Iranian Journal of Microbiology
Volume 17 Number 1 (February 2025) 80-91
DOI: http://doi.org/10.18502/ijm.v17i1.17804

ORIGINAL ARTICLE

Evaluation of the relatedness between the biofilm-associated genes and antimicrobial resistance among *Acinetobacter baumannii* isolates in the southwest Iran

Nafiseh Hosseinzadeh Shakib[1,2], Zahra Hashemizadeh[1], Abolfazl Rafati Zomorodi[1,2], Reza Khashei[1,3], Yeganeh Sadeghi[1], Abdollah Bazargani[1*]

[1]Department of Bacteriology and Virology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran
[2]Student Research Committee, Shiraz University of Medical Sciences, Shiraz, Iran
[3]Department of Microbiology, School of Medicine, Sabzevar University of Medical Sciences, Sabzevar, Iran

Received: May 2024**, Accepted:** December 2024

ABSTRACT

Background and Objectives: Increasing antimicrobial resistance among *Acinetobacter baumannii (A. baumannii)* strains poses a significant challenge, particularly in intensive care units (ICUs) where these bacteria are common causes of hospital infections. Biofilm production is recognized as a key mechanism contributing to this resistance. This study aims to explore the relationship between biofilm production, the presence of biofilm-associated genes, and antibiotic resistance patterns in *A. baumannii* isolates obtained from ICU patients.

Materials and Methods: We collected 100 *A. baumannii* isolates from ICU patients at Nemazee Hospital in Shiraz, Iran. Antimicrobial susceptibility testing (AST) was performed using the Kirby-Bauer disk diffusion method, and biofilm production potential was assessed through the tissue culture plate (TCP) method. Additionally, we investigated eleven biofilm-related genes (*ompA*, *bap*, *csuE*, *epsA*, bla_{per-1}, *bfmS*, *pgaB*, *csgA*, *fimH*, *ptk*, and *kpsMII*) in all isolates using polymerase chain reaction (PCR). The REP-PCR technique was utilized to analyze the genetic relatedness of the isolates (Fig. 4).

Results: All isolates displayed multi-drug resistance, with the highest resistance rates observed against ceftazidime, cefotaxime, and trimethoprim/sulfamethoxazole (100%). Gentamicin and amikacin showed the lowest resistance rates at 70% and 84%, respectively. A total of 98% of the isolates were capable of biofilm production, with 32% categorized as strong biofilm producers. The most frequently detected biofilm-associated genes included *csuE* (99%), *bfmS* (98%), *ompA* (97%), and *pgaB* (89%).

Conclusion: Biofilm production significantly contributes to the prevalence of multi-drug resistant *A. baumannii* strains. It is essential to implement effective antimicrobial stewardship and develop innovative anti-biofilm strategies to address this global health issue.

Keywords: *Acinetobacter baumannii*; Antibiotic stewardship; Multi-drug resistant; Intensive care units pathogens; REP-polymerase chain reaction

*Corresponding author: Abdollah Bazargani, Ph.D, Department of Bacteriology and Virology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran. Tel: +98-7132304356 Email: bazargania@sums.ac.ir

Copyright © 2025 The Authors. Published by Tehran University of Medical Sciences.
This work is licensed under a Creative Commons Attribution-Non Commercial 4.0 International license (https://creativecommons.org/licenses/by-nc/4.0/). Noncommercial uses of the work are permitted, provided the original work is properly cited.

INTRODUCTION

Worryingly, *Acinetobacter baumannii* is becoming more multidrug-resistant (MDR), extensively drug-resistant (XDR), and even pan-drug-resistant (PDR). It is one of the most important bacteria that causes infections in hospitalized patients in intensive care units (ICUs) worldwide (1). Systematic reviews and meta-analyses have recently reported an annual pooled prevalence of 72% to 88% for MDR *A. baumannii* isolates among hospitalized patients globally (2, 3). The mortality rate of health-acquired *A. baumannii* infections was estimated to range from 23% to 68%. Hence, the World Health Organization (WHO) has mentioned *A. baumannii* as a critical priority agent in hospital and device-associated infections (4).

Several features promote resistance of *A. baumannii* strains against different classes of antibiotics; biofilm production is one of the most remarkable. Indeed, it promotes resistant mechanisms by degrading enzyme activity, altering permeability, and excluding antibiotics, making difficulties in treating related infections. Also, biofilm-producing *A. baumannii* strains colonize on various surfaces, specifically medical equipment, and subsequently adapt and survive in harsh hospital conditions (5).

Indeed, biofilm formation on medical equipment provides a dual benefit for bacteria: it protects them from antimicrobial agents and facilitates the exchange of resistance genes among the bacteria present within the biofilm (6). In addition, biofilm production enhances pathogenicity by conferring antibiotic resistance, undermining treatment strategies, and facilitating the colonization of *A. baumannii* isolates in clinical settings and hospitals. Consequently, biofilm-producing *A. baumannii* isolates exhibit resistance to most antimicrobials. Unfortunately, only a limited number of effective treatments are available, exacerbating the spread of this bacterium in healthcare settings due to biofilm formation on surfaces and the expression of multidrug resistance (7, 8).

Biofilm formation in *A. baumannii* is mediated by a variety of virulence factors, including outer membrane protein A (OmpA), biofilm-associated protein (Bap), a class A extended β-lactamase $bla_{\text{PER-1}}$ enzyme, and the CsuA/BABCDE chaperone-usher pili assembly complex. Furthermore, studies have demonstrated the significant role of the csuE gene in this phenomenon. The regulation of the *csuE* operon is mediated by a two-component system (*bfmR-bfmS*); the *bfmS* component acts as a sensor-kinase and activates the response regulator (*bfmR*) (6, 8).

Epidemiological studies help to assess the clonal relationship between various bacterial strains and increase knowledge about cross-transmission, detection of virulent strains, and distribution of virulence genes. Many typing methods can discriminate bacteria strains, but the repetitive extragenic palindromic polymerase chain reaction-based (REP-PCR) method for molecular characterization of *A. baumannii* has gotten a lot of attention in the past because it is cost-effective, simple, and rapid and has high discrimination and reproducibility compared to other techniques (9).

Thus, the present study investigated the correlation between biofilm-producing, the presence of biofilm-associated genes, and antibiotic resistance patterns among *A. baumannii* isolates from ICU-admitted patients. The REP-PCR method was also assessed to evaluate the dispersion pattern of isolates.

MATERIALS AND METHODS

Bacterial collection and identification. In this cross-sectional study, from May to September 2021, a total of 100 non-duplicate *A. baumannii* isolates were isolated from 186 various specimens (including blood, urine, endotracheal tubes, sputum, wounds, and endotracheal aspirates); all specimens were obtained from ICU-admitted patients at Nemazee Hospital in Shiraz, southwest Iran. Primary identification of *A. baumannii* isolates was performed using the biochemical standard tests, as explained earlier (1). Briefly, the specimens were cultured on MacConkey agar and blood agar (Merck, Germany) for overnight incubation at 37°C. Then, the presumptive colonies were tested using standard biochemical tests comprising oxidase, citrate, urea urease, malonate consumption, oxidation and fermentation of sugars, motility, and indole production.

DNA extraction. The boiling method was utilized to extract the DNA from all isolates. However, there are several disadvantages, including the potential for DNA fragmentation, contamination by proteins or RNA, and limitations regarding certain samples or bacterial types. Nonetheless, the method is considered simple, cost-effective, rapid, and not requiring

specialized equipment. Consequently, it has been reported as a convenient and popular method for routine investigations by authors worldwide (10). As explained previously (11), pick up one-three single colonies of fresh subcultured *A. baumannii* onto the non-selective media and resuspend into the sterile 1.5 mL microtube containing 500 µL ultrapure distilled water. After homogenization, the microtubes were transferred into the water bath at 95°C for 10 minutes. Then, the microtubes were immediately cooled and placed onto the ice box for 5 min, followed by centrifuging the microtubes for 5 min at 800 rpm, and finally, 100 µL of supernatant was taken gently. The purity of the extracted DNA was evaluated by measuring the 260/280 and 230/280 ratios using a photometric evaluation (NanoDrop 1000, Thermo Fisher, Waltham, Massachusetts, USA).

Molecular confirmation of *A. baumannii* isolates. The amplification of the $bla_{\text{OXA-51-like}}$ gene was carried out using the polymerase chain reaction (PCR) technique to confirm the identified isolates as *A. baumannii* through biochemical standard tests. The oligonucleotide primer sequences were Forward: 5'-TAATGCTTTGATCGGCCTTG-3' and Reverse: 5'-TGGATTGCACTTCCTGG-3'. The PCR conditions as described in the prior study (12) were as follows: initial denaturation for 3 min at 94°C, continuing the 35 cycles consisting each of 45 s at 94°C, the annealing temperature at 57°C for 45 s, and 1 min extension at 72°C; a final extension of 5 min at 7°C was conducted.

Antimicrobial susceptibility testing. The antimicrobial susceptibility of isolates against 11 antibiotics was assessed using the Kirby-Bauer disk diffusion method recommended by the Clinical and Laboratory Standards Institute (CLSI 2021) (13). The 11 tested antibiotics were as follow: ceftazidime (30 µg), cefotaxime (30 µg), cefepime (30 µg), imipenem (10 µg), meropenem (10 µg), piperacillin/tazobactam (100/10 µg), gentamycin (10 µg), amikacin (30 µg), tetracycline (30 µg), ciprofloxacin (5 µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg) (MAST Group, UK). The MDR isolates were identified as being previously defined as one isolate that is non-susceptible to at least one antibiotic in ≥3 antimicrobial categories (11). The *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 strains were used as quality control.

Tissue culture plate (TCP). As a quantitative method, the TCP method was used to evaluate the potency of biofilm-producing among *A. baumannii* isolates, as described in prior work (Fig. 1). The *Staphylococcus epidermidis* ATCC 35984 and Staphylococcus epidermidis ATCC 12228 strains were subjected as positive and negative controls, respectively. The isolates were sought in triplicate and repeated three times. The isolates were classified as non-, weak-, moderate-, and strong-biofilm producers. The interpretation was performed according to the below criteria:

$OD_{cut} = OD_{avg}$ of negative control + 3 × standard deviation (SD) of ODs of the negative control. Then, if $OD \leq OD_{cut}$ (non-biofilm producer), if $OD_{cut} < OD \leq 2 \times OD_{cut}$ (weak-biofilm producer), if $2 \times OD_{cut} < OD \leq 4 \times OD_{cut}$ (moderate-biofilm producer), and if $OD > 4 \times OD_{cut}$ (strong-biofilm producer) (14).

Molecular detection of biofilm genes. The frequency of eleven biofilm-associated genes comprising *ompA, bap, csuE, epsA,* $bla_{\text{per-1}}$, *bfmS, pgaB, csgA, fimH, ptk,* and *kpsMII* was assessed using the monoplex PCR method. All PCR reactions (including sections 2.3 and 2.7) were set up in a final 25 µL volume consisting of 12.5 µL of PCR 2× Master Mix (Amplicon, Danmark) comprising Taq DNA Polymerase, reaction buffer, dNTPs mixture, a protein stabilizer, and the convenience for use was optimized by adding sediment for electrophoresis, and 2× solution of loading dye, 1 µL of each primer (2 µM), 2 µL of template DNA (200ng/µl) and up to 25 µL final volume added nuclease-free water. the sequence of used primers is listed in Table 1.

REP-PCR. Though REP-PCR is a useful technique for bacterial typing, offering notable advantages such as high sensitivity, cost-effectiveness, and rapid results, it is essential to recognize its limitations regarding discriminatory power and the complexity of analysis. Consequently, this method is frequently employed alongside other molecular typing techniques to achieve a more comprehensive understanding of bacterial diversity and their interrelations (9). The specific REP primers designed previously, were applied to investigate the relatedness of *A. baumannii* isolates. The sequences of primers were REP1: 5'-IIIGCGCCGICATCAGGC-3' and REP2: 5'- ACGTCTTATCAGGCCTAC-3'. Also, the PCR condition started with an initial denaturation at 95°C for 3 min in following the 30 cycles consisting each of 30 s

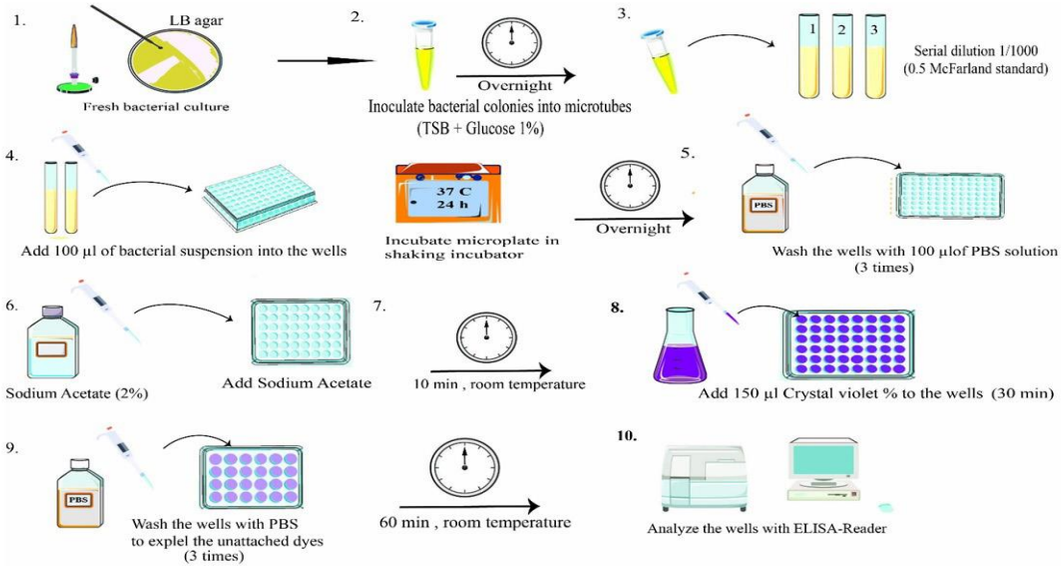

Fig. 1. A single colony, from fresh subcultured of *A. baumannii* isolates onto the LB, was inoculated into the sterile plastic microtube containing 2 mL Tryptic Soy Broth (TSB) supplemented with 1% glucose; the tubes incubated aerobically at 37°C for 24 h. After that, 0.2 mL of each suspension was aliquoted into the wells of a flat-bottomed 96-well Microtiter Microplate separately upon sterile condition; the microplates were incubated at 37°C for 24 h in a shaking incubator. Then, the wells' contents were eliminated by trapping the plates gently; subsequently, the wells were washed with 100 µl of phosphate-buffered saline (pH 7.2) three times. In the next step, the sodium acetate (HiMedia) in 2% concentration (for 10 minutes) and crystal violet (Merck, Germany) in 0.1% concentration (for 30 minutes) were applied for biofilm fixation and staining the plates, respectively. This is followed by removing the wells' contents and washing the wells according to mentioned above. At the end, let dry the plates in the room temperature (for ~1 h), then, measuring of biofilm production was evaluated through reading of absorbance in a spectrophotometer at 620 nm OD. Designing the figure was done using some pictures from Servier Medical Art. Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/).

at 90°C, the annealing temperature at 45°C for 1 min, and 8 min extension at 65°C, and final extension at 72°C for 16 min (9).

Statistical analysis. The data were analyzed using SPSS software version 22 (SPSS Inc., Chicago, IL, USA). The Chi-square statistical test was performed to analyze the data, and a p-value ≤ 0.05 was considered statistically significant.

Ethical approval. This study was approved by the Ethics Committee of Shiraz University of Medical Sciences with an ethical code number (Approval No). IR.SUMS.REC.1400.164. The samples were taken as part of the regular procedure and were isolated anonymously.

RESULTS

Isolation and identification. Generally, 100 *A. baumannii* isolates were identified using routine biochemical standard testing; all identified isolates were confirmed as *A. baumannii* due to harboring the $blaq_{\text{OXA-51-like}}$ gene (Fig. 2). The gender proportion of participating patients was 57% and 43% for male and female, respectively. The *A. baumannii* isolates were frequently isolated from sputum (45%), followed by endotracheal tubes (24%), blood (12%), wound (10%), urine (5%), and throat (4%) (Table 2).

Antimicrobial susceptibility testing. The highest resistance was against ceftazidime, cefotaxime, and trimethoprim/sulfamethoxazole 100%, followed by piperacillin/tazobactam, ciprofloxacin, and imipenem with a frequency of 98%, 97%, and 92%, respectively (Graph 1). In addition, all isolates were MDR.

Characterization of biofilm producers. The recorded OD values of isolates ranged from 0.009 - ≥ 0.260. Overall, 98% of isolates were biofilm producers; 33% were determined as strong biofilm-produc-

Table 1. Oligonucleotide primer properties used for PCR amplification.

Genes	Primer sequences (5´-3´)	Annealing Tm (◦C)	Amplicon size (bp)	Reference
bap	F-ATGCCTGAGATACAAATTAT	53	650	7
	R-GTCAATCGTAAAGGTAACG			
pgaB	F-AAGAAAATGCCTGTGCCGACCA	65	490	7
	R-GCGAGACCTGCAAAGGGCTGAT			
bfmS	F-TTGCTCGAACTTCCAATTTATTATAC	58	127	10
	R-TTATGCAGGTGCTTTTTTATTGGTC			
epsA	F-AGCAAGTGGTTATCCAATCG	54	451	7
	R- ACCAGACTCACCCATTACAT			
kpsMII	F- GCGCATTTGCTGATACTGTTG	60	272	7
	R- CATCCAGACGATAAGCATGAGCA			
bla-$_{per1}$	F-ATGAATGTCATTATAAAAGC	60	340	7
	R- AATTTGGGCTTAGGGCAAGAAA			
csuE	F-ATGCATGTTCTCTGGACTGATGTTGAC	55	168	7
	R-CGACTTGTACCGTGACCGTATCTTGATAG			
ompA	F-CGCTTCTGCTGGTGCTGAAT	58	531	7
	R- CGTGCAGTAGCGTTAGGGTA			
ptk	F-GGCTGAGCATCCTGCAATGCGT	65	597	7
	R- ACTTCTGGAGAAGGGCCTGCAA			
fimH	F-TGCAGAACGGATAAGCCGTGG	65	870	7
	R- GCAGTCACCTGCCCTCCGGTA			
csgA	F- ACTCTGACTTGACTATTACC	53	200	7
	R- GATGCAGTCTGGTCAAC			
bla$_{OXA-like-51}$	F- TAA TGC TTT GAT CGG CCT TG	57	353	37
	R- TGG ATT GCA CTT CCTGG			
REP-PCR	REP1- IIIICGICGICATCIGGC	45	-	19
	REP2- ICGICTTATCIGGCCTAC			

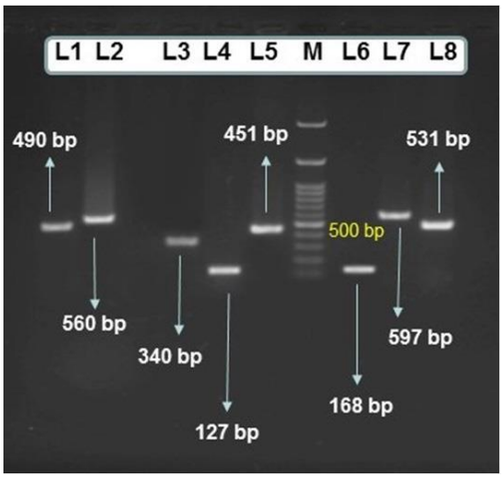

Fig. 2. Demonstration of the PCR amplified products using agarose gel electrophoresis (1.5% agarose). Lane 1: *pgaB* gene; Lane 2: *bap* gene; Lane 3: *bla*$_{per-1}$ gene; Lane 4: *bfmS* gene; Lane 5: *epsA* gene; Lane 6: *csuE* gene; Lane 7: *ptk* gene; Lane 8: *ompA* gene; Lane M: DNA marker (100 bp DNA Ladder).

ing, followed by 45% and 20% as moderate and weak biofilm-producing, respectively.

Distribution of biofilm-related genes. All *A. baumannii* isolates in this study carried almost all biofilm-related genes, except the *csgA*, *fimH*, and *KpsMII* genes that were not detected. The highest frequency of studied genes was determined for *csuE* (99%), *bfmS* (98%), *ompA* (97%), and *ptk* (96%), and the least belonged to *bap* (71%), *pgaB* (89%), *epsA* (88%), and *bla*$_{per-1}$ (88%). Furthermore, all isolates were categorized under 16 profiles based on harboring biofilm-associated genes (Table 3). Moreover, the presence of biofilm-associated genes was analyzed among antibiotic-resistant isolates of *A. baumannii*. There was only significant correlation between harboring the *csuE* and resistance against cefepime (p = 0.006) (Table 4).

Correlation of biofilm-producing and harboring biofilm-associated genes. As presented in Table 5,

Table 2. The demographic information of *A. baumannii* isolates regarding gene profiling (N= 100).

Demographic	Gene Profiles (N= %)																
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Total (N=%)
Gender																	
Male	23%	9%	3%	3%	4%	2%	3%	2%	-	3%	1%	1%	-	1	1%	-	56%
Female	27%	4%	2%	3%	3%	-	1%	1%	1%	-	-	-	1%	-	-	1%	44%
Age group																	
0-5	9%	3%	1%	2%	1%	-	1%	1%	-	-	-	-	-	-	-	-	18%
5-25	2%	1%	-	1%	-	-	-	-	-	2%	-	-	-	-	-	-	6%
25-45	3%	2%	-	-	1%	1%	-	-	-	-	-	-	-	-	-	-	7%
45-65	9%	4%	1%	1%	1%	1%	2%	-	-	-	-	1%	-	1%	-	1%	22%
>65	27%	3%	3%	2%	4%	-	1%	2%	1%	1%	1%	-	1%	-	1%	-	47%
Samples																	
Sputum	27%	4%	1%	2%	4%	-	2%	1%	1%	2%	-	-	-	-	1%	-	45%
Urine	2%	1%	-	-	-	-	1%	-	-	-	-	-	-	1	-	-	5%
Blood	4%	2%	1%	1%	2%	1%	-	1%	-	-	-	-	-	-	-	-	12%
Wound	5%	2%	-	-	-	-	-	-	-	1%	-	1%	-	-	-	1%	1-%
ETT	11%	3%	2%	3%	1%	1%	1%	1%	-	-	-	-	1	-	-	-	24%
Throat	1%	1%	1%	-	-	-	-	-	-	-	1%	-	-	-	-	-	4%

Table 3. The gene profile patterns and frequency of isolates in each profile are based on biofilm-producing potency (N=100).

Profile	Gene profile	Biofilm producing N = %				Total N = %
		Strong N= 32	Moderate N= 46	Weak N= 20	Non* N= 2	
A	*bap, ompA, pgaB, csuE, epsA, bfmS, ptk, bla-*$_{per1}$	22	27	1	-	50
B	*ompA, pgaB, csuE, epsA, bfmS, ptk, bla-*$_{per1}$	4	8	1	-	13
C	*bap, ompA, csuE, epsA, bfmS, ptk, bla-*$_{per1}$	2	1	2	-	5
D	*bap, ompA, pgaB, csuE, bfmS, ptk, bla-*$_{per1}$	1	2	3	-	6
E	*bap, ompA, pgaB, csuE, epsA, bfmS, ptk*	1	3	3	-	7
F	*pgaB, csuE, epsA, bfmS, ptk, bla-*$_{per1}$	1	-	1	-	2
G	*ompA, csuE, epsA, bfmS, ptk, bla-*$_{per1}$	1	1	2	-	4
H	*ompA, pgaB, csuE, bfmS, ptk, bla-*$_{per1}$	-	2	1	-	3
I	*ompA, pgaB, csuE, epsA, bfmS, ptk*	-	1	-	-	1
J	*ompA, pgaB, csuE, epsA, bfmS, bla-*$_{per1}$	-	-	3	-	3
K	*ompA, csuE, epsA, bfmS, bla-*$_{per1}$	-	-	-	1	1
L	*Bap, ompA, epsA, ptk*	-	-	-	1	1
M	*bap, pgaB, csuE, bfmS, ptk, bla-*$_{per1}$	-	-	1	-	1
N	*bap, ompA, pgaB, csuE, bfmS, bla-*$_{per1}$	-	1	-	-	1
O	*ompA, pgaB, csuE, epsA, ptk, bla-*$_{per1}$	-	-	1	-	1
P	*ompA, pgaB, csuE, bfmS*	-	-	1	-	1

* Non-biofilm producing.

Table 4. Antimicrobial resistance patterns among *A. baumannii* isolates harboring biofilm-mediated genes.

Antibiotics		No. of positive gene (N=%)															
		bap **(71%)**	**p value***	***ompA*** **(97%)**	**p value**	***pgaB*** **(89%)**	**p value**	***csuE*** **(99%)**	**p value**	***ptk*** **(96%)**	**p value**	***bla*** $_{\text{per-1}}$ **(88%)**	**p value**	***epsA*** **(88%)**	**p value**	***bfmS*** **(98%)**	**p value**
CAZ	R	71	-**	97	-	89	-	99	-	96	-	88	-	88	-	98	-
	I	0		0		0		0		0		0		0		0	
	S	0		0		0		0		0		0		0		0	
CTX	R	71	-	97	-	89	-	99	-	96	-	88	-	88	-	98	-
	I	0		0		0		0		0		0		0		0	
	S	0		0		0		0		0		0		0		0	
CPM	R	61	0.113	86	0.7	87	0.074	89	0.006	85	0.621	85	0.523	77	0.431	88	0.209
	I	1		2		0		2		2		0		2		2	
	S	9		9		2		8		9		3		9		8	
IMP	R	67	0.197	89	0.874	83	0.291	91	0.92	88	0.711	81	0.866	80	0.553	90	0.845
	I	1		1		1		1		1		1		1		1	
	S	3		7		5		7		7		6		7		7	
MER	R	64	0.468	86	0.826	79	0.56	88	0.889	86	0.3	77	0.224	79	0.788	87	0.79
	I	4		5		4		5		4		5		4		5	
	S	3		6		6		6		6		6		5		6	
PIP	R	69	0.361	95	0.8	87	0.616	97	0.84	94	0.685	86	0.472	86	0.472	96	0.775
	I	0		0		0		0		0		0		0		0	
	S	2		2		2		2		2		2		2		2	
CIP	R	69	0.867	94	0.757	87	0.209	96	0.805	93	0.619	85	0.377	85	0.377	95	0.726
	I	0		0		0		0		0		0		0		0	
	S	2		3		2		3		3		3		3		3	
GEN	R	47	0.4	68	0.151	62	0.614	69	0.698	67	0.743	63	0.627	60	0.437	68	0.486
	I	6		6		7		7		7		6		6		7	
	S	18		23		20		23		22		19		22		23	
TET	R	63	0.53	89	0.842	81	0.263	89	0.899	86	0.65	79	0.696	80	0.317	88	0.808
	I	5		1		5		7		7		6		5		7	
	S	3		7		3		3		3		3		3		3	
AMK	R	59	0.656	81	0.745	73	0.3	83	0.839	80	0.49	76	0.202	73	0.59	82	0.703
	I	6		7		7		7		7		5		7		7	
	S	6		9		9		9		9		7		8		9	
SXT	R	71	-	97	-	89	-	99	-	96	-	88	-	88	-	98	-
	I	0		0		0		0		0		0		0		0	
	S	0		0		0		0		0		0		0		0	

Abbreviation: CAZ, ceftazidime; CTX, cefotaxime; CPM, cefepime; IMP, imipenem; MER, meropenem; PIP, piperacillin/tazobactam; CIP, ciprofloxacin; GEN, gentamycin; TET, tetracycline; AMK, amikacin; SXT, trimethoprim/sulfamethoxazole; R, resistant; I, intermediated; S, sensitive.

* p value ≤ 0.05 is significant.

** Not applicable.

Table 5. The relationship between the prevalence of biofilm-mediated genes and biofilm-producing *A. baumannii* isolates

No. of positive genes (N=%)	Biofilm producing N (%)				Total	p value
	Strong N= 32	Moderate N= 46	Weak N= 20	Non N= 2		
bap (71%)	25 (35.2%)	35 (49.2%)	10 (28.1%)	1 (1.5%)	71 (100%)	0.10
ompA (97%)	31 (31.9%)	46 (47.4%)	18 (18.5%)	2 (2.2%)	97 (100%)	0.18
pgaB (89%)	29 (32.5%)	44 (49.6%)	16 (17.9%)	-	89 (100%)	0.001
csuE (99%)	32 (32.6%)	46 (46.5%)	19 (19.3%)	2 (2%)	99 (100%)	0.001
ptk (96%)	32 (33.3%)	44 (45.8%)	19 (19.7%)	1 (1.2%)	96 (100%)	0.006
bla_{per-1} (88%)	31 (35.2%)	43 (48.8%)	13 (14.7%)	1 (1.1%)	88 (100%)	0.001
epsA (88%)	30 (34%)	42 (47.7%)	14 (15.9%)	2 (2.4%)	88 (100%)	0.04
bfmS (98%)	32 (32.6%)	46 (47.1%)	19 (19.3%)	1 (1%)	98 (100%)	0.001

* p value < 0.05 is significant.

there is a significant correlation between carrying the *ptk, bfmS, pgaB, csuE, epsA,* and bla_{per-1} genes and biofilm-producing (P-value ≤0.05). However, there were no significant differences in presence of the bap and ompA genes with biofilm-producing.

REP-PCR analyzing. The GelJ software (version 2.0) analyzed the gel images from REP-PCR amplification to generate a dendrogram. A dendrogram was drawn using a similar matrix with the Dice method. According to the dendrogram, 56 fingerprint patterns were revealed for all isolates (Fig. 3). The obtained multiple DNA bands ranged from 220 bp to 3000 bp; the most common bands were 310, 750, and 1600 bp. Regarding less than 80% similarities, the 97 *A. baumannii* isolates were classified into 7 clusters and two singletons; three isolates were non-typeable. Cluster 2 was the biggest one, containing 37 isolates; in contrast, the smallest one was clusters 5 and 6, with two isolates. Also, clusters 1, 3, 7, and 4 contain 23, 19, 7, and 5 isolates, respectively.

DISCUSSION

According to the Centers for Disease Control and Prevention, MDR *A. baumannii* isolates cause 20% of infections in ICUs, leading to significantly rising mortality rates globally (3). The frequency of MDR isolates in this study was 100%, compared to 58%, 74.75%, and 95% in previous studies (15-17). This suggests that the number of MDR *A. baumannii* isolates in Iran is rising. According to previous reports, the high frequency of MDR *A. baumannii* isolates has changed over time in different regions. In 2008, Asia demonstrated a higher frequency than Europe and the USA, while in 2014, Africa had the highest frequency (18).

Carbapenems have been considered as one of the last treatment options (11); nevertheless, 92% and 89% of *A. baumannii* isolates were resistant against imipenem and meropenem, respectively; these findings are in correlation with earlier surveys in Iran (19), Italy and Spain (20), Malaysia (21). Furthermore, 100% and 98% resistance against tested cephalosporins (including ceftazidime, cefotaxime, and cefepime) and piperacillin/tazobactam, respectively, have proposed the ineffective of these antibiotics for the treatment of infections due *A. baumannii*. confirmed that developing of novel antibiotics based on new β-lactamase inhibitors (cefepime/enmetazobactam, cefepime/zidebactam, cefoperazone/sulbactam, ceftazidime/avibactam, and ceftolozane/tazobactam) is essential (22). The lowest resistance among tested antibiotics was against gentamycin (70%) and amikacin (84%). However, they are not recommended as an appropriate choice, as comprehensive review investigations have significantly proposed aminoglycosides resistance among *A. baumannii* strains worldwide (23, 24).

Several studies have presented biofilms as the structure conferring resistance to antimicrobial and host defense response (25). The highlighted resistance against different families of antibiotics already used might be related to biofilm production. Notably, 98% of *A. baumannii* isolates were biofilm

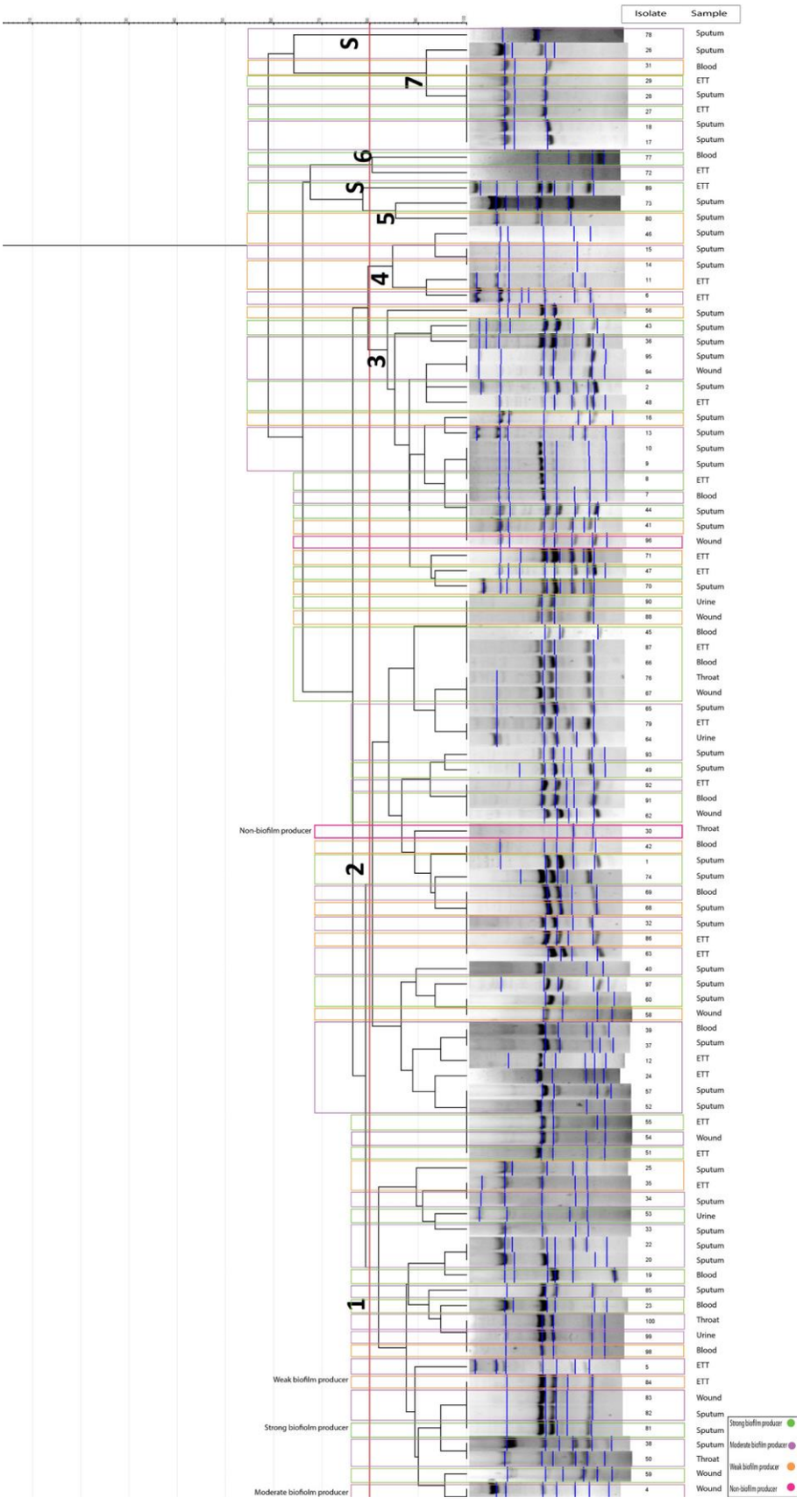

Fig. 3. The dendrogram analysis of REP-PCR fingerprinting of *A. baumannii* isolated from ICU-admitted patients (n=100). S: singleton

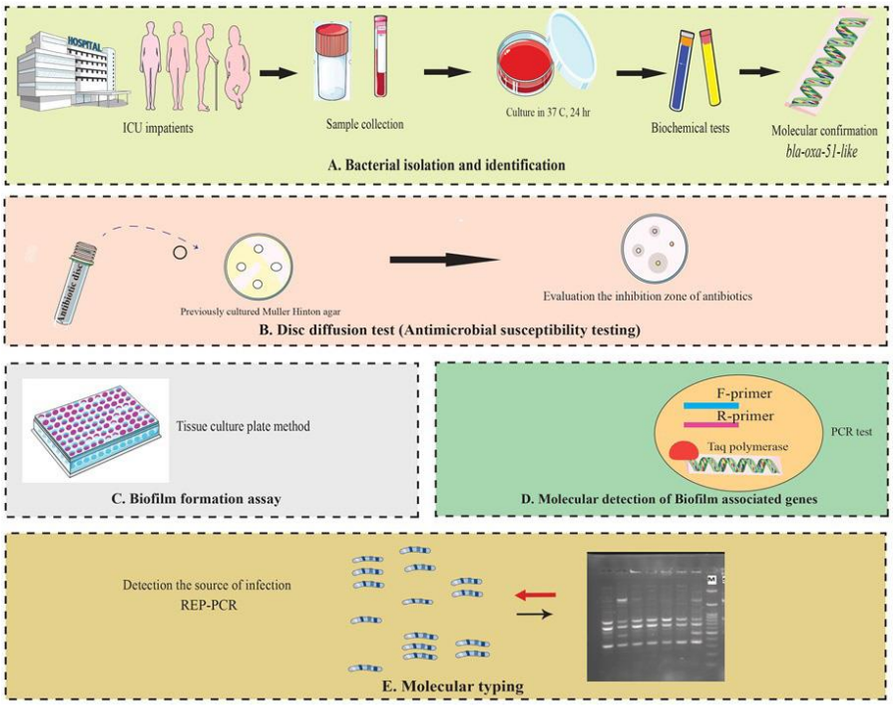

Fig. 4. Graphical abstract demonstrating the significant points of the manuscript

producers. The frequency of strong- and moderate-biofilm producers was 32% and 46%, respectively; that was in line with previous reports in Iran (26), China (27), and Egypt (28). Biofilm-associated resistance significantly impacts hospital infections, leading to treatment failures, extended hospital stays, and rising healthcare costs. Developing comprehensive strategies to prevent biofilm formation and creating effective treatments specifically targeting biofilm-associated infections is essential for enhancing patient outcomes in clinical settings. Research initiatives, monitoring efforts, and healthcare practices must confront the challenges posed by biofilm-related resistance to mitigate its effects on public health (29).

Among detected biofilm-associated genes, the *csuE* and the *bap* were the highest and lowest frequencies, respectively. This value was found in other investigations that stated *csuE* as the most pervasive biofilm-associated gene (5, 6). The high prevalence of the *csuE* gene among *A. baumannii* isolates in the current study is consistent with findings from previous studies. This indicates that the *csuE* gene represents a promising candidate for targeted therapies and preventive strategies aimed at addressing the challenges posed by biofilm formation.

Several studies have pointed out bla_{per-1} and *epsA* as crucial factors for biofilm producer *A. baumannii* isolates (8, 30). This confirmed our results that demonstrated the *epsA* and bla_{per-1} genes were absent among weak biofilm-producing isolates. Generally, statistical analysis has revealed a significant relationship between biofilm formation and *csuE*, bla_{per-1}, *epsA*, *bfmS*, and *ptk* genes (P-value $\leq$ 0.05); earlier survey results supported this value (31).

The high frequency of bla_{per-1} (39%) was observed among isolates from the sputum, which is correlated with other studies (30, 32). Moreover, the previous investigation determined that the strong and moderate biofilm-producing *A. baumannii* isolates are mostly isolated from patients with pneumonia and respiratory diseases (33); subsequently, it seems the role of bla_{per-1} is highlighted to attach the respiratory tract, produce biofilm, and intensify infections. Nonetheless, more information could be received by assessing higher sample sizes from different geographical regions. Further investigations should be done to evaluate antimicrobial susceptibility and the value of biofilm-associated gene expression.

The analyzing REP-PCR results showed that *A. baumannii* isolates discriminated regarding biofilm-producing potency among various clusters. Indeed, the strains with identical genotypes produce biofilms with similar severity. In this regard, 15 and 5 out of 32 isolates with strong biofilm-producing ability

have been placed in clusters 2 and 1, respectively. Also, 16 and 14 out of 46 moderate biofilm-producing isolates have been classified into clusters 2 and 1, respectively. In agreement with our results, several reports from studies indicate the high discriminatory power of the REP-PCR method for genotyping of *A. baumannii* isolates (34).

CONCLUSION

In summary, the frequency of MDR *A. baumannii* isolates was remarkable (100%). Also, most of the subjected *A. baumannii* isolates were frequently resistant to carbapenems, imipenem (92%), and meropenem (89%). Therefore, representing a new combination of β-lactam/β-lactamase inhibitors seems necessary to prevent and control severe nosocomial infections. Producing biofilm is one of the most highlighted mechanisms for raising the frequency of MDR *A. baumannii* strains. Therefore, efficient antimicrobial stewardship and designing novel anti-biofilm combating strategies are necessary to control this global concern.

ACKNOWLEDGEMENTS

This work is supported by the Vice-Chancellor for Research of Shiraz University of Medical Sciences as Nafiseh Hosseinzadeh Shakib MSc thesis and financially supported by there under Grant Number (1400.02.18.22204).

REFERENCES

1. Meshkat Z, Salimizand H, Amini Y, Mansury D, Zomorodi AR, Avestan Z, et al. Detection of efflux pump genes in multiresistant *Acinetobacter baumannii* ST2 in Iran. *Acta Microbiol Immunol Hung* 2021; 68: 113-120.
2. Bialvaei AZ, Kouhsari E, Salehi-Abargouei A, Amirmozafari N, Ramazanzadeh R, Ghadimi-Daresajini A, et al. Epidemiology of multidrug-resistant *Acinetobacter baumannii* strains in Iran: a systematic review and meta-analysis. *J Chemother* 2017; 29: 327-337.
3. Keikha M, Karbalaei M, Rahimi F, Abadi ATB. The prevalence of antibiotic-resistant *Acinetobacter baumannii* infections among the Iranian ICU patients: a systematic review and meta-analysis. *Gene Rep* 2023; 30: 101731.
4. Falcone M, Tiseo G, Giordano C, Leonildi A, Menichini M, Vecchione A, et al. Predictors of hospital-acquired bacterial and fungal superinfections in COVID-19: a prospective observational study. *J Antimicrob Chemother* 2021; 76: 1078-1084.
5. Zeighami H, Valadkhani F, Shapouri R, Samadi E, Haghi F. Virulence characteristics of multidrug resistant biofilm forming *Acinetobacter baumannii* isolated from intensive care unit patients. *BMC Infect Dis* 2019; 19: 629.
6. Khoshnood S, Savari M, Abbasi Montazeri E, Farajzadeh Sheikh A. Survey on genetic diversity, biofilm formation, and detection of colistin resistance genes in clinical isolates of *Acinetobacter baumannii*. *Infect Drug Resist* 2020; 13: 1547-1558.
7. Gedefie A, Alemayehu E, Mohammed O, Bambo GM, Kebede SS, Kebede B. Prevalence of biofilm producing *Acinetobacter baumannii* clinical isolates: a systematic review and meta-analysis. *PLoS One* 2023; 18(11): e0287211.
8. Monfared AM, Rezaei A, Poursina F, Faghri J. Detection of genes involved in biofilm formation in MDR and XDR *Acinetobacter baumannii* isolated from human clinical specimens in Isfahan, Iran. *Arch Clin Infect Dis* 2019; 14(2): e85766.
9. Rafati Zomorodi A, Rad M, Hashemi Tabar GR, Salimizand H. Molecular typing of cephalosporin resistant serovars of *Salmonella enterica* from poultry and farm animals. *Bulg J Vet Med* 2020; 23: 178-186.
10. Shin SK, Lee Y, Kwon H, Rhee JS, Kim JK. Validation of direct boiling method for simple and efficient genomic DNA extraction and PCR-based macroalgal species determination. *J Phycol* 2021; 57: 1368-1372.
11. Zomorodi AR, Mohseni N, Hafiz M, Nikoueian H, Hashemitabar G, Salimizand H, et al. Investigation of mobile colistin resistance (*mcr*) genes among carbapenem resistance *Pseudomonas aeruginosa* isolates from bovine mastitis in Mashhad, Iran. *Gene Rep* 2022; 29: 101695.
12. Sales AJ, Naebi S, Bannazadeh-Baghi H, Saki M. Antibiotic resistance pattern and prevalence of bla_{OXA-51}, bla_{NDM}, bla_{VIM}, bla_{PER}, bla_{VEB}, bla_{CTX}, *tetA* and *tetB* Genes in *Acinetobacter baumannii* isolated from clinical specimens of hospitals in Tabriz city, Iran. *J Clin Res Paramed Sci* 2021; 10(2): e118521.
13. Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Susceptibility Testing.31st ed. CLSI supplement M100. Clinical and Laboratory Standards Institute, USA, 2021.
14. Singh AK, Prakash P, Achra A, Singh GP, Das A, Singh RK. Standardization and classification of in vitro biofilm formation by clinical isolates of *Staphylococcus*

aureus. J Glob Infect Dis 2017; 9: 93-101.
15. Sobouti B, Mirshekar M, Fallah S, Tabaei A, Mehrabadi JF, Darbandi A. Pan drug-resistant *Acinetobacter baumannii* causing nosocomial infections among burnt children. *Med J Islam Repub Iran* 2020; 34: 24.
16. Mirzaei B, Bazgir ZN, Goli HR, Iranpour F, Mohammadi F, Babaei R. Prevalence of multi-drug resistant (MDR) and extensively drug-resistant (XDR) phenotypes of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* isolated in clinical samples from Northeast of Iran. *BMC Res Notes* 2020; 13: 380.
17. Hazhirkamal M, Zarei O, Movahedi M, Karami P, Shokoohizadeh L, Taheri M. Molecular typing, biofilm production, and detection of carbapenemase genes in multidrug-resistant *Acinetobacter baumannii* isolated from different infection sites using ERIC-PCR in Hamadan, west of Iran. *BMC Pharmacol Toxicol* 2021; 22: 32.
18. Lob SH, Hoban DJ, Sahm DF, Badal RE. Regional differences and trends in antimicrobial susceptibility of *Acinetobacter baumannii*. *Int J Antimicrob Agents* 2016; 47: 317-323.
19. Rashvand P, Peymani A, Mohammadi M, Karami AA, Samimi R, Hajian S, et al. Molecular survey of aminoglycoside-resistant *Acinetobacter baumannii* isolated from tertiary hospitals in Qazvin, Iran. N*ew Microbes New Infect* 2021; 42: 100883.
20. Nowak J, Zander E, Stefanik D, Higgins PG, Roca I, Vila J, et al. High incidence of pandrug-resistant *Acinetobacter baumannii* isolates collected from patients with ventilator-associated pneumonia in Greece, Italy and Spain as part of the MagicBullet clinical trial. *J Antimicrob Chemother* 2017; 72: 3277-3282.
21. Woon JJ, Teh CSJ, Chong CW, Abdul Jabar K, Ponnampalavanar S, Idris N. Molecular characterization of carbapenem-resistant *Acinetobacter baumannii* isolated from the Intensive care unit in a tertiary teaching hospital in Malaysia. *Antibiotics (Basel)* 2021; 10: 1340.
22. Liu P-Y, Ko W-C, Lee W-S, Lu P-L, Chen Y-H, Cheng S-H, et al. In vitro activity of cefiderocol, cefepime/enmetazobactam, cefepime/zidebactam, eravacycline, omadacycline, and other comparative agents against carbapenem-non-susceptible *Pseudomonas aeruginosa* and *Acinetobacter baumannii* isolates associated from bloodstream infection in Taiwan between 2018–2020. *J Microbiol Immunol Infect* 2022; 55: 888-895.
23. Karakonstantis S, Kritsotakis EI, Gikas A. Pandrug-resistant Gram-negative bacteria: a systematic review of current epidemiology, prognosis and treatment options. *J Antimicrob Chemother* 2020; 75: 271-282.
24. Karakonstantis S, Kritsotakis EI, Gikas A. Treatment options for *K. pneumoniae*, *P. aeruginosa* and *A. baumannii* co-resistant to carbapenems, aminoglycosides, polymyxins and tigecycline: an approach based on the mechanisms of resistance to carbapenems. *Infection* 2020; 48: 835-851.
25. Gebreyohannes G, Nyerere A, Bii C, Sbhatu DB. Challenges of intervention, treatment, and antibiotic resistance of biofilm-forming microorganisms. *Heliyon* 2019; 5(8): e02192.
26. Asadian M, Azimi L, Alinejad F, Ostadi Y, Lari AR. Molecular characterization of *Acinetobacter baumannii* isolated from ventilator-associated pneumonia and burn wound colonization by random amplified polymorphic DNA polymerase chain reaction and the relationship between antibiotic susceptibility and biofilm production. *Adv Biomed Res* 2019; 8: 58.
27. Shenkutie AM, Yao MZ, Siu GK, Wong BKC, Leung PH. Biofilm-induced antibiotic resistance in clinical *Acinetobacter baumannii* isolates. *Antibiotics (Basel)* 2020; 9: 817.
28. Asaad AM, Ansari S, Ajlan SE, Awad SM. Epidemiology of biofilm producing *Acinetobacter baumannii* nosocomial isolates from a tertiary care hospital in Egypt: a cross-sectional study. *Infect Drug Resist* 2021; 14: 709-717.
29. Assefa M, Amare A. Biofilm-associated multi-drug resistance in hospital-acquired infections: A review. *Infect Drug Resist* 2022; 15: 5061-5068.
30. Altınok Ö, Boral B, Ergin A, Eser ÖK. Existence of biofilm and biofilm-associated virulence genes in multi-drug resistant invasive *Acinetobacter baumannii* isolates. *Mikrobiyol Bul* 2020; 54: 40-49.
31. Yang C-H, Su P-W, Moi S-H, Chuang L-Y. Biofilm formation in *Acinetobacter baumannii*: genotype-phenotype correlation. *Molecules* 2019; 24: 1849.
32. Badmasti F, Siadat SD, Bouzari S, Ajdary S, Shahcheraghi F. Molecular detection of genes related to biofilm formation in multidrug-resistant *Acinetobacter baumannii* isolated from clinical settings. *J Med Microbiol* 2015; 64: 559-564.
33. Alamri AM, Alsultan AA, Ansari MA, Alnimr AM. Biofilm-formation in clonally unrelated multidrug-resistant *Acinetobacter baumannii* isolates. *Pathogens* 2020; 9: 630.
34. Leelasupasri S, Santimaleeworagun W, Jitwasinkul T. Antimicrobial susceptibility among colistin, sulbactam, and fosfomycin and a synergism study of colistin in combination with sulbactam or fosfomycin against clinical isolates of carbapenem-resistant *Acinetobacter baumannii*. *J Pathog* 2018; 2018: 3893492.