

Determining the Antibiotic Resistance Pattern and the Frequency of the Most Common Resistance Genes in *Acinetobacter baumannii* Complex Isolates from Two Medical Centers in Tehran

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ABSTRACT

Background: *Acinetobacter baumannii* is a major cause of nosocomial infections. Today, the increasing trend of antibiotic resistance in this bacterium has created many therapeutic challenges. This study aimed to assess the antibiotic resistance pattern and the presence of the most important resistance genes in *A. baumannii* complex clinical isolates collected from two medical centers in Tehran.

Materials & Methods: In this study, 73 clinical isolates of *A. baumannii* complex belong to patients hospitalized in Sina and Shariati hospitals in 2018 were obtained from the university's microbial collection. Standard biochemical tests and PCR (polymerase chain reaction) detection of the 16srRNA gene were performed to confirm *A. baumannii* complex isolates. Antibiotic sensitivity test was done using disk diffusion method. Isolates with resistance to three or more antibiotic classes were defined as multidrug-resistant (MDR). The frequency of genes encoding aminoglycoside-modifying enzymes and those responsible for resistance to beta-lactam antibiotics was determined using PCR method.

Findings: A total of 73 isolates were confirmed as *A. baumannii* complex. The isolates showed the highest resistance (100%) to ciprofloxacin, cefotaxime, and ceftazidime. They also showed high resistance to other antibiotics. Ninety-three percent of the isolates were classified as MDR. Genetic analysis confirmed the presence of *bla*_{OXA-2'}, *aphA6*, and *bla*_{VIM} genes in 100% of *A. baumannii* complex isolates. Furthermore, the isolates contained *ant* (87.67%), *bla*_{IMP} (65.75%), *aacC1* (76.71%), *aadA1* (35.61%), and *aadB* (61.64%) genes.

Conclusion: The prevalence of antibiotic-resistant *A. baumannii* complex strains has increased among hospitalized patients, leading to significant therapeutic challenges.

Keywords: *Acinetobacter baumannii*, Multidrug-resistant, Beta-lactamase, Antibiotic resistance genes

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Introduction

Members of the genus *Acinetobacter* are Gram-negative bacteria that are present almost everywhere in nature and cause infections in hospitalized patients, especially in the intensive care unit (ICU) [1]. *A. baumannii* is the most important species of this genus, which has emerged as a global threat in medical centers [2]. This bacterium is commonly isolated from the sputum or secretions of patients hospitalized in ICU, especially those who are intubated or have intravenous lines [3]. In fact, this bacterium is the most important species of the *Acinetobacter* genus in the *Moraxellaceae* family, which is associated with various hospital infections [4]. This bacterium has a strong ability to survive for a long time in hospital environments, increasing the possibility of its transmission among patients through human reservoirs and inanimate objects in the hospital. Over the past years, *A. baumannii* has acquired and developed antibiotic resistance mechanisms, which has made it a significant challenge in the field of antibiotic resistance [2]. Although only a few virulence factors of this bacterium are known, antimicrobial resistance is one of the key aspects of its severe pathogenicity [5]. Some resistant strains of *A. baumannii* exhibit resistance to all available antibiotics, necessitating a global effort to address the issue. The emergence of antibiotic resistance in this bacterium has enabled it to persist in hospital environments for a long time, leading to hospital infections [2]. One of the main challenges associated with *A. baumannii* is the emergence of multidrug-resistant (MDR) strains that are resistant to at least three classes of antibiotic classes, including beta-lactams, aminoglycosides, and fluoroquinolones [6,7]. Resistance to aminoglycosides is mediated by several genes, including *aacC1* (encoding acetyltransferase that causes

resistance to gentamicin), *aadA1* (encoding adenylyltransferase that causes resistance to streptomycin and spectinomycin), *aadB* (encoding adenylyltransferase that causes resistance to gentamicin, tobramycin, and kanamycin), and *aphA6* (encoding phosphotransferase that causes resistance to gentamicin, amikacin, kanamycin, and neomycin) [8]. Also, *bla_{VIM}* and *bla_{IMP}* genes confer resistance to beta-lactam antibiotics like imipenem and meropenem by activating efflux pumps and producing metallo-beta-lactamases. Another factor contributing to resistance to carbapenems such as meropenem and imipenem is the presence of OXA genes, which confer resistance by producing carbapenemases [9,10].

Objectives: This study aimed to investigate the antibiotic resistance pattern and the abundance of *bla_{VIM}*, *bla_{IMP}*, *ant*, *aphA6*, *aadB*, *aadA*, *aacC1*, and *bla_{OXA-2}* genes in *A. baumannii* complex clinical isolates collected from two medical centers in Tehran.

Materials and Methods

Identification of bacterial isolates: In this study, 73 clinical isolates of *A. baumannii* complex belonging to patients hospitalized in Sina and Shariati hospitals including emergency room, operating room, general, oncology, women's surgery, men's internal medicine, endocrinology, and ICU were obtained from the university's microbial collection. The isolates were collected from bronchial lavage (BAL), wound, urine, sputum, blood, tissue, and tracheal samples in 2018. Then the isolates were identified with biochemical tests including culture on MacConkey agar and OF agar (Ibresco, Iran), oxidase (Padtan Teb, Iran), SIM, MRVP, TSI, urease (Merck, Germany), and catalase (Bahar Afshan, Iran) tests.

DNA extraction: Boiling method was used to extract bacterial DNA. For this purpose, a loopful of fresh bacterial culture on brain

heart infusion agar was suspended in 200 µL of distilled water under completely sterile conditions. The sample was heated at 95 °C for 15 min. After centrifugation at 12,000 rpm for 10 min, the supernatant was collected and used as a DNA template in polymerase chain reaction (PCR).

Determining the genotypic identity of the samples: In order to confirm the genotypic identity of *A. baumannii* complex, PCR test was performed to detect the 16SrRNA gene [11]. The primer sequences used to detect this gene are presented in Table 1. Clinical isolates from our microbial collection and distilled water were used as positive and negative controls, respectively.

The PCR reaction was carried out in a final volume of 25 µL, including 12.5 µL of master mix, 0.5 µL of forward primer, 0.5 µL of reverse primer, 9.5 µL of sterile distilled water, and 2 µL of template DNA. The PCR reaction was performed in a thermocycler (Sensoquest, Germany) under the following thermal cycling conditions: an initial denaturation

step at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, and elongation at 72 °C for 1 min and a final elongation step at 72 °C for 5 min. PCR products were electrophoresed on 1% agarose gel and analyzed in a transilluminator (Cleaver Scientific Ltd, UK) for the presence or absence of the 16srRNA gene.

Antibiotic susceptibility test: The antibiotic sensitivity of each isolate was evaluated by the standard disk diffusion method on Mueller-Hinton agar (MHA) medium (Merck, Germany) using the following antibiotic disks, including amikacin (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), cefotaxime (30 µg), ceftazidime (30 µg), imipenem (10 µg), meropenem (10 µg) and tobramycin (10 µg). All of them were prepared from Padtan Teb Company, Iran. The results were analyzed according to the instructions of the Clinical and Laboratory Standards Institute (CLSI) version 2017.

Table 1) Sequences of primers used to identify *Acinetobacter baumannii* complex and resistance genes

Gene	Primer (5'→3')	Annealing (c°)	(bp)	Reference
16srRNA	F: -CAGCTCGTGTCTGAGATGT R: -CGTAAGGGCCATGATGACTT	55	150	[11]
<i>bla</i> _{IMP}	F: AACCAGTTTTGCCTTACCAT R: CTACCGCAGCAGAGTCTTTG	57	587	[12]
<i>bla</i> _{VIM}	F: ATTGGTGTTTGTCTGCATATC R: TGGGCCATTCAGCCAGATC	59	510	[13]
<i>bla</i> _{OXA-2}	F: AAGAAACGCTACTCGCCTGC R: CCACTCAACCCATCCTACCC	61.5	486	[14]
<i>ant</i>	R: GCTCACGCAACTGGTCCAGA F: GGCACGCAAGACCTCAACCT	61	719	[15]
<i>aacC1</i>	F: ATGGGCATCATTCGCACATGTAGG R: TTAGGTGGCGGTACTTGGGTC	52	456	[16]
<i>aphA6</i>	F: ATGGAATTGCCCAATATTATTC R: TCAATTCAATTCATCAAGTTTTA	55	797	[16]
<i>aadA1</i>	F: ATGAGGGAAGCGGTGATCG R: TTATTTGCCGACTACCTTGGTG	52	792	[16]
<i>aadB</i>	F: ATGGACACAACGCAGGTCGC R: TTAGGCCGCATATCGCGACC	55	534	[16]

Antibiotic resistance genes: All *A. baumannii* complex isolates were investigated for the presence of resistance genes conferring resistance to beta-lactams (*bla*_{VIM}, *bla*_{IMP}, and *bla*_{OXA-2}) and aminoglycosides (*aphA6*, *aacC1*, *aadA1*, *aadB*, and *ant*) using PCR reactions. The primer sequences used to detect these genes are presented in Table 1.

The PCR reaction was performed in a final volume of 25 µL, including 12.5 µL of master mix (SMO BIO, China), 0.5 µL of forward primer, 0.5 µL of reverse primer (Pishgam, Iran), 9.5 µL of sterile distilled water, and 2 µL of template DNA.

The PCR reaction was performed in a thermocycler under the following thermal cycling conditions: an initial denaturation step at 95 °C for 4 min, followed by 30 cycles of denaturation at 95 °C for 35 s, annealing at the specific temperature for each primer for 35 s, and elongation at 72 °C for 40 s and a final elongation step at 72 °C for 5 min. PCR products were then electrophoresed on 1% agarose gel and examined in a transilluminator for the presence or absence of the mentioned genes.

Statistical analysis: Data was analyzed using SPSS software version 22.0 (SPSS Inc.,

USA). Variables are represented as frequency and percentage.

Findings

Identification of bacterial isolates: Based on the results of biochemical tests and PCR detection of the 16SrRNA gene, all of 73 isolates were identified as *A. baumannii* complex. The frequency of *A. baumannii* complex isolates in different clinical samples and hospital departments was investigated. Based on the results, most of the isolates were collected from BAL samples (n=27, 36.9%), followed by wound, urine culture, sputum, blood culture, tissue, and tracheal samples, respectively. In addition, most of the isolates were collected from ICU wards (n=41, 56.1%) which was significantly higher compared to the samples isolated from other wards (Figures 1 and 2). In addition, the frequency distribution of bacterial isolates was almost the same in both genders: 36 (49.3%) from male and 37 (50.6%) from female patients. Furthermore, most of the patients belonged to the age group of 50-70 years (n=27, 36.9%), followed by 30-50 years (n=23, 31.5%), 70-95 years (n=19, 26%), and <30 years (n=4, 5.4%).

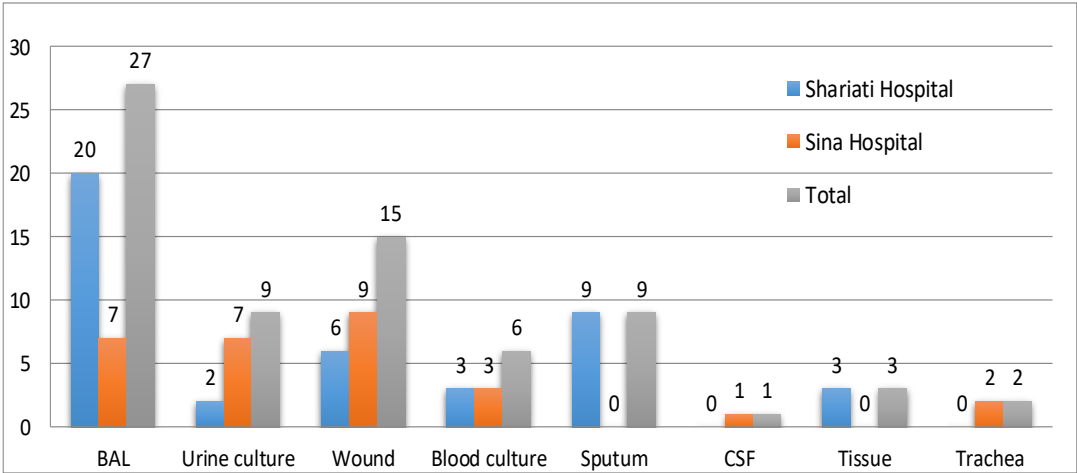


Figure 1) Frequency of *Acinetobacter baumannii* complex isolates according to the type of clinical samples from Shariati and Sina hospitals. The vertical axis expresses the number of isolates in each sample. Bronchial lavage (BAL) was the most common sample type, followed by wound, urine culture, sputum, blood culture, tissue, tracheal and cerebrospinal fluid (CSF) samples, respectively.

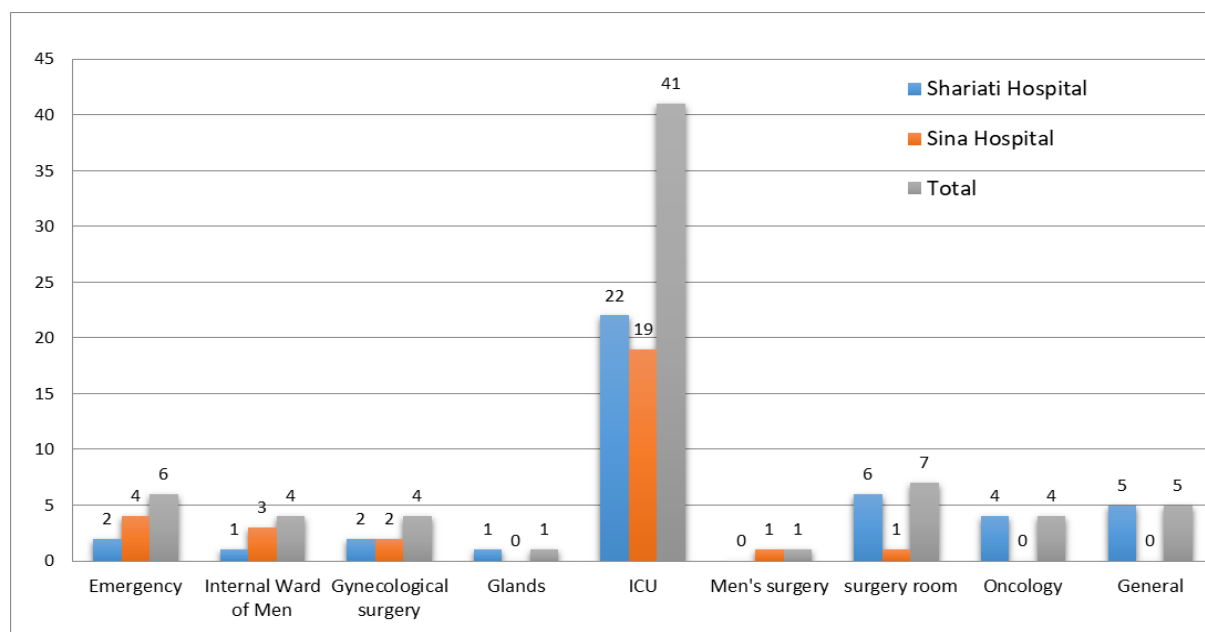


Figure 2) Total frequency of *Acinetobacter baumannii* complex isolates in different departments of Shariati and Sina hospitals. The vertical axis expresses the number of isolates in each ward. Most of the isolates belonged to ICU ward.

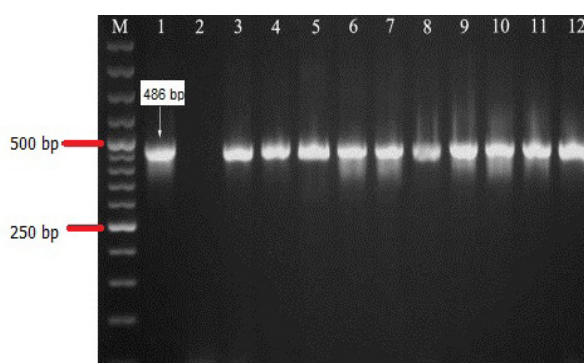


Figure 3) Amplification of *aphA6* resistance gene by PCR. M. size marker (1 kb DNA ladder). Lane1: positive control. Lane2: negative control. Lanes 3-12: isolates with *aphA6* gene.

Antibiotic sensitivity pattern: The isolates showed the highest resistance (100%) to ciprofloxacin, cefotaxime and ceftazidime. The rates of resistance to other antibiotics were as follows: amikacin (n= 68, 93.1%), gentamicin (n= 66, 90.4%), imipenem (n= 71, 97.3%), meropenem (n= 72, 98.7%), and tobramycin (n= 60, 82.2%). A total of 68 isolates (93%) showed resistance to three classes of antibiotics (MDR isolates).

Identification of resistant genes: The frequency of resistance genes in *A. baumannii*

complex isolates was investigated by PCR (Figure 3). Accordingly, all isolates harbored *aphA6*, *bla*_{OXA-2} and *bla*_{VIM} genes. Furthermore, the isolates also contained *ant* (n=64, 87.67%), *aacC1* (n=56, 76.71%), *bla*_{IMP} (n=48, 65.75%), *aadB* (n=45, 61.64%), and *aadA1* (n=26, 35.61%) genes.

Discussion

Nosocomial infections are common and important causes of death, disability, increased length of hospitalization, imposition and increase of hospital costs, and occurrence of health problems. Over the past decade, *A. baumannii* has been the most successful nosocomial opportunistic pathogen worldwide, which has recently become one of the most important causes of severe infections in ICU. Its inherent resistance to antibiotics could be attributed to its long-term survival in the hospital environment [17]. This bacterium causes a wide range of infections, leading to a mortality rate of 8-40% in nosocomial infections [18]. Many studies conducted in hospitals during sudden outbreaks have

shown that hospital environments are the source of *A. baumannii* infections in most cases [19].

Treatment of *A. baumannii* infections is very difficult due to the emergence of MDR strains and their rapid spread in hospital environments. Currently, this bacterium is considered as one of the most important causes of mortality of patients hospitalized in different hospital departments, especially ICU, and therefore one of the main concerns of the medical community [20]. The antibiotic sensitivity test results show that hospital isolates have higher antibiotic resistance, which increases the probability of bacterial survival in hospital environments. In the ICU, where patients take a large amount of antibiotics such as carbapenems, the risk of developing resistant infections increases [4]. In addition, most pathogenic bacteria have become almost completely resistant to some new antibiotics such as broad-spectrum cephalosporins, including cefotaxime and ceftazidime. Previously, imipenem was the most effective drug against infections caused by this bacterium, but recently, the spread of imipenem-resistant strains has also been documented [6].

In this study the average age of patients hospitalized in Shariati and Sina hospitals was reported to be 55 years. Also, most of the patients belonged to the age group of 50-70 years (n=27, 36.9%). Out of 73 isolates, 36 (49.31%) strains were isolated from male patients, and 37 (50.68%) strains were isolated from female patients. Among 73 *A. baumannii* complex isolates, most of them (36.9%) were isolated from BAL samples. Previous studies have shown that bacterial samples are mostly isolated from the skin surface and the pharyngeal area (larynx) [21, 22]. In this study, 36.9% of the samples were isolated from the lungs of hospitalized patients. On the other hand, among hospital departments, *A. baumannii* complex strains

were mostly isolated from the ICU (56.1%), which is the most suitable and important department for the spread of these bacteria. In a study in Turkey, Merik et al. (2005) reported *Acinetobacter* species as the second most common organism in the ICU (26.8%) [23]. According to other studies and reports, ICU is one of the most important hospital departments where infections caused by this bacterium are very common [24,25].

In this research, the antibiotic resistance of 73 *A. baumannii* complex isolates from Sina and Shariati hospitals was investigated against different antibiotics, including ciprofloxacin, imipenem, cefotaxime, ceftazidime, meropenem, gentamicin and tobramycin. Also, the abundance of resistance genes conferring resistance to carbapenems and beta-lactams (*bla_{IMP}*, *bla_{VIM}*, and *bla_{OXA-2}*) as well as aminoglycosides (*aacC1*, *aphA6*, *aadA1*, *aadB*, and *ant*) was investigated in these isolates using PCR. Due to the high resistance of these isolates to different antibiotic classes, 93% of them were reported to be MDR.

According to the findings, *A. baumannii* complex isolates showed 100% resistance to beta-lactam antibiotics such as cefotaxime and ceftazidime. Resistance to these antibiotics has also been reported to be high in other studies. In the study conducted by Seyedi Abhari et al. (2021) in Tehran resistance to ceftazidime has been reported to be 96.9% [26]. In other study by Teimourpour and colleagues (2020) in Ardabil, resistance to cefotaxime and ceftazidime has been reported to be 94.6% and 89.6%, respectively [27]. In addition, the frequency of *bla_{VIM}*, *bla_{OXA-2}*, and *aphA6* genes was 100%, while the frequency of *bla_{IMP}* gene was 65.5%. The *bla_{IMP}* gene has been reported to be more prevalent among metallo-beta-lactamase genes, especially in Iran [2, 28]. In a study by Fallah et al. (2014), the prevalence of *bla_{VIM}* and *bla_{IMP}* was 17.4

and 3.5%, respectively [2]. In another study by Sarhaddi et al. (2016), the prevalence rate of *bla*_{VIM} and *bla*_{IMP} resistance genes was reported as 65.6 and 70.4%, respectively [29]. Therefore, according to these investigations, the prevalence of beta-lactam resistance genes has increased dramatically, and this may be the reason for the high resistance of *A. baumannii* isolates to ceftazidime and cefotaxime antibiotics. Oxacillinases, which are recognized as Ambler class D, play a key role in antimicrobial resistance among *Acinetobacter* species [30].

OXA-2 type beta-lactamases possess the ability to hydrolyze oxacillin and cloxacillin at a high level [30]. There is limited information about this gene in the literature.

Asgin et al. (2019) reported the frequency of *bla*_{OXA-2} among bacterial isolates as 21.7% [31]. However, in our study, the frequency of this gene was high. Thus, *A. baumannii* strains producing beta-lactamases are a serious threat in the ICU. By careful screening of metallo-beta-lactamase-producing strains, the spread of MDR *A. baumannii* strains and related infections could be reduced [2].

The present study results showed significant resistance of *A. baumannii* complex isolates to aminoglycoside antibiotics, such as gentamicin (90.4%), amikacin (93.15%), and tobramycin (82.19%). These results are higher compared to other studies results. Ardabili et al. (2012) reported 86, 94, and 62% resistance to gentamicin, amikacin, and tobramycin in the isolates obtained from burn wounds of patients in Shahid Motahari hospital, respectively [32].

Additionally, lower resistance rates to these antibiotics have been reported in the studies by Amini et al. (2016) in the ICU department of Imam Reza hospital in Kermanshah and Khashai et al. (2018) in the ICU department of Namazi hospital in Shiraz [33, 34]. Taken together, these studies indicate the increasing trend of

aminoglycoside resistance in *A. baumannii* isolates in different regions. Resistance to aminoglycosides in *A. baumannii* is mainly due to inactivation of antibiotics by specific modifying enzymes such as adenylyltransferases, phosphotransferases, and acetyltransferases [35, 36].

According to the molecular analysis performed to detect aminoglycoside resistance genes, the following genes were detected in the isolates: *aphA6* (100%), *ant* (87.67%), *aacC1* (76.71%), *aadB* (61.64%), and *aadA1* (35.61%). In a study by Tawakkal and Mumtaz (2015), the prevalence of *aadA1* gene was reported to be 55.37%, which is higher than the present study result [37]. In another study in Tabriz on *A. baumannii* isolates, the frequency of *aphA6* (48.5%) and *aadB* (53.15%) genes was lower than the present study results [38].

In another study, Ali Akbarzade et al. (2014) in Kerman reported lower rates for genes encoding resistance to aminoglycosides, including *aadB* (18.6%), *aphA6* (60.46%), *aacC1* (65.11%) and *aadA1* gene (27.9%), which indicates an increase in the prevalence of these genes in our study [39].

The *aphA6* gene encodes phosphotransferase, which is responsible for resistance to gentamicin, amikacin, kanamycin, and neomycin antibiotics. In this study, 66 isolates (90%) harboring the *aphA6* gene were resistant to amikacin and gentamicin. On the other hand, the *aadB* gene encodes adenylyltransferase, which is responsible for resistance to antibiotics such as gentamicin, tobramycin and kanamycin. In almost all the studies mentioned in this article, the resistance rate of *A. baumannii* isolates to aminoglycosides was lower compared to the present study, indicating the increasing trend of resistance to aminoglycoside antibiotics in these isolates.

Resistance of *Acinetobacter* to aminoglycosides is an important therapeutic prob-

lem. This study results show that bacterial isolates from hospitalized patients have different types of genes encoding aminoglycoside-modifying enzymes, and timely identification of these genes could lead to a reduction in severe complications and mortality rate. Due to budget limitations, we were unable to determine the clonality of the isolates.

Conclusion

A. baumannii is known as an important opportunistic pathogen involved in hospital infections. This study results indicate an increase in the antibiotic resistance and the prevalence of MDR strains of this bacterium compared to previous studies. Additionally, the prevalence of resistance genes such as *aphA6*, *aadB*, *aacC1*, *aadB*, *bla_{VIM}*, *bla_{IMP}*, and *bla_{OXA-2}* in clinical isolates of *A. baumannii* complex is also increasing. These results underscore the critical importance of identifying these antibiotic resistance genes. Appropriate use of antibiotics is critical to control the spread of antibiotic resistance. On the other hand, the isolation of high levels of resistant strains from ICU is worrisome, and this problem needs further investigation.

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Consent to participate: Consent was obtained

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