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Original Article

Modifying enzymes related aminoglycoside: analyses of resistant *Acinetobacter* isolates

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Abstract: Enzymatic modification of aminoglycosides by nucleotidyltransferases, acetyltransferases and/or phosphotransferases accounts for the majority of aminoglycoside-resistant *Acinetobacter* isolates. In this study, we investigated the relationship between aminoglycoside resistance and the presence of aminoglycoside-modifying enzymes in *Acinetobacter baumannii* clinical isolate groups with different resistance profiles. Thirty-two clinical *A. baumannii* isolates were included in this study. *Acinetobacter* isolates were divided into 4 groups according to results of susceptibility testing. The presence of genes encoding the following aminoglycoside-modifying enzymes; *aph (3')-V1, aph (3')-Ia, aac (3)-Ia, aac (3) IIa, aac (6')-Ih, aac (6')-Ib* and *ant (2')-Ia* responsible for resistance was investigated by PCR in all strains. The acetyltransferase *(aac (6')-Ib, aac (3)-Ia)* and phosphotransferase *(aph (3')-Ia)* gene regions were identified in the first group, which comprised nine imipenem, meropenem, and gentamicin-resistant isolates. The acetyltransferase *(aac (6')-Ib, aac (3)-Ia),* phosphotransferase *(aph (3')-VI)* and nucleotidyltransferase *(ant2-Ia)* gene regions were identified in the second group, which was composed of nine imipenem-resistant, meropenem-resistant and gentamicin-sensitive isolates. The acetyltransferase *(aac (3)-Ia)* and phosphotransferase *(aph (3')-Ia)* regions were identified in the fourth group, which comprised eight imipenem-sensitive, meropenem-sensitive and gentamicin-resistant isolates. Modifying enzyme gene regions were not detected in the third group, which was composed of six imipenem, meropenem and gentamicin-sensitive isolates. Our data are consistent with previous reports, with the exception of four isolates. Both acetyltransferases and phosphotransferases were widespread in *A. baumannii* clinical isolates in our study. However, the presence of the enzyme alone is insufficient to explain the resistance rates. Therefore, the association between the development of resistance and the presence of the enzyme and other components should be investigated further.

Keywords: *Acinetobacter baumannii,* aminoglycoside-modifying enzymes, resistance

Introduction

Resistance rates to all the clinically useful aminoglycoside antibiotics are higher in *Acinetobacter spp.* than in most other groups of pathogens [1, 2] Most aminoglycoside resistance in *Acinetobacter spp*. involves production of aminoglycoside-modifying enzymes, and all three classes of aminoglycoside-modifying enzymes have been found in *Acinetobacter spp*.. Enzymatic modification of aminoglycosides by acetyltransferases, nucleotidyltransferases and/or phosphotransferases accounts for the majority of aminoglycoside-resistant *Acinetobacter* isolates [3] Several aminoglycoside-modifying enzymes (AMEs) have been detected in different *Acinetobacter* species (*A. baumannii*, *A. pittii*, *A. nosocomialis*, and *A. johnsonii*), including: the phosphotransferases *aph (3')-Ia, aph (3')-VIa, aph (3')-II*, the acetyltransferases *aac (3)-Ia, aac (3)-IIa, aac (6')-Ib, aac (6')-Iad, aac (6')-Im* and *aac (6')-II* and the nucleotidyltransferases *ant (2")-Ia, ant (3")-Ia*, and *ant (3")-Id* [3-8].

This study investigated the relationship between aminoglycoside resistance and the presence of aminoglycoside-modifying enzymes in *Acinetobacter baumannii* clinical isolate groups with different resistance profiles.

Materials and methods

Thirty-two *A. baumannii* strains present in the laboratory archieve and which have been isolated from the samples sent from various clin-

Table 1. Groups of Our Isolates

Group	Gentamicin	Imipenem	Meropenem
Group 1	R	R	R
Group 2	S	R	R
Group 3	S	S	S
Group 4	R	S	S

R: Resistance, S: Susceptible.

ics to Sakarya University Training and Research Hospital Microbiology Laboratory (Turkey) were included in this study. The isolates were identified using the GN ID card (PN 21341, bioMérieux, ABD) and their susceptibility profiles assessed using the AST-N262 (bioMérieux, ABD) card of Vitek2 automated system (bioMérieux, Fransa). Densi-Check 2 system was used to calibrate the turbidity to 0.5 McFarland standards. Species identification was performed using conventional molecular methods with OXA_{51} primer. Acinetobacter isolates were divided into 4 groups according to results of susceptibility testing. The first group, consisted of 9 strains that are identified as resistant gentamicin, Imipenem and Meropenem. The second group, consisted of 9 strains that are identified as sensitive gentamicin and resistant imipenem and meropenem. The third group, consisted of 6 strains that are identified as sensitive gentamicin, imipenem and meropenem. The forth group, consisted of 8 strains that are identified as resistant gentamicin and sensitive imipenem and meropenem (**Table 1**). *Acinetobacter baumannii ATCC19606* was used as quality control strains.

The presence of genes encoding the following aminoglycoside-modifying enzymes was investigated by PCR:, phosphotransferases *aph (3')-V1* and *aph (3')-Ia*, acetyltransferases *aac (3)-Ia, aac (3)-Iia, aac (6')Ih* and *aaa (6')-Ib*, and nucleotidyltransferases *ant (2')-Ia*. The primers were those given **Table 2**.

DNA was extracted from fresh culture of *A. baumannii* colonies according to the following protocol performed by GeneJET Genomic DNA Purification Kit (Thermo Scientific, USA). The RNA extraction was performed by GeneJET RNA Purification Kit (Thermo Scientific, USA) extraction kit. In this study, 16S rRNA gene was used as housekeeping gene.

Optimization studies were performed using conventional polymerase chain reaction (PZR). The transcription product levels of blaOXA-51-like, m13 were identified with real-time polymerase chain reaction (qPRC) method using a Fluorion Instrument (Iontek, Turkey). The presence of genes encoding the following aminoglycoside-modifying enzymes; *aph (3')-V1, aph (3')-Ia, aac (3)-Ia, aac (3)IIa, aac (6')-Ih, aac (6')-Ib* and *ant (2')-Ia* responsible for resistance was investigated by PCR in all strains. All PCR methods used the following concentrations of reagents unless otherwise indicated. The reaction volume calculated as 25 µl and prepared by 12.5 µl master mix, 1 µl F primer, 1 µl R primer, 2 µl sample and 8 µl distilled water without DNA of each primer. All PCR followed a standard PCR protocol of a 5-min hot start. All had a 10 min final extension step at 72°C. PCR reactions were performed on a Sensoquest Labcycler cooled thermocycler. To be able to evaluate the similarity among the strains the arbitrarily primed polymerase chain reaction (AP-PCR) were used [9, 10]

Electrophoresis was applied for the analysis of the amplicons by using ORTE (Salubris, Turkey) Real time electrophoresis. Agarose gel (2%) was prepared. PCR products were visualized over led illuminator.

Hospital-isolated carbapenem-resistant *A. baumannii* strains were investigated to determine whether there was an association between the presence of aminoglycoside-modifying enzymes (AMEs) and carbapenem resistance.

Results

All isolates were OXA_{51} positive. OXA_{51} that is located in the structural gene region of A. baumannii conventional and molecular species identification was confirmed. The acetyltransferase *(aac (6')-Ib, aac (3)-Ia)* and phosphotransferase *(aph (3')-Ia)* gene regions were identified in the first group, which comprised nine imipenem, meropenem, and gentamicin-resistant isolates. The acetyltransferase *(aac (6')-Ib, aac (3)-Ia)*, phosphotransferase *(aph (3')-VI)* and nucleotidyltransferase *(ant2-Ia)* gene regions were identified in the second group, which was composed of nine imipenem-resistant, meropenem-resistant and gentamicin-sensitive isolates. The acetyltransferase *(aac (3)-Ia)* and phosphotransferase *(aph (3')-Ia)* regions were identified in the fourth group, which comprised eight imipenem-sensitive, meropenem-sensitive and gentamicin-resistant isolates. Modifying enzyme gene regions

Table 2. Primer sets used in our study

Primer Sets	Nucleotide sequence (5'-3')	Target DNA	Genbank accession number	Size of amplicon (bp)	Reference
1	F: TCAGCAAGAGGCACAGTTTG	bla_{OXA-51}	EU255296.1	188 bp	[17]
	R: GCTGAACAACCCATCCAGTT				
2	F: GACATAAGCCTGTTCGGTT	*aac (3)-Ia*	X15852	372 bp	[18]
	R: CTCCGAACTCACGACCGA				
3	F: ATGCATACGCGGAAGGC	*aac (3)-IIa*	M62833	822 bp	[18]
	R: TGCTGGCACGATCGGAG				
4	F: ATCTGCCGCTCTGGAT	*ant (2')-Ia*	U17586	404 bp	[18]
	R: CGAGCCTGTAGGACT				
5	F: TGCCGATATCTGAATC	*aac (6') Ih*	L29044.1	407 bp	[18]
	R: ACACCACACGTTCAG				
6	F: TATGAGTGGCTAAATCGAT	*aaa (6')-Ib*	M21682.1	395 bp	[18]
	R: CCCGCTTTCTCGTAGCA				
7	F: CGGAAACAGCGTTTTAGA	*aph (3')-V1*	X07753.1	716 bp	[18]
	R: TTCCTTTTGTCAGGTC				
8	F: ATCGCGTATTTCGTCTCGCT	*aph (3')-Ia*	J01839	292 bp	This Study
	R: GGAGAAAACTCACCGAGGCA				
9	F: GAC GAT CTG TAG CGG GTC TG	16S rRNA	FJ855135.1	791 bp	This Study
	R: CCC AAC ATC TCA CGA CAC GA				
10	GAGGGTGGCGGTTCT	M13			[19]

F, forward primer; R, reverse primer.

were not detected in the third group, which was composed of six imipenem, meropenem and gentamicin-sensitive isolates. None of the strains showed *aac (3')_IIa* enzyme positivity. Modifying enzyme gene regions were not detected in *A. baumannii ATCC 19606* strains (**Table 3**).

Eight *aac (3)_Ia*, two *aph (3)_Ia*, and two *aac (6)_Ib* enzyme profiles were identified in our study. Additionally, the genes for seven *aac (3)_Ia* and *aph (3)_Ia* resistance profiles were identified simultaneously (**Table 4**).

The data obtained in this study were consistent with previous reports, with the exception of four isolates. Two isolates that were amikacin-resistant and gentamicin-sensitive were negative for AME genes. Also, two amikacin and gentamicin-sensitive isolates were AME positive. According to AP-PZR results band profiles were compared for the similarities between strains and 10 different patterns were observed (**Table 4**).

Discussion

Although aminoglycosides present ototoxicity and nephrotoxicity risks and problems with resistance, they are important agents and frequently used to treat infections. In general, aminoglycosides such as gentamicin, tobramycin, amikacin, netilmicin, isepamicin, dibekacin and arbekacin have a broader antibacterial spectrum than the older agents such as streptomycin and kanamycin [11]. Concentration-dependent bactericidal activity, post-antibiotic activity, favorable pharmacokinetics, and synergistic interactions with other antibiotics favor the future usage of aminoglycosides [12].

In recent years, broad-spectrum antibiotics including cephalosporins, fluoroquinolones, and carbapenems have been used in combination with many other agents for treatment of *A. baumannii* infection [12-14]. However, improper antibiotic use and the acquisition of novel genetic factors by *A. baumannii* have contributed to an increase in aminoglycoside resistance [15]. AMEs are the source of this resistance [16].

The genes encoding AMEs can be disseminated via integrons, and expression of AMEs enable bacteria to catalyze the modification of amino and hydroxyl groups on sugar moieties, such as aminoglycosides [17, 18]. This ability is a major cause of aminoglycoside resistance in many bacteria [19, 20]. In addition, efflux pumps and 16s rRNA methylases are also

Table 3. PCR results

Isolates	Resistance group	Genta-micin	Ami-kacin	Imipe-nem	Merope-nem	AP-PCR groups	Aminoglycoside-modifying enzymes Primers					
							ant2_Ia	aph (3)_VI	aph (3)_Ia	aac (3)_Ia	aac (6)_Ib	aac (3)_Ih
							Result	Result	Result	Result	Result	S result
A1	1	R	R	R	R	S1					Positive	
A2	1	R	R	R	R	S2				Positive		
A3	1	R	R	R	R	S2				Positive		
A4	1	R	R	R	R	S2				Positive		
A5	1	R	R	R	R	S2				Positive		
A6	1	R	R	R	R	S2				Positive		
A7	1	R	S	R	R	S1				Positive		
A8	1	R	S	R	R	S2			Positive	Positive		
A9	1	R	S	R	R	S2				Positive		
A10	2	S	R	R	R	S1					Positive	
A11	2	S	R	R	R	S2			Positive			
A12	2	S	R	R	R	S4						
A13	2	S	R	R	R	S5	Positive	Positive				
A14	2	S	R	R	R	S5						
A15	2	S	S	R	R	S3						
A16	2	S	S	R	R	S2			Positive			
A17	2	S	S	R	R	S2				Positive		
A18	2	S	S	R	R	S1						
A19	3	S	S	S	S	S6						
A20	3	S	S	S	S	S7						
A21	3	S	S	S	S	S8						
A22	3	S	S	S	S	S9						
A23	3	S	S	S	S	S7						
A24	3	S	S	S	S	S2						
A25	4	R	R	S	S	S7			Positive	Positive		Positive
A26	4	R	R	S	S	S1	Positive		Positive			
A27	4	R	R	S	S	S3			Positive	Positive		
A28	4	R	R	S	S	S7			Positive	Positive		
A29	4	R	R	S	S	S3			Positive	Positive		
A30	4	R	R	S	S	S7			Positive	Positive		
A31	4	R	R	S	S	S7			Positive	Positive		
A32	4	R	R	S	S	S3			Positive	Positive		
ATCC1		S	S	S	S	S10						
ATCC2		S	S	S	S	S10						

important resistance factors [21]. Recently, 16S rRNA methylation via ArmA has been shown to cause very high aminoglycoside resistance in clinical *A. baumannii* isolates [22].

AMEs in *A. baumannii* isolates were first classified in 1993 [23]. A wide array of aminoglycoside-modifying enzymes have previously been reported in *A. baumannii* [24]. The largest published data set evaluated AMEs detected by a combination of phenotypic inference and DNA hybridization in 1,189 *Acinetobacter spp.* isolates from South Africa, Europe, China, Latin America, and Mediterranean countries [1]. The predominant AME was an aac (3)-class enzyme, which occurred in nearly 50% of the isolates.

Noppe-Leclercq et al. analyzed the aminoglycoside resistance genes of 40 *A. baumannii* strains that were found to synthesize several types of AME, including aminoglycoside phosphotransferases, acetyltransferases, and nucleotidyltransferases (75%, 62.5% and 12.5%, respectively) [25]. Similar results were obtained in this study, and no isolates were positive for *aac (3')_IIa* resistance profiles.

Table 4. Enzymes detected in *A. baumannii* isolates

Enzymes	Number	(%)
aac (3)_Ia	8	24.2
aph (3)_Ia	2	6.1
aac (6)_Ib	2	6.1
aac (3)_Ia + *aph (3)_Ia*	7	21.2
aph (3)_VI + *ant2_Ia*	1	3.0
aph (3)_Ia + *ant2_Ia*	1	3.0
aph (3)_Ia + *aac (3)_Ia* + *aac (3)_Ih*	1	3.0

More recently, a South Korean study reported a different prevalence of AME genes in a polyclonal group of *A. baumannii* isolates: *aac (3)-Ia* in 14.8%, *aac (6')-Ib* in 83.6%, *ant (3")-Ia* in 85.2%, *aph (3')-Ia* in 88.5%, and *aph (3')-VI* in 1.6% [8]. Another study reported AMEs that acetylates to be less common (42.6%) among their isolates. The *ant (2")-Ia* adenylase was the predominant gene present in their isolates (62.6%) and the only one statistically correlated with resistance to each of the aminoglycosides tested [24].

Cho et al. reported that more than 80% of isolates were positive for nucleotidyltransferase, acetyltransferase, and phosphotransferase activity [8]. Akers et al. identified nucleotidyltransferases (62.6%) as the most prevalent AME genes [24].

In a study of 50 *Acinetobacter* isolates, phosphotransferases were identified in 88% of isolates and acetyltransferases in 4% of isolates [26]. In another study examining AMEs in Gram-negative bacteria, acetyltransferases were identified in 65.2%, and nucleotidyltransferases in 40% of *Pseudomonas* spp. [27]. In this study 19 (57.6%) isolates were positive for acetyltransferase genes, and 12 (36.4%) were positive for nucleotidyltransferase genes.

There have been few reports on the presence of multiple AMEs in the literature. Miller et al. found that 11.3% of isolates were positive for both aac *(3)-ant (2")-I* and *aph (3')-VI-ant (2")-I* resistance profiles [1]. Cho et. al. report three enzymes high positivity for all enzymes but have not to clarify enzymes association in resistant isolates [8]. Therefore, the presence of multiple enzymes, and the relationship between AMEs and aminoglycoside resistance has yet to be fully elucidated. This study identified the following resistance profiles: seven *aac (3)_Ia* and *aph (3)_Ia*, one *aph (3)_VI* and *ant2_Ia*, and one *aph (3)_Ia* and ant2_Ia. Additionally we found genes for *aph (3)_Ia, aac (3)_Ia*, and *aac (3)_Ih* enzymes in a single isolate that was aminoglycoside-resistant and carbapenem-sensitive.

The data obtained in this study were consistent with previous reports, with the exception of four isolates. Two isolates that were amikacin-resistant and gentamicin-sensitive were negative for AME genes. Also, two amikacin- and gentamicin-sensitive isolates were AME positive. These results suggest that efflux pumps and outer membrane proteins should be investigated to explain the aminoglycoside resistance in *A. baumannii* isolates. The presence or absence of AME genes is unlikely to be sufficient to explain the resistance profiles detected.

AP-PCR method used in this study was not sufficient to molecularly distinguish *A. baumannii* isolates. Therefore, the various resistance groups and AP-PCR group were grouped together. We suggest that pulsed-field gel electrophoresis and a molecular typing method should be used to analyze *A. baumannii* isolates.

In this study, hospital isolated carbapenem-resistant *A. baumannii* strains were investigated to determine whether there is an association between AMEs and carbapenem-resistance. We detected no relationship between carbapenem-sensitivity and the presence of AME genes. In conclusion, both acetyltransferases and phosphotransferases were widespread in *A. baumannii* clinical isolates in our study. However, the presence of the enzyme alone is insufficient to explain the resistance rates. Therefore, the association between the development of resistance and the presence of the enzyme and other components should be investigated further.

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This study was granted approval by the local Ethics in Research Committee.

Disclosure of conflict of interest

None.

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