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Multidrug-Resistant Strains of *Acinetobacter baumannii* from Intensive Care Unit Patients Show Genetic Diversity and Distribution of Genes Associated with Aminoglycoside Resistance

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ABSTRACT

Background & Objective: As a significant cause of nosocomial infections, *Acinetobacter baumannii* has been linked to opportunistic infections. The assessment of clonal relatedness of *A. baumannii* isolates using typing methods like ERIC-PCR is beneficial for controlling conditions due to these resistant isolates. This research aims to study *Acinetobacter baumannii* resistant isolates to multidrug using typing methods like ERIC-PCR in clinics of Zanjan city.

Materials & Methods: In all, one hundred immunocompromised patients in ICU were included in the study and isolates of *A. baumannii* were extracted from their samples, and molecular typing using ERIC-PCR was performed on patients who were positive for aminoglycoside resistance-related genes (aph(2")-Id, ant(4")-Ia, ant(3")-I, aac(6")-Ib, aac(3)-I, aph(3")-I, aph(2")-Ib and aph (2")-Ic).

Results: 67% of isolates had gentamicin resistance, and 63% had tobramycin resistance. The isolates tested positive for multidrug resistance (MDR) were all labeled as MDR strains. Furthermore, all antibiotics tested were ineffective against 32% of the isolates, while 91% could be deemed extensively drug-resistant (XDR). The aminoglycoside resistance gene aac(6′)-Ib accounted for 79% of the cases, followed by ant(3")-I and aph(2")-Id (47%). Sixty-four percent of the isolates carried three or more aminoglycoside resistance genes simultaneously. A total of six types and 20 subtypes of patterns were obtained from ERIC-PCR.

Conclusion: In this study, aminoglycoside-resistant *A. baumannii* was found in a high percentage of ICU patients, mainly with the enzyme-modified aminoglycosides like aac(6′)-Ib, aph(2")-Id and ant(3")-I. ERIC-PCR has also shown an increased level of diversity in *A. baumannii* isolates. Therefore, genetic diversity or clonal relatedness of *A. baumannii* isolates in clinical settings can be assessed using ERIC-PCR.

Keywords: Acinetobacter baumannii, Molecular typing, Antibiotic resistance, ERIC PCR

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Introduction

Due to limited therapeutic options, *Acinetobacter baumannii* accounts for a high mortality rate induced by opportunistic nosocomial infections. *A. baumanni*-induced infections include meningitis, wounds, ventilator-associated pneumonia, peritonitis, bacteremia, and soft-tissue infections (1, 2). Nosocomial infections resulting from *Acinetobacter baumannii* are an increasing concern around the world, as they are resistant to many antimicrobial agents and can survive in different hospital environments (2, 3). Due to MDR, at least three groups of antibiotics consisting of quinolones, beta-lactams, carbapenems, and aminoglycosides are ineffective on *A. baumannii*. Therefore, healthcare systems in Iran are facing a significant challenge (2, 4). Additional studies have reported various pan drug-resistant (PDR) *A. baumannii* strains (5, 6). Limited therapeutic options have made treating MDR *A. baumannii* strain infections difficult (2). To fight *A. baumannii* infections in hospitalized patients, aminoglycosides, whether taken alone or in combination with β-lactams, have been used and remain an essential tool in the fight against MDR strain infections(7). The specific interaction of antimicrobial agents with the 30S ribosomal subunit components, 16S rRNA, blocks translation initiation (8). Aminoglycoside- modifying enzymes (AMEs) such as aminoglycoside O-nucleotidyltransferases (ANTs) (5, 9), aminoglycoside N-acetyltransferases (AACs), and aminoglycoside O-phosphotransferases (APHs) in *A. baumanni* cause aminoglycoside resistance. APHs are correlated with increased gentamicin resistance (5). *A. baumannii* has been reported to have a variety of AMEs, consisting of phosphotransferase variants: APH (3')-I,

APH (3')-II, and APH(2")-Ib, as well as variants of the nucleotidyltransferases ANT(3)-I, ANT(4')-I, ANT(2")-I and the acetyltransferases AAC(3)-I, AAC(6')-I, AAC(6')-II, and AAC(6')-III(10). In addition, methylation of 16S rRNA contributes to the inactivation of aminoglycosides (8). Hyper resistance to aminoglycosides, except streptomycin, is caused by ArmA, RmtA, RmtB, RmtC, and RmtD (8،9). *Acinetobacter baumannii* usually acquires aminoglycoside resistance genes via transposons, plasmids, or class 1 integrons (Int-1) containing single or multiple gene cassettes (5, 6). Thus, these resistance genes can be transmitted to other bacterial species.

The genotyping of *A. baumannii* is an essential method for the specification of the connection between genetics and epidemiology. ERIC-PCR (Enterobacterial repetitive intergenic consensus polymerase chain reaction) is a reliable and fast method for determining the genetic diversity or clonality of A. baumannii isolates (11, 12).

We examined the aminoglycoside resistance genes (aph (2")-Id, ant (4′)-Ia, ant (3")-I, aac (6′)-Ib, aac (3)-I, aph (3′)-I, aph (2")-Ib, aph (2")-Ic). We used ERIC-PCR to molecularly type MDR A. baumannii gathered from ICU patients with an immune deficiency.

Materials and Methods

baumannii samples

A prior study obtained 100 clinical isolates from urine, chest, wound swabs, blood, tube secretions, sputum taken from immunodeficiency patients in ICU. (Ethical approval code: 13820507972007)(2). To detect *A. baumannii* isolates, biochemical experiments and polymerase chain reaction with primers specific for the bla OXA-51 gene were performed (Table 1).

Table 1. Primers sequences and the annealing temperatures used in this study

Target Genes	Primers sequences (5-3)	Annealing Temperature (°c)	DNA amplicon Size (bp)	Reference
OXA-51	TAATGCTTTGATCGGCCTTG TGGATTGCACTTCATCTTGG	60	324	(2)
aph(2")-Id	AATCGGTAGAAGCCCAA GCACCTGCCATTGCTA	58	642	(10)
ant(4′)-Ia	CTGCTAAATCGGTAGAAGC CAGACCAATCAACATGGCACC	58	172	(10)
ant(3")- I	GAAGTACGCAGAAGAGA ACATGGCAAGCTCTAGGA	60	284	(10)
aac(6′)-Ib	TATCCAGCTAAGCGCGAACT ATTTGCCGACTACCTTGGTC	57	490	(10)
aac(3)-I	CTTCAGGATGGCAAGTTGGT TCATCTCGTTCTCCGCTCAT	57	402	(10)
aph(3′)-I	GCTCACGCAACTGGTCCA GA GGCACGCAAGACCTCAACCT	57	816	(10)
aph(2")-Ib	CTTGGACGCTGAGATATATGAGCAC GTTTGTAGCAATTCAGAAACACCCTT	60	867	(10)
aph(2")-Ic	CCACAATGATAATGACTCAGTTCCC CCACAGCTTCCGATAGCAAGAG	58	444	(10)

Susceptibility testing of antimicrobial agents

As described in a previous study, sensitivity to antimicrobial drugs was determined by disk diffusion method (2). The antimicrobial disks used are as follows: gentamicin (10µg), ampicillin-sulbactam (10/10), ceftazidime (30µg), imipenem (10µg), tobramycin (10µg), doxycycline (30µg), ciprofloxacin (5µg), co-trimoxazole (1.25/23.75µg), piperacillin (100µg), levofloxacin (5µg), and cefepime (30µg) (MAST, Merseyside, UK). Interpretations were made under CLSI guidelines (13). Multidrug-resistant bacteria are resistant to more than two categories of antibiotics. *Acinetobacter baumannii* was one such organism. Those isolates of *A. baumannii* that were resistant to more than one antimicrobial agent in all but two antimicrobial categories were considered extensively drugresistant (XDR) (14).

DNA extraction

QIAGEN DNA Mini Kit was used to isolate genomic DNA from bacterial cells after overnight culture.

(QIAGEN Inc., Valencia, CA). At 260 and 260/280 nm, a spectrophotometer (ND-1000, Nano-Drop Technologies, Wilmington, DE) was used to measure the amount of DNA in each sample.

Genes associated with aminoglycoside resistance

We used the PCR method disclosed previously to examine the genes associated with aminoglycoside resistance of aph(2")-Id, ant(4")-Ia, ant(3")-I, aac(6")-Ib, aac(3)-I, aph(2")-I, aph(2")-Ib, and aph(2")-Ic(8). The sequence of primer pairs (Metabion, Germany) is shown in Table 1. The PCR experiments were conducted with PCR Master Mix (Amplicon, Denmark), containing Taq DNA polymerase, deoxynucleotides, MgCl2, and the proper buffer. It took 25 µl for each reaction, containing 12.5 µl of master mix, one µl each of reverse and forward primers (at 100 nM each), 1µl of DNA sample of 200 ng/µl concentration and nuclease-free water for PCR to reach the final volume. The Gene Atlas 322 system (ASTEC) was used for amplification. PCR steps were as follows: initial denaturation step (94ºC, 5 min), then, 94ºC, 1 min for denaturation (30 cycles), 1-minute annealing (Table 1 shows Tm for each primer pairs), and extension at 72ºC for 1 min, then a final extension at 72ºC for 10 minutes. 1% agarose gel electrophoresis was performed to separate DNAs according to their size. Neutral Red (Sigma Aldrich, Germany) was used for DNA staining, UV transillumination was then used to visualize DNA fragments.

ERIC-PCR

ERIC-PCR was performed according to the protocol described previously (15). The oligonucleotide pairs ERIC-1 5'-ATGTAAGCTCCTGGGGATTCAC-3' and ERIC-2 5'-AAGTAAGTGACTGGGGG-3' were utilized to amplify the ERIC like sequences in *A. baumannii* DNA. The following PCR program was performed: incipient denaturation (94ºC, 10 min), followed by denaturation at 94ºC for 1 min (30 cycles), 1 min annealing at 52ºC, extension (65ºC, 8 minutes) and 16 min extension at 65ºC. For electrophoresis of amplicons generated by PCR, 20 µl of each amplified sample was loaded in 2% agarose gel. Numerical taxonomy and multivariate analysis were used (NTSYS version 2.1; Exeter Software, New York, NY, USA) to analyze ERIC patterns. The ERIC PCR gels Dendrograms were generated using Dice similarity coefficients, arithmetic averages, 1% optimization, and 1% position tolerance. The most similar isolates were deemed clonally related if their similarities exceeded 96%.

Statistical analysis

The version 17.0 of SPSS (SPSS, Inc., Chicago, IL) was used to analyze the data. The statistical significance of the information was calculated by an X2 test. P <0.05 was regarded as significant.

Results

Isolates: their features

Out of the100 *A.baumannii* isolates, 26%, 24%, 25%, 15%, 10% were recovered from blood, sputum, secretions collected from thoracic tubes (thoracic catheters), wound swabs, and urine, respectively.

Susceptibility to antimicrobial agents

As described previously (2), 67% and 63% of isolates showed gentamicin and tobramycin resistance, respectively. *A. boumannii* isolates showing resistance to three antimicrobial agents or more were considered MDR. The isolates were also 32% resistant to all antibiotics tested, and 91% were classified as XDRs. The most common resistance pattern was "ampicillin/sulbactam-ceftazidime-imipenem-gentamicin-tobramycin-doxycycline-ciprofloxacin-levofloxacin-cotrimoxazole-piperacillin-cefepime" with 32% frequency.

Genes involved in aminoglycoside resistance

Each isolate contained one or more aminoglycoside resistance genes is shows the frequency of resistance-related genes (Table2). There were three common aminoglycoside resistance genes: aac(6′)-Ib (79%), ant(3")-I and aph(2")-Id (47%). All isolates resistant to aminoglycoside had aac(6′)-Ib gene. The phenotypic patterns of aminoglycoside resistance genes in isolates of *A. baumannii* are shown in Table 3. 64% of isolates had three or more aminoglycoside resistance-related genes simultaneously. Also, 6% of isolates had 6 aminoglycoside resistance genes whose patterns were "aph(2")-Ib+ aph(2")-Ic+ aph(2")-Id+ ant(4′)-Ia+ ant(3")-I+ aac(6′)-Ib"(Table3).

Table 2. **Frequency of aminoglycoside resistance genes in *A. baumannii* isolates**

Aminoglycoside resistance genes	No = (%) of isolates
aph(2'')-Id	47
aph(2'')-Ib	37
aph(2'')-Ic	39
aac(6′)-Ib	79

Aminoglycoside resistance genes	No = (%) of isolates
aac(3)-I	5
ant(3'')-I	47
ant(4′)-Ia	38
aph(3′)-I	9

Table 3. Patterns of aminoglycoside resistance genes among *A. baumannii* isolates

No. of resistance genes	Patterns of aminoglycoside resistance genes	Percent (%) of *A. baumannii*	Total (%)
1 gene	aac(6′)-Ib	**18**	**29**
	aph(2'')-Ib	**1**	
	ant(3'')-I	**6**	
	aph(2'')-Ic	**1**	
	aph(2'')-Id	**2**	
	ant(4′)-Ia	**1**	
2 genes	aph(2'')-Ib+ ant(4′)-Ia	**1**	**7**
	aph(2'')-Ib+ ant(3'')-I	**1**	
	aph(2'')-Ib+ aac(6′)-Ib	**1**	
	ant(4′)-Ia+ aac(3)-I	**1**	
	aac(6′)-Ib+ ant(3'')-I	**2**	
	aph(2'')-Id + ant(4′)-Ia	**1**	
3 genes	aph(2'')-Ib+ aph(2'')-Ic+ aac(6′)-Ib	**2**	**24**
	aph(2'')-Ib+ aph(2'')-Ic+ ant(3'')-I	**2**	
	aph(2'')-Ic+ aac(6′)-Ib+ ant(3'')-I	**3**	
	ant(3'')-I+ aac(6′)-Ib+ aph(3′)-I	**2**	
	aph(2'')-Ib+ aph(2'')-Id+ aac(6′)-Ib	**1**	
	aph(2'')-Ib+ ant(3'')-I+ aac(6′)-Ib	**1**	
	aph(2'')-Id + ant(4′)-Ia+ aac(6′)-Ib	**4**	
	aph(2'')-Ic+ aph(2'')-Id+ aac(6′)-Ib	**2**	
	aph(2'')-Ic+ aph(2'')-Id+ aac(6′)-Ib	**2**	
	aph(2'')-Id+ ant(3'')-I+ aac(6′)-Ib	**4**	
	aph(2'')-Ib + aph(2'')-Ic+ aph(2'')-Id	**1**	
4 genes	aph(2'')-Ib + aac(6′)-Ib+ ant(3'')-I+ aph(3′)-I	**2**	
	aph(2'')-Ib+ aph(2'')-Ic+ aph(2'')-Id+ ant(3'')-I	**1**	
	aph(2'')-Ib+ aph(2'')-Ic+ ant(3'')-I+ aac(6′)-Ib	**2**	
	ant(4′)-Ia+ aac(6′)-Ib+ ant(3'')-I+ aac(3)-I	**2**	

No. of resistance genes	Patterns of aminoglycoside resistance genes	Percent (%) of *A. baumannii*	Total (%)
	aph(2")-Id+ ant(4′)-Ia+ ant(3")-I+ aac(3)-I	2	
	aph(2")-Ib+ aph(2")-Ic+ aph(2")-Id+ aac(6′)-Ib	2	18
	aph(2")-Ic+ aph(2")-Id+ aac(6′)-Ib+ aph(3′)-I	1	
	aph(2")-Id+ ant(4′)-Ia + aac(6′)-Ib+ ant(3")-I	5	
	aph(2")-Ib + aph(2")-Id+ ant(4′)-Ia + aac(6′)-Ib	1	
5 genes	aph(2")-Ib+ aph(2")-Id+ ant(4′)-Ia + aac(6′)-Ib+ ant(3")-I	2	16
	aph(2")-Ib + aph(2")-Ic+ aph(2")-Id+ aac(6′)-Ib+ aph(3′)-I	2	
	aph(2")-Ib + aph(2")-Ic+ aac(6′)-Ib+ ant(3")-I+ ant(4′)-Ia	4	
	aph(2")-Ic+ aph(2")-Id+ aac(6′)-Ib+ ant(3")-I+ ant(4′)-Ia	2	
	aph(2")-Ib +aph(2")-Ic+aph(2")-Id+aac(6′)-Ib+ ant(4′)-Ia	4	
	aph(2")-Ic+ aph(2")-Id+ aac(6′)-Ib+ ant(4′)-Ia + aph(3′)-I	2	
6 genes	aph(2")-Ib+ aph(2")-Ic+ aph(2")-Id+ ant(4′)-Ia+ ant(3")-I+ aac(6′)-Ib	6	6

ERIC-PCR

ERIC-PCR produced multiple bands which were different in size ranging from 100to 3000 bp. The patterns are sorted into six types (A-F) and 20 subtypes. Band patterns illustrated that 16 out of 20 subtypes were distinctive ERIC-PCR clusters and four singleton isolates. 13% and 10% of sets were A3/A4 and B3, respectively, the most common among *A. baumannii* isolates (Table4, Figure 1).

Table 4. **ERIC-PCR patterns among A. baumannii isolates**

ERIC Types	Subtypes	Size of ERIC-PCR products (bp)	Total isolates number (%)
A (2 bands) (40%)	A1	550, 1000	3
	A2	100, 800	8
	A3	150, 900	13
	A4	900, 1500	13
	A5	900, 2500	3
B (3 bands) (19%)	B1	900, 1200, 1500	3
	B2	200, 850, 1500	5
	B3	500, 850, 1000	10
	B4	400, 550, 800	1
C (4 bands) (20%)	C1	300, 700, 1000, 1500	3
	C2	700, 850, 1500, 2500	5
	C3	600, 850, 950, 1500	3
	C4	150, 350, 850, 1400	5
	C5	150, 650, 1000, 1500	4

ERIC Types	Subtypes	Size of ERIC-PCR products (bp)	Total isolates number (%)
D (5 bands) (15%)	D1	600, 700, 900, 1200, 1500	8
	D2	300, 600, 900, 1000, 1500	6
	D3	150, 550, 900, 1400, 2000	1
E (6 bands)	E1	100, 400, 700, 1000, 1500, 3000	1
(5%)	E2	300, 550, 700, 850, 1000, 1500	4
F (7 bands) (1%)	F1	150, 600, 800, 950, 1500, 2000, 2500	1

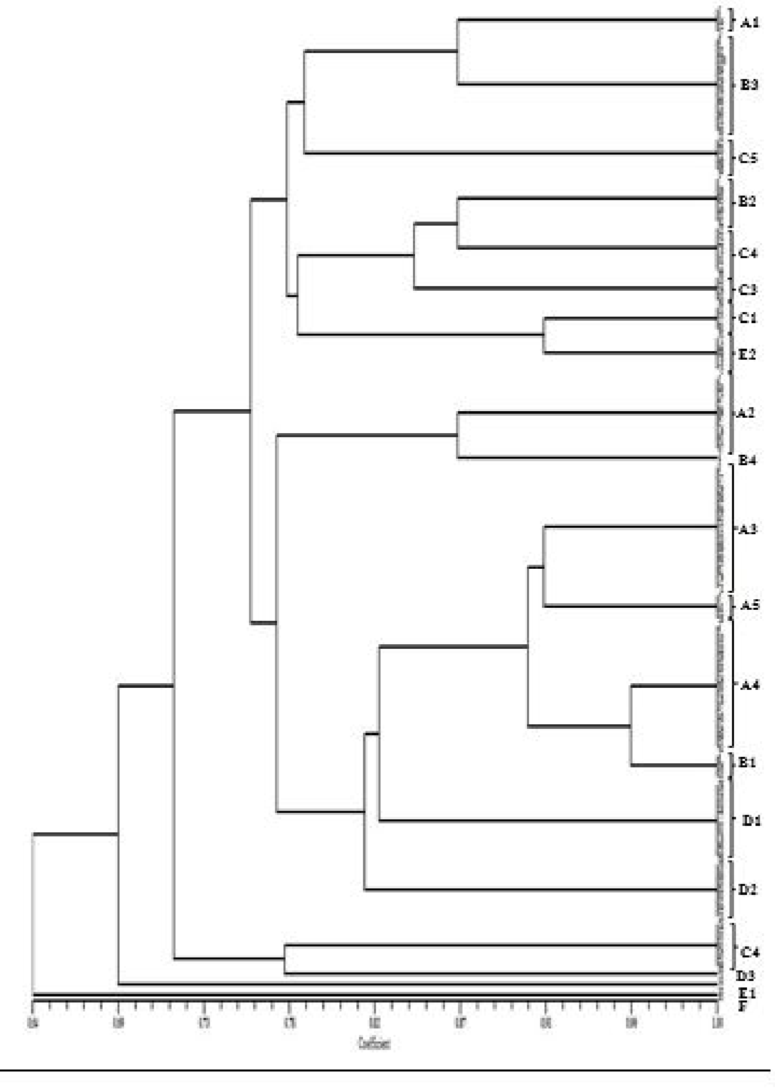

Figure 1. Dendrogram of enterobacterial repetitive intergenic consensus–polymerase chain reaction (ERIC–PCR) of A. *baumannii* isolates. Scale represents percentages of similarity.

Discussion

A. baumannii is known as the leading cause of nosocomial infections in hospitals as it can acquire a wide spectrum of antimicrobial resistance (2, 3). Limited treatment options have made MDR *A. baumanni*i hard to cure in many environments (10, 16-18). Colistin and tigecycline are the ultimate treating choices (2). This work revealed that 100% isolates of *A. baumannii* were MDR that were in line with publications from Iran .Studies conducted in Iran (10, 19, 20). In concordance with these results, Safari et al.,

(2017) found 98% of *A. baumannii* isolates to be XDR **(20)**. Resistance to Tobramycin and Gentamicin was seen in 67% and 63% of isolates, respectively. The high frequency of aminoglycoside-resistant *A. baumannii* in our study and previous reports may be due to acquiring AMEs **(21)**. It has been reported that *A. baumannii* has a wide range of aminoglycoside resistance-related genes. Various risk factors are indicated to be related to the emergence of aminoglycoside-resistant *A. baumannii*, such as widespread use of antimicrobial agents and inappropriate prescription of aminoglycosides **(10)**. Moreover, the aac(6′)-Ib, aph(2")-Id, and ant(3")-I genes showed high prevalence than other genes in this study. Furthermore, the co-existing aac(6′)-Ib and ant(3")-I was discovered in 33% of isolates. Isolates of *A. baumannii* in China showed that aac(6′)-Ib and ant(3")-I are high frequency genes associated with resistance to aminoglycosides found, supporting this study's results **(22)**. According to Haldorsen et al., study (2014) aac(6′)-Ib enzyme could alter amikacin, even in isolates that were phenotypically susceptible to amikacin- of E. coli and Klebsiella spp**.** **(23)**.

A study conducted by Helmy and Kashef (2017) illustrated aac(6′)-Ib gene in 84.4% of aminoglycoside-resistant Enterobacteriaceae **(21)**. A gene cassette, aac(6′)-Ib, is mainly associated with insertion sequences like IS26 and truncated integrons, class 1 integrons, which probably explains its frequency among *A. baumannii* isolates **(24)**. Our findings are in line with previous pieces of literature which reported the aac(6′)-Ib and ant (3")-I as high frequency genes in isolates of *A. baumannii* **(9, 10, 22)**. In contrast, aac(3)-I and aph(3′)-I genes were less common in this study **(8, 22)**. Similar to this study's results, Xiao et al., (2014) also reported the lower frequency of aac(3)-I and aph(3′)-I genes **(16)**.

The molecular methods for typing *A. boumannii* are the most critical procedures to determine the association between genetics and epidemiology. ERIC-PCR is a reliable and rapid technique for evaluating genetic differences or clonal relatedness of *A. baumannii* isolates **(11, 12)**. The ERIC-PCR displayed 2-7 bands (100-3000 bp) based on our findings. The patterns produced by ERIC-PCR were sorted into six types (A-F) and 20 subtypes. They have implied 16 distinctive clusters and four singleton isolates. The most frequent clusters among *A. baumannii* isolates were A3/A4 (13%) and B3 (10%). In a study conducted by Viana et al., (2011), typing by ERIC–PCR showed a high range of clonal diversity (38 different clones), indicating that *A. baumannii* spreading depends on multiple factors not entirely attributed to a predominant clone **(11)**. In agreement with our findings, Codjoe et al., (2019) have shown that ERIC-PCR typing of Gram-negative bacilli resistant to carbapenem generates some bands (1 to 8) with different sizes (50-800 bp). Among the 111 carbapenem-resistant (CR) isolates studied, 93 complex dissimilarities were detected, while 18 similar band patterns were observed in pairs **(12)**. Additional studies indicated that *A. baumannii* showed genetic diversity and heterogeneity. Falah et al., (2019), by identifying 14 different ERIC patterns (ERIC-types) including 11 regular types and three unique types, reported clonal diversity in 80 *A. baumannii* isolates **(25)**. This study was contrary to Ece et al., (2015) who reported a clonal relationship between strains of *A. baumannii* collected from a tertiary care center in Turkey. Their results revealed that many *A. baumannii* clusters in the ICU were the main clusters **(26)**.

Conclusion

Our work indicated the abundance of aminoglycoside-resistant *A. baumannii* in patients in ICU, as well as the high frequency of aminoglycoside-modifying enzymes: aac(6′)-Ib, ant(3")-I and aph(2")-Id. ERIC-PCR has also illustrated an increased level of diversity in *A. baumannii* isolates. Thus, it can be a convenient method for investigating *A. baumanni* to determine its isolates' genetic differences or clonal relatedness in clinical environments.

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Conflict of Interest

The authors declare that they have no competing interests.

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