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Identification of aac(2')-I Type b aminoglycoside-modifying enzyme genes in resistant *Acinetobacter baumannii*

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ABSTRACT. The aim of this study was to investigate the mechanism underlying the drug resistance of *Acinetobacter baumannii* toward aminoglycosides. A total of 32 *A. baumannii* strains were identified by molecular identification and subsequently isolated. The isolates were then amplified by polymerase chain reaction to analyze the 9 aminoglycoside-modifying enzyme genes and 7 16S rRNA methylase genes. Five types of aminoglycoside-modifying enzyme genes and 1 type of 16S rRNA methylase gene were detected in the 32 drug-resistant *A. baumannii* strains. Positive genes included 7 detection modes, of which the all-6-gene-positive mode *aac*(*2'*)-*Ib*+*aac*(*3*)-*I*+*aac*(*6'*)-*Ib*+*ant*(*3''*)-*I*+*aph*(*3'*)-*I*+*armA* exhibited the largest number of strains (12, 37.5%). The resistance of *A. baumannii* against aminoglycosides resulted from the presence of 5 types of aminoglycoside-modifying enzyme genes and the 16S rRNA methylase gene *armA*. This study is the first to isolate the *aac*(*2'*)-

Ib aminoglycoside-modifying enzyme gene from *A. baumannii* in a domestic clinical setting.

Key words: 16S rRNA methylase gene; *Acinetobacter baumannii*; Aminoglycosides; Aminoglycoside-modifying enzyme gene; Molecular identification; Resistance

INTRODUCTION

According to the 2nd annual report of Ministry of Health National Antimicrobial Resistance Investigation Net, *Acinetobacter baumannii* is the most commonly isolated pathogenic bacteria in the intensive care unit (Xiao, 2010; Xiao et al., 2012). The monitoring report of another collaboration group (Wang et al., 2011) showed that the resistance rate of clinically isolated *A. baumannii* was 2-fold higher than clinically isolated *Pseudomonas aeruginosa* (Wang et al., 2011). The resistance of clinically isolated *A. baumannii* is continuously increasing and has triggered widespread concern in global medical communities (Perez et al., 2007; Munoz-Price and Weinstein, 2008; Peleg et al., 2008; Potron et al., 2009).

Bacteria can acquire drug resistance-related genes from movable genetic elements such as plasmids, integrons, and transposon and insertion sequences. Moreover, their own genes and the genes they carry (including resistance genes) can be transferred within the same bacterial species or to different bacterial strains; these phenomena led to rapid distribution of the resistance gene (Bennett, 2008; Potron et al., 2009). More than a decade has passed since the appearance of the multidrug-resistant *A. baumannii* in the mid-1990s, to the pan-drug-resistant *A. baumannii*, until the first appearance of the extreme drug-resistant *A. baumannii* (Wang et al., 2012b). Clinical experience and *in vitro* studies have both suggested that a combination of aminoglycosides and β-lactam drugs remains the most effective drug therapy for infective diseases caused by *A. baumannii* (Huang and Liu, 2013). However, resistance to β-lactams increases with increased resistance of the clinically isolated *A. baumannii* against aminoglycosides. National monitoring has shown that the resistance of clinically isolated strains of *A. baumannii* against aminoglycosides has remained high for 2 consecutive years (Xiao et al., 2012).

The resistance mechanism of *A. baumannii* against aminoglycosides is thought to mainly involve the acquisition of the *aac*, *ant*, and *aph* genes, which encode *N*-acetyltransferase, *O*-nucleoside transferase, and *O*-phosphate transferase, respectively, through mobile genetic elements. These enzymes can modify specific aminoglycoside groups that enter bacterial cells; this modification results in a loss of aminoglycoside biological activities (Mingeot-Leclercq et al., 1999). In addition, the mechanism is related to the 16S rRNA methylase (Mingeot-Leclercq et al., 1999; Yu et al., 2010) and drug "efflux pumps" (Sun et al., 2010; Luo et al., 2011), which have been widely examined in recent years.

Chinese researchers have also studied the resistance mechanisms of *A. baumannii* against aminoglycosides (Potron et al., 2009; Wang et al., 2012a). In China, the problems associated with the 16S rRNA methylase gene *armA*-mediated multiple resistance against aminoglycoside antibiotics have become serious. The presence of *A. baumannii* strains in 25

hospitals in 6 domestic provinces and cities was observed, with the *armA* gene reported in 23 hospitals; moreover, the positive rate of the *armA* gene increased to 47.7%, which is considerably higher than that in other countries and regions (Adams et al., 2009). Recently, Doi et al. (2007) detected aac(2')-Ib aminoglycoside-modifying enzymes, which can modify gentamicin and tobramycin, among others, in *A. baumannii*.

The antimicrobial drug resistance of clinically isolated *A. baumannii* strains in our hospital has become serious with the detection of multidrug-resistant and pan-drug resistance *A. baumannii* strains. To investigate the mechanism underlying the resistance of these microorganisms to aminoglycosides, the presence of 9 types of aminoglycoside-modifying enzyme genes and 7 types of 16S rRNA methylase genes was determined in 32 clinically isolated specimens of *A. baumannii*.

MATERIAL AND METHODS

Strain source, strain molecular identification, and drug susceptibility test

The 32 strains were isolated from hospitalized patients in Huai'an First Hospital, Jiangsu Province, China, from January 2013 to March 2013. The samples collected included 28 specimens of sputum, 1 specimen of blood, 1 of pus, 1 of oozing fluid, and 1 of cerebrospinal fluid. All strains were identified as *A. baumannii* using a Medical Bacterial Identification Instrument (Zhuhai Deere Biological Engineering Co., Ltd., Shen Zhen, China) and an integrated 96-E bacterial identification card. The 32 strains were subjected to 16S-23S rDNA *A. baumannii*-specific polymerase chain reaction (PCR) amplification to identify the molecular species. The results confirmed that all strains were *A. baumannii*. The primer sequences used for the 16S-23S rDNA *A. baumannii*-specific PCR amplification were P1: 5'-CATTATCA CGGTAATTAGTG-3' and P2: 5'-AGAGCACTGTGCACTTAAG-3' (Chen et al., 2007). A genetic testing kit was provided by the Wuxi Institute of Cloning Genetic Technologies (Wuxi, China). The drug susceptibility test kit was used with a Zhuhai Deere Medical bacterial identification instrument and the integrated 96-E bacterial identification card. Standard susceptibility of the antimicrobial drug was determined according to the US Clinical and Laboratory Standards Institute (2011).

Bacterial treatment

Single colonies were selected and placed in 0.5-mL Eppendorf centrifuge tubes (Hamburg, Germany) containing 400 μL freshly prepared 200 μg/L proteinase K solution. The tubes were heated in a water bath at 56°C for 2 h. Next, the water bath temperature was increased to 95°C for 10 min. The supernatant was used as the genetic testing template and stored at -20°C until use.

Genetic testing

Nine types of aminoglycoside-modifying enzyme genes [*aac*(*2'*)-*Ib*, *aac*(*3*)-*I*, *aac*(*3*)-*II*, *aac*(*6'*)-*Iad*, *aac*(*6'*)-*Ib*, *aac*(*6'*)-*II*, *ant*(*3''*)-*I*, *ant*(*2''*)-*I*, and *aph3'*-*I*] and 7 types of 16S rRNA methylase genes (*armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD*, *rmtE*, and *npmA*) were detected using the

PCR assay. The primer sequences for *aac* (*2'*)-*Ib* were P1: 5'-ATGCAGTTCAAAATTATCGC AGCC-3' and P2: 5'-CTACCAAAGTTCACCTTCTCGCC-3' (product length: 543 base pairs); and the primer sequences for *rmtE* were P1: 5'-ATGAATATTGATGAAATGGTTG-3' and P2: 5'-TCATTGATTTCCTCCGTTTTTGG-3' (product length: 822 base pairs). The use of these primer sequences was authorized by Zuhuang Mi, Wuxi Institute of Cloning Genetic Technologies. These sequences were used to complete the design according to the conserved regions of the *aac* (*2'*)-*Ib* and *rmtE* sequences that were downloaded from www.ncbi.nlm.nih.gov/nucleotide before January 1, 2013. The other primer sequences for the aminoglycoside-modifying enzyme genes and 16S rRNA methylase genes for PCR detection are listed in previous reports (Huang et al., 2008; Qu et al., 2008). The PCR amplification system for the various targeted genes (per 20-μL reaction system) consisted of the following: 1 μL P1 primer (1.0 μM), 1 μL P2 primer (1.0 μM), 2 μL dNTPs (2 mM), 2 μL 10X buffer [10 mM KCl, 8 mM $(NH4)_2SO_4$, 2 mM $MgCl_2$, 10 mM Tris-HCl, pH 9.0, 0.5% NP_{40}, and 0.02% bovine serum albumin (wt/vol)], 1 U *Taq* DNA polymerase (excluding the volume), 9 μL ultrapure water, and 5 μL template solution. The thermal cycling parameters for PCR amplification were as follows: denaturation at 93°C for 2 min, followed by 93°C for 60 s → 55°C for 60 s → 72°C for 60 s, for 35 cycles, and finally a 72°C cycle extension of 5 min. The PCR products were analyzed by 2% agarose gel electrophoresis. The appearance of targeted bands equal in size to the molecular weight of the positive control was considered positive. The genetic testing kit and the positive-control DNA were provided by the Wuxi Institute of Cloning Genetic Technologies.

DNA sequencing

The full-length forward and reverse sequences of the *aac*(*2'*)-*Ib* aminoglycoside modifying-enzyme gene were sent to Shanghai Boshang Biotechnology Co., Ltd. (Shanghai, China) and subjected to PCR direct automatic fluorescent sequencing.

Sequence comparison

The Chromas software (Tehnelysium, South Brisbane, Australia) was used for sequence analysis. Chromas sequencing results were then directly compared to those of BLAST.

RESULTS

Drug susceptibility test

The drug susceptibility test results for the 32 *A. baumannii* strains are shown in Table 1. The resistance rates of the 32 strains to 14 commonly used antimicrobial drugs, including gentamicin and amikacin, all exceeded 90%; high resistance was also observed against imipenem and meropenem. These strains exhibited higher sensitivity only to polymyxin B at a resistance rate of 3.13%.

Table 1. Drug susceptibility test results of 32 strains of *Acinetobacter baumannii* (%)

Drug	Resistance (R)	Middle (I)	Susceptable (S)
Ampicillin /Sulbactam	29 (90.63)	0 (0.00)	3 (9.38)
Piperacillin	30 (93.75)	0 (0.00)	2 (6.25)
Piperacillin /Tazobactam	29 (90.63)	0 (0.00)	3 (9.38)
Ceftriaxone	29 (90.63)	0 (0.00)	3 (9.38)
Cefotaxime	30 (93.75)	0 (0.00)	2 (6.25)
Ceftazidime	29 (90.63)	1 (3.13)	2 (6.25)
Cefepime	29 (90.63)	0 (0.00)	3 (9.38)
Imipenem	29 (90.63)	1 (3.13)	6 (18.75)
Meropenem	29 (90.63)	3 (9.38)	3 (9.38)
Amikacin	29 (90.63)	0 (0.00)	3 (9.38)
Gentamycin	29 (90.63)	0 (0.00)	3 (9.38)
Polymyxin B	1 (3.13)	0 (0.00)	31 (96.88)
Ciprofloxacin	29 (90.63)	0 (0.00)	3 (9.38)
Levofloxacin	29 (90.63)	0 (0.00)	3 (9.38)
Tetracycline	29 (90.63)	0 (0.00)	3 (9.38)

Genetic testing

The sequence of the *aac*(*2'*)-*I* gene PCR product is shown in Figure 1. The 32 *A. baumannii* strains all contained *aac*(*2'*)-*Ib* aminoglycoside-modifying enzyme genes and other aminoglycoside-modifying enzyme genes, including *aac*(*3*)-*I*, *aac*(*6'*)-*I b*, *ant*(*3''*)-*I*, and *aph*(*3'*)-*I* as well as the 16S rRNA methylase gene *armA*. The detection rates of the different aminoglycoside-modifying enzyme genes and 16S rRNA methylase gene are shown in Table 2. A total of 5 aminoglycoside-modifying enzyme genes, including *aac*(*2'*)-*Ib*, *aac*(*3*)-*I*, *aac*(*6'*)-*Ib*, *ant*(*3''*)-*I*, and *aph*(*3'*)-*I*, and 1 16S rRNA methylase gene (*armA*) were found in the 32 *A. baumannii* strains. The *aac*(*2'*)-*Ib* gene was found in all 32 strains and thus had a positive detection rate of 100% (32/32). The positive rate of the 16S rRNA methylase gene *armA* was 78.1% (25/32), whereas those of *ant*(*3''*)-*I* and *aac*(*6*)-*Ib* were 62.5% (20/32) and 59.4% (19/32), respectively.

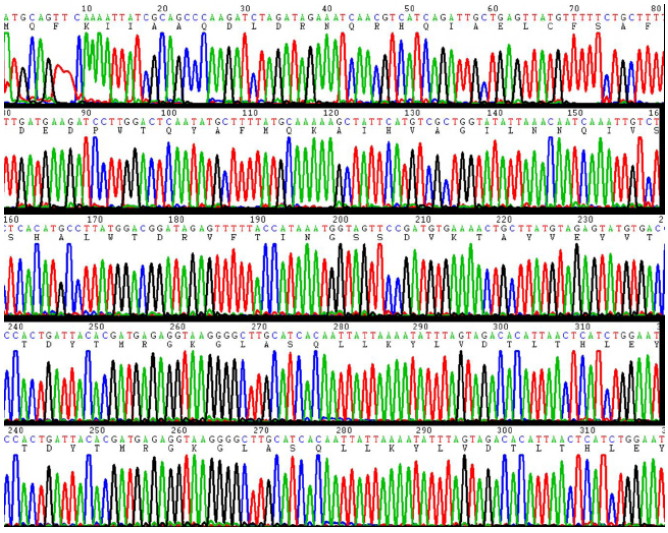

Figure 1. Sequencing of *aac* (*2'*)-*Ib* PCR product.

Table 2. Detection rates of aminoglycoside-modifying enzyme genes and 16SrRNA methylase genes in 32 strains of *Acinetobacter baumannii*

Gene name	Positive ratio (%)
aac(*2*')-*Ib*	32 (100.0)
aac(*3*)-*I*	15 (46.9)
aac(*6*')-*Ib*	19 (59.4)
ant(*3*'')-*I*	20 (62.5)
aph(*3*')-*I*	19 (59.4)
armA	25 (78.1)

The detection modes of the aminoglycoside-modifying enzyme genes and 16S rRNA methylase gene are shown in Table 3. The aminoglycoside-modifying enzyme genes were found in all 32 *A. baumannii* strains. Only 4 strains showed a single detection of the *aac*(*2*')-*Ib* gene (12.3%, 4/32); the remaining strains contained more than 2 types of genes. The highest detection mode was observed for *aac*(*2*')-*Ib*+*aac*(*3*)-*I*+*aac*(*6*')-*Ib*+*ant*(*3*'')-*I*+*aph*(*3*')-*I*+*armA*, which had a positive rate of 37.5% (12/32).

Table 3. Detection modes of positive aminoglycoside-modifying enzyme genes and 16SrRNA methylase genes in 32 strains of *Acinetobacter baumannii*

Gene detection mode	Strains (%)
aac(2')-Ib+aac(3)-I+aac(6')-Ib+ant(3'')-I+aph(3')-I+armA	12 (37.5)
aac(2')-Ib+aac(6')-Ib+ant(3'')-I+aph(3')-I+armA	4 (12.3)
aac(2')-Ib+aac(3)-I+ant(3'')-I+armA	1 (3.1)
aac(2')-Ib+armA	8 (25.0)
aac(2')-Ib+aac(3)-I+aac(6')-Ib+ant(3'')-I+aph(3')-I	2 (6.3)
aac(2')-Ib+aac(6')-Ib+ant(3'')-I+aph(3')-I	1 (3.1)
aac(2')-Ib	4 (12.3)

DISCUSSION

Acinetobacter baumannii is a major pathogenic bacteria responsible for nosocomial infection in our hospital. The number of *A. baumannii* strains resistant to quinolones, including imipenem and aminoglycosides, is increasing. Thus, the mechanism by which *A. baumannii* acquires aminoglycoside-resistant genes should be thoroughly examined.

Aminoglycosides inhibit bacterial protein synthesis. The small 30S ribosomal subunit is the platform for the translation and synthesis of bacterial protein. This subunit is the target of aminoglycosides and contains 3 binding sites, including the aminoacyl site, peptidyl site, and exit site. Aminoglycosides can bind to the A site to impede bacterial protein synthesis, resulting in bacterial death (Mingeot-Leclercq et al., 1999).

Currently, the known resistance mechanism of Gram-negative bacillus to aminoglycosides involves the acquisition of *aac*, *ant*, and *aph* genes through mobile genetic elements. These genes encode *N*-acetyltransferase, *O*-nucleoside transferase, and *O*-phosphate transferase, respectively. These aminoglycoside-modifying enzymes can modify specific groups of aminoglycosides that enter bacterial cells; these modifications result in the loss of aminoglycoside biological activities (Fan et al., 2006).

Several foreign studies have been conducted to examine the resistance mechanism underlying multidrug resistance of *A. baumannii* to aminoglycosides (Adams et al., 2009; Wang et al., 2012b). The aminoglycoside-modifying enzymes mediated by plasmids main-

ly include aac(3)-I (aacCl), aac(3)-II(aac2), aaC(3)III-(aac3), aac(6')-I(aacA4), and aph(3') (aphAl). Adams et al. (2009) found that in the complete genomic sequence of the *A. baumannii* AB307-0294 strain (NCBI No.: CP001172, 2011-11-21), the median locus tag ABBFA-003344 (sequence, 3557653-3558195; 543 bp) encoded the aac(2')-Ib aminoglycoside-modifying enzyme. We detected the genes of 9 aminoglycoside-modifying enzymes in 32 clinically isolated strains of *A. baumannii*. Table 2 shows that 5 genes, including *aac*(*2*')-*Ib*, *aac*(*3*)-*I*, *aac*(*6*')-*Ib*, *ant*(*3*'')-*I*, and *aph*(*3*')-*I*, were found in the 32 strains. Of these, *aac*(*2*')-*Ib* was highly prevalent, with a positive rate of 100% (32/32). This study is the first to isolate aac(2')-Ib aminoglycoside-modifying enzyme genes in China. These genes have been isolated from *Mycobacterium tuberculosis* abroad and from *A. baumannii*. The aac(2')-Ib aminoglycoside-modifying enzyme can modify gentamicin and tobramycin (Perez et al., 2007). The positive rates of ant(3'')-I and aac(6')-Ib were 62.5% (20/32) and 59.4% (19/32), respectively. These results are similar to those of Huang et al. (2008), but differ from the results of Shi et al. (2005) and Vila et al. (1999).

In recent years, Gram-negative bacilli have been found to obtain various types of 16S rRNA methylase genes and express methylases that act upon the targets of aminoglycosides. As a result, aminoglycosides cannot bind to the target sites, resulting in resistance (Fan et al., 2006). The genes coding 16S rRNA methylases include *rmtA*, *rmtB*, *rmtC*, *rmtD*, *rmtE*, *npmA*, and *armA*. In 2010, Davis et al. (2010) reported on the *rmtE* gene. In the current study, 7 types of 16S rRNA methylase genes were detected in 32 clinically isolated multidrug-resistant *A. baumannii*. Table 2 shows that only 1 gene (*armA*) was found in the 32 strains. The *armA* gene had a positive rate of 78.1% (25/32), which was higher than the 47.7-68.6% detected in many domestic hospitals. This value was also higher than those reported by Adams et al. (2009), but lower than the 84.5% reported by Liu et al. (2012). The other 6 types of 16S rRNA methylase and the *rmtE* gene detected in this study were not detected in previous studies. This finding indicates that *armA* is the dominant gene for 16S rRNA methylase. Table 3 shows that only 4 strains showed single detection of the *aac*(*2*')-*Ib* gene (12.3%, 4/32); the remaining strains were found to contain more than 2 types of genes. The highest detection mode was observed for *aac*(*2*')-*Ib*+*aac*(*3*)-*I*+*aac*(*6*')-*Ib*+*ant*(*3*'')-*I*+*aph*(*3*')-*I*+*armA*, which showed a positive rate of 37.5% (12/32).

In this study, the high detection rates of various aminoglycoside-modifying enzyme genes in *A. baumannii* and the presence of the 16S rRNA methylase gene *armA* were consistent with previously reported results and with the high resistance of *A. baumannii* to aminoglycosides. The high detection rates of aminoglycoside-resistance genes, particularly the linkage communication of the 16S rRNA methylase gene and β-lactamase gene, may lead to the evolution of super-bacteria for which no treatment is currently available. This possibility deserves urgent clinical attention. An epidemiological investigation of the existence of the *aac*(*2*')-*Ib* gene in clinically isolated strains of *A. baumannii* must be conducted.

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