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In vivo emergence of colistin resistance in *Acinetobacter baumannii* clinical isolates of sequence type 357 during colistin treatment☆,☆☆

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ABSTRACT

This study was performed to investigate the mechanisms of in vivo acquisition of colistin resistance in *A. baumannii* during colistin treatment. Three colistin-susceptible/resistant pairs of *A. baumannii* were recovered from patients who underwent colistin treatment. All of the 6 isolates included in this study shared an identical sequence type (ST), ST375, and they showed identical *SmaI*-macrorestriction patterns by pulsed-field gel electrophoresis. The individual colistin-resistant isolates harbored distinct mutations in the *pmrB* gene. Mutations detected in the *pmrB* gene were Ala227Val, Pro233Ser, and frame shift from Phe26. In matrix-assisted laser desorption ionization–time of flight analysis, colistin-resistant isolates were different from their colistin-susceptible counterparts, and they showed additional distinct peaks at 1852 m/z, 1937 m/z, 1954 m/z, 1975 m/z, 2034 m/z, and 2157 m/z. In vivo selection of colistin-resistant *A. baumannii* occurred independently in strains of ST357 during colistin treatment, and the strains acquired colistin resistance via mutations in the *pmrB* gene resulting in modification of lipid A components.

1. Introduction

The extensive use of antimicrobial agents, combined with the propensity of *Acinetobacter baumannii* to accumulate antimicrobial resistance, has resulted in the development of multi-drug resistant (MDR), extreme-drug resistance (XDR), and pan-drug resistant (PDR) *A. baumannii* (Falagas and Karageorgopoulos, 2008; Gordon and Wareham, 2010; Peleg et al., 2008). The persistent increase of these *A. baumannii* is a global issue. Highly resistant *A. baumannii* is considered to be a serious threat in clinical practice because the limited therapeutic options. XDR *A. baumannii* are non-susceptible to at least 1 agent in all but 2 or fewer antimicrobial categories, and anti-microbial agents such as extended-spectrum cephalosporins, carbapenems, penicillins with beta-lactamase inhibitors, folate pathway inhibitors, tetracyclines, and fluoroquinolones have shown poor performances against XDR *A. baumannii* (Magiorakos et al., 2012). Therefore, polymyxin B and polymyxin E (colistin) have been introduced in clinical practice to treat highly resistant *Acinetobacter* infections due to the lack of new antibiotics (Durante-Mangoni and Zarrilli, 2011; Falagas and Kasiakou, 2005).

Polymyxins are cyclic peptides with long hydrophobic tails. Having a positive charge, these drugs displace Mg^{2+} or Ca^{2+} ions and bind to the lipid A components of lipopolysaccharide (LPS), resulting in changes to the outer membranes of bacteria (Falagas and Kasiakou, 2005; Falagas et al., 2010; Newton, 1956; Schindler and Osborn, 1979). Polymyxins are selectively active against Gram-negative pathogens including *A. baumannii*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella* spp., and other Enterobacteriaceae. However, due to concerns over nephrotoxicity and neurotoxicity, the use of polymyxins has been limited (Falagas et al., 2010).

Resistance mechanisms against polymyxins vary among different pathogens and include modifications of the bacterial outer membrane, reductions in the overall negative charge of the LPS, overexpression of efflux pump systems, overproduction of capsule polysaccharide, and colistinase production (Bengoechea and Skurnik, 2000; Campos et al., 2004; Falagas et al., 2010; Ito-Kagawa and Koyama, 1980; Kline et al., 2008; Raetz and Whitfield, 2002; Schindler and Osborn, 1979). Previous in vitro studies have shown that colistin resistance in *A. baumannii* is mediated by a complete loss of LPS production via mutations in LPS-producing genes (*lpxA*, *lpxC*, and *lpxD*) or by modification of lipid A components of LPS via mutations in the *pmrA* and *pmrB* genes encoding 2-component signaling proteins (Adams et al., 2009; Moffatt et al., 2010), but any mutations in LPS-producing genes and a complete loss of LPS production were not reported in vivo studies

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with clinical isolated *A. baumannii* from patients who have been treated with colistin.

The exact mechanisms of in vivo selection of colistin-resistant *A. baumannii* are not well understood. Here, we studied the alterations occurring in *A. baumannii* as they changed from colistin-susceptible strains to colistin-resistant counterparts during colistin therapy in three Korean patients.

2. Materials and methods

2.1. Patient information

Colistin-susceptible/resistant pairs of *A. baumannii* were recovered from 3 separated patients who were first infected by colistin-susceptible *A. baumannii* strains and who underwent intravenous colistin therapy for at least 7 consecutive days.

2.1.1. Case 1

A 75-year-old man with diffuse large B cell lymphoma was admitted for his second round of chemotherapy on December 3, 2010, and received the chemotherapy on the following day. On day 11, he developed fever and chemotherapy-induced neutropenia, so treatment with cefepime 500 mg/d was started. On day 14, antibiotics were changed to equal quantities of imipenem and cilastatin (500 mg/6 h) to treat persistent neutropenic fever and the newly developed pneumonia. On day 31, he was transferred to the intensive care unit (ICU) as the symptoms of pneumonia became aggravated. On day 39, MDR *A. baumannii* was first isolated from a respiratory tract specimen, and we used the colistin-susceptible MDR *A. baumannii* isolate AB0019, which was recovered from this patient on day 41, for this study. Intravenous administration of colistimethate sodium (300 mg/d) was started on day 42. After 7 days, colistin-resistant *A. baumannii* isolate AB0023 was isolated from a sputum specimen from this patient. On day 54, antibiotics were changed to meropenem (500 mg/8 h), but chest X-ray and computerized tomography findings showed persistent pneumonia. Colistin-resistant *A. baumannii* strain was recovered again from a sputum specimen that was collected on day 109. Finally, the patient died of uncontrolled septic shock on day 111 (Table 1).

2.1.2. Case 2

A 47-year-old woman with systemic lupus erythematosus was admitted on December 30, 2010. She was transferred to the ICU on day 10 for the management of a seizure event, pulmonary alveolar hemorrhage, and acute renal failure. MDR *A. baumannii* isolate AB0026 was isolated from her sputum specimen on day 25. Treatment with intravenous colistimethate sodium (300 mg/d) started on day 28. After 19 days, colistin-resistant *A. baumannii* isolates were recovered from her sputum and urine specimens. On day 55, antibiotics were changed to linezolid (600 mg/12 h), cefoperazone/sulbactam (2 g/8 h), doxycycline (100 mg/12 h), and rifampin (450 mg/d), but colistin-resistant *A. baumannii* isolates were persistently recovered from sputum samples until day 85, and isolate AB0111, which was recovered from her sputum on day 73, was used for this study. Her family refused further treatments and then she was discharged on day 127 (Table 1).

2.1.3. Case 3

A 65-year-old man with hepatocellular carcinoma was admitted on December 27, 2010. On day 15, MDR *A. baumannii* was isolated from a respiratory tract specimen, and rifaximin and piperacillin/tazobactam were prescribed. He was transferred to the ICU on day 22 for the management of acute kidney injury and MDR *A. baumannii* pneumonia. Antibiotics were changed to meropenem on day 28. For this study, we used the colistin-susceptible MDR isolate AB0028, which was recovered from the patient's sputum specimen on day 26. On day 33, MDR *A. baumannii* strain was isolated from a sample of the patient's blood. Intravenous colistimethate sodium (300 mg/d) therapy was started on day 53. Because of the elevated creatinine level of this patient (1.87 mg/dL), the daily dose of colistimethate sodium was changed from 300 mg/d to 150 mg/d on day 55. Colistin-resistant *A. baumannii* strain AB0075 was isolated from a urine specimen from this patient on day 60. Symptoms were not improved, and he died of hepatic encephalopathy on day 72 (Table 1).

Table 1
Characteristics of the patients with colistin-resistant *A. baumannii* infection.

Variable	Case 1	Case 2	Case 3
Age/gender	75/male	47/female	65/male
APACHE II score[a]	24	29	25
CRP[b], mg/L	174.96	19.3	1.53
WBC count, $\times 10^9$/L[b]	11.83	10.65	4.35
Mechanical ventilation	applied	applied	applied
Colistin therapy			
Daily dose (mg/d)	300	300	300/150
Daily dose (mg/kg/d)	4.62	6	5.88/2.94
Concomitant drug	Teicoplanin, 200 mg/d	Teicoplanin, 200 mg/d	Teicoplanin, 200 mg/d
Duration of colistin therapy[c]	7 d	19 d	8 d
Length of hospital stay (d)	111	127	72
Length of ICU stay (d)	89	117	50
Time to death (d)[d]	89	improved	50

[a] APACHE II score was evaluated with the data collected during the initial 24-hour periods of ICU admission.
[b] C-reactive protein and white blood cell were evaluated on the first day of ICU admission.
[c] Duration of colistin therapy before the emergence of colistin-resistant strains.
[d] Time to death (days) indicates the period of time from the first day of ICU admission until death.

2.2. Strains

This study included 3 colistin-susceptible MDR *A. baumannii* isolates (AB0019, AB0026, and AB0028) and their colistin-resistant counterparts (isolates AB0023, AB0111, and AB0075, respectively) (Table 2). The isolates were identified as *A. baumannii* by Vitek GNI system (bio-Mérieux, Marcy l'Etoile, France) and sequencing of RNA polymerase β-subunit (*rpoB*) gene (La Scola et al., 2006). PCR experiment of the bla_{OXA-51}–like gene was performed as described previously (Woodford et al., 2006). *A. baumannii* ATCC 19606 was used as a reference strain.

2.3. Antimicrobial susceptibility testing

Antimicrobial susceptibilities were tested using antibiotic-containing discs (Becton Dickinson, Sparks, MD, USA) on Mueller-Hinton agar (Difco Laboratories, Detroit, MI, USA). MICs of meropenem and imipenem were determined by agar dilution method, and the MIC of colistin was determined by E-test (bio-Mérieux) according to the Clinical and Laboratory Standards Institute guidelines (Schindler, Osborn). PCR and sequencing experiments were performed to detect the bla_{OXA-23} gene (Woodford et al., 2006) and the presence of the *armA* gene also evaluated (Cho et al., 2009).

2.4. Multi-locus sequence typing (MLST)

PCR and sequencing experiments were performed on the 7 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 (Bartual et al., 2005; Lee et al., 2011). Nucleotide sequences of both strands were determined. Allelic profiles of the 7 housekeeping genes and sequence types (STs) of the strains were