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Distribution of AdeABC Efflux System Genes in *Acinetobacter baumannii* Isolated from Blood Cultures of Hospitalized Patients and Their Relationship with Carbapenem and Aminoglycoside Resistance

Kan Kültürlerinden İzole Edilen *Acinetobacter baumannii* İzolatlarında AdeABC Efluks Pompası Genlerinin Dağılımı ve Karbapenem-Aminoglikozit Direnci ile İlişkisinin Araştırılması

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Abstract

Introduction: The increasing emergence of multidrug-resistant (MDR) *Acinetobacter* infections has become a significant challenge for physicians and clinical microbiologists owing to the difficulties arising during therapy. The major efflux mechanism associated with MDR in *A. baumannii* is the chromosomally encoded tripartite efflux pump, AdeABC, which has been reported worldwide. AdeABC belongs to the resistance-nodulation-division efflux pump family and has a three-component structure: AdeB forms the transmembrane component, AdeA forms the inner membrane fusion protein, and AdeC forms the outer membrane protein. AdeABC is chromosomally encoded and is regulated by a two-component system containing a sensor kinase (AdeS) and its associated response regulator (AdeR). Point mutations in these components are associated with the overexpression of AdeABC, thereby leading to multiple drug resistance. The purpose of this study was to investigate the distribution of the AdeABC efflux pump genes and their relationship with carbapenem and multiple drug resistance in *A. baumannii* strains isolated from the blood cultures of hospitalized patients.

Materials and Methods: A total of 97 *A. baumannii* strains that were isolated from the blood cultures of hospitalized patients in different departments, were included in the study. The Phoenix Automated System was used to identify and determine antibiotic susceptibility patterns. The susceptibility of the study strains to carbapenems, ciprofloxacin, trimethoprim-sulfamethoxazole, amikacin, gentamicin, and netilmicin were determined according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria. AdeRS mutations and *adeB* gene expression of drug efflux genes were analyzed by sequencing and qPCR, respectively. The 16S rRNA gene was used as a housekeeping gene, and the *A. baumannii* ATCC 19606 standard strain was also used to normalize the expression results of *adeB* gene.

Results: Of the 97 isolates, 61 were found to be carbapenem resistant. The resistance rates of carbapenem-resistant *A. baumannii* (CRAB) isolates were found to be 100% for ceftazidime; 96.7% for cefepime, piperacillin-azobactam, ciprofloxacin, and trimethoprim-sulfamethoxazole; 86.8% for amikacin; and 75.4% for gentamicin and netilmicin. The significant overexpression (3.45-52.18 fold) of *adeB* was observed in 49 CRAB isolates, whereas less increased levels were observed in only 12 CRAB isolates (0.23-0.54 fold) and non-CRAB isolates (0.109-0.783 fold). In total, 80.3% of the CRAB isolates were positive for the *adeRS* genes. The p.Val120Ile change in the AdeR aminoacid sequence was determined in 42.8% of the *adeB*-overexpressing CRAB isolates. The p.His158Leu and p.Pro116Ser changes were found in 36.7% of these isolates. None of the non-CRAB isolates had p.Val120Ile, p.His158Leu, and p.Pro116Ser changes. In the AdeS aminoacid sequence, p.Gly293Ser, p.Leu105Phe, and His227Asp changes were most commonly observed in *adeB*-overexpressing CRAB isolates, whereas pGly293Ser change was detected in only 8% of the non-CRAB isolates.

Conclusion: These data showed that AdeABC efflux pump overexpression (both *adeB* expression and AdeRS mutation) was higher than expected in our *A. baumannii* isolates. They were significantly associated with the AdeABC efflux system and both CRAB and MDR isolates. The overexpression

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of *adeB* and aminoacid changes in the AdeRS regions led to an increase resistance to different antibiotics; therefore, *A. baumannii* strains should be monitored to ensure the correct treatment, especially in nosocomial MDR.
Keywords: Proteomics, efflux system genes, aminoglycoside resistance, blood stream infections, carbapenem resistance

Öz

Giriş: Çok ilaca dirençli *Acinetobacter* enfeksiyonlarının artması, hekimler ve klinik mikrobiyologlar için tedavide büyük zorluklar oluşturmaktadır. AdeABC, kromozomal olarak kodlanan ve son zamanlarda tüm dünyadan bildirilen çoklu ilaç direnci ile ilişkili efflux mekanizmasıdır. AdeABC üç bileşenli bir yapıya sahiptir. AdeB, transmembran bileşenini, AdeA, iç membran füzyon proteinini ve AdeC, dış membran proteinini oluşturur. AdeABC bir sensör kinaz (AdeS) ve bir regülatör (AdeR) içeren iki bileşenli bir sistem tarafından düzenlenir. Bu bileşenlerde nokta mutasyonları, çoklu ilaç direncine yol açan AdeABC'nin aşırı ekspresyonu ile ilişkilidir. Bu çalışmanın amacı, hastanede yatan hastaların kan kültürlerinden izole edilen *A. baumannii*'de AdeABC efflux pompa genlerinin dağılımını ve bunların karbapenem ve çoklu ilaç direnciyle ilişkilerini araştırmaktır.
Gereç ve Yöntem: Hastanenin farklı bölümlerinde yatan hastaların kan kültürlerinden izole edilen toplam 97 *A. baumannii* kökeni çalışmaya dahil edildi. Çalışma kökenlerinin antibiyotik duyarlılık paternleri Phoenix Otomatik Sistemi ile belirlendi. Suşların karbapenemler, siprofloksasin, trimetoprim-sülfametoksazol, amikasin, gentamisin ve netilmisine duyarlılıkları "European Committee on Antimicrobial Susceptibility Testing" (EUCAST) kriterlerine göre belirlendi. *AdeRS* mutasyonları ve *adeB* gen ekspresyonu dizileme ve qPCR ile analiz edildi. Referans gen olarak 16s rRNA geni ve *A. baumannii* ATCC 19606 standart suşu kullanıldı.
Bulgular: Doksan yedi kökenin 61'i karbapenem dirençliydi. Karbapenem dirençli *A. baumannii* (CRAB) izolatlarının direnç oranları seftazidime %100; sefepime, piperasilin-tazobaktam, siprofloksasin ve trimetoprim-sülfametoksazole %96,7; amikasine %86,8; gentamisin ve netilmisine %75,4 olarak bulundu. Kırk dokuz CRAB izolatında *adeB* aşırı ekspresyonu (3,45-52,18 kat) gözlendi, ancak sadece 12 CRAB izolatında (0,23-0,54 kat) ve CRAB olmayan izolatlarda (0,109-0,783 kat) daha az artış gözlendi. CRAB izolatlarının %80,3'ü *adeRS* genleri için pozitifti. AdeR aminoasit dizisindeki p.Val120Ile değişimi, *adeB*-aşırı eksprese eden CRAB izolatlarının %42,8'inde belirlendi.
Sonuç: Verilerimiz *A. baumannii* izolatlarımızda AdeABC efflux pompası aşırı ekspresyonunun beklenenden daha yüksek olduğunu gösterdi. AdeABC efflux sistemi; hem CRAB hem de çoklu ilaç dirençli izolatlar ile anlamlı şekilde ilişkiliydi. *AdeRS* bölgelerinde *adeB* ve aminoasit değişikliklerinin aşırı ekspresyonu, farklı antibiyotiklere karşı artan bir direnç ortaya çıkmasına neden olmuştur, bu nedenle özellikle nozokomiyal çoklu ilaç direncinde doğru tedaviyi sağlamak için *A. baumannii* kökenleri izlenmelidir.
Anahtar Kelimeler: Proteomik, efflux genleri, aminoglikozit direnci, kan dolaşımı enfeksiyonları, karbapenem direnci

Introduction

Acinetobacter baumannii is a ubiquitous, Gram-negative coccobacillus, which is an important nosocomial pathogen that causes various infections, such as wound infections, bloodstream infections, ventilator-acquired pneumonia, central nervous system infections, and urinary tract infections. In fact, *A. baumannii* is considered as an opportunistic pathogen. The increasing emergence of multidrug-resistant (MDR) *Acinetobacter* spp. infections has become a significant challenge for physicians and clinical microbiologists due to the difficulties arising during therapy[1-4]. The major efflux mechanism associated with MDR in *A. baumannii* is the chromosomally encoded tripartite efflux pump, AdeABC, which has been reported globally. AdeABC belongs to the resistance-nodulation-division efflux pump family and has a three-component structure: AdeB forms the transmembrane component, AdeA forms the inner membrane fusion protein, and AdeC forms the outer membrane protein. AdeABC is chromosomally encoded and is regulated by a two-component system containing a sensor kinase (AdeS) and its associated response regulator (AdeR). Point mutations in these components are associated with the overexpression of AdeABC, thereby leading to multiple drug resistance. This major efflux mechanism is associated with carbapenems, aminoglycosides, fluoroquinolones, tetracyclines, amphenicols, macrolides, and trimethoprim sulfamethoxazole[5-9].

The purpose of this study was to investigate the distribution of the AdeABC efflux pump genes and their relationship with carbapenems and aminoglycosides susceptibility in *A. baumannii* strains isolated from the blood cultures of hospitalized patients.

Materials and Methods

According to the "Ethical Principles for Medical Research Involving Human Subjects" of the principles of the World Medical Association Declaration of Helsinki (amended in October 2013), the İstanbul University-Cerrahpaşa, Cerrahpaşa Faculty of Medicine Ethics Committee of Clinical Research (decision no: 2015/147) granted the approval to this study. The written informed consent was obtained from the study participants.

Sample Collection

Study strains were the 97 *A. baumannii* strains that were isolated from the blood cultures of hospitalized patients in different departments (intensive care 49%, surgery 19.6%, hematology 9.8%, orthopedics and traumatology 3%, and internal medicine 18%) of İstanbul University-Cerrahpaşa, Cerrahpaşa Faculty of Medicine Hospital, İstanbul, Turkey.

Antimicrobial Susceptibility Testing

The BD Phoenix™ automated identification and susceptibility pattern-testing system (Becton-Dickinson Company, Franklin Lakes, NJ, USA) was used to identify and determine the antibiotic susceptibility. The concentration gradient-based E-test (bioMérieux, France) strip method was employed to measure the minimum inhibitory concentration (MIC) values in imipenem, meropenem, and colistin *in vitro* susceptibility tests. The susceptibilities of the strains to carbapenems, ciprofloxacin, trimethoprim-sulfamethoxazole, amikacin, gentamicin, and netilmicin were determined according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria[10].

adeB Gene Expression

The ribonucleic acid (RNA) samples were isolated by using the High Pure RNA isolation kit (Roche Diagnostics GmBH, Mannheim, Germany) from *A. baumannii* strains produced in the Luria Bertani (LB) medium (Sigma-Aldrich, St. Louis, MO, USA) in accordance with the manufacturer's instructions. The obtained RNA samples were stored at -80 °C until they were processed by qPCR LightCycler 480 II (Roche Diagnostics GmBH). Prior to qPCR runs, the ratios of RNAs at A260/A280 nm were examined on a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) for the calculation of the quantities and purity values. Complementary DNA (cDNA) synthesis was performed by using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics GmBH) by adhering to the manufacturer's instructions. Each sample weighed 50 ng. The *16S rRNA* gene was used as a housekeeping gene. The primers were ordered from IDT (Integrated DNA Technologies Inc., Skokie, IL, USA) and were used for qPCR steps for the *adeB* and *16S rRNA* genes. These primers are presented in Table 1.

The qPCR experiments were performed by using the LightCycler 480 SYBR Green I Master kit (Roche Diagnostics GmBH) to detect the *adeB* and *16S rRNA* genes in accordance with the manufacturer's instructions. The qPCR protocol consisted of enzyme activation for 10 min at 95 °C. After 45 cycles, there was an amplification phase of 10 s at 95 °C, 20 s at 60 °C, and 3 s at 72 °C, which was followed by 1 s of denaturation at 95 °C, 60 s at 65 °C, and continuous reading up to 97 °C. The fluorescence data were obtained automatically, and the *adeB* and 16S rRNA Cp values were generated for each isolate by using the ΔCt method. The *A. baumannii* ATCC 19606 standard strain was also used to normalize the *adeB* gene expression results[11]. Each isolate was tested in duplicate samples in two independent experiments.

AdeRS mutations

Deoxyribonucleic acid (DNA) isolations were performed by using the High Pure PCR Template preparation kit (Roche Diagnostics GmBH) from the *A. baumannii* strains generated in the LB medium in accordance with the manufacturer's instructions. The obtained DNA sample was stored at -20 °C until the process of sequencing. DNA sequencing for the detection of mutations in the *adeR* and *adeS* genes was performed by using the primers shown in Table 1. Primers were obtained from IDT (Integrated DNA Technologies Inc.). DNA sequencing was performed by using the automated MegaBACE 1000 (Amersham Biosciences, CA, USA) sequencing system in accordance with the manufacturer's instructions. At the end of the process, the chromatogram files obtained for the *AdeS* and *AdeR* gene regions were compared with the sequences obtained by downloading them after being converted to the FASTA format (See Supplementary Table 1 for GenBank Accession Numbers-placed after the references).

Results

Of the 97 isolates, 61 were found to be carbapenem resistant. The MIC values were found to range between 8-12 mg/ml for imipenem and meropenem.

The resistance rates of carbapenem-resistant *A. baumannii* (CRAB) isolates were found to be 100% for ciprofloxacin and trimethoprim-sulfamethoxazole, 86.8% for amikacin, and 75.4% for gentamicin and netilmicin (Figure 1). The significant overexpression (3.45-52.18 fold) of *adeB* was observed in the 49 CRAB isolates, whereas only 12 CRAB isolates (0.23-0.54 fold) and non-CRAB isolates (0.109-0.783 fold) had less increased levels (Figures 2, 3). Of the 97 isolates, 42 were resistant to aminoglycosides. The significant overexpression (21.38-51.68 fold) of *adeB* was displayed in the 34 aminoglycoside-resistant isolates and was found to be positive for the *AdeRS* gene. Eight aminoglycoside-resistant isolates had low levels (0.23-0.54) of *adeB*, which were found to be negative for the *adeRS* gene (Table 2).

Approximately 80.3% of the CRAB isolates were found to be positive for the adeRS gene, the p.Val120Ile change in the AdeR amino acid sequence was determined in the 21 (42.8%) isolates of *adeB*-overexpressing CRAB isolates. In total, 14 of these

Table 1. Oligonucleotide sequences used for the *adeB*, 16S rRNA, *adeS*, and *adeR* genes

Target	Oligonucleotide sequences	References
adeB—F	AACGGACGACCATCTTTGAGTATT	Peleg et al.[9]
adeB—R	CAGTTGTTCCATTTCACGCATT	Peleg et al.[9]
16S rRNA—F	ACTCCTACGGGAGGCAGCAGT	Selasi et al.[11]
16S rRNA—R	TATTACCG CGGCTGCTGGC	Selasi et al.[11]
adeR—F	AGCGTATGATGAGTTGAAGCA	Bratu et al.[12]
adeR—R	AATCCAGCCTTTTTCAATCG	Bratu et al.[12]
adeS—F	CGTGGCGTGGGATATAGACT	Bratu et al.[12]
adeS—R	AGGAAAATGCCACAAAATGG	Bratu et al.[12]

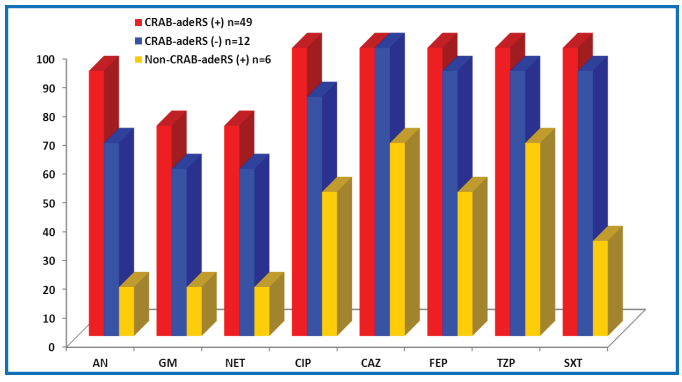

Figure 1. Comparison of antibiotic resistance of carbapenem-resistant *A. baumannii* (CRAB) *adeRS*+, CRAB *adeRS*–, and nonCRAB *adeRS*+

AK: Amikacin, GM: Gentamicin, NET: Netilmicin, CIP: Ciprofloxacin, CAZ: Ceftazidime, FEP: Cefepime, TZP: Piperacillin-tazobactam, SXT: Trimethoprim-sulfamethoxazole, CRAB: Carbapenem-resistant *A. baumannii*

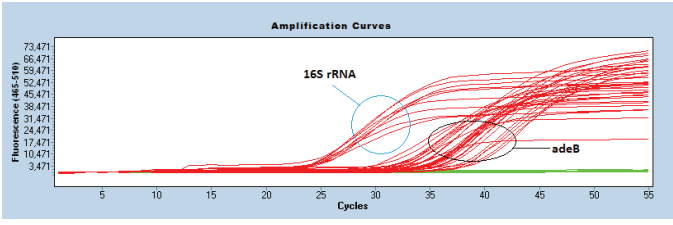

Figure 2. qPCR amplification curves of *adeB* and 16S rRNA genes in *A. baumannii* isolates

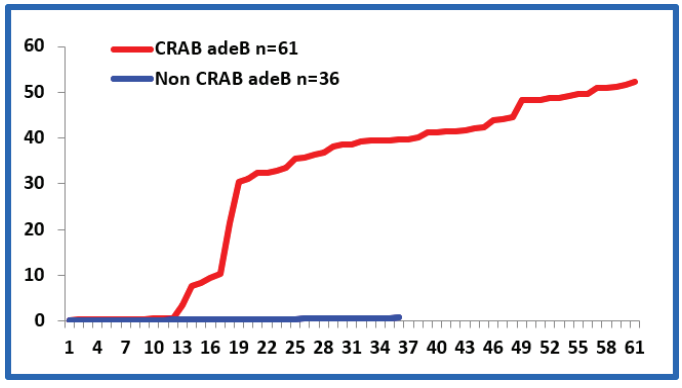

Figure 3. Comparison of *adeB* gene expression fold of carbapenem-resistant *A. baumannii* (CRAB) and non-CRAB isolates

CRAB: Carbapenem-resistant *A. baumannii*

isolates were aminoglycoside-resistant isolates. The p.His158Leu and p.Pro116Ser changes were observed in 36.7% of the CRAB isolates. Eight of the aminoglycoside-resistant isolates showed change in p.His158Leu, and 15 of them showed p.Pro116Ser change. None of the non-CRAB isolates and aminoglycoside-susceptible isolates showed p.Val120Ile, p.His158Leu, and p.Pro116Ser changes. In the AdeS amino acid sequence, p.Gly293Ser, p.Leu105Phe, and p.His227Asp changes were most commonly observed in the *adeB*-overexpressing CRAB isolates and aminoglycoside-resistant isolates. The p.Gly293Ser change was detected in only 8% of the non-CRAB isolates (Table 3).

Discussion

The RND family is a multidrug efflux pump and plays a vital role in the antimicrobial resistance of *A. baumannii.* The first characterized RND system in *A. baumannii* samples was the AdeABC efflux pump. The expression is controlled by the two-component regulatory system known as *adeS* and *adeR*[12-14]. The MDR phenotype against antimicrobials is more expressed than the natural isolates at this pump[13,15].

Aminoglycosides are the antibiotics most affected by the ABC-type pumps[16-18]. For this reason, we tried to measure the expression level of *adeB* gene and investigate the mutation of *adeR* and *adeS* genes, the regulatory compartments, and their relationship with carbapenemase production in *A. baumannii* samples isolated from the blood cultures of hospitalized patients. This study found that the *adeB* gene expression increased 52-fold with p.Val120Ile aminoacid change. In addition, there was a 50-fold increase with p.Pro116Ser and p.His158Leu aminoacid changes for the *AdeR* region. The aminoacid changes of p.Gly293Ser, p.Leu105Phe, and p.His227Asp were observed most frequently in the cases of *adeB* overexpression for the *AdeS* region. The most frequent p.Val120Ile change was observed in the 97 isolates for the *AdeR* region, whereas the most frequent p.Gly293Ser change was observed for the *AdeS* region.

Qiu et al.[19] reported that the *adeB* expression of CRAB isolates was 10.4-62.3 times higher than that of non-CRAB isolates. Our study found similar results. The CRAB isolates had at least 3.45-52.18 times higher overexpression pattern for the *adeB* gene. Coyne et al.[20] reported that the *adeB* overexpressing strains were less susceptible to gentamicin and had a 12-fold increase in MICs. We found similar resistance results for amikacin, gentamicin, and netilmicin, and the *adeB* overexpression in this resistant isolates was found to be 21.38-51.68 fold higher than that in the susceptible isolates. Lari et al.[21] suggested that the efflux-based system AdeABC was an important contributor to reduced susceptibility to antibiotics of choice for treatment, including ciprofloxacin and cefepime, in the *A. baumannii* isolates.

Ardebili et al.[22] reported that p.His158Leu, p.Pro116Ser, p.Val120Ile, and p.Ala136Val were the most common aminoacid changes in the *AdeR* regions, whereas the p.Lys84Glu, p.Ala97Ser, and p.Gly103Asp were the most common aminoacid changes in the *AdeS* regions. They also reported that these changes had caused increased ciprofloxacin MICs, similar with aminoglycosides. We also found similar results for these mutations in ciprofloxacin. Richmond et al.[23] reported that the strains in p.Ala94Val mutation in the *AdeS* region were observed to have 91-fold higher *adeB* expression than the non-mutagenic strains. Similarly, several studies have shown the same results[24-26].

Table 2. Aminoacid substitutions in the *adeR* and *adeS* genes of 34 aminoglycoside-resistant isolates in the displayed levels of *adeB* overexpression

No. of isolates	Levels of *adeB* gene expression	Changes in the *adeR* aminoacid sequence	Changes in the *adeS* aminoacid sequence
2	44.54	p.Val120Ile, p.Leu142Ile, p.His158Leu	p.Lys84Glu, p.Gly103Asp
3	48.65	p.Pro116Ser, p.Ala136Val	p.Ala97Ser, p.Gly103Asp, p.Gly293Ser
5	41.23	p.Val120Ile, p.Leu142Ile	p.Ala94Val, p.His227Asp
9	49.54	p.Val120Ile	p.Gly103Asp, p.Val279Ala
10	39.43	p.Leu142Ile, p.His158Leu	p.Val186Gly, p.Val279Ala
13	48.37	p.Pro116Ser, p.Ala136Val	p.Ala97Ser, p.His227Asp, p.Val279Ala, p.Gly293Ser
19	51.68	p.Pro116Ser, p.Val120Ile	p.Leu105Phe, p.His227Asp
20	48.25	p.Pro116Ser, p.Ala136Val, p.Lys84Glu	p.Ala97Ser, p.Val279Ala
22	39.54	p.Val120Ile	p.Val279Ala, p.Gly293Ser
23	39.65	p.Pro116Ser, p.Ala136Val	p.Lys84Glu, p.Ala97Ser
24	30.43	p.Gly36Val, p.Pro116Ser	p.Leu105Phe
25	33.59	p.Gly36Val, p.Pro116Ser	p.Asp60Tyr, p.Val279Ala
26	48.24	p.Val120Ile, p.Leu142Ile	p.Ala97Ser, p.Gly103Asp
27	41.39	p.Val120Ile, p.Leu142Ile	p.Ala94Val, p.Gly293Ser
28	40.13	p.Gly36Val, p.Pro116Ser	p.Gly103Asp, p.Gly293Ser
29	32.35	p.Pro116Ser	p.Val59Ile, p.Ala94Val
30	32.42	p.Val120Ile, p.Leu142Ile, p.His158Leu	p.Ala94Val, p.His227Asp
31	38.251	p.Met88Leu, p.His158Leu	p.Val186Gly
32	38.69	p.His158Leu	p.Lys84Glu, p.Gly293Ser
33	31.16	p.Val120Ile, p.Leu142Ile	p.Asp60Tyr, p.His227Asp
34	21.38	p.Ala136Val	p.Leu105Phe, p.Gly293Ser
35	39.15	p.Val120Ile, p.Leu142Ile	p.Leu105Phe, p.Gly293Ser
36	41.58	p.Val120Ile, p.Ala136Val	p.Gly103Asp, p.Gly293Ser
37	32.83	p.Val120Ile, p.Leu142Ile	p.Lys84Glu, p.Gly293Ser
38	39.59	p.Met88Leu, p.His158Leu	p.Lys84Glu, p.Val245Ile
39	42.09	p.Val120Ile, p.Ala136Val	p.Ala97Ser, p.Gly293Ser
50	51.03	p.Pro116Ser, p.His158Leu	p.Ala94Val
51	51.03	p.Pro116Ser, p.His158Leu	p.Ala94Val
52	48.75	p.Pro116Ser	p.Asp60Tyr, p.Val245Ile
55	38.54	p.Pro116Ser	p.His227Asp, p.Val245Ile
57	49.56	p.Pro116Ser, p.His158Leu	p.Ala97Ser, p.Gly293Ser
58	41.26	p.Val120Ile, p.Leu142Ile	p.Ala94Val, p.Val245Ile, p.Gly293Ser
59	42.39	p.Pro116Ser	p.Val59Ile
61	36.45	p.Met88Leu, p.His158Leu	p.Val186Gly

To the best of our knowledge, this is the first study in our country to detect both mutations in AdeRS and the expression level in *adeB* on the clinical *A. baumannii* isolates.

Conclusion

These results demonstrated that AdeABC efflux pump overexpression (both *adeB* expression and AdeRS mutation) is higher than expected in our *A. baumannii* isolates. They were significantly associated with the AdeABC efflux system and both CRAB and MDR isolates. The overexpression of *adeB* and aminoacid changes in the AdeRS regions lead to an increase in resistance to different antibiotics. Nosocomial *A. baumannii* strains especially the MDR strains should be monitored to ensure correct treatment.

Table 3. Aminoacid substitutions in the *adeR* and *adeS* genes of 49 carbapenem-resistant *A. baumannii* isolates in the displayed levels of adeB overexpression

No. of isolates	Levels of *adeB* gene expression	Changes in the *adeR* aminoacid sequence	Changes in the *adeS* aminoacid sequence
1	36.85	p.Val120Ile, p.His158Leu	p.Lys84Glu, p.Ala97Ser, p.Val279Ala
2	44.54	p.Val120Ile, p.Leu142Ile, p.His158Leu	p.Lys84Glu, p.Gly103Asp
3	48.65	p.Pro116Ser, p.Ala136Val	p.Ala97Ser, p.Gly103Asp, p.Gly293Ser
5	41.23	p.Val120Ile, p.Leu142Ile	p.Ala94Val, p.His227Asp
7	3.45	p.Val120Ile, p.Leu142Ile	p.Leu105Phe, p.Gly293Ser
8	41.49	p.Pro116Ser, p.Ala136Val	p.Lys84Glu, p.Ala94Val
9	49.54	p.Val120Ile	p.Gly103Asp, p.Val279Ala
10	39.43	p.Leu142Ile, p.His158Leu	p.Val186Gly, p.Val279Ala
12	51.15	p.Val120Ile, p.Leu142Ile	p.Leu105Phe, p.His227Asp, p.Gly293Ser
13	48.37	p.Pro116Ser, p.Ala136Val	p.Ala97Ser, p.His227Asp, p.Val279Ala, p.Gly293Ser
14	7.65	p.Val120Ile, p.Leu142Ile	p.Leu105Phe, p.Val279Ala
15	10.35	p.Val120Ile, p.Ala136Val	p.Val186Gly, p.His227Asp, p.Val279Ala
19	51.68	p.Pro116Ser, p.Val120Ile	p.Leu105Phe, p.His227Asp
20	48.25	p.Pro116Ser, p.Ala136Val	p.Lys84Glu, p.Ala97Ser, p.Val279Ala
22	39.54	p.Val120Ile	p.Val279Ala, p.Gly293Ser
23	39.65	p.Pro116Ser, p.Ala136Val	p.Val59Ile, p.Ala94Val
24	30.43	p.Gly36Val, p.Pro116Ser	p.Leu105Phe
25	33.59	p.Gly36Val, p.Pro116Ser	p.Asp60Tyr, p.Val279Ala
26	48.24	p.Val120Ile, p.Leu142Ile	p.Ala97Ser, p.Gly103Asp
27	41.39	p.Val120Ile, p.Leu142Ile	p.Ala94Val, p.Gly293Ser
28	40.13	p.Gly36Val, p.Pro116Ser	p.Gly103Asp, p.Gly293Ser
29	32.35	p.Pro116Ser	p.Val59Ile, p.Ala94Val
30	32.42	p.Val120Ile, p.Leu142Ile, p.His158Leu	p.Ala94Val, p.His227Asp
31	38.251	p.Met88Leu, p.His158Leu	p.Val186Gly
32	38.69	p.His158Leu	p.Lys84Glu, p.Gly293Ser
33	31.16	p.Val120Ile, p.Leu142Ile	p.Asp60Tyr, p.His227Asp
34	21.38	p.Ala136Val	p.Leu105Phe, p.Gly293Ser
35	39.15	p.Val120Ile, p.Leu142Ile	p.Leu105Phe, p.Gly293Ser
36	41.58	p.Val120Ile, p.Ala136Val	p.Gly103Asp, p.Gly293Ser
37	32.83	p.Val120Ile, p.Leu142Ile	p.Lys84Glu, p.Gly293Ser
38	39.59	p.Met88Leu, p.His158Leu	p.Lys84Glu, p.Val245Ile
39	42.09	p.Val120Ile, p.Ala136Val	p.Ala97Ser, p.Gly293Ser
41	52.18	p.Val120Ile, p.His158Leu	p.Gly103Asp, p.Gly293Ser
42	49.14	p.Val120Ile, p.Leu142Ile, p.His158Leu	p.His227Asp, p.Gly293Ser
44	35.49	p.Met88Leu, p.His158Leu	p.Gly293Ser
46	39.56	p.Met88Leu, p.His158Leu	p.Gly293Ser
47	43.81	p.Pro116Ser	p.Ala94Val, p.Gly293Ser
48	8.36	p.His158Leu	p.Val59Ile, p.Gly293Ser
49	9.43	p.His158Leu	p.Val59Ile, p.Val245Ile
50	51.03	p.Pro116Ser, p.His158Leu	p.Ala94Val
51	51.03	p.Pro116Ser, p.His158Leu	p.Ala94Val
52	48.75	p.Pro116Ser	p.Asp60Tyr, p.Val245Ile
55	38.54	p.Pro116Ser	p.His227Asp, p.Val245Ile
56	35.78	p.His158Leu	p.Val245Ile, p.Gly293Ser
57	49.56	p.Pro116Ser, p.His158Leu	p.Ala97Ser, p.Gly293Ser
58	41.26	p.Val120Ile, p.Leu142Ile	p.Ala94Val, p.Val245Ile, p.Gly293Ser
59	42.39	p.Pro116Ser	p.Val59Ile
60	44.12	p.Pro116Ser	p.Val59Ile, p.Ala94Val, p.Val245Ile
61	36.45	p.Met88Leu, p.His158Leu	p.Val186Gly

Ethics

Ethics Committee Approval: The study approved by the Clinical Research Ethics Committee of İstanbul University-Cerrahpaşa, Cerrahpaşa Faculty of Medicine (decision no: 2015/147).

Informed Consent: The written informed consent was obtained from the study participants.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Concept: O.A., H.A., M.D., F.K.Ç., Design: O.A., H.A., M.D., F.K.Ç., Data Collection or Processing: O.A., H.A., M.D., Analysis or Interpretation: M.D., F.K.Ç., Literature Search: O.A., M.D., F.K.Ç., Writing: O.A., M.D., F.K.Ç.

Conflict of Interest: No conflict of interest was declared by the authors.

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Supplementary Table 1. Genbank accesion number

	Number	abal	AdeB	AdeR	AdeS	Genbank Accession No
1	90	0,379	0,179	-	-	GU647217.1
2	104	0,104	0,175	-	-	GU647217.1
3	154	0,269	0,306	-	p.Val186Gly	GU647217.1
4	178	0,302	0,591	-	p.Val245Ile, p.Gly293Ser	GU647216.1
5	252	0,315	0,643	-	p.Val245Ile	GU647217.1
6	275	0,158	0,615	p.Gly36Val	p.Val245Ile	GU647217.1
7	301	0,105	0,783	p.Gly36Val	p.Asp60Tyr, p.Val245Ile	GU647217.1
8	309	0,468	0,593	p.Gly36Val	-	GU647217.1
9	321	0,259	0,653	-	p.Val186Gly, p.Val245Ile	GU647217.1
10	325	0,198	0,485	p.Ala136Val	-	GU647217.1
11	598	0,169	0,690	-	p.Val186Gly	GU647217.1
12	1242	0,196	0,259	-	-	GU647217.1
13	1399	0,233	0,364	-	p.Val186Gly, p.Val245Ile	GU647217.1
14	1475	0,109	0,109	-	-	GU647217.1
15	1611	0,391	0,264	-	p.Asp60Tyr, p.Val245Ile	GU647217.1
16	1798	1,608	21,380	p.Ala136Val	p.Leu105Phe, p.Gly293Ser	KF147860.1
17	2641	0,194	0,109	-	p.Asp60Tyr, p.Val245Ile	KF147860.1
18	3269	1,351	49,140	p.Val120Ile, p.Leu142Ile, p.His158Leu	p.His227Asp, p.Gly293Ser	KF147860.1
19	3410	0,952	40,130	p.Gly36Val, p.Pro116Ser	p.Gly103Asp, p.Gly293Ser	GU647217.1
20	3415	0,176	0,331	-	p.Asp60Tyr, p.Val279Ala	GU647217.1
21	5034	0,115	0,245	-	-	HM440348.1
22	5140	0,257	0,391	-	-	GU647217.1
23	5544	1,739	39,150	-	-	GU647217.1
24	5888	1,526	43,810	p.Pro116Ser	p.Ala94Val, p.Gly293Ser	GU647217.1
25	6039	0,301	0,652	p.Gly36Val	-	GU647217.1
26	6534	1,115	8,360	-	p.Val59Ile, p.Gly293Ser	GU647217.1
27	8039	1,609	41,580	p.Val120Ile, p.Ala136Val	p.Gly103Asp, p.Gly293Ser	KF147860.1
28	8179	1,350	32,830	p.Val120Ile, p.Leu142Ile	p.Lys84Glu, p.Gly293Ser	KF147860.1
29	9226	0,875	42,090	p.Val120Ile, p.Ala136Val	p.Ala97Ser, p.Gly293Ser	KF147860.1
30	9289	1,756	52,180	p.Val120Ile, p.His158Leu	p.Gly103Asp, p.Gly293Ser	KF147860.1
31	9476	1,424	39,590	p.Met88Leu, p.His158Leu,	p.Lys84Glu, p.Val245Ile	KF147860.1
32	11067	0,357	0,357	-	p.Val279Ala	KF147860.1
33	14911	0,109	0,482	-	p.Asp60Tyr, p.Val186Gly	GU647217.1
34	16791	1,322	0,430	-	-	GU647217.1
35	21122	0,736	39,650	p.Pro116Ser, p.Ala136Val	-	GU647217.1
36	25035	0,865	39,540	p.Val120Ile	p.Val279Ala, p.Gly293Ser	GU647217.1
37	25037	0,209	0,713	-	p.Val245Ile	GU647217.1
38	25683	1,699	32,420	p.Val120Ile, p.Leu142Ile, p.His158Leu	p.Ala94Val, p.His227Asp	GU647217.1
39	25790	1,753	48,240	p.Val120Ile, p.Leu142Ile	p.Ala97Ser, p.Gly103Asp	GU647217.1
40	25936	0,642	41,390	p.Val120Ile, p.Leu142Ile	p.Ala94Val, p.Gly293Ser	GU647217.1
41	26911	0,814	0,310	-	-	KF147860.1
42	27443	1,304	30,430	p.Gly36Val, p.Pro116Ser	p.Leu105Phe	GU647217.1
43	28453	1,103	33,590	p.Gly36Val, p.Pro116Ser	p.Asp60Tyr, p.Val279Ala	GU647217.1
44	28825	0,379	0,639	-	p.Val279Ala	KF147860.1
45	29532	1,819	35,490	p.Met88Leu, p.His158Leu,	p.Gly293Ser	EU290753.1
46	29696	0,979	32,350	p.Pro116Ser	-	KF147860.1
47	30171	0,912	0,430	-	-	KF147860.1
48	31014	0,350	0,374	-	p.Val279Ala	GU647217.1
49	32485	1,531	38,690	p.His158Leu	p.Lys84Glu, p.Gly293Ser	GU647217.1
50	32556	0,209	0,324	-	p.Val245Ile	GU647217.1
51	33285	0,316	0,119	-	p.Val245Ile	GU647217.1
52	33679	0,301	0,301	-	p.Val245Ile, p.Gly293Ser	GU647217.1

53	34988	0,424	0,168	-	p.Val245Ile, p.Val279Ala	EU290753.1
54	35100	0,189	0,217	-	-	EU290753.1
55	35204	0,184	0,358	-	p.Asp60Tyr, p.Gly293Ser	KF147860.1
56	35204	0,304	0,403	-	-	GU647217.1
57	35329	0,206	0,248	-	p.Val186Gly, p.Gly293Ser	KF147860.1
58	36745	0,136	0,376	-	p.Val245Ile	KF147860.1
59	37929	0,406	0,677	p.Ala136Val	p.Val245Ile	GU647217.1
60	38640	1,690	31,160	p.Val120Ile, p.Leu142Ile	p.Asp60Tyr, p.His227Asp	GU647217.1
61	42501	1,084	39,560	-	-	GU647217.1
62	46718	0,351	0,351	-	-	GU647217.1
63	552118	1,562	51,680	p.Pro116Ser, p.Val120Ile	p.Leu105Phe, p.His227Asp	KF147860.1
64	5123284	1,901	0,401	-	-	GU647217.1
65	5136889	1,539	10,350	p.Val120Ile, p.Ala136Val	p.Val186Gly, p.His227Asp, p.Val279Ala	GU647217.1
66	5137368	1,453	44,540	p.Val120Ile, p.Leu142Ile, p.His158Leu	p.Lys84Glu, p.Gly103Asp	GU647217.1
67	5137630	1,696	7,650	-	p.Leu105Phe, p.Val279Ala	KF147860.1
68	5151414	1,542	41,490	p.Pro116Ser, p.Ala136Val	p.Lys84Glu, p.Ala94Val	GU647217.1
69	5173984	1,119	48,250	p.Pro116Ser, p.Ala136Val	p.Lys84Glu, p.Ala97Ser, p.Val279Ala	GU647217.1
70	5177284	1,634	48,650	p.Pro116Ser, p.Ala136Val	p.Ala97Ser, p.Gly103Asp, p.Gly293Ser	GU647217.1
71	5178320	1,195	51,150	p.Val120Ile, p.Leu142Ile	p.Leu105Phe, p.His227Asp, p.Gly293Ser	GU647217.1
72	5187267	1,113	49,540	p.Val120Ile	p.Gly103Asp, p.Val279Ala	KF147860.1
73	5287358	0,987	0,540	-	-	GU647217.1
74	5290338	1,432	0,230	-	-	GU647217.1
75	5310874	1,824	3,450	-	p.Leu105Phe, p.Gly293Ser	KF147860.1
76	5375595	0,743	0,370	-	-	GU647217.1
77	5469297	1,853	36,850	p.Val120Ile, p.His158Leu	p.Lys84Glu, p.Ala97Ser, p.Val279Ala	GU647217.1
78	5506625	1,317	41,230	p.Val120Ile, p.Leu142Ile	p.Ala94Val, p.His227Asp	GU647217.1
79	5596058	1,395	0,491	-	-	GU647217.1
80	5601323	1,091	0,540	-	-	GU647217.1
81	5624172	1,712	0,390	-	-	GU647217.1
82	5643804	1,175	39,430	p.Leu142Ile, p.His158Leu	p.Val186Gly, p.Val279Ala	GU647217.1
83	5713144	1,845	48,370	p.Pro116Ser, p.Ala136Val	p.Ala97Ser, p.His227Asp, p.Val279Ala, p.Gly293Ser	GU647217.1
84	5970545	1,574	38,540	p.Pro116Ser	p.His227Asp, p.Val245Ile	GU647217.1
85	5983935	1,648	35,780	p.His158Leu	p.Val245Ile, p.Gly293Ser	GU647217.1
86	5984229	0,988	0,510	-	-	GU647217.1
87	6026977	1,391	42,390	p.Pro116Ser	p.Val59Ile	GU647217.1
88	6098203	0,858	0,480	-	-	GU647217.1
89	6112218	1,658	48,750	p.Pro116Ser	p.Asp60Tyr, p.Val245Ile	GU647217.1
90	6162108	1,209	41,260	p.Val120Ile, p.Leu142Ile	p.Ala94Val, p.Val245Ile, p.Gly293Ser	GU647217.1
91	6235443	1,150	36,890	-	p.Leu105Phe, p.Val245Ile	GU647217.1
92	6306281	1,766	9,430	p.His158Leu	p.Val59Ile, p.Val245Ile	GU647217.1
93	6309830	1,109	49,560	p.Pro116Ser, p.His158Leu	p.Ala97Ser, p.Gly293Ser	GU647217.1
94	6430344	1,310	51,030	p.Pro116Ser, p.His158Leu	p.Ala94Val	GU647217.1
95	6451132	1,694	44,120	p.Pro116Ser	p.Val59Ile, p.Ala94Val, p.Val245Ile	GU647217.1
96	28684	1,817	38,251	p.Met88Leu, p.His158Leu,	p.Val186Gly	GU647217.1
97	28685	1,767	36,450	p.Met88Leu, p.His158Leu,	p.Val186Gly	GU647217.1
98	ATCC19606	1,000	1,000			GU647217.1