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Multidrug-resistant genes of aminoglycoside-modifying enzymes and 16S rRNA methylases in *Acinetobacter baumannii* strains

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ABSTRACT. We examined the distribution of genes of aminoglycoside-modifying enzymes and 16S rRNA methylases in multidrug-resistant *Acinetobacter baumannii* to explore the association of these genes with drug resistance. Strains isolated from clinical specimens were screened using an automatic microbial identification system, and 9 aminoglycoside-modifying enzyme and 6 16S rRNA methylase genes were analyzed using polymerase chain reaction and verified by DNA sequencing. Next, sequence alignment was carried out using the Chromas software and a susceptibility test was performed using the Kirby-Bauer disk diffusion method. Genes encoding aminoglycoside-modifying enzymes were detected in all 20 strains of multidrug-resistant *A. baumannii*. The positive rates of *aac(3')-I*, *aac(6')-Ib*, *ant(3'')-I*, and *aph(3')-I* were 90.0, 90.0, 85.0, and 35.0%, respectively. However, genes encoding 16S rRNA methylases were not positively detected in the 20 strains of multidrug-resistant *A. baumannii*. The resistance of multidrug-resistant *A. baumannii*

may be associated with aminoglycoside-modifying enzyme genes but not with 16S rRNA methylase genes.

Key words: *Acinetobacter baumannii*; 16S rRNA methylases; Aminoglycoside-modifying enzymes; Multidrug-resistant

INTRODUCTION

Acinetobacter baumannii is a parasite that is widely distributed in nature and in the human body. Because of extensive application of broad-spectrum antibiotics, an increasing number of multi-drug resistant strains of *A. baumannii* have appeared, which have become important pathogens of nosocomial infection (Fishbain and Peleg, 2010; Gordon and Wareham, 2010; Hou et al., 2012). These strains show strong resistance to the external environment and can acquire multidrug resistance quickly by carrying many movable genetic elements for multiple resistance genes (Golanbar et al., 2011; Taherikalani et al., 2011). The increasing occurrence of drug-resistant *A. baumannii* has caused concern in global medicine (Baumgart et al., 2010; Tasbakan et al., 2011).

β-Lactams drugs are bactericides used in the multiplication stage, whereas aminoglycoside are bactericides used during the rest period. It is commonly understood that these 2 drugs have a synergistic effect. Thus, a combination of aminoglycosides (such as Gentamicin) and β-lactams (such as Imipenem) has been commonly used for treating *A. baumannii* infections. However, due to its production of OXA-23-type β-lactamase, imipenem-resistant *A. baumannii* has become a worldwide problem (Mugnier et al., 2010; Poirel et al., 2010; Kim et al., 2012). Along with the increasing rate of drug resistance to β-lactams, drug resistance to aminoglycoside is also increasing, and clinically isolated aminoglycoside-resistant strains have become common (Brigante et al., 2012). There are 2 main reasons for Gram-negative bacilli resistance to aminoglycosides. The first is that aminoglycosides entering bacteria are modified by aminoglycoside-modifying enzymes and become non-functional. The second is that methylation of target sites is induced by 16S rRNA methylases, leading to decreased affinity of 16S rRNA for aminoglycosides (Galimand et al., 2000; Davis et al., 2010; Zhou et al., 2010).

Regional differences in the drug resistance of bacteria have been observed; the resistance gene carried by some strains also varies from location to location. Genes encoding aminoglycoside-modifying enzymes and 16S rRNA methylase in multidrug-resistant *A. baumannii* should be examined to investigate their relationship to the development of drug resistance. In this study, 9 aminoglycoside-modifying enzyme genes and 6 16S rRNA methylase genes were investigated in 20 strains of multidrug-resistant *A. baumannii* isolated from the Lianyungang region in China. The objective of this study was to increase understanding of the prevalence of multidrug-resistant *A. baumannii* in this region, provide guidance for research and development of new drugs, and facilitate clinical anti-infection treatment and reasonable use of antimicrobial agents for early rehabilitation of patients.

MATERIAL AND METHODS

Collection of the strains

Twenty strains of multidrug-resistant *A. baumannii* were isolated from sputum

specimens of patients hospitalized in the Second People's Hospital, Lianyungang, Jiangsu, China, from January 2011 to December 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 City. Written informed consent was obtained from all participants.

Strain identification

Because biochemical identification methods cannot distinguish between *A. baumannii* and *Acinetobacter calcoaceticus*, strain identification was conducted using an automatic microbial identification system (Siemens Healthcare Diagnostics; Malvern, PA, USA) for initial screening and amplification of the *gyrA* and *parC* genes using a polymerase chain reaction (PCR) technique in this study. After DNA sequencing of the PCR products and matching their sequences against the National Center for Biotechnology Information database (www.ncbi.nlm.nih.gov), the strains confirmed as *A. baumannii* were selected for further analysis.

Susceptibility test

The NC31 Gram-negative bacteria identification and susceptibility board (Siemens) was used to preliminarily assess the minimum inhibitory concentration (MIC) susceptibility results of the strains against the 14 drugs recommended by the Clinical and Laboratory Standards Institute (CLSI), including piperacillin, cefotaxime, ceftazidime, ceftriaxone, cefepime, imipenem, ampicillin/sulbactam, ticarcillin/clavulanic acid, gentamicin, tobramycin, amikacin, ciprofloxacin, levofloxacin, and compounded sulfamethoxazole. Subsequently, the Kirby-Bauer disk diffusion method (Oxoid Microbiology Products; Basingstoke, UK) was used to verify multidrug resistance. Antimicrobial susceptibility determination was based on the criteria outlined in the 2011 edition of the CLSI. The Mueller-Hinton (M-H) medium was obtained from Oxoid Microbiology Products.

DNA extraction

One single colony of each strain was picked from the purified culture and transferred into a 0.5-mL EP tube containing 400 μL 200 ng/mL freshly prepared proteinase K. After incubation at 56°C for 2 h to destroy the bacterial membrane and release the DNA, the tube was then incubated at 95°C for 10 min to inactivate proteinase K. Before PCR, all DNA samples were stored at -20°C.

Gene detection

Nine aminoglycoside-modifying enzyme genes and 6 16S rRNA methylase genes were detected using PCR according to manufacturer instructions of the PCR kit (Wuxi Institute of Cloning and Genetic Technology; Jiangsu, China). All gene-specific primers were designed and provided by Zuhuang Mi, Department of Bioinformatics, Wuxi Institute of Cloning and Genetic Technology, Jiangsu, China (Table 1).

Table 1. Primer design for detection of target genes.

Class	Gene	Primer	Product size (bp)
Aminoglycoside-modifying enzymes	*aac(3)-I*	P1: ACCTACTCCCAACATCAGCC; P2: ATATAGATCTCACTACGCGC	169
	aac(3)-II	P1: ACTGTGATGGGATACGCGTC; P2: CTCCGTCAGCGTTTCAGCTA	237
	aac(6')-I ad	P1: ATGATTAGAAAAGCAACTGTCCAAG; P2: TTAAAGTTGCTTTGTAAAACAAATC	435
	aac(6')-I b	P1: ATGACTGAGCATGACCTTGC; P2: TTAGGCATCACTGCGTGTTC	519
	aac(6')-II	P1: TTCATGTCCGCGAGCACCCC; P2: GACTCTTCCGCCATCGCTCT	178
	ant(3")-I	P1: TGATTTGCTGGTTACGGTGAC; P2: CGCTATGTTCTCTTGCTTTTG	284
	ant(2")-I	P1: GAGCGAAATCTGCCGCTCTGG; P2: CTGTTACAACGGACTGGCCGC	320
	ant(4')-I	P1: CGTGGAGCGATATCGATTTCG; P2: TCTGGTTCGGCGGCCGGATGC	266
	aph(3')-I	P1: ATGTGCCATATTCAACGGGAAACG; P2: TCAGAAAAACTCATCGAGCATCAA	816
16S rRNA methylases	*rmtA*	P1: CCTAGCGTCCATCCTTTCCTC; P2: AGCGATATCCAACACACGATGG	315
	rmtB	P1: TGCGGCGCATCCTGACTGAGGA; P2: GGGAAAGCTGACAGCCATGCGC	542
	rmtC	P1: ATGAAAACCAACGATAATTATC; P2: TTACAATCTCGATACGATAAAATAC	846
	rmtD	P1: ATGAGCGAACTGAAGGAAAAACTGCT; P2: TCATTTTCGTTTCAGCACGTAAAACAG	744
	armA	P1: ATGGATAAGAATGATGTTGTTAAG; P2: TTATTTCTGAAATCCACTAGTAATTA	774
	npmA	P1: TTGGGTACTGGAGACGGTAG; P2: CAGCTTTGTATTGTTCGCTC	421

DNA sequencing

Positive PCR products were sent to Shanghai Boshang Biological Technology, Ltd., Co. for DNA sequencing. An Applied Biosystems 3730xl DNA Sequencer (Applied Biosystems; Foster City, CA, USA) was used.

Sequence alignment

DNA sequences were read using the Chromas software and aligned using BLAST search tools.

RESULTS

Drug resistance of *A. baumannii*

The susceptibility of the 20 strains of *A. baumannii* against the 14 antibiotics was similar according to the results of two different susceptibility test methods (Table 2). All strains were fully resistant to piperacillin, ceftazidime, ceftriaxone, cefepime, and imipenem. The resistance rate against aminoglycosides, fluoroquinolones, and trimethoprim-sulfamethoxazole was more than 90.0%. For instance, the resistance of all 20 strains of *A. baumannii* against

gentamicin, tobramycin, amikacin, and other aminoglycoside drugs was more than 90%. All 20 strains of *A. baumannii* showed resistance to 3 or more different classes of antibiotics simultaneously; thus, they conformed to the criteria of multi-drug resistance.

Table 2. Susceptibility of the 20 strains of *Acinetobacter baumannii* to 14 antibiotics.

Drugs	Resistant (R%)	Intermediate (I%)	Sensitive (S%)
Piperacillin	20 (100.0	0 (0)	0 (0)
Cefotaxime	18 (90.0)	2 (10.0)	0 (0)
Ceftazidime	20 (100.0)	0 (0)	0 (0)
Ceftriaxone	20 (100.0)	0 (0)	0 (0)
Cefepime	20 (100.0)	0 (0)	0 (0)
Imipenem	20 (100.0)	0 (0)	0 (0)
Ampicillin/Sulbactam	19 (95.0)	1 (5.0)	0 (0)
Ticarcillin/Clavulanic acid	19 (95.0)	1 (5.0)	0 (0)
Gentamicin	18 (90.0)	2 (10.0)	0 (0)
Tobramycin	18 (90.0)	2 (10.0)	0 (0)
Amikacin	18 (90.0)	2 (10.0)	0 (0)
Ciprofloxacin	18 (90.0)	2 (10.0)	0 (0)
Levofloxacin	18 (90.0)	2 (10.0)	0 (0)
Trimethoprim+Sulfamethoxazole	18 (90.0)	2 (10.0)	0 (0)

Aminoglycosides-modifying enzyme genes in the 20 strains of *A. baumannii*

All 9 aminoglycoside-modifying enzyme genes were positively detected, among which the positive rates *of aac(3')-I*, *aac(6')-Ib*, *ant(3")-I*, and *aph(3')-I* were 90.0% (18/20), 90.0% (18/20), 85.0% (17/20), and 35.0% (7/20), respectively. One strain contained *aac(3')-I* and *aac(6')-Ib*, 2 strains contained only *aac(3')-I*, 2 strains contained *aac(6')-Ib*, *ant(3")-I*, and *aph(3')-I*, 5 strains contained *aac(3')-I*, *aac(6')-Ib*, *ant(3")-I*, and *aph(3')-I*, and 10 strains contained *aac(3')-I*, *aac(6')-Ib*, and *ant(3")-I* (Table 3). DNA sequencing results of the PCR products were confirmed using the BLASTn tool (www.ncbi.nlm.nih.gov/BLASTn) (Figure 1).

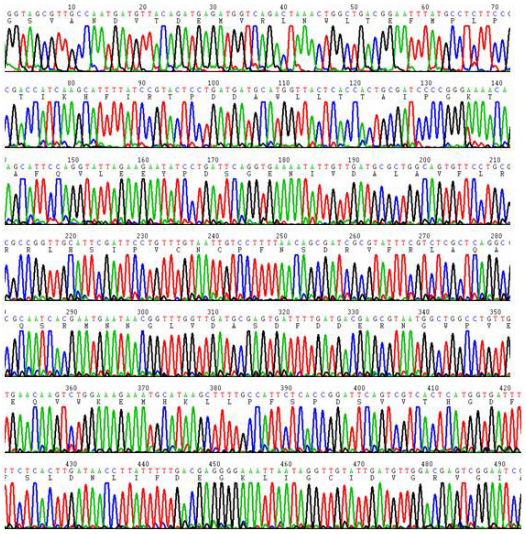

Figure 1. DNA sequence of the PCR product of the *aph(3')-I* gene in the #8 strain, confirmed by DNA alignment using the BLASTn tool (www.ncbi.nlm.nih.gov/BLASTn).

Table 3. Positive patterns of the aminoglycoside-modifying enzyme genes in *Acinetobacter baumannii*.

Gene-positive patterns	Positive number (%)
aac(3')-I + aac(6')-Ib + ant(3")-I	10 (50.0%)
aac(3')-I + aac(6')-Ib + ant(3")-I + aph(3')-I	5 (25.0%)
aac(6')-Ib + ant(3")-I + aph(3')-I	2 (10.0%)
aac(3')-I	2 (10.0%)
aac(3')-I + aac(6')-Ib	1 (5.0%)

16S rRNA methylase genes in the 20 strains of *A. baumannii*

None of the 6 16S rRNA methylase genes was detected positively in the 20 strains of *A. baumannii.*

DISCUSSION

Since aminoglycosides (Streptomycin) were first identified 70 years ago, aminoglycoside drugs have been commonly used for treatment. Most aminoglycosides are natural antibacterial substances produced by microorganisms, but a few, such as amikacin and isepamicin, are semi-synthetic derivatives of natural compounds. Aminoglycosides are broad-spectrum bactericidal agents. They have killing effects on most pathogens and opportunistic pathogens isolated clinically, except for anaerobes. This may be because aminoglycosides carry protons, which can easily interact with the negative charge on a bacterial cell surface, facilitating binding to nucleic acids. When increasing numbers of aminoglycosides bind to the A site of the bacterial ribosome, which is not only the aminoacyl-tRNA binding site, but also the codon-anticodon recognition region, mistranslation of mRNA into an amino acid sequence can occur, resulting in the production of abnormal proteins, and bacterial cell death.

The drug resistance of *A. baumannii* has become increasingly important and causes great difficulties in the clinical treatment of bacterial infections (Shahcheraghi et al., 2011; Doi, 2012; Wang et al., 2012). The 20 strains of *A. baumannii* analyzed in this study were isolated from sputum specimens from in-patients. They were all insensitive to the commonly used cephalosporins, including imipenem, and the cephalosporin complex preparations. Their resistance to aminoglycosides, fluoroquinolones, and trimethoprim-sulfamethoxazole reached greater than 90%, among which resistance against gentamicin, tobramycin, amikacin, and other aminoglycoside-based drugs was more than 90%. Thus, the resistance of the 20 strains of *A. baumannii* isolated in this study is very alarming. Garnacho-Montero and Amaya-Villar (2010) found that polymyxin has a clear curative effect on multidrug-resistant *A. baumannii*. However, a study by Moffatt et al. (2010) showed that *A. baumannii* has heterogeneous resistance to polymyxin, and that the heterogeneous resistance strain can partially recover its sensitivity to other antibacterial drugs. Therefore, polymyxin combined with β-lactam antibiotics or tigecycline is a potential alternative for inhibiting *A. baumannii*, but this requires confirmation through large-scale clinical studies.

Aminoglycoside-modifying enzymes can be divided into 3 categories according to their functions: 1) acetyltransferase (AACs), whose related genes include *aac(3)-I*, *aac(3)-II*, and *aac(6')-Ib*, among others, and can cause acetylation of free hydroxyl groups; 2) phosphotransferase (APH), including *aph(3')-I*, leads to phosphorylation of free hydroxyl groups;

3) nucleotide transferase (ANT), also known as adenosine transferase (AAD), includes *ant(3")-I* and *ant(2")-I*, which can lead to nucleosidation of free hydroxyl groups. More than 30 aminoglycoside-modifying enzyme genes and 7 16S rRNA methylase genes have been identified to date. In Europe, the most commonly observed aminoglycoside-modifying enzyme genes include *aac(3)-I*, *aac(3)-II*, *aac(6')-Ib*, *aph(3')-I*, and *ant(3")-I* (Nemec et al., 2004). In this study, we detected 9 aminoglycoside-modifying enzyme genes from all 20 strains of *A. baumannii.* We found that all of the 20 strains of *A. baumannii* carried at least one type of aminoglycoside-modifying enzyme gene, among which both *aac(3')-I* and *aac(6')-Ib* showed a positive rate of 90% (18/20), *ant(3")-I* showed a positive rate of 85% (17/20), and *aph(3')-I* showed a positive rate of 35% (7/20). These results are consistent with those of Nemec et al. (2004). Based on the positive patterns of the 20 strains of *A. baumannii* shown in Table 3, most *A. baumannii* possess ≥2 aminoglycoside-modifying enzyme genes, except for the 2 strains possessing only one gene, *aac(3')-I*. Seven 16S rRNA methylase genes have been previously identified, including *armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD*, *rmtE*, and *npmA* (Davis et al., 2010; Zhou et al., 2010). The *rmtE* gene was recently isolated from *Escherichia coli* from bovine (Davis et al., 2010). However, in the present study, the 6 16S rRNA methylase genes tested were undetected in the 20 strains of *A. baumannii.* This is different from the results of previous studies (Davis et al., 2010; Zhou et al., 2010), which may be related to regional differences.

A cluster analysis conducted by Chinese researchers showed that aminoglycoside-modifying enzyme genes and 16S rRNA methylase genes are often associated with genetic markers of moveable genetic elements (Kansakar et al., 2011), i.e., these genes are removable rear-mediated genetic elements (Wu et al., 2009; Asadollahi et al., 2011). This acquired resistance mechanism facilitates horizontal spread. Genomic studies have revealed that multidrug-resistant *A. baumannii* is rich in the efflux pump gene (Rajamohan et al., 2010; Zhou et al., 2011), and high expression of this gene plays an important role in the multidrug resistance of *A. baumannii* (Coyne et al., 2011). In future studies, mobile genetic elements and the efflux pump will be further investigated to determine their correlation with multidrug resistance.

In conclusion, the multidrug resistance of *A. baumannii* isolated from in-patients of our hospital was related to aminoglycoside-modifying enzyme genes but not to 16S rRNA methylase genes.

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