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Prevalence of Aminoglycoside and Carbapenemase Resistance Genes and Biofilm Formation among Clinical Isolates of *Acinetobacter baumannii* in Iran

İran'da *Acinetobacter baumannii* Klinik İzolatlarında Aminoglikozid ve Karbapenemaz Direnç Genleri ve Biyofilm Oluşumunun Prevalansı

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Abstract

Introduction: Nowadays, due to the increasing resistance of *Acinetobacter baumannii* to antibiotics, it has been a problematic agent in clinical settings. As a significant nosocomial pathogen, *A. baumannii* isolates use resistant genes and biofilm development as means of survival. The aim of this study was to determine antimicrobial resistance patterns, aminoglycoside and carbapenems resistance genes, and biofilm formation among clinical isolates of *A. baumannii.*

Materials and Methods: A cross-sectional study was conducted from April 2018 to March 2019. In all, 133 nonduplicated isolates of *A. baumannii* were isolated from Yasuj and Bandar Abbas, located in Iran. Antimicrobial susceptibility was determined using disk diffusion. Carbapenem- and aminoglycoside-resistant genes were investigated by the polymerase chain reaction method. The ability to generate biofilms was evaluated using the microtiter plate method.

Results: In this study, all isolates contained $bla_{OXA\text{-}51\text{-}like}$ and were confirmed as *A. baumannii*. High-level resistance was observed for carbapenems and aminoglycosides. The prevalence of oxacillinase genes $blaOXA_{\text{-}23\text{-}like}$ and $bla_{OXA\text{-}24\text{-}like}$ was 89 (66.9%) and 46 (34.6%), respectively. Moreover, the co-occurrence of $bla\text{-}_{OXA\text{-}23\text{-}like}$ and $bla\text{-}_{OXA\text{-}24\text{-}like}$ was 10 (7.5%). A total of 73 (54.9%) and 69 (51.9%) were positive for aac (3)-I and aph (3')-I, respectively. Furthermore, the coexistence of two genes was obtained in 55 (41.4%) isolates. The result demonstrates that 129 (97%) of isolated were strong, three (2.3%) moderate, and one (0.8%) weak biofilm producer.

Conclusion: Results revealed that $bla\text{-}_{OXA\text{-}23\text{-}like}$ and aac (3)-I genes were the most prevalent resistance genes. Since a vast majority of isolates were drug-resistant with strong biofilms, infection control programs and policies should be frequently upgraded to control the transmission of drug-resistant *A. baumannii* isolates in the future.

Keywords: Antimicrobial resistance, *Acinetobacter baumannii*, OXA carbapenemase, aminoglycosides, biofilms

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Öz

Giriş: Acinetobacter baumannii antibiyotiklere karşı artan direnci nedeniyle günümüzde sorunlu bir etken olarak karşımıza çıkmaktadır. Direnç genleri ve *A. baumannii* izolatları tarafından biyofilm üretimi, organizmanın önemli bir hastane patojeni olmasını sağlayan hayatta kalma yollarıdır. Bu çalışmanın amacı, *A. baumannii* klinik izolatlarında antimikrobiyal direnç paternlerini, aminoglikozid ve karbapenem direnç genlerini ve biyofilm oluşumunu belirlemektir.

Gereç ve Yöntem: Nisan 2018'den Mart 2019'a kadar yürütülen bu kesitsel çalışmada; İran'da Yasuj ve Bandar Abbas bölgelerinde bulunan hastanelerden toplam 133 *A. baumannii* izolatı izole edildi. Antimikrobiyal duyarlılık disk difüzyon kullanılarak belirlendi. Karbapenem ve aminoglikozid direnç genleri polimeraz zincir reaksiyonu yöntemi ile araştırıldı. Biyofilm oluşturma yeteneği, mikrotitre plak yöntemi kullanılarak değerlendirildi.

Bulgular: Bu çalışmada tüm izolatlar $bla_{OXA\text{-}51\text{-}like}$ içermekteydi ve *A. baumannii* olarak doğrulandı. Karbapenemler ve aminoglikozidler için yüksek düzeyde direnç gözlemlendi. Oksasilinaz genleri olan $bla_{OXA\text{-}23\text{-}like}$ ve $bla_{OXA\text{-}24\text{-}like}$ prevalansları sırasıyla 89 (%66,9) ve 46 (%34,6) idi. Ayrıca, $bla_{OXA\text{-}23\text{-}like}$ ve $bla_{OXA\text{-}24\text{-}like}$ birlikteliği 10 (%7,5) izolatta saptanmıştır. Sırasıyla aac (3)-I ve aph (3')-I 73 (%54,9) ve 69 (%51,9) izolatta pozitif bulundu. Ayrıca izolatların 55'inde (%41,4) iki gen bir arada bulundu. Sonuçlar, izolatların 129'unun (%97) güçlü, üçünün (%2,3) orta ve birinin (%0,8) zayıf biyofilm üreticisi olduğunu göstermiştir.

Sonuç: Sonuçlar, $bla_{OXA\text{-}23\text{-}like}$ ve aac (3)-I genlerinin en yaygın direnç genleri olduğunu ortaya koydu. İzolatların büyük çoğunluğu güçlü biyofilm üreticisi ve ile ilaca dirençli olduğundan, gelecekte ilaca dirençli *A. baumannii* izolatlarının bulaşmasını kontrol etmek için enfeksiyon kontrol programları ve politikaları sıklıkla güncellenmelidir.

Anahtar Kelimeler: Antimikrobiyal direnç, *Acinetobacter baumannii*, OXA karbapenemaz, aminoglikozidler, biyofilmler

Introduction

In recent years *Acinetobacter baumannii* (*A. baumannii)* a challenging and known organism has gained much clinical attention worldwide[1,2]. The *Acinetobacter* species were responsible for a wide range of nosocomial infections principally in patients who were admitted to intensive care units[2]. Todays, because of resistance to different classes of antibiotics, multidrug-resistant (MDR) and extensively drug-resistant *A. baumannii* are problematic agents worldwide[3]. Combination therapy is essential for the effective treatment of such isolates[4]. Furthermore, as a result of synergistic bactericidal activity, aminoglycoside and carbapenem were sometimes used in combination for the treatment of drug-resistant *A. baumannii* infections[5-7]. Unfortunately, *A. baumannii* can become resistant to these agents. Carbapenem-hydrolyzing enzymes belong to class B metallo-b-lactamases and class D beta-lactamases (oxacillinases) $bla_{OXA\text{-}23\text{-}like}$, $bla_{OXA\text{-}24\text{-}like}$ and $bla_{OXA\text{-}58\text{-}like}$ are the most common mechanism of carbapenem resistance in *A. baumannii*[3,7]. Several mechanisms that are involved in the aminoglycoside resistance phenotype have been suggested in *A. baumannii* isolates, but the main mechanism is related to aminoglycoside-modifying enzyme (AME) genes such as acetyltransferases (AACs), nucleotidyltransferases (ANTs), and/or phosphotransferases (APHs)[6,8]. The APHs *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* are linked to aminoglycoside resistance[8]. It has been established that *A. baumannii* has different virulence factors and the most attractive one is bacterial biofilm formation[9,10]. It should be noted that biofilm formation contributes to resistance in *A. baumannii* and may lead to the development of MDR strains[10]. It has been observed that the production of biofilm, can increase the persistence of A. *baumannii* isolates in hospital environments, including patients, and may lead to epidemics in healthcare systems[11,12].

Our study aimed to investigate the prevalence of selected genes ($bla_{OXA\text{-}23\text{-}like}$, $bla_{OXA\text{-}24\text{-}like}$, $blaOXA_{\text{-}58\text{-}like}$), aac (3)-I, aac (6')-Ib, aph (3')-I in clinical isolates of *A. baumannii.* In addition, we evaluated biofilm formation ability via the microtiter plate method by crystal violet staining.

Materials and Methods

Bacterial Isolation and Identification

This cross-sectional study, which was carried out from April 2018 to March 2019, included 133 nonduplicated isolates of *A. baumannii* that were obtained from clinical specimens at three major hospital centers connected to Yasuj and Hormozgan Universities of Medical Sciences. Conventional microbiology techniques were initially used to identify *A. baumannii* isolates. Finally, samples were confirmed by polymerase chain reaction (PCR) for intrinsic $bla\text{-}_{OXA\text{-}51\text{-}like}$ using specific primers listed in Table 1[13,14].

DNA Extraction

As previously mentioned, the DNA template was extracted by the boiling method[15]. The DNA concentration and purity were estimated by UV-spectrophotometer (Photo Biometer, Eppendorf, Germany) at 260/280 nm[7,15].

Amplification of *bla-*$_{OXA\text{-}51\text{-}like}$

Each reaction had a final volume of 25 µL and was carried out using the following ingredients: 1× PCR buffer, 1.5 mM $MgCl_2$, 200 µM of dNTP, 10 pmol of each primer, 1 U Taq polymerase (Sina Clon, Bioscience Co, Iran), and 1 µL of extracted DNA. Furthermore, *A. baumannii* ATCC 19606 was used as a positive control. The amplification parameters were programmed in a thermal cycler (Bio-Rad My Cycler Thermal Cycler) with the following conditions: Initial denaturation at 94 °C for 4 min; 35 cycles of amplification consisting of denaturation at 94 °C for 45 s, annealing at 57 °C for 45 s, extension 72 °C for 1 min, and final extension 72 °C for 5 min[14]. The PCR products were separated on 1.5% (w/v) agarose gel (Sina Clon, Iran) in 1× TBE (Tris-Borate-EDTA, pH=8.2) buffer, stained with 5× GelRed (Biotium, USA), and then visualized on a UV transilluminator.

Antimicrobial Susceptibility Tests

By the recommendations of the Clinical and Laboratory Standards Institute, which was updated in 2019, susceptibility was assessed using the disk diffusion method on Mueller-Hinton agar (Merck, Germany) plates. All isolates were tested for imipenem 10 µg, meropenem 10 µg, gentamicin 10 µg, amikacin 30 µg, and tobramycin 10 µg (MAST, Group Ltd., Merseyside, UK). *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 were used as quality control strains[16].

Molecular Characterization of Oxacillinase Genes

Multiplex PCR method was carried out for identification of *bla-*$_{OXA\text{-}23\text{-}like,}$ *bla-*$_{OXA\text{-}24\text{-}like,}$ and bla-OXA-58-like as previously described[17]. Each multiplex PCR reaction was prepared in a final volume of 25 µL with 1× PCR buffer, 2 mM MgCl2, 1 U Taq polymerase, 0.2 µM of each primer (TAG, Copenhagen A/S Denmark), 200 µM of dNTP (Sina Clon, Iran), and 1 µL of template DNA. Target fragments were amplified by a thermal cycler (Bio-Rad My Cycler Thermal Cycler) as follows: Initial denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 53 °C for 40 s, and 72 °C for 50 s, and a final extension at 72 °C for 6 min. *A. baumannii* NCTC 13304 and *A. baumannii* NCTC 13302 were used as a positive control for *bla-*$_{OXA\text{-}23\text{-}like}$ and *bla-*$_{OXA\text{-}24\text{-}like}$, respectively. All amplicons were separated using electrophoresis on 1.5% (w/v) agarose gel (Sina Clon, Iran) in 1× TBE buffer, stained with 5× GelRed (Biotium, USA). A 100 bp DNA ladder (Sina Clon, Iran) was used for the comparison of PCR products.

PCR Amplification of Aminoglycoside Resistance Genes

Using the specific primers specified in Table 1, all isolates were screened for resistance genes, aac (3)-I, aac (6 ')-Ib, and aph (3 ')-I as previously described[18]. The PCR mixture was performed in a total volume of 25 µL including 1× PCR buffer, 200 µM of dNTP, 1.5 mM $MgCl_2$, 10 pmol of each primer (TAG, Copenhagen A/S Denmark), 1 U Taq polymerase (SinaClon, Bioscience Co, Iran), and 1 µL of extracted DNA. The PCR analysis was done by a thermal cycler (Bio-Rad My Cycler Thermal Cycler) under the following program: Initial denaturation at 95 °C for 5 min; followed by 35 cycles at 95 °C for 30 s, 55 °C or 58 °C for 30 s, and 72 °C for 1 min, and final extension at 72 °C for 5 min [58 °C for aac(3)-I and aac(6')-Ib and 55 °C for aph(3')-I was performed][18]. All amplicons were analyzed by electrophoresis on 1.5% (w/v) agarose gel (Sina Clon, Iran) in 1× TBE buffer, stained with 5X GelRed (Biotium, USA). A 100 bp DNA ladder (Sina Clon, Iran) was used for the comparison of PCR products. In addition, positive PCR products of two isolates containing aac (3)-I and aph(3')-I were sequenced by an ABI 3730XL DNA analyzer (Bioneer, South Korea) and after confirmation used as a positive control in each PCR.

Biofilm Formation Assay

The quantitative technique previously described was used to test each isolate's capacity to generate biofilms. Each isolate was inoculated in Trypticase Soy Broth at 37 °C for 24 h. Following growth, a dilution ratio of 1:100 was prepared and 150 µL of this dilution was inoculated in sterile 96-well flat-bottomed microtiter polystyrene plates. Each test was carried out in triplicate. Then, without shaking, the infected plates were incubated at 37 °C for 24 h. Then, after incubation time, the supernatants were gently removed and the pellets were washed three times with 200 µL of phosphate-buffered saline.

Table 1. Primers sequences used in current research

Target gene	Primer sequence		Product size (bp)
	Forward (5′-3′)	Reverse (5′-3′)	
bla-$_{OXA\text{-}51\text{-}like}$	TAATGCTTTGATCGGCCTTG	TGGATTGCACTTCATCTTGG	353
bla-$_{OXA\text{-}23\text{-}like}$	GATCGGATTGGAGAACCAGA	ATTTCTGACCGCATTTCCAT	501
bla-$_{OXA\text{-}24\text{-}like}$	GGTGTTGGCCCCCTATAA	AGTTGAGCGAAAAGGGGATT	249
bla-$_{OXA\text{-}58\text{-}like}$	AAGTATTGGGGCTTGTGCTG	CCCCTCTGCGCTCTACATACTA	599
aac(3)-I	TTACGCAGCAGCAACGATGT	GTTGGCCTCATGCTTGAGGA	402
aac(6')-Ib	CATGACCTTGCGATGCTCTA	GCTCGAATGCCTGGCGTCTT	490
aph(3')-I	ATGTGCCATATTCAACGGGAAACG	TCAGAAAAACTCATCGAGCATCAA	816

In addition, to drying, the plates were placed at an inverted position at room temperature. To fix the attached bacteria, 100 µL of 99% methanol were added to each well and after 15 min the wells were emptied and allowed to air dry. Then, the wells were stained with 150 µL of 1% crystal violet for 20 min. The excess dye was removed and for solubility of bound crystal violet, 150 µL of acetic acid was added to each well[19]. Using a microtiter plate, the optical density (OD) for each well was measured at 620 nm. Biofilm formation was determined according to the following category[20]: A: non-biofilm producer=OD≤ODc, B: weak biofilm producer=ODc<OD≤2ODc, C: medium biofilm producer = 3ODc<OD≤4ODc, D: strong biofilm producer=4OD<ODc.

This study was approved by the Yasuj University of Medical Sciences (YUMS) Ethics Committee (approval ID: IR.YUMS.REC.1398.011).

Nucleotide Sequence Accession Number

The two novel nucleotide sequences aac (3)-I and aph (3')-I detected in this research were submitted to the BankIt nucleotide sequence database and are available under the accession numbers MW429276 and MW429277.

Results

A total of 133 nonrepeated *A. baumannii* isolates were gathered from three teaching hospitals in Yasuj and Bandar Abbas between April 2018 and March 2019.

Antimicrobial Susceptibility Testing

The result of the antimicrobial susceptibility test for 133 *A. baumannii* isolates is shown in Table 2. According to the results, the highest resistance rate was observed for carbapenems. Moreover, among aminoglycosides, high susceptibility was related to tobramycin *in vitro* and the high resistance rate was related to gentamicin.

Distribution of Oxacillinase Genes

Results of PCR revealed that all isolates contained intrinsic *bla*-$_{\text{OXA-51-like}}$ and confirmed as *A. baumannii*. Among 133 isolates, 89 isolates (66.9%) were positive for *bla*-$_{\text{OXA-23-like}}$ and this gene was the most prevalent oxacillinase in studied isolates. Furthermore, *bla*-$_{\text{OXA-24-like}}$ was detected in 46 isolates (34.6%). No *bla*-$_{\text{OXA-58-like}}$ was determined among isolates. Co-occurrence of *bla*-$_{\text{OXA-23-like}}$ and *bla*-$_{\text{OXA-24-like}}$ was observed in 10 isolates (7.5%).

Prevalence of Aminoglycoside Resistance Genes

Based on the results of PCR, aac (3)-I was found in 73 isolates (54.9%) and aph (3')-I was present in 69 isolates (51.9%). The aac (6')-Ib gene was not detected in any of the strains. Coexistence of aac (3)-I and aph (3')-I was detected in 55 isolates (41.4%). The percentage of aminoglycoside-resistant genes and aminoglycoside antibiotics are shown in Table 3.

Biofilm Formation

According to the mentioned criteria, the microtiter plate assay results were interpreted. The isolates were divided into four categories including strong, moderate, weak, and nonbiofilm producers. All isolates were biofilm producers. According to the standard microtiter plate method no significant differences were found. The majority of isolates, 129 of 133 (97%) were strong biofilm producers. The prevalence of moderate biofilm production was 2.3% (3 isolates). The minority of isolates 0.8% (1 isolate) was weak biofilm producers.

Table 2. Antimicrobial susceptibility result of *A. baumannii* isolates

Antibiotics	Resistant (%)	Intermediate (%)	Sensitive (%)
Imipenem	126 (94.7)	0	7 (5.3)
Meropenem	126 (94.7)	0	7 (5.3)
Gentamicin	125 (94)	0	8 (6)
Amikacin	122 (91.7)	2 (1.5)	9 (6.8)
Tobramycin	112 (84.2)	0	21 (15.8)

Table 3. The percentage of aminoglycoside-resistant genes

Aminoglycoside-resistant genes	Amikacin			Gentamicin			Tobramycin		
	R (%)	I (%)	S (%)	R (%)	I (%)	S (%)	R (%)	I (%)	S (%)
aac(3)-I	65 (48.9)	1 (0.8)	7 (5.3)	66 (49.6)	0 (0)	7 (5.3)	61 (45.9)	0 (0)	12 (9)
aac(6')-Ib	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
aph(3')-I	61 (45.9)	1 (0.8)	7 (5.3)	62 (46.6)	0 (0)	7 (5.3)	52 (39.1)	0 (0)	17 (12.8)

R: Resistant, I: Intermediate, S: Sensitive

Discussion

Resistance to antimicrobial agents in *A. baumannii* is a serious global concern[21,22]. In the past, carbapenems were more effective against *A. baumannii* isolates, but nowadays, carbapenem-resistant *A. baumannii* is increasingly reported worldwide[23,24]. As expected, in our study the highest rate of resistance was observed to carbapenems and 126 (94.7%) of isolates were resistant to imipenem and meropenem. Additionally, we investigated bla_{OXA} carbapenemase genes because they are the main mechanism of carbapenem resistance in *A. baumannii*[24]. Among the OXA carbapenemase, $bla\text{-}_{OXA\text{-}23\text{-}like}$ reported as the most prevalent[25,26] and *A. baumannii* harboring this gene have been reported from different parts of the world[24]. Ranjbar and Farahani[27] showed that $bla\text{-}_{OXA\text{-}23\text{-}like}$ and $bla\text{-}_{OXA\text{-}40\text{-}like}$ carrying isolates had a high level of minimum inhibitory concentration value for imipenem and meropenem. In another study[26], there was a significant relationship between the presence of $bla\text{-}_{OXA\text{-}23\text{-}like}$ and resistance to carbapenems which is in agreement with our findings.

In our study, based on PCR results, the $bla\text{-}_{OXA\text{-}23\text{-}like}$ was the most detected carbapenemase, and 89 (66.9%) of isolates harbored this gene which is in accordance with other studies[28]. Furthermore, in the present study, the rate of $bla\text{-}_{OXA\text{-}24\text{-}like}$ was 46 (34.6%) which is in the range of 0% to 85.43% that has been reported for this gene[24]. While $bla\text{-}_{OXA\text{-}58\text{-}like}$ previously reported from 0% to 84.92%[24,29-34] none of our isolates was positive for this gene. Additionally, we investigated the aminoglycosides disks on *A. baumannii* isolates and we found despite high amikacin and gentamicin resistance, tobramycin was the most effective aminoglycoside *in vitro* and 15.8% (n=21) of isolates were sensitive to this agent. By our findings, Kulah et al.[25,35] found that tobramycin was the most effective agent against *A. baumannii* isolates. Resistance to aminoglycosides mainly is due to AMEs[25]. A markedly different aminoglycoside-resistant gene has been reported from different parts of the world[36]. Our results revealed that 54.9% (n=73) and 51.9% (n=69) of isolates were carried aac (3)-I and aph (3')-I, respectively. These genes are predominant in studied *A. baumannii*. Our finding indicates that aminoglycoside resistance in our isolates is associated with aac (3)-I and aph (3')-I. This finding is in agreement with previous studies that reported aac (3) class enzyme and aph (3')-I genes are the most common AME genes in *A. baumannii* isolates[7,37-39]. We did not identify any aac (6)I-b, despite reports of its prevalence ranging from 80.9% to 83.6%[40,41]. Interestingly, 55 (41.4%) of isolates harbored aac (3)-I and aph (3')-I. This phenomenon is shown in other studies[7,42]. Results of the study showed a significant association between biofilm formation and antimicrobial resistance phenotypes. They believe that this phenomenon could be a result of inadequate penetration of antimicrobial agents into the biofilms[27]. Similar to the previous study, in our research, 129 (97%) of the 133 isolates were strong biofilm producers. Notably, various studies have proved that survival in the environment and antibiotic resistance in *A. baumannii* is related to the biofilm formation of this organism[22]. Accordingly, the obtained data from our study indicated that the presence of these resistance genes, along with biofilm production, plays an important role in creating resistance to both carbapenems and aminoglycosides classes in *A. baumannii* isolates.

Study Limitations

Our study had the following limitations:

First: Lack of sufficient information about patients.

Second: Limitations to studying other resistance-related genes.

Third: We did not have any information about the different wards of the hospital in terms of infection prevalence.

Conclusion

Among OXA carbapenemase, $bla_{\text{-OXA-23-like}}$ was the most prevalent in *A. baumannii* isolates. The genes related to AME were also detected in the isolates and aac (3)-I and aph (3') was more prevalent. Moreover, the coexistence of aminoglycoside-resistant genes was found. Furthermore, the majority of *A. baumannii* isolates were strong biofilm producers. It seems that carbapenem and aminoglycoside cannot be used as a treatment for *A. baumannii* isolates in studied hospitals. The high prevalence of drug-resistant *A. baumannii* isolates with multiple resistance mechanisms makes this bacterium a major clinical and public health concern. Infection control programs and policies should be frequently reviewed to control the transmission of drug-resistant *A. baumannii* isolates in the future.

Ethics

Ethics Committee Approval: This study was approved by the Yasuj University of Medical Sciences (YUMS) Ethics Committee (approval ID: IR.YUMS.REC.1398.011).

Informed Consent: Cross-sectional study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: S.G., S.S., F.M., A.S., Concept: S.G., S.S., M.A.G., S.S.K., A.S., Design: S.G., S.S., F.M., F.R., S.S.K., S.A.K., A.S., Data Collection or Processing: S.G., S.S., F.M., M.A.G., Analysis or Interpretation: S.G., S.S., F.M., F.R., S.S.K., S.A.K.,

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