CHARACTERIZATION OF AMINOGLYCOSIDE RESISTANCE MECHANISMS IN *ACINETOBACTER BAUMANNII* ISOLATES FROM BURN WOUND COLONIZATION

*MÉCANISMES DE RÉSISTANCE AUX AMINOSIDES D'*ACINETOBACTER BAUMANNII *ISOLÉ DE ZONES BRÛLÉES*

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SUMMARY. Clinical isolates of *Acinetobacter baumannii* have a tendency to develop antimicrobial resistance against commonly prescribed antimicrobial agents, including aminoglycoside agents, particularly in hospitalized patients worldwide. Resistance mechanisms of the bacterium to aminoglycosides are diverse and commonly involve production of aminoglycoside-modifying enzymes and efflux systems. The aim of this study was to investigate the frequency of gene encoding aminoglycoside-modifying enzymes and expression level of *adeB* efflux gene in *A. baumannii* isolates recovered from burn wound colonization. A total of 47 clinical isolates of *A. baumannii* were obtained from burned patients admitted to the Burns Teaching Hospital, Tehran, in 2018. Standard antimicrobial susceptibility screening was performed to determine resistance pattern. A polymerase chain reaction (PCR) assay was performed to determine aminoglycoside-modifying genes *ACC(6')*, *aph(3')-Via*, *aph(3')-IIb*, *aadA1*, *aphA1* and *aph6*. Semi-quantitative RT-PCR was also carried out to quantify the expression level of the *adeB* gene. According to the results of the present study, the *acc(6')* was the predominant aminoglycoside-modifying enzyme gene (80.9%), followed by *aph(3')-via*, *aph6*, *aph(3')-IIb* and *aphA1,* which was detected in 59.6%, 42.6%, 14.9% and 14.9% of isolates, respectively. None of the *A. baumannii* isolates harboured the *aadA1* gene. The up regulation of *adeB* gene expression was observed in 63.8% of strains. Moreover, we indicated that there is a relationship between *adeB* expression and high resistance to gentamicin. Our results revealed that aminoglycoside resistance could be explained by the production of one or a combination of known aminoglycoside-modifying enzymes rather than overexpression of *adeB.*

Keywords: *Acinetobacter baumannii*, aminoglycoside, burned patients, *adeB* gene, efflux pump

RÉSUMÉ. Acinetobacter baumannii *(AB) est de plus en plus fréquemment isolé de prélèvements cliniques de par le monde. Il est très susceptible de développer des résistances aux antibiotiques, parmi lesquels les aminosides, en particulier dans les hôpitaux. Les mécanismes sont variables, le plus souvent enzymatiques ou par efflux. Le but de cette étude était d'évaluer les fréquences des gènes codant pour des enzymes modifiant les aminosides et le niveau d'expression du gène de pompe d'efflux* adeB *chez 47 AB isolés de zones brûlées dans le CTB du CHU de Téhéran. Les gènes codant pour* AAC(6'), aph(3')-Via, aph(3') IIb, aadA1, aphA1 et aph6 *ont été recherchés par PCR. Le niveau d'expression du gène* adeB *a été étudié par PCR semi-quantitative* : aac(6') *était le gène le plus fréquemment retrouvé (80,9%), suivi par* aph(3')-Via (59,6%), aph6 (42,6%), aph(3') IIb (14,9%) *et* aphA1 (14,9%). *Nous n'avons pas mis en évidence* aadA1. *Une surexpression de* adeB *a été observée chez 63,8 % des souches, reliée à une résistance élevée à la gentamicine. Ces résultats montrent que la résistance de AB aux aminosides est plus d'origine enzymatique que liée à un efflux.*

Mots-clés *:* Acinetobacter baumannii, *aminosides, brûlés, efflux, gène* adeB

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Introduction

Acinetobacter spp., especially *Acinetobacter baumannii*, have indeed emerged as opportunistic pathogens responsible for causing a variety of severe life-threatening nosocomial infections in immunocompromised individuals, particularly in burn patients.[1,2] The bacteria are capable of surviving in dehydrated and harsh environmental conditions for prolonged periods of time and might colonize in hospitalized patients, which could cause delayed wound healing, sepsis, and even death. *A. baumannii* can cause complicated infections, such as wound infections, hospital-acquired pneumonia, osteomyelitis and bacteremia among patients with burns.[3,4]

In recent decades, clinical isolates of *A. baumannii* have gained antimicrobial resistance against commonly prescribed antimicrobial agents, exclusively in hospitalized patients worldwide. It has accurately been described that one of the most important factors contributing to the high mortality of patients with nosocomial infections caused by *A. baumannii* is the ability for the acquisition of a wide variety of antibiotic resistance genes and rapid development of multidrug- resistant (MDR), extensively drug-resistant (XDR) and even pan-drug resistant (PDR) strains.[5,6] The emergence and spread of drug resistant *A. baumannii* strains has significantly limited the choice of available therapeutic options for treatment of infections caused by the microorganisms and their worse clinical outcome.[7,8] Numerous mechanisms are responsible for the development of multidrug resistance in *A. baumannii*, including decreased membrane permeability due to loss of porins, acquisition of extended-spectrum β - lactamase, and multidrug efflux systems.[9]

The antibiotic class of aminoglycosides is widely used to treat hospital-acquired infections caused by gram-negative bacilli, including *A. baumannii* strains. However, high resistance to traditional aminoglycoside agents such as gentamicin and kanamycin is common among clinical isolates of *A. baumannii*. In addition, strains of *A. baumannii* resistant to newer semisynthetic aminoglycosides such as amikacin, tobramycin, isepamicin and sisomicin are increasingly being reported in many countries worldwide.[10,11] The resistance mechanisms of *A. baumannii* to aminoglycoside agents are diverse and commonly involve production of aminoglycoside-modifying enzymes, which can be classified into aminoglycoside acetyltransferases (AAC), aminoglycoside phosphotransferases (APH), and/or aminoglycoside nucleotidyltransferases (ANT or AAD). The genes for this group of enzymes are commonly present on mobile elements, such as plasmids and transposons, and are transferred among the *A. baumannii* population.[12] The synthesis of AAC(3)-I, APH(3')-VI and ANT(3'')-I has been found to be predominant by several investigations on *A. baumannii* isolates, but there are considerable regional variances in their genotypes.[13,14]

Moreover, aminoglycoside resistance mediated by efflux systems has been reported in *A. baumannii* strains. Among these efflux systems, AdeABC in the resistance-nodulation-division (RND) superfamily has been well demonstrated to be associated with high resistance to aminoglycoside agents.[15]

In Iran, although the prevalence of aminoglycoside resistance has been estimated to be high, especially among MDR *A. baumannii* isolates,[16] the overall prevalence of aminoglycoside resistance genes and the mechanisms of resistance among *A. baumannii* clinical isolates from burn infections have not been well elucidated experimentally. Therefore, the aim of the present study was to provide an insight into the frequency of gene encoding aminoglycoside-modifying enzymes and expression level of AdeB efflux gene in *A. baumannii* isolates recovered from burn infections.

Material and methods

Samples and bacterial isolation

The present study was performed on 47 clinical isolates of *A. baumannii* recovered from burn patients admitted to the Burns Teaching Hospital, Tehran, during June 2018 and August 2018. The study protocol was approved by the Ethics Committee of the National Institute for Medical Research Development (NIMAD). Bacterial identification was carried out using conventional biochemical tests such as growth on MacConkey agar (Merck co., Germany), Gram-staining, oxidase test, oxidative-

fermentative (OF) test, growth in 44°C and motility. In addition, to confirm *A. baumannii* identification, amplification and sequencing of intrinsic *bla*OXA-51-like genes were carried out using specific primers, as previously described.[17] All strains were preserved in Tryptic Soy Broth (TSB; Merck, Germany) containing 20% glycerol at -70°C for further analysis.

Antibiotic susceptibility testing

In vitro susceptibility testing was performed using a panel of three representative aminoglycosides via Kirby–Bauer disc diffusion method according to the Clinical and Laboratory Standards Institute (CLSI 2018) guidelines. The antimicrobial agents tested included, gentamicin, amikacin and tobramycin. *Escherichia coli* ATCC 25922 was used as a quality control strain in every test run.

Detection of aminoglycoside resistance genes

Genomic DNA of the isolates was extracted using the boiling method as described previously.[18] The existence of aminoglycoside resistance genes *ACC(6')*, *aph(3')-Via*, *aph(3')-IIb*, *aadA1*, *aphA1* and *aph6* was evaluated using PCR via specific primers presented in *Table I*. The PCR products were detected by agarose gel electrophoresis (1.5%) then they were stained with ethidium bromide and visualized under (UV) light (UVItec, Cambridge, UK).

Semi-quantitative Real-Time RT-PCR

Semi-quantitative RT-PCR was performed to quantify the expression rates of the *adeB* gene among *A. baumannii* isolates. Accordingly, RNA extraction was carried out using the RNeasy Mini Kit (Roche Co., Germany) according to the manufacturer's instructions with the addition of an extra DNase treatment (CinnaGen Co., Iran) following RNA purification. The absence of DNA contamination was verified by PCR amplification of the housekeeping *16srRNA* gene. The relative expression was compared to the value of the standard strain ATCC19606. Reverse transcription was carried out using the cDNA synthesis kit (Wizbio Co., South Korea) according to the manufacturer's instructions. Real-time PCR assays were carried out using the SYBR green PCR master mix (Amplicon Co., Denmark) via specific primers (*Table I*). The level of gene expression of the efflux pump was normalized with the 16S ribosomal RNA gene as an internal gene control. Amplification was designed including 10 of SYBR Green qPCR MasterMix, 0.5 µL of primers, 8 µL water, and 1 µL cDNA in a 20 µL total volume. The reaction conditions were 95°C for 5 minutes for the first denaturation; 95°C for 20 seconds for denaturation, 60°C for one minute for annealing for 40 cycles, and melt curve at 72 to 95°C.

Statistical analysis

The data were analyzed with SPSS version 22.0 (IBM Corp., USA).

Results

The results of antimicrobial susceptibility testing indicated that 97.9%, 89.5% and 87.5% of the tested isolates were resistant to amikacin, gentamicin and tobramycin, respectively. Our data also showed that 79.1% of isolates were non-susceptible to all of the tested aminoglycoside agents.

According to the results of the present study, the *acc(6')* was the predominant aminoglycoside-modifying enzyme gene (80.9%), followed by *aph(3')-via*, *aph6*, *aph(3')-IIb*, and *aphA1,* which was detected in 59.6%, 42.6%, 14.9% and 14.9% of isolates, respectively. None of the *A. baumannii* isolates harboured the *aadA1* gene. The distribution of aminoglycoside resistance genes among *A. baumannii* isolates is shown in *Table II*. Furthermore, the co-existence of aminoglycoside resistance genes is also indicated in *Table III*.

To understand whether the expression of *adeB* efflux pump gene effects aminoglycoside resistance, the *adeB* expression was analyzed by qRT-PCR in the isolates. The expression levels of *adeB* upregulated in 63.8% of isolates. Accordingly, our analysis showed that there is no statistically significant correlation between *adeB* expression level and resistance rate to amikacin, gentamicin and tobramycin (*p value 0.5*) (*Table IV*).

Table I - Oligonucleotide primers used in this study

Target	Sequence (5′→3′)	Annealing temperature (C°)	Product size (bp)	Reference
Acc(6)	F: TTGCGATGCTCTATGAGTGGCTA R: CTCGAATGCCTGGCGTGTTT	63	482	(32)
Aph3-Lib	F: ATGCATGATGCAGCCACCTCC R: CTAGAAGAACTCGTCCAATAGCCT	64	807	(25)
AphVia	F: AGCGAAAATGTTGAGTTGGCT R: TCCGTGATATCGCCATGAGA	57	339	(33)
aadA1	F: GTGGATGGCGGCCTGAAGCC R: AATGCCCAGTCGGCAGCG	63	527	(34)
AphA1	F: AAACGTCTTGCTCGAGGC R: CAAACCGTTATTCATTCGTGA	56	461	(35)
Aph6	F: GAGCGCACCTTCGACTATGC R: GCCATGGCGTTTACGGCCAG	63	248	(25)
AdeB	F: AACGGACGACCATCTTTGAGTATT R: CAGTTGTTCCATTTCACGCATT	60	84	(36)

Table II - The distribution of aminoglycoside resistance genes in 48 A. baumannii isolated from burn infections

	Non-susceptible			Susceptible		
Aminoglycoside resistance genes	**Amikacin**	**Gentamicin**	**Tobramycin**	**Amikacin**	**Gentamicin**	**Tobramycin**
acc(6') *n=38*	97.3%	86.8%	86.8%	2.7%	13.2%	13.2%
aph(3')-Via *n=28*	96.4%	82.1%	78.6%	3.6%	17.9%	21.4%
aph6 *n=20*	100%	95%	90%	0%	5%	10%
aph(3')-IIb *n=7*	100%	100%	100%	0%	0%	0%
aphA1 *n=7*	100%	100%	100%	0%	0%	0%
aadA1 *n=0*	-	-	-	-	-	-

Table III - Aminoglycoside resistance gene profiles of the Acinetobacter baumannii isolates from burn infections

	Resistance rate		
Aminoglycoside resistance genes	**Amikacin**	**Gentamicin**	**Tobramycin**
acc(6') + aph(3')-Via + aph(3')-IIb + aph6 *n=1*	100%	100%	100%
acc(6') + aph(3')-Via + aph(3')-IIb + aphA1 *n=1*	100%	100%	100%
acc(6') + aph(3')-Via + aph6+ aphA1 *n=1*	100%	100%	100%
acc(6') + aph(3')-Via + aph6 *n=4*	100%	75%	75%
acc(6') + aph(3')-Via + aph(3')-IIb *n=1*	100%	100%	100%
acc(6') + aph(3')-IIb + aph6 *n=1*	100%	100%	100%
acc(6') + aph(3')-Via *n=12*	91.6%	66.6%	75%
aphA1 + aph(3')-Via *n=4*	100%	100%	100%
aph(3')-Via + aph6 *n=2*	100%	50%	50%
acc(6') + aph(3')-IIb *n=2*	100%	100%	100%

Table IV - The correlation between the expression level of adeB efflux pump gene and aminoglycoside resistance

Antimicrobial susceptibility	**Non-susceptible**			**Susceptible**		
	Amikacin	**Gentamycin**	**Tobramycin**	**Amikacin**	**Gentamycin**	**Tobramycin**
***adeB*-Upregulation**	100%	86.6%	89.2%	0%	14.4%	10.8%
***adeB*-Downregulation**	88.2%	94.1%	94.1%	11.8%	5.9%	5.9%

Discussion

Although aminoglycosides present nephrotoxicity risks and other side effects, they are considered to be important antimicrobial agents and are used to treat nosocomial infections. The high rates of aminoglycoside resistance could cause a serious issue for combination therapy of aminoglycoside with broad-spectrum β-lactams including cephalosporins and carbapenems against *A. baumannii* infections.[19] Aminoglycoside-modifying enzymes and efflux pumps are the most important sources of aminoglycoside resistance among *A. baumannii* isolates. The genes encoding these aminoglycoside resistance mechanisms can be distributed mobile elements.[20] Previously, a high rate of resistance to aminoglycosides was reported by several investigators in Iran.[21-24]

Antimicrobial susceptibility screening revealed that 98.9% (46/47) of isolates were fully resistant to at least one of the tested aminoglycosides and at least one aminoglycoside-modifying enzyme gene was detected in these isolates. Furthermore, our findings indicated

that resistance against amikacin is more related to the existence of aminoglycoside-modifying enzyme genes among *A. baumannii* isolates.

Our results showed that the *acc(6')* gene was the predominant aminoglycoside-modifying gene among the *A. baumannii* isolates from burn patients. Nie and colleagues suggested that the *acc(6')* is related to high level aminoglycoside resistance in *A. baumannii* strains.[25] To the best of our knowledge, there are insufficient data on the frequency of *acc(6')* in clinical isolates of *A. baumannii* in Iran. In this study approximately half of the investigated clinical isolates of *A. baumannii* contained *aph6* gene. This finding is in accordance with the results reported by Asadollahi and colleagues in Tehran.[26]

Our findings also indicated that the presence of the phosphotransferases genes *aphA1* and *aph(3')-IIb* was correlated with high resistance against amikacin, gentamicin and tobramycin. The prevalence of *aphA1* and *aph(3')-IIb* genes in this study was in accordance with the findings reported by Aliakbarzade et al. in Tabriz.[22] However, Farsiani et al. reported a high prevalence of *aphA1* (75%) among nosocomial *A. baumannii* isolates in the north-east of Iran.[27] Among the aminoglycoside-modifying enzyme genes, *aadA1* was not detected in the examined *A. baumannii* isolates. On the contrary, more recently, Salimizand et al. reported that *aadA1* is the most frequent aminoglycoside-modifying enzyme among nosocomial *A. baumannii* isolates from Iranian patients.[28] Considering these differences, it appeared that the aminoglycoside resistance genes were distributed among various genotypically distinct groups of *A. baumannii* strains. Thus, these data could reflect a widespread occurrence and clonal variation in nosocomial *A. baumannii* isolates in various hospitals in Iran.

It has been clearly established that AdeABC efflux pumps are associated with high resistance against aminoglycoside agents.[29] Magnet et al. first described that the *adeB* gene encoded an RND-type efflux pump involved in aminoglycoside resistance in *A. baumannii* strain BM4454.[30] However, they stated that among the aminoglycoside agents, amikacin and kanamycin appeared to be less efficiently transported than other agents by AdeB efflux pump, because these compounds contain the highest density of hydroxyl groups and, consequently, are the most hydrophilic.

Therefore, we then evaluated the potential association between the expression level of the *adeB* and aminoglycoside resistance among clinical isolates of *A. baumannii*. Our findings revealed that all *A. baumannii* isolates with *adeB* overexpression were highly resistant to gentamicin (MIC90≥32). Previously, Rumbo et al. described that the overexpression of AdeABC system is associated with high-level resistance with a MIC above 8.0 μg/ml against gentamicin in MDR- *A. baumannii* isolates. However, they reported that these systems are not significantly associated with resistance to netilmicin or tobramycin.[31] Accordingly, the resulting data obtained in this study indicated that there is no clear relationship between *adeB* expression and resistance to tobramycin among *A. baumannii* isolates.

Conclusion

A. baumannii clinical isolates collected from burn patients in Tehran showed high levels of aminoglycoside resistance. Several resistant aminoglycoside-modifying enzyme genes were also detected in the isolates, and coexistence of resistance genes was found in most strains. We also characterized the *adeB* expression level in *A. baumannii* clinical isolates, which exhibited that aminoglycoside resistance could be explained by production of one or a combination of known aminoglycoside-modifying enzymes rather than overexpression of *adeB.* Furthermore, exploration of other resistance mechanisms to aminoglycoside compounds, such as alteration of the ribosome-binding site and reduced uptake, can help determine the correct correlation between genotype and phenotype pattern.

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