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A novel variant of the β-lactamase *ADC-61* gene in multi-drug resistant *Acinetobacter baumannii*

Y. Zhou, S.-J. Teng, L. Yang, S.-B. Li and Y. Xu

Clinical Laboratory Department,
The Second People's Hospital of Lianyungang City, Lianyungang, Jiangsu, China

Corresponding author: S.-J. Teng
E-mail: shijieteng@yeah.net

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ABSTRACT. The aim of this study was to investigate the existence of a β-lactamase gene in a group of multi-drug resistant *Acinetobacter baumannii*. Twenty strains of multi-drug resistant *A. baumannii* were isolated. Thirty-four β-lactamase genes and the *ISaba1-OXA-23* linkage were analyzed in these strains by polymerase chain reaction (PCR) and verified by DNA sequencing. Three kinds of β-lactamase genes (*TEM*, *ADC*, and *OXA-23*) were identified, among which the sequence of strain No. 20, *ADC*, was different from *ADC* subtypes recorded by GenBank, and was identified as a new variant of β-lactamase genes (named *ADC-61* and registered in GenBank: accession No. JQ753702); all the other 19 strains were *ADC-30*. Eighteen strains of the *OXA-23* group were all positive as indicated by detection of *ISaba1-OXA-23* linkage. Gene sequencing indicated that the *TEM* gene was *TEM-1*. These results suggest that the three kinds of β-lactamase genes identified in this study, *TEM*, *ADC*, and *OXA-23*, play a key role in drug resistance in this group of *A. baumannii*. To our knowledge, this is the first report of an

emergent new mutation of the β-lactamase gene, *ADC-61*, in China or abroad.

Key words: *Acinetobacter baumannii*; Multi-drug resistant; *ADC-61*; β-lactamase gene; Novel variant

INTRODUCTION

Acinetobacter baumannii, an important opportunistic pathogen, is a species of glucose-non-fermenting, Gram-negative bacteria that exists extensively in nature and in humans. The prevalence of a multi-drug resistant strain of *A. baumannii,* which has become one of the main pathogenic bacteria causing nosocomial infection, is increasingly due to the wide use of broad-spectrum antibiotics in recent years (Fishbain and Peleg, 2010; Gordon and Wareham, 2010). The increasing clinical separation rate and drug resistance of *A. baumannii* have attracted extensive medical attention around the world (Munoz-Price and Weinstein, 2008; Peleg et al., 2008).

According to previous reports, the mechanisms underlying the resistance of *A. baumannii* to β-lactam antibiotics can be described as follows: 1) generation of A-D types of β-lactamase that hydrolyze β-lactam drugs (Bush, 2010; Ogbolu et al., 2011); 2) the outer membrane protein (OMP) variants with molecular weights of 29 and 43 kDa are related to carbapenem resistance (Limansky et al., 2002; Dupont et al., 2005); 3) target sites of β-lactam drugs, or penicillin-binding proteins (PBPs) change, e.g. the resistance of *A. baumannii* to carbapenems correlates with downregulation of PBPs (Perez et al., 2007; Zarrilli et al., 2009); and 4) it has been reported that overexpression of efflux pumps, such as adeABC, adeIJK, and adeFGH on the inner membrane, is associated with increasing minimal inhibitory concentration (MIC) of some β-lactam drugs (Cortez-Cordova and Kumar, 2011; Coyne et al., 2011). Recent research in China and abroad has primarily focused on *A. baumannii* strains carrying β-lactamase genes, and new genotypes have been discovered continually, such as metal beta-lactamase (*SIM-1*) (Lee et al., 2005), carbapenems hydrolyzing extended spectrum beta-lactamases (ESBL) (*GES-11*) (Moubareck et al., 2009), first carbenicillin hydrolyzing (CARB) type ESBL enzyme (*CARB-10/RTG-4*) (Potron et al., 2009), and extended-spectrum AmpC β-lactamases (*ADC-33*) (Rodríguez-Martínez et al., 2010). Most research, however, has focused on only one or a few β-lactam genes in *A. baumannii,* so more research is needed regarding the features of β-lactam at the genome level to understand the regional characteristics of gene variation. In our study, we detected 34 kinds of β-lactam gene variants in locally isolated multidrug-resistant *A. baumannii* strains, and investigated whether there was a linkage between the *ISaba1* and *OXA-23* genes.

Drug resistance of bacteria and the genes they carry display regional differences. The changes in drug resistance seen in China attracts our attention to the issue of genovariation. Therefore, we investigated β-lactamase genes in local multi-drug resistant *A. baumannii* to study the gene mutations, with the aim to better understand the prevalence of *A. baumannii* drug resistance, and to provide guidance for new drug development, as well as to facilitate anti-infection treatment in the clinic, which permits a cure for the patients through rational application of antimicrobial agents. The results of this research are presented in the following sections.

MATERIAL AND METHODS

Bacterial strain isolation

More than twenty strains of *A. baumannii* with multi-antibiotic resistance were isolated from the sputum specimens of patients from the Second People's Hospital, Lianyungang, Jiangsu Province, China between January and December of 2011. This study was conducted in accordance with the Declaration of Helsinki and with approval from the Ethics Committee of the Second People's Hospital of Lianyungang. Written informed consent was obtained from all participants.

Bacterial strain identification

As *A. baumannii* and *A. calcoaceticus* cannot be distinguished by biochemical methods, automatic bacterial identification and drug susceptibility testing were carried out using the MicroScan WalkAway 96SI system (Siemens Healthcare USA, Malvern, PA, USA). The *gyrA* and *parC* genes of the strains isolated were amplified using standard reaction conditions (AmpliTaq core reagents, ABI, Carlesbad, CA, USA) with 0.6 μM of each primer and the following reaction parameters: 5 min at 95°C, 30 cycles (94°C for 15 s, 50°C for 30 s, 72°C for 30 s), 7 min at 72°C. Amplicons were sequenced according to the standard protocol of gene sequencing, and sequences compared on the NCBI website using the Basic Local Alignment Search Tool for nucleotides (BLASTn). *A. baumannii* strains were verified and selected as the experimental group. The *gyrA* primer (P1: 5'-AAA TCT GCC CGT GTC GTT GGT-3', P2: 5'-GCC ATA CCT ACG GCG ATA CC-3'), and *parC* primers (P1: 5'-AAA CCT GTT CAG CGC CGC ATT-3', P2: 5'-AAA GTT GTC TTG CCA TTC ACT-3') were provided by Huma Bioinformatics Workshop, New District, Wuxi.

Drug sensitivity testing

For drug sensitivity testing, we utilized the NC31 Identification plate from Siemens Healthcare. The minimum inhibitory concentration (MIC) method was employed to acquire preliminary results of the drug sensitivity of the isolated strains against fourteen antibiotics recommended by the Clinical Laboratory Standards Institute (CLSI). Disk diffusion was subsequently performed to verify the multi-drug resistance status. The sensitivity of antibiotic resistant strains was judged according to CLSI 2010. Test drugs included piperacillin, cefotaxime, ceftazidime, ceftriaxone, cefepime, imipenem, ampicillin/sulbactam, ticarcillin/clavulanic acid, gentamicin, tobramycin, amikacin, ciprofloxacin, levofloxacin, and compound sulfamethoxazole. Drug sensitivity discs and M-H medium were both obtained from Oxoid (Basingstoke, Hampshire, United Kingdom).

Bacterial culture

Single colonies of bacteria were picked up from a pure culture and placed into a 0.5 mL Eppendorf tube with 400 μL fresh 200 ng/mL proteinase K solution. Samples were incubated in a 56°C water bath for 2 h to digest the bacterial cell membrane and expose the genomic DNA, followed by incubation in a 95°C water bath for 10 min to inactivate the

proteinase K. After centrifugation at 15,000 rpm for 30 s, the supernatant was isolated as the template solution for genetic testing, and reserved at -20°C for further experiments.

Genetic testing

Twenty strains of *A. baumannii* were analyzed for the presence of 34 β-lactamase genes (A-D class) and *ISaba1-OXA-23* linkage by the polymerase chain reaction (PCR). The PCR test kit and positive controls were provided by the Cloning and Genetic Technology Institute, Wuxi, China, and experiments were conducted following the protocol provided. All PCR primers were designed and authorized by the Huma Bioinformatics Workshop, New District, Wuxi, China. Primer sequences and the corresponding amplicon lengths are listed in Table 1.

Gene sequencing

Amplicons from positive PCR experiments were sequenced using a model 3730 Capillary Automatic Sequencing Instrument from ABI (Applied Biosystems, Inc., Foster City, CA, USA) by the Boshang Biotechnology Limited Company, Shanghai, China.

Gene identification

Gene sequences were read and compared using BLAST searches (NCBI) with the Chromas software (technelysium.com.au).

RESULTS

Drug sensitivity testing

Twenty strains of multi-drug resistant *A. baumannii* were tested against fourteen antibiotics by two drug sensitivity testing methods. All twenty strains were completely resistant to piperacillin, ceftazidime, ceftriaxone, cefepime, and imipenem; and 90% resistant to aminoglycosides, fluoroquinolones, and bactrim. Twenty strains of *A. baumannii* were simultaneously resistant to more than three antibiotics with different structures, which accorded with the standard for multiple drug-resistant bacteria.

β-Lactamase genetic testing

Twenty strains of multi-drug resistant *A. baumannii* were analyzed by PCR and three kinds of β-lactamase genes, including *TEM*, *ADC*, and *OXA-23*, were identified with detection rates of 85.0% (17/20), 100% (20/20), and 90.0% (18/20), respectively. There were sixteen strains carrying three genes, nineteen strains carrying two genes, and one strain carrying a single β-lactamase gene. The PCR products of the *ADC* genes were sequenced, from which it was identified that strain No. 20 was different from the *ADC* subtypes recorded by GenBank; this was identified as a new variant of β-lactamase gene, named *ADC-61*, and registered in GenBank (accession No. JQ753702); all of the other nineteen strains were *ADC-30*. The molecular evolution of the sequence of the *ADC-61* gene and the associated fraction of *ADC* subtype genes is shown in Figure 1, the cladogram was generated using the software tool for

Table 1. Primer sequences for β-lactamase PCR and amplicon length.

Classification	Gene name	Primer sequence (5'→3')	Product length (bp)
Class A β-lactamase	TEM	P1: AGGAAGAGTATGATTCAACA	535
		P2: CTCGTCGTTTGGTATGGC	
	SHV	P1: TGCGCAAGCTGCTGACCAGC	305
		P2: TTAGCGYTGCCAGTGCTCGA	
	CTX-M-1 group	P1: ATGGTTAAAAAATCACTGCGYCAGTTC	876
		P2: TCACAAACCGTYGGTGACGATTTTAGCCGC	
	CTX-M-2 group	P1: ATGATGACGCAGAGCATTCGCCGCTCA	876
		P2: TCAGAAACCGTGGGTTACGATTTTCGC	
	CTX-M-8 group	P1: ATGATGAGACATCGCGTTAAGCGG	876
		P2: TTAATAACCGTCGGTGACGATTTTCGCG	
	CTX-M-9 group	P1: ATGGTGACAAAGAGAGTGCAACGG	876
		P2: TTACAGCCCTTCGGCGATGATTCTCGC	
	CTX-M-25 group	P1: ATGATGAGAAAAAGCGTAAGGCGGGCG	876
		P2: TTAATAACCGTCGGTGACAATTCTGGC	
	PER	P1: AGTCAGCGGCTTAGATA	978
		P2: CGTATGAAAAGGACAATC	
	GES	P1: ATGCGCTTCATTCACGCAC	846
		P2: CTATTTGTCCGTGCTCAGG	
	VEB	P1: GCGGTAATTTAACCAGA	961
		P2: GCCTATGAGCCAGTGTT	
	CARB	P1: AAAGCAGATCTTGTGACCTATTC	588
		P2: TCAGCGCGACTGTGATGTATAAAC	
	RTG	P1: TATGTCTCACGCTATCATTAAATGC	338
		P2: ATAATGTGGCCTGACACAGCTCT	
	KPC	P1: ATGTCACTGTATCGCCGTCTA	882
		P2: TTACTGCCCGTTGACGCCCAA	
	SCO	P1: ATGACAAGATCTGCCCTTTTGAT	882
		P2: TTATTCCAGAACTTCGGCAGCA	
Class B β-lactamase	IMP	P1: CGGCCKCAGGAGMGKCTTT	587
		P2: AACCAGTTTTGCYTTACYAT	
	VIM	P1: ATTCCGGTCGGMGAGGTCCG	633
		P2: GAGCAAGTCTAGACCGCCCG	
	SPM	P1: CTGCTTGGATTCATGGGCGCG	786
		P2: CCTTTTCCGCGACCTTGATCG	
	GIM	P1: CCTGTAGCGTTGCCAGCTTTA	562
		P2: CAGCCCAAGAGCTAATTGAGG	
	SIM	P1: ACAAGGGATTCGGCATCGTT	355
		P2: TTATCTTGAGTGTGTCCTGG	
	AIM	P1: CGTCGCTTCACCCTGCTGGGCAGC	535
		P2: AGGCGAGGCGACCGCCGTCAGGCC	
	NDM	P1: TCAGCGCAGCTTGTCGGCCATGCG	813
		P2: GCAACCGCGCCCAACTTTGGCCCG	
	KHM	P1: ATGAAAATAGCTCTTGTTATATCG	726
		P2: TCACTTTTTAGCTGCAAGCGCTTC	
	DIM	P1: ATGAGAACACATTTTACAGCGTTA	756
		P2: TCAATCAGCCGACGCGTTAGCGTT	
	TMB	P1: TATGCCTCAGCGCTGACTAAT	400
		P2: TCAGCGGTCGCCGTGATTGGC	
Class C β-lactamase	DHA group	P1: AACTTTCACAGGTGTGCTGGGT	405
		P2: CCGTACGCATACTGGCTTTGC	
	ADC	P1: ATGCGATTTAAAAAAATTTCTTGTYTA	1152
		P2: CTAAGASTTGGTCRAARGGT	
Class D β-lactamase	OXA-1 group	P1: CTGTTGTTTGGGTTTCGCAAG	440
		P2: CTTGGCTTTTATGCTTGATG	

Continued on next page

Table 1. Continued.

Classification	Gene name	Primer sequence (5'→3')	Product length (bp)
	OXA-2 group	P1: CAGGCGCYGTTCGYGATGAGTT P2: GCCYTCTATCCAGTAATCGCC	233
	OXA-10 group	P1: GTCTTTCRAGTACGGCATTA P2: GATTTTCTTAGCGGCAACTTA	822
	OXA-20 group	P1: TTGATAATCCGATTTCTAGCAC P2: CTAGTTGGGTGGCAAAGCAT	801
	OXA-23 group	P1: ATGAATAAATATTTTACTTGCTATGTG P2: TTAAATAATATTCAGCTGTTTTAATGA	822
	OXA-24 group	P1: CAAGAGCTTGCAAGACGGACT P2: TCCAAGATTTTCTAGCRACTTATA	420
	OXA-51 group	P1: ATGAACATTAAAGCACTCTTACTT P2: CTATAAAATACCTAATTGTTCTAA	825
	OXA-58 group	P1: TCGATCAGAATGTTCAAGCGC P2: ACGATTCTCCCCTCTGCGC	530
Gene linkage test	ISaba1 OXA-23	P1: GATGTGTCATAGTATTCGTCG P2: TCACAACAACTAAAAGCACTG	Variable

PCR = polymerase chain reaction.

molecular evolution, MEGA5.0 (www.megasoftware.net); this analysis revealed that subtype 61 of *ADC* had the strongest homology to subtypes 56, 30, 60, 57, and 59. Eighteen strains of the *OXA-23* group were all positive as indicated by detection of *ISaba1-OXA-23* linkage. Gene sequencing confirmed the PCR product amplified from *TEM* as *TEM-1*.

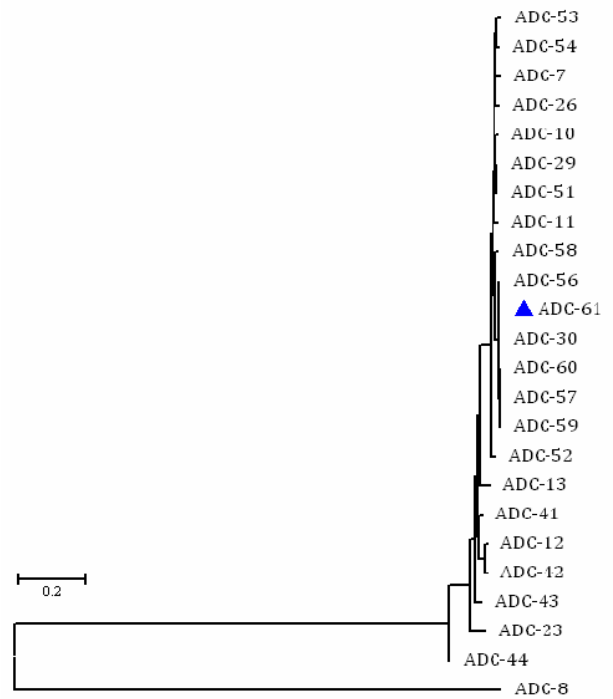

Figure 1. Molecular evolution of the *ADC-61* gene sequence and the corresponding fraction of *ADC* gene subtypes.

DISCUSSION

The resistance of *A. baumannii* to anti-bacterial drugs has become increasingly serious and has resulted in great difficulties in the fight against infections in the clinic (Shahcheraghi et al., 2011; Doi, 2012). Twenty strains of multi-drug resistant *A. baumannii* isolated from sputum specimens of patients were found to be resistant to commonly used cephalosporin drugs including imipenem, as well as to cephalosporins in compound preparation, and were also found to be 90% resistant to aminoglycosides, fluoroquinolones, and bactrim, indicating the severe drug resistance of bacterial strains isolated for our research.

Twenty strains of *A. baumannii* were analyzed by PCR and DNA sequencing for 34 β-lactamase genes (A-D class); *TEM*, *ADC*, and *OXA-23* were identified in the isolated strains, with detection rates of 85.0, 100, and 90.0%, respectively. *TEM* genes we detected were *TEM-1*. The *TEM-1* gene, belonging to class A β-lactamases, is a penicillin enzyme, and only contributes marginally to the resistance to the third and fourth generations of cephalosporin and carbapenems drugs. *ADC*, a specific AmpC enzyme of *A. baumannii*, belongs to class C β-lactamases, thus earning its name as acinetobacter-derived cephalosporinases (ADC) (Figueiredo et al., 2009a). This type of enzyme hydrolyzes penicillins, the first and third generations of cephalosporins, and monocyclic β-lactam antibiotics, which cannot be inhibited by classic β-lactamase inhibitors (Hujer et al., 2005). New variants of the AmpC enzyme have been continuously discovered, such as carbapenems hydrolyzing extended-spectrum AmpC β-lactamases (ADC-33) (Rodríguez-Martínez et al., 2010), extended-spectrum AmpC β-lactamases (ADC-56) that hydrolyze the fourth generation of cephalosporins, cefepime (Tian et al., 2011), and ADC-57, whose ability to hydrolyze ertapenem was identified through calculation of binding free energy during molecular docking (Zhou et al., 2012). The strains in our study carried variants of the *ADC* gene, among which the variant carried by strain No. 20 was different from *ADC* subtypes recorded in GenBank and was identified as a new variant of β-lactamase genes (named *ADC-61* and registered in GenBank). The molecular evolution of the *ADC-61* gene sequence and the corresponding fraction of *ADC* subtype genes are shown in Figure 1.

OXA-23 is class D carbapenem-hydrolyzing β-lactamase, which can be mildly inhibited by β-lactamase inhibitors. The OXA-23 enzyme has been found in plasmids and is prevalent all around the world including in China (Kim et al., 2012). Our study reports the first *OXA* gene to be found in *A. baumannii*. As a general mechanism, the insertion of genetic sequence carrying its own promoter into the genome often results in overexpression of adjacent genes; for example, it has been reported that insertion of sequence in *A. baumannii* leads to overexpression of class C and D β-lactamase genes (Héritier et al., 2006; Figueiredo et al., 2009a,b). The eighteen strains of isolated *A. baumannii* in the *OXA-23* group were all positive for this gene as indicated by detection of *ISaba1-OXA-23* linkage, which demonstrated that *OXA-23* expression was mediated by insertion of *ISaba1* sequence.

Due to the high positive rate for *ADC* and *OXA-23* genes carried by multi-drug resistant strains of *A. baumannii*, we speculated that the *ADC* and *OXA-23* types of β-lactamases provide a greater contribution to the resistance to the third and fourth generations of cephalosporin and carbapenems drugs.

The mechanism underlying the resistance of *A. baumannii* to β-lactam antibiotics is not only reliant upon the presence of β-lactamase. Therefore, the next direction of our research

will be to analyze the 29 and 43 kDa variants of the OMP protein, and the corresponding changes of target sites for the β-lactam PBP drugs.

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