks at 1729 m/z and 1911 m/z in negative-ion MALDI-TOF mass spectra. These two major peaks most likely correspond to *bis*-phosphorylated hexa- and hepta-acylated lipid A, respectively. Spectra for the lipid A structure from the PDR *A. baumannii* AB003 isolate were distinct from those of AB001 and AB002, as it showed additional peaks at 2034 m/z, 2157 m/z, 2261 m/z, and 2384 m/z (Fig. 2).

DISCUSSION

The extensive use of antimicrobial agents has led to a considerable increase in MDR, XDR, and PDR pathogenic bacteria. Colistin has resurfaced as effective last resort for treatment of infections caused by XDR *A. baumannii*. Many studies evaluated the resistance mechanisms against polymyxins, and analysed effectiveness of colistin monotherapy as well as the combination therapy to reduce the colistin resistance and toxicity of colistin.[8,10,25] Despite the efforts, colistin therapy-related colistin-resistant *A. baumannii* strains have been isolated.[10,26,27] A complete loss of LPS production via mutations in LPS-producing genes (*lpxA*, *lpxC*, and *lpxD*) and the modification of lipid A components via mutations in the *pmrAB* gene have been evaluated in various studies with *in vitro* manipulated colisitn-resistant *A. baumannii.*[12,13] Between two colistin-resistance mechanisms, only mutations in the *pmrA* (Met12Ile; Met12Lys) or *pmrB* gene (Gln270Pro; Gln277Lys) were found in clinical isolates from patient who has been treated with colistin. In our study, AB003 harbored Glu229Asp mutation in the *pmrB* gene, while the isolate carried wild-type LPS-producing genes (*lpxA*, *lpxC*, and *lpxD*). The mutant *pmrAB* genes are involved in LPS modification by adding 4-amino-4-deoxy-L-arabinose (Ara4N) and/or phosphoethanolamine (pEtN) to lipid A.[23,28,29] In MALDI-TOF analysis, we observed the expected spectra ion peaks of 1728 m/z and 1912 m/z in all three isolates, AB001, AB002, and AB003. However, the peaks of 2034 m/z, 2157 m/z, 2261 m/z, and 2384 m/z were detected only in the AB003 strain, which contained the *pmrB* mutation. Possibly, these peaks were generated by the addition of one or two pEtN (123 m/z) residues to *bis*-phosphorylated hepta- and octa-acylated lipid A molecules. These modifications should affect the electrostatic interaction of certain cationic antimicrobial-peptide resistance determinants with the bacterial cell surface, and act as one of the colistin-resistant mechanisms.[30,31]

In our study, the AB003 acquired a mutation in the *pmrB* gene after five days of colistin therapy. It should be a short period of time to obtain a resistance, but colistin resistant *A. baumannii* were collected from patients who received colistin therapy for 7–19 days.[26,32]

The AB003 strain also harbored the *armA* gene and showed resistance to amikacin (<9 mm of disk diffusion test). After admission, the patient did not received aminoglycoside agents, such as tobramycin and amikacin. Therefore, it is highly likely that the *armA* is located on plasmids was incidentally transferred from another *A. baumannii* clone.[18,33]

In our study, all three isolates showed identical *Sma*I-macrorestriction patterns by PFGE and identified as an identical ST, ST 138 (1-3-3-2-2-50-3), indicating that they are a clone. All three isolates shared the *gyrA* gene with a same mutation, and they carried identical resistance genes IS*Aba1*-*bla*$_{AmpC}$ and IS*Aba1*-*bla*$_{OXA-23}$. However, AB003 carried only the *pmrB* gene with a mutation of Glu229Asp and the *armA* gene. Therefore, AB003 might acquire resistance to colistin and aminoglycosides by *in vivo* selection during colistin treatment from the XDR parental strains (AB001 and AB002).

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