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Expression of *bap* gene in multidrug-resistant and biofilm-producing *Acinetobacter baumannii* clinical isolates

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Abstract

Introduction *Acinetobacter baumannii* is a significant biofilm-producer and antibiotic-resistant pathogen associated with various infections caused in humans. This study aimed to investigate the expression level of the *bap* gene in multidrug-resistant and biofilm-producer clinical isolates of *A. baumannii*.

Materials and methods One Hundred *A. baumannii* clinical isolates were collected from hospitalized patients and identified by phenotypic and genotypic tests. The antibiotic resistance pattern of the isolates was determined by the disk agar diffusion method. The ability of biofilm production was investigated using the microtiter plate test. This study employed the Real-time PCR method to evaluate the expression level of the *bap* gene.

Results Ninety nine percent *A. baumannii* isolates were MDR. However, the highest resistance rate was observed against ciprofloxacin (100%), while ceftazidime was the most effective drug. Also, 49%, 49%, and 2% of the isolates were strong, moderate, and weak biofilm-producing, respectively. However, we detected no strain without the ability to produce biofilm. Most strong and moderate biofilm-former isolates were non-susceptible to all tested antibiotics. An increased expression level of the *bap* gene was detected in 99% of the isolates. The results of the present study suggest a correlation between the *bap* gene expression level and the development of multidrug resistance and biofilm formation in *A. baumannii* isolates.

Conclusion This research emphasizes the importance of biofilm formation in the emergence of multidrug-resistant *A. baumannii* strains in healthcare settings, making them progressively difficult to control. The *bap* gene may be a considerable target for the development of novel anti-*A. baumannii* treatment option and eradication of the biofilm formation by this organism.

Keywords *Acinetobacter baumannii*, Multidrug resistance, Biofilm, *Bap*

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Introduction

Several factors, such as misuse or overuse of antibiotics and the emergence of biofilm in patients with chronic infectious diseases or recurrent infections can contribute to the emergence of antimicrobial resistance (AMR) in different bacteria. Besides, the antibiotic resistance level is more reported in low and middle-income countries [1]. Due to the genomic plasticity of *Acinetobacter baumannii*, the number of multidrug-resistant (MDR) isolates of this organism is increased, resulting in limited treatment options and an increased mortality rate [2]. This organism is reported as a top 10 threat due to the high-level antibiotic resistance and potential pathogenic characteristics, such as environment persistence, surface adhesion, host cell invasion, and escape from the host's immune system [1]. *A. baumannii* is a gram-negative opportunistic pathogen causing different clinical infections, predominantly in immunocompromised and hospitalized patients [3]. This organism has developed a high resistance to traditional antibiotics, making it difficult to eradicate in clinical settings [4]. Biofilm formation is a crucial mechanism used by *A. baumannii* to survive and colonize different biotic and abiotic surfaces, such as wounds, medical devices, and catheters, in healthcare settings [5, 6]. Biofilm protects bacteria when exposed to antibiotics, environmental stresses, temperature, pH fluctuations, desiccation, and the host immune system, boosting bacterial virulence [7]. Also, biofilms provide nutrients to bacteria and facilitate the transfer of resistance genes, resulting in the emergence of multidrug-resistant (MDR) clones [8]. A 26–60% morbidity and mortality rate was reported by *A. baumannii*, while this rate is higher when the MDR isolates are distributed in hospitals [9]. These characteristics have led to the introduction of this organism as one of the most significant high-priority critical pathogens by WHO [9].

Several microbial structures, including adhesins, polysaccharides, surface appendages, proteins, chaperon-usher pilus, etc., are significant in the creation and maintenance of the *A. baumannii* biofilms [6].

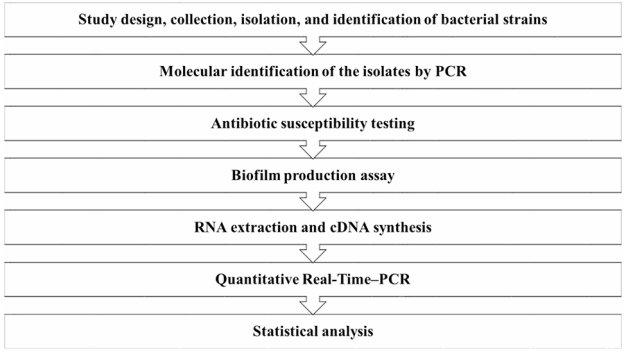

Fig. 1 All the procedures that are done in Materials and Methods

Biofilm-associated protein (Bap) is a high-molecular-weight protein exposed on the surface of *A. baumannii*, assisting cell-cell adhesion, interactions between the bacteria and environment, and formation and maturation of biofilm [10]. The Bap in *A. baumannii* is associated with a strong biofilm formation. This protein is secreted by a type I secretion system and targets the host cells' carbohydrates [9]. The *bap* gene, encoding the biofilm-associated protein, is a molecular marker found in the genome of *A. baumannii* and has been identified as a potential target for managing multidrug resistance in *A. baumannii* [11, 12]. The overexpression of the *bap* gene is associated with increased adherence of the bacteria to the human epithelial cells, biofilm production, and the emergence of multidrug resistance phenotype by *A. baumannii* [9]. Antimicrobial resistance by biofilm-embedded cells is due to diffusion inhibition of the antibiotics, decreased growth rate of the bacteria, antimicrobial tolerance of the persister cells in biofilm, and change in the bacterial morphology due to environmental stresses [9]. Several studies have shown a positive correlation between antibiotic resistance and biofilm formation [13–15] and the emergence of MDR isolates among high biofilm-producer *A. baumannii* [16, 17]. These data suggest that the overexpression of the *bap* gene has significant implications in clinical practice, making it more challenging to prevent and treat infections caused by biofilm-producer *A. baumannii* [18]. Thus, we aimed to assess the role of *bap* gene overexpression in the ability of biofilm production and the emergence of MDR phenotype in clinical isolates of *A. baumannii*.

Materials and methods

All the procedures done in the Materials and Methods section are shown in Fig. 1. The *A. baumannii* isolates were collected from hospitals and were identified by phenotypic and genotypic methods. Then, the antibiotic susceptibility pattern of the isolates was detected using the disk agar diffusion method, and the biofilm-producer isolates were identified by microtiter plate test. Next, the Real-time method was used to assess the expression level of the *bap* gene in isolates.

Study design, collection, isolation, and identification of bacterial strains

This study was approved by the Ethics Committee of Mazandaran University of Medical Science (MAZUMS), Iran) with the ethics number IR.MAZUMS.REC.1401.14299. This cross-sectional study was conducted from March 2022 to August 2022 in Sari city, North of Iran. One Hundred non-duplicated *A. baumannii* clinical isolates were collected in the present study. The bacteria were isolated from different clinical samples at a burn hospital affiliated with Mazandaran University

of Medical Sciences in Iran. The isolates were identified again as *A. baumannii* using microbiological and biochemical standard tests and were confirmed by testing the presence of bla_{OXA-51} gene using Polymerase Chain Reaction (PCR) [19].

Molecular identification of the isolates by PCR

The genomic DNAs of the *A. baumannii* clinical isolates were extracted using the alkaline lysis method, employing Sodium Dodecyl Sulfate and NaOH [20]. The quality of the extracted DNAs was determined by the evaluation of the optical density (OD) of the DNAs using a NanoDrop (ND1000, USA) and electrophoresis of the products on a 1% agarose gel (Wizbiosolutions, South Korea). The PCR test was performed using a specific primer pair, including bla_{OXA-51}-F-5′-TAATGCTTTGATCGGCCTTG-3′, and bla_{OXA-51}-R-5′-TGGATTGCACTTCATCTTGG-3′, as previously described [21]. The PCR reaction was conducted in a final volume of 15 µl (µL) containing 7.5 µL of Master Mix, 0.5 µL of each primer, 5.5 µL of distilled water, and 300 ng (1 µL) of DNA. The initial denaturation step was performed at 94 °C for three min. Then, 35 cycles of denaturation at 94 °C for 45 s, annealing at 60 °C for one min, and extension at 72 °C for one min, along with a final amplification step at 72 °C for five min, were done. The PCR products were electrophoresed on a 1% agarose gel (Wizbiosolutions) with 1.5 µl of Safe Stain (SinaClon, Iran).

Antibiotic susceptibility testing

The antibiotic resistance pattern of the isolates was determined using the Kirby-Bauer disk agar diffusion method, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [22]. The antibiotics tested in this study included: Doripenem (10 µg), Meropenem (10 µg), Imipenem (10 µg), Trimethoprim/Sulfamethoxazole (1.25/23.75 µg), Piperacillin (100 µg), Piperacillin/Tazobactam (100/10 µg), Cefepime (30 µg), Ceftazidime (30 µg), Ceftriaxone (30 µg), Levofloxacin (5 µg), Ciprofloxacin (5 µg), Gentamicin (10 µg), and Tobramycin (10 µg) (Liofilchem, Italy). *Escherichia coli* ATCC 25,922 was used as a quality control strain in antibiotic susceptibility testing [22]. The *Acinetobacter baumannii*-non-susceptible to at least one drug in at least 3 of the following categories was defined as MDR isolate.

(1) Extended-spectrum cephalosporins (cefepime, ceftazidime, ceftriaxone, cefotaxime) (2) Fluoroquinolones (ciprofloxacin, levofloxacin) (3) Aminoglycosides (amikacin, gentamicin, tobramycin) (4) Carbapenems (imipenem, meropenem, doripenem) (5) Piperacillin/tazobactam (6) Ampicillin/sulbactam [23].

Biofilm production assay

The ability of *A. baumannii* clinical isolates to produce biofilms was detected using the microtiter plate method. The bacterial cells were grown overnight at 37 °C in 10 mL of Trypticase Soy Broth (TSB) (Merck, Germany). A 96-well flat-bottomed polystyrene tissue culture plate (three wells for each strain) was filled with 20 µL of overnight culture (equivalent to 0.5 McFarland standard) and 180 µL of TSB medium and incubated overnight at 37 °C. Then, the wells were rinsed with Phosphate Buffer Saline (PBS) three times to remove free-floating bacteria. The adhered cells were fixed by adding the absolute methanol (Merck) for 15 min, and then the methanol was discarded, and the wells were air-dried. Next, the wells were stained with 0.2 mL of 0.1% (v/v) Hucker crystal violet for 15 min and then washed with tap water. After drying the plates in the air, the dyes attached to the adhered cells were dissolved in 200 µL of 33% (v/v) glacial acetic acid (Merck) at 37 °C for 15 min. Then, the optical density of each well was measured at 630 nm using an enzyme-linked immunosorbent assay (ELISA) reader (Stat Fax 2100). The biofilm-producing reference strain *A. baumannii* ATCC 19,606 was used as a positive control [24]. Also, the uninoculated wells containing the sterile broth medium were used as negative controls. An optical density cut-off (ODc) value was recognized as three standard deviations above the average of negative control optical density. The isolates were divided into four groups according to their OD results, including non-biofilm producer ($OD \leq ODc$), weak biofilm producer ($ODc < OD \leq 2\times ODc$), moderate biofilm producer ($2\times ODc < OD \leq 4 \times ODc$), and strong biofilm producer ($4\times ODc < OD$) [14].

RNA extraction and cDNA synthesis

The total RNAs of the isolates were extracted manually using a pure YTzol RNA solution (Wizbiosolutions). The 24-hour pure cultured bacteria were homogenized by stirring in one mL of sterile normal saline and were centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant was removed, and 200 µL of cold YTzol solution was added to the bacterial sediment. Then, the suspension was vortexed for five seconds to homogenize and incubated for five min at 4 °C. Subsequently, 200 µL of chloroform solution was added, and the vial was shake until a milky color changed. The sample was then incubated for 3 min and centrifuged at 12,000 rpm for 15 min at 4 °C. This process established three distinct phases, and the supernatant, containing the RNA molecules, was transferred to a separate vial. Isopropanol (500 µL) was added, and the sample was placed on ice for 10 min and then centrifuged at 12,000 rpm for 10 min (4 °C). The supernatant was discarded and replaced with one mL of 70% ethanol and centrifuged at 7500 rpm for five min (4 °C).

The ethanol was discarded, and the sample was air-dried under a biological hood. Finally, 50 µL of Diethyl pyrocarbonate (DEPC) treated water was added to the extracted RNA and vortexed for five sec. The optical density of the RNA was measured at 260/280 nm, and the products were stored at -70 °C. Then, the extracted RNAs were treated with RNase-free DNase I (Wizbiosolutions). The complementary DNAs (cDNAs) were synthesized using the Wizbiosolutions kit for cDNA synthesis, according to the manufacturer's instructions. Five µg of the extracted RNA were added in a 20 µL final volume to synthesize the cDNA using M-mulv reverse transcriptase and random hexamer primer (Wizbiosolutions). The synthesized cDNAs were stored at -20 °C until use.

Quantitative real-time–PCR

The qRT-PCR using the specific primer pair, including *bap*-F-5′-TAGACGCAATGGATAACG-3′ and *bap*-R-5′-TTAGAACCGATAACGATACC-3′, was used to detect the expression level of the *bap* gene, as previously described [12]. The *16 S rRNA* gene was used as a reference normalizing gene in RT-PCR, as previously designated by Sepahvand et al. [25]. The primer pair used to determine the *16 S rRNA* expression level included *16 S-rRNA*-F-5′-TCAGCTCGTGTCGTGAGATG-3′, and *16 S-rRNA*-R-5′-CGTAAGGGCCATGATG-3′. The fold-change increase of the *bap* gene expression was quantified using the $RQ = 2^{-\Delta\Delta Ct}$ (Livak method) compared to the reference strain *A. baumannii* ATCC 15,308 [26]. All qRT-PCR reactions were carried out in a final volume of 12.5 µl by an ABI 7300 thermocycler (Applied Biosystems, Foster City, CA, USA). Each qRT-PCR reaction contained 6.25 µl of 2X Syber Green Mastermix (Takara, Japan), one µl of each primer, 0.2 µl of ROX dye, 3.05 µl of nuclease-free water, and one µl of template cDNA. Both qRT-PCRs were done in two steps, including an initial denaturation at 94 °C for 5 min, followed by 45 cycles, containing a denaturation at 94 °C for 20 s (*16 S rRNA*) and 25 s (*bap*), annealing at 58 °C for 20 s (*16 S rRNA*) and 62 °C for 40 s (*bap*), and an extension step at 72 °C for 20 s (*16 S rRNA*) and 50 s (*bap*).

A melting curve cycle was achieved on the SYBR channel at gain 70, using a ramping rate of 0.5 °C/10 s for 65–95 °C. Also, To verify the accuracy of the qRT-PCR and the absence of false-positive reactions, a no-template control (NTC) consisting of a negative control sample without cDNA was incorporated into each step of the experimental procedure. To ascertain amplification efficiency, the qRT-PCR was done using serial dilutions of the cDNA of the standard strain.

Table 1 Antibiotic susceptibility profile of *Acinetobacter baumannii* isolates in this study ($n = 100$)

Antibiotics	Resistant	Intermediate resistant	Susceptible
Imipenem	97	1	2
Meropenem	98	1	1
Doripenem	97	0	3
Trimethoprim/Sulfamethoxazole	99	0	1
Piperacillin	97	1	2
Piperacillin/Tazobactam	95	2	3
Cefepime	99	0	1
Ceftazidime	93	0	7
Ceftriaxone	99	1	0
Levofloxacin	97	1	2
Ciprofloxacin	100	0	0
Gentamicin	94	0	6
Tobramycin	94	2	4

Statistical analysis

Data were assessed, checked for accuracy, and analyzed using the Statistical Package for the Social Sciences (SPSS) version 24 (IBM Corp, Armonk, NY.). The chi-square and Fisher's exact tests were used to determine the relationship between the gene expression, the ability of biofilm formation, and antibiotic resistance phenotypes of *A. baumannii*. The total frequency of biofilm-related genes in isolates was measured, and their association with biofilm formation was further explored using the Kruskal–Wallis H test. All analyses were conducted with 95% confidence, and P-*values* < 0.05 were considered statistically significant.

Results

Isolates identification and distribution

During the six months of the study period, 100 *A. baumannii* clinical isolates were collected from patients hospitalized in teaching and therapeutic hospitals. In the present study, the age of the patients ranged from six months to 88 years (mean age, 42.08 ± 25.08). Also, 50 (50%) isolates were obtained from males and others from females. The majority of clinical isolates were collected from adult burn wards (34%), Intensive Care Units (29%), surgical wards (21%), and pediatric burn units (16%). The bacterial isolates in this study were collected from the wound (73%), urine (15%), and blood (12%) samples.

Antibiotic resistance profile of the isolates

It was detected that 99% of the *A. baumannii* isolates were MDR. However, the highest resistance rate was observed against ciprofloxacin (100%), trimethoprim-sulfamethoxazole (99%), and ceftriaxone (99%). Ceftazidime was the most effective drug in this study, while 93% of the isolates were resistant to this antibiotic. The antibiotic resistance pattern of the isolates is shown in Table 1.

Relationship between antibiotic susceptibility and biofilm formation

The biofilm formation capacity of the 100 *A. baumannii* clinical isolates in this study showed that 49%, 49%, and 2% of isolates were strong, moderate, and weak biofilm producers, respectively (Fig. 2).

However, we detected no strain without biofilm production in this study. According to the relationship between the biofilm formation ability and resistance to a class of the antibiotics tested in this study, all the strong biofilm-producer isolates ($n=49$) were non-susceptible (resistant or intermediate resistant) against all classes, except for aminoglycosides and cephalosporins, where 47 (95.91%) and 46 (93.87%) isolates showed this properties, respectively (Fig. 3).

We found that most strong- and moderate biofilm-producing isolates of *A. baumannii* were non-susceptible against all antibiotics tested in the present study (Table 2). However, there was a significant relationship between the ability of biofilm production and non-susceptibility to all tested antibiotics (P‹0.05).

Biofilm formation capacity and its relation to clinical sample types and hospital wards

In this study, 34, 29, 21, and 16 isolates of *A. baumannii* were collected from the burn, intensive care units (ICUs), surgery, and pediatric wards in the studied hospitals. Moreover, 73, 15, and 12 isolates were collected from the wound, urine, and blood samples. However, 40 (54.79%) and 9 (60%) isolates collected from wound and urine samples grouped as the strong biofilm-producer *A. baumannii*, respectively (Fig. 4). None of the *A. baumannii* isolated from blood cultures were strong biofilm producers (Fig. 4). Also, 24 (70.58%) isolates collected from the patients hospitalized in adult burn wards were strong biofilm producers, while 20 (95.23%) isolates collected in surgery sections were moderate biofilm producers

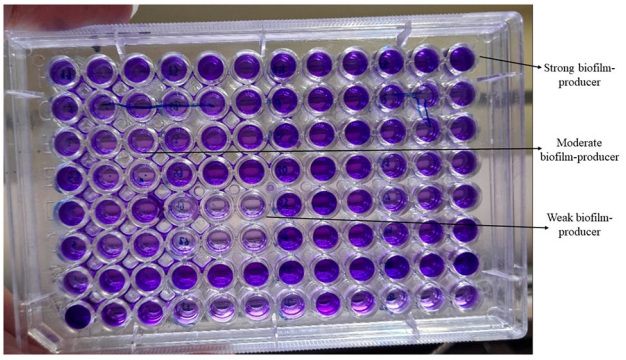

Fig. 2 Result of the biofilm formation assay using the microtiter plate test

(Fig. 5). However, the weak biofilm-producer isolates were not detected in adult burn wards and ICUs.

The bap gene expression level and its relation to biofilm formation ability

Among 100 clinical isolates of *A. baumannii* in the present study, 99 showed increased expression of the *bap* gene (Fig. 6). However, 46 (46.46%) isolates had a 2-5-fold increased expression of the *bap* gene, while 44 (95.65%) belonged to the moderate biofilm-producer group. Also, 15 (15.15%) isolates showed a 6–10-fold increase in expression, 15 (15.15%) isolates exhibited an 11–15-fold increased expression, and a 16–20-fold increased expression of this gene was detected in 17 (17.17%) isolates. All isolates in these three groups were detected as strong biofilm producers. However, 7 (7.07) isolates exhibited no change in the expression level of the *bap* gene, from which five isolates were moderate biofilm producers, and two others were weak biofilm producers. The strains exhibiting strong biofilm formation ability showed more *bap* gene expression levels. The Kruskal-Wallis H test revealed a statistically significant association between the expression of the *bap* gene and the biofilm formation

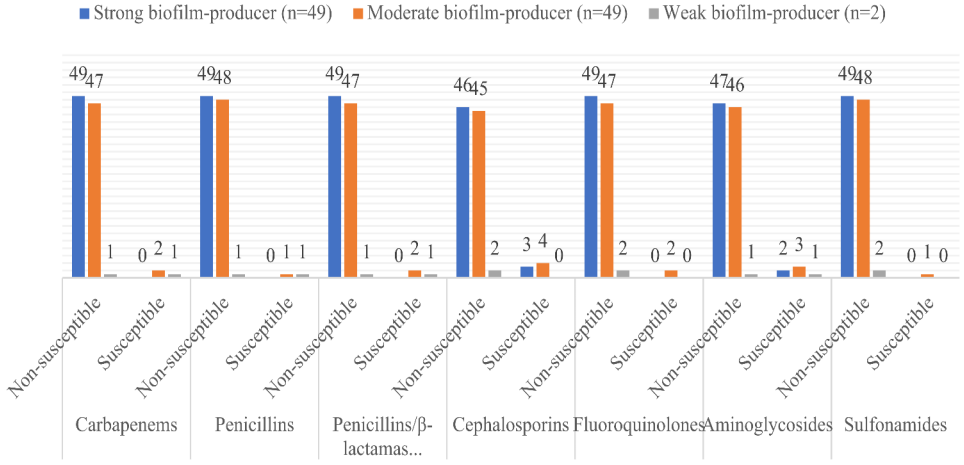

Fig. 3 Relationship between the biofilm formation ability and resistance to a class of antibiotics

Table 2 Relationship between the antibiotic resistance pattern and the ability of biofilm production of *Acinetobacter baumannii* isolates

Antibiotics	Strong (*N* = 49)		Moderate (*N* = 49)	Weak (*N* = 2)	*P*-Value
Doripenem	NS	49 (100%)	47 (95.91%)	1 (50%)	**0.030**
	S	0	2 (4.08%)	1 (50%)	
Meropenem	NS	49 (100%)	48 (97.95%)	2 (100%)	**0.027**
	S	0	1 (2.04%)	0	
Imipenem	NS	49 (100%)	48 (97.95%)	1 (50%)	**0.027**
	S	0	1 (2.04%)	1 (50%)	
Trimethoprim/Sulfamethoxazole	NS	49 (100%)	48 (97.95%)	2 (100%)	**0.027**
	S	0	1 (2.04%)	0	
Piperacillin	NS	49 (100%)	48 (97.95%)	1 (50%)	**0.027**
	S	0	1 (2.04%)	1 (50%)	
Piperacillin-tazobactam	NS	49 (100%)	47 (95.91%)	1 (50%)	**0.030**
	S	0	2 (4.08%)	1 (50%)	
Cefepime	NS	49 (100%)	48 (97.95%)	2 (100%)	**0.027**
	S	0	1 (2.04%)	0	
Ceftazidime	NS	46 (93.87%)	45 (91.83%)	2 (100%)	**0.048**
	S	3 (6.12%)	4 (8.16%)	0	
Ceftriaxone	NS	49 (100%)	49 (100%)	2 (100%)	**0.001**
	S	0	0	0	
Levofloxacin	NS	49 (100%)	47 (95.91%)	2 (100%)	**0.030**
	S	0	2 (4.08%)	0	
Ciprofloxacin	NS	49 (100%)	49 (100%)	2 (100%)	**0.001**
	S	-	-	-	
Gentamicin	NS	47 (95.91%)	46 (93.87%)	1 (50%)	**0.037**
	S	2 (4.08%)	3 (6.12%)	1 (50%)	
Tobramycin	NS	48 (97.95%)	47 (95.91%)	1 (50%)	**0.033**
	S	1 (2.04%)	2 (4.08%)	1 (50%)	

Abbreviations: NS, non-susceptible; S, susceptible; N, number

capacity of the *A. baumannii* clinical isolates in this study (*P*-value < 0.001).

Relationship between *bap* gene expression and antibiotic resistance pattern of the isolates

The highest resistance rate against tested antibiotics in the present study was in groups with an 11–15 and 16-20-fold increased level of the *bap* gene. All isolates in these two groups were resistant to all antibiotics. However, there was a significant statistical relationship between the overexpression of the *bap* gene and resistance against antibiotics (Table 3).

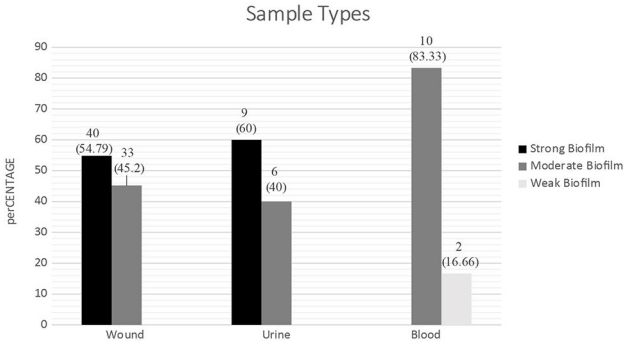

Fig. 4 Number (%) of biofilm-producing *A. baumannii* isolates among different samples

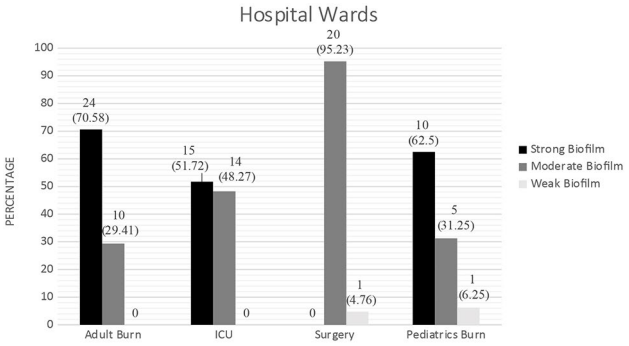

Fig. 5 Number (%) of biofilm-producing *A. baumannii* isolates among different hospital wards

Discussion

Acinetobacter baumannii is an opportunistic pathogen colonizing various anatomical sites, including the skin, oral cavities, respiratory system, conjunctiva, and urinary and gastrointestinal tracts [27]. Due to the long-time persistency in the environment, the transmission of nosocomial infections typically occurs through direct healthcare or indirect environmental contacts [28]. Biofilm formation causing the persistency of this pathogen in different infections is related to multiple factors, among which the Bap protein plays a significant role [29]. The present study aimed to assess the biofilm formation ability of *A. baumannii* isolated from wounds, bloodstream, and urinary tract infections. Additionally, this study aimed to explore the significance of the *bap* gene in the emergence of multidrug-resistant and biofilm-producing clinical *A. baumannii* isolates

Overall, 99% of the *A. baumannii* isolates in the present study were MDR, and all were detected as biofilm-producers, indicating that most MDR isolates are biofilm-producers, as previously reported [30, 31]. The high prevalence of antibiotic-resistant and biofilm-producer *A. baumannii* was reported by other studies, indicating the

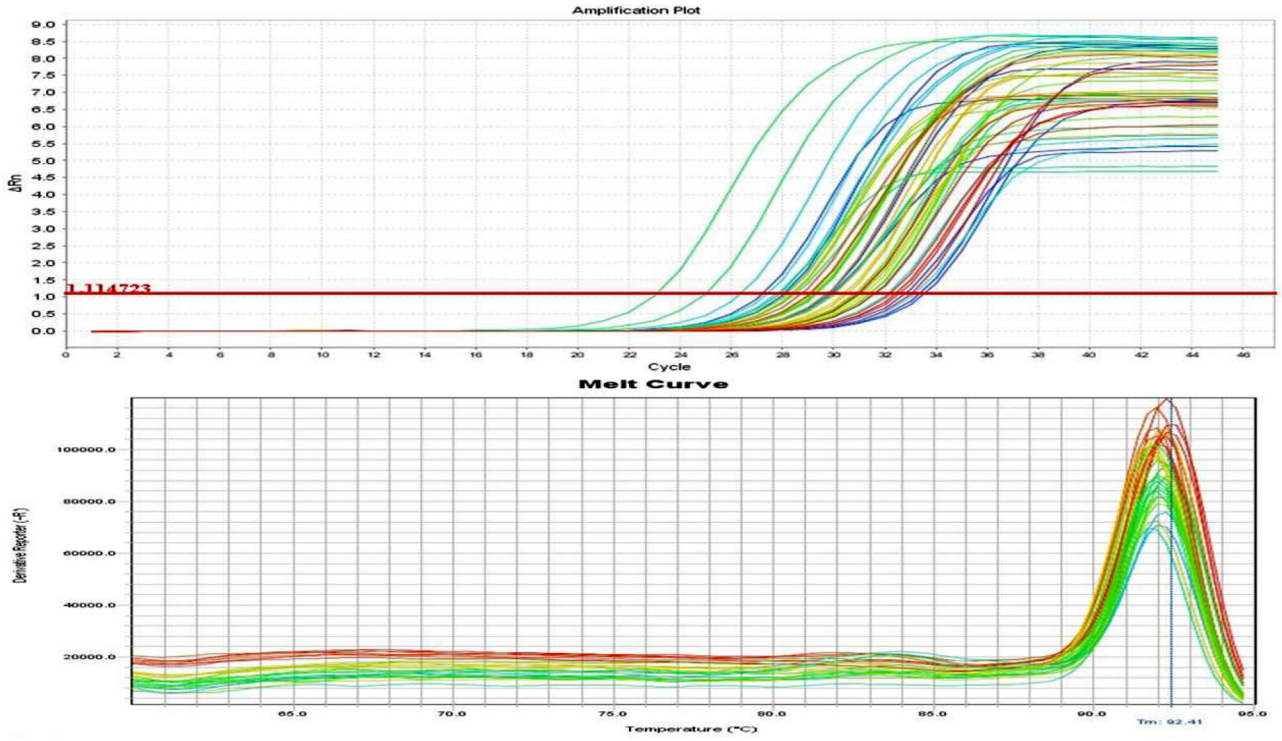

Fig. 6 Amplification plot and melting curve related to *bap* gene in clinical isolates of *Acinetobacter baumannii* in real-time PCR test. This curve is based on ΔRn and the number of cycles

significance of this pathogen in clinical settings [8, 12, 32, 33]. However, the lower prevalence of biofilm-producer *A. baumannii* in some studies may be due to the different sources, time of study, and different rates of antibiotic consumption [30, 34, 35]. Biofilm production is a significant strategy by *A. baumannii* to overcome the antibiotics [8, 12, 30]. The complex mixture of biofilms enclosed by polysaccharides makes them resistant to different classes of antibiotics [32]. Biofilms can cause the bacteria bind the biotic and abiotic surfaces [32]. Also, the capacity of the *A. baumannii* clinical isolates to produce biofilm could be related to the pathogenicity of the isolates [36]. Kaihang et al. reported that 13.3%, 66.7%, and 20% of isolates collected from survived patients were weak, medium, and strong biofilm-producing, while these rates in dead persons were 6.7%, 83.3%, and 10%, respectively [36]. However, we found that all strong biofilm-producer *A. baumannii* were non-susceptible to tested antibiotics, except gentamicin, tobramycin, and ceftazidime. Also, we reported that 91% to almost 98% of the moderate biofilm-producing isolates were non-susceptible to antibiotics. These data indicate the relationship between antibiotic resistance and the power of biofilm formation, which may be due to the imperfect diffusion of antibiotics in biofilm, neutralization of the antibiotics, various functions, and slow growth rate of the bacteria in the depth of biofilm [8]. Inappropriate antibiotic stewardship, easy access, and arbitrary use of antibiotics are the possible reasons for high-level antibiotic resistance and biofilm production in *A. baumannii* clinical isolates in the present study. However, bacterial exposure to certain antibiotics can cause increased biofilm formation ability [8]. On the other hand, the selective pressure due to indiscriminate use of broad-spectrum antibiotics causes a high-level resistance in some specific hospital wards [30]. However, 50% and 29% of our isolates were collected from the burn wards and ICUs, respectively

The frequency of the *bap* gene has been investigated in several studies using the PCR method. In research by Saadati et al., 2021, 89% of *A. baumannii* isolates were *bap*-positive [37]. They reported an 84% prevalence of biofilm-producer *A. baumannii*, while 3.57% of these isolates exhibited a strong capacity for biofilm formation. These discrepancies with our study may be due to the source of the isolates, while 73% of our isolates were collected from wound infections [32]. Another research conducted by Rouhi et al. in 2020 reported a 53.42% prevalence of the *bap* gene, while 6.84%, 34.24%, and 10.95% of the isolates were weak, moderate, and strong-biofilm-producers, respectively [38]. This discrepancy may be due to the different clinical samples and antibiotic stewardship. However, the antibiotic resistance rates were significantly different between the two studies. Also, Mahmoudi et al. in 2019 detected that 70.3% of their *A. baumannii* isolates contained the *bap* gene, while 28.0%, 31.4%, and 13.6% of the isolates exhibited a weak,

Table 3 Association between *bap* gene expression and antibiotic resistance pattern of the isolates

Increased fold change of bap gene expression	No. (%) of the isolates non-susceptible to antibiotics											
	Doripenem (NS = 97)	Meropenem (NS = 99)	Imipenem (NS = 98)	Trime-thoprim- sul-famethoxa-zole (NS = 99)	Piperacillin (NS = 98)	Piperacillin-tazobactam (NS = 97)	Cefepime (NS = 99)	Ceftazi-dime (NS = 93)	Ceftri-axone (NS = 100)	Levofloxa-cin (NS = 98)	Cipro-floxacin (NS = 100)	Gentamicin (NS = 94)	Tobra-mycin (NS = 96)
No change	5 (5.15%)	7 (7.07%)	5 (5.10%)	7 (7.07%)	6 (6.12%)	5 (5.15%)	7 (7.07%)	5 (5.37%)	7 (7%)	7 (7.14%)	7 (7%)	5 (5.31%)	5 (5.20%)
2–5	45 (46.39%)	45 (45.45%)	46 (46.93%)	45 (45.45%)	45 (45.91%)	45 (46.39%)	45 (45.45%)	44 (47.31%)	46 (46%)	44 (44.89%)	46 (46%)	44 (46.80%)	45 (46.87%)
6–10	15 (15.46%)	15 (15.15%)	15 (15.30%)	15 (15.15%)	15 (15.30%)	15 (15.46%)	15 (15.15%)	12 (12.90)	15 (15%)	15 (15.30%)	15 (15%)	13 (13.82%)	14 (14.58%)
11–15	15 (15.46%)	15 (15.15%)	15 (15.30%)	15 (15.15%)	15 (15.30%)	15 (15.46%)	15 (15.15%)	15 (16.12%)	15 (15%)	15 (15.30%)	15 (15%)	15 (15.95%)	15 (15.62)
16–20	17 (17.52%)	17 (17.17%)	17 (17.34%)	17 (17.17%)	17 (17.34%)	17 (17.52%)	17 (17.17%)	17 (18.27%)	17 (17%)	17 (17.34%)	17 (17%)	17 (18.08%)	17 (17.70%)
P value	0.000	0.001	0.000	0.001	0.000	0.000	0.001	0.000	0.001	0.001	0.001	0.000	0.000

Abbreaviations: NS: non-susceptible

moderate, and strong ability to produce biofilm, respectively [39]. Other Iranian studies observed a 97.67% and 98% prevalence of the *bap* gene among *A. baumannii* clinical isolates [29, 40]. Also, Khoshnood et al. reported a 91.4% frequency of the *bap* gene in *A. baumannii* isolates, while all strong and moderate biofilm producers contained this gene [41]. This result was concordant with our study that 98% of the isolates were strong and moderate biofilm-producers, and the *bap* gene overexpression was detected in 99% of the isolates. There was a direct correlation between the intensity of biofilm production and *bap* gene expression in the present study. We found that the strong-biofilm-producer isolates had the highest levels of *bap* gene expression. The Bap protein is associated with a strong biofilm formation in *A. baumannii* [9]. However, there are several genes related to the biofilm production in *A. baumannii* other than the *bap* gene [6]

On the other hand, the majority of the wound and urine isolates were strong biofilm producers, and none of the *A. baumannii* isolates from bloodstream infections show this phenotype, as shown in Fig. 4. However, Vijayakumar et al. reported that their blood isolates produced weak biofilm compared to the sputum isolates that were strong biofilm-formers [42]. This result shows the importance of biofilm production for the adherence of the bacteria to burned skin and urinary tracts. Probably the blood flow can prevent bacterial attachment to the surfaces and formation of the biofilms. Contrary to our results, Vijayakumar et al. did not find a correlation between the biofilm formation capacity and the MDR phenotype [42]. However, Shakib et al. detected a significant difference between the expression of the *bap* gene and the type of clinical samples [29]. They found that the *A. baumannii* isolated from the sputum and wound samples have the highest expression level of the *bap* gene ($P < 0.0001$) [29]

Our findings suggest that the *bap* gene may be a potential target and new strategy to prevent and treat the infections caused by the MDR and biofilm-producing *A. baumannii*, as this relationship has been demonstrated by other studies [12, 29]. Also, Goh et al. showed that 23 (95.83%) carbapenem-resistant ST92 clinical isolates of *A. baumannii* collected from hospital outbreaks in Australia overexpressed the *bap* gene [43]. They reported a significant correlation between the expression of the *bap* gene and the strong biofilm formation by *A. baumannii*. Nevertheless, it is imperative to acknowledge that substantial knowledge is yet to be acquired regarding the complex relationships between bacterial genes and environmental factors in these mechanisms. One limitation of our study is that we focused solely on the expression of the *bap* gene and did not investigate other potential factors associated with antibiotic resistance and biofilm formation. However, our findings suggest that the *bap* gene may serve as a diagnostic marker or therapeutic target for

multidrug-resistant and biofilm-producing *A. baumannii* infections

Conclusion

The investigation of *bap* gene expression in multidrug-resistant and biofilm-producing clinical *Acinetobacter baumannii* has yielded important insights into the mechanisms underlying antibiotic resistance and biofilm formation in this pathogen. Although several virulence factors are related to biofilm production and the emergence of antibiotic resistance, our results indicate that an increased *bap* gene expression is linked to multidrug resistance and biofilm formation in *A. baumannii* isolates. However, the *bap* gene may be a considerable target to develop a novel anti-*A. baumannii* treatment option, as suggested by our findings. Furthermore, this research emphasizes the significance of continual surveillance of *A. baumannii* strains in medical facilities, given their propensity to rapidly acquire resistance to numerous antibiotics, making them progressively challenging to manage. In summary, this study improves our understanding of the pathogenic nature of *A. baumannii*, resulting in a basis for future investigations focused on devising innovative therapeutic approaches to counteract this significant pathogen associated with health care

Limitations

Considering the multifactoriality of biofilm production in *Acinetobacter baumannii*, one limitation of this study was that we did not investigate other virulence factors related to biofilm production. Also, this study could have been performed in hospitals with different specialties to compare with the burn center

Abbreviations

MDR	Multidrug Resistant
WHO	World Health Organization
bap	Biofilm-associated Protein
PCR	Polymerase Chain Reaction
OD	Optical Density
DNA	Deoxy Ribonucleic Acid
CLSI	Clinical and Laboratory Standards Institute
ATCC	American Type Culture Collection
TSB	Trypticase Soy Broth
PBS	Phosphate Buffer Saline
ELISA	Enzyme-linked Immunosorbent Assay
ODc	Optical Density Cut-off
DEPC	Diethyl Pyrocarbonate
cDNA	Complementary DNAs
RNA	Ribonucleic Acid
qRT-PCR	Quantitative Real-Time–PCR
NTC	No-template Control
SPSS	Statistical Package for the Social Sciences

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Author contributions

MK contributed to the acquisition of data and drafting of the manuscript. MG contributed to the analysis, interpretation of data, and revision of the article. MA contributed to the acquisition of data, and revision of the article. MAE contributed to the analysis, interpretation of data, and revision of the article. MS contributed to the analysis, interpretation of data, and revision of the article. FR contributed to the analysis, interpretation of data, and revision of the article. HRG contributed to the study concept and design, acquisition of data, analysis, and interpretation of data, review, and approval of the final article.

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Data availability

All data created or considered during this study are involved in published article.

Declarations

Ethics approval and consent to participate

This study was approved by the Iran National Committee for Ethics in Biomedical Research, with the national ethical code IR.MAZUMS.REC.1401.283. This study was directed according to the Declaration of Helsinki. However, a written informed consent form was provided by the patients or close relatives. Also, the classifying data of patients was kept secret.

Consent for publication

Not Applicable.

Competing interests

The authors declare no competing interests.

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References

1. Algammal A, Hetta HF, Mabrok M, Behzadi P. Emerging multidrug-resistant bacterial pathogens superbugs: a rising public health threat. Front Microbiol. 2023;14:1135614.
2. Sarshar M, Behzadi P, Scribano D, Palamara AT, Ambrosi C. *Acinetobacter baumannii*: an ancient commensal with weapons of a pathogen. Pathogens. 2021;10(4):387.
3. Nocera FP, Attili A-R, De Martino L. *Acinetobacter baumannii*: Its Clinical Significance in Human and Veterinary Medicine. Pathogens. 2021;10(2):127.
4. Lin MF, Lan CY. Antimicrobial resistance in *Acinetobacter baumannii*: from bench to bedside. World J Clin Cases. 2014;2(12):787–814.
5. Amin M, Navidifar T, Shooshtari FS, Rashno M, Savari M, Jahangirmehr F, Arshadi M. Association between biofilm formation, structure, and the expression levels of genes related to biofilm formation and biofilm-specific resistance of *Acinetobacter baumannii* strains isolated from burn infection in Ahvaz, Iran. Infect Drug Resist 2019:3867–81.
6. Eze EC, Chenia HY, El Zowalaty ME. *Acinetobacter baumannii* biofilms: effects of physicochemical factors, virulence, antibiotic resistance determinants, gene regulation, and future antimicrobial treatments. Infect drug Resist. 2018;11:2277.
7. Gedefie A, Demsis W, Ashagrie M, Kassa Y, Tesfaye M, Tilahun M, Bisetegn H, Sahle Z. *Acinetobacter baumannii* biofilm formation and its role in disease pathogenesis: a review. Infect Drug Resist 2021:3711–9.
8. Yang C-H, Su P-W, Moi S-H, Chuang L-Y. Biofilm formation in *Acinetobacter Baumannii*: genotype-phenotype correlation. Molecules. 2019;24(10):1849.
9. Roy S, Chowdhury G, Mukhopadhyay AK, Dutta S, Basu S. Convergence of biofilm formation and antibiotic resistance in *Acinetobacter baumannii* infection. Front Med 2022, 9.
10. De Gregorio E, Del Franco M, Martinucci M, Roscetto E, Zarrilli R, Di Nocera PP. Biofilm-associated proteins: news from Acinetobacter. BMC Genomics. 2015;16(1):1–14.

11. Skariyachan S, Taskeen N, Ganta M, Venkata Krishna B. Recent perspectives on the virulent factors and treatment options for multidrug-resistant *Acinetobacter baumannii*. Crit Rev Microbiol. 2019;45(3):315–33.
12. Azizi O, Shahcheraghi F, Salimizand H, Modarresi F, Shakibaie MR, Mansouri S, Ramazanzadeh R, Badmasti F, Nikbin V. Molecular analysis and expression of bap gene in biofilm-forming multi-drug-resistant *Acinetobacter baumannii*. Rep Biochem Mol Biology. 2016;5(1):62.
13. Thummeepak R, Kongthai P, Leungtongkam U, Sitthisak S. Distribution of virulence genes involved in biofilm formation in multi-drug resistant *Acinetobacter baumannii* clinical isolates. Int Microbiol. 2016;19(2):121–9.
14. Bardbari AM, Arabestani MR, Karami M, Keramat F, Alikhani MY, Bagheri KP. Correlation between ability of biofilm formation with their responsible genes and MDR patterns in clinical and environmental *Acinetobacter baumannii* isolates. Microb Pathog. 2017;108:122–8.
15. 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 2021:709–17.
16. Nahar A, Anwar S, Miah MRA. Association of biofilm formation with antimicrobial resistance among the *Acinetobacter* species in a tertiary care hospital in Bangladesh. J Med. 2013;14(1):28–32.
17. Emami S, Eftekhar F. The correlation between biofilm formation and drug resistance in nosocomial isolates of *Acinetobacter baumannii*. *Avicenna Journal of Clinical Microbiology and Infection* 2015, 2(2).
18. 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(Pt5):559–64.
19. Raut S, Rijal KR, Khatiwada S, Karna S, Khanal R, Adhikari J, Adhikari B. Trend and characteristics of *Acinetobacter baumannii* infections in patients attending Universal College of Medical Sciences, Bhairahawa, Western Nepal: a longitudinal study of 2018. Infect Drug Resist 2020:1631–41.
20. Šipošová NŠ, Liptáková V, Kvasnová S, Kosorínová P, Pristaš P. Genetic diversity of *Acinetobacter Spp.* adapted to heavy metal polluted environments. Nova biotechnologica et chimica; 2017.
21. Turton JF, Woodford N, Glover J, Yarde S, Kaufmann ME, Pitt TL. Identification of *Acinetobacter baumannii* by detection of the Bla OXA-51-like carbapenemase gene intrinsic to this species. J Clin Microbiol. 2006;44(8):2974–6.
22. Wayne P. Clinical and laboratory standards institute: performance standards for antimicrobial susceptibility testing: twenty-fourth informational supplement, M100-S30. Clin Lab Stand Inst 2020, 34(1).
23. Zhuo J, Liang B, Zhang H, Chi Y, Cai Y. An overview of gram-negative bacteria with difficult-to-treat resistance: definition, prevalence, and treatment options. Expert Rev Anti-infective Therapy. 2023;21(11):1203–12.
24. Sherif MM, Elkhatib WF, Khalaf WS, Elleboudy NS, Abdelaziz NA. Multidrug Resistant *Acinetobacter baumannii* Biofilms: evaluation of phenotypic–Genotypic Association and susceptibility to cinnamic and gallic acids. Front Microbiol. 2021;12:716627.
25. Sepahvand S, Davarpanah MA, Roudgari A, Bahador A, Karbasizade V, Kargar Jahromi Z. Molecular evaluation of colistin-resistant gene expression changes in *Acinetobacter baumannii* with real-time polymerase chain reaction. Infect Drug Resist 2017:455–62.
26. Livak KJ. TD Schmittgen 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2– ΔΔCT method. Methods 25 4 402–8.
27. Jouybari MA, Ahanjan M, Mirzaei B, Goli HR. Role of aminoglycoside-modifying enzymes and 16S rRNA methylase (ArmA) in resistance of *Acinetobacter baumannii* clinical isolates against aminoglycosides. Rev Soc Bras Med Trop 2021, 54.
28. 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. Archives Clin Infect Dis 2019, 14(2).
29. Shakib P, Halimi S, Rezaei F, Delfani S. Expression of bap Gene in Clinical *Acinetobacter baumannii* Isolates in Khorramabad, Iran. *Gene, Cell and Tissue* 2022(In Press).
30. Ramakrishnan M, Bai SP, Babu M. Study on biofilm formation in burn wound infection in a pediatric hospital in Chennai, India. Annals Burns fire Disasters. 2016;29(4):276.
31. Sanchez CJ, Mende K, Beckius ML, Akers KS, Romano DR, Wenke JC, Murray CK. Biofilm formation by clinical isolates and the implications in chronic infections. BMC Infect Dis. 2013;13(1):1–12.
32. Babapour E, Haddadi A, Mirnejad R, Angaji S-A, Amirmozafari N. Biofilm formation in clinical isolates of nosocomial *Acinetobacter baumannii* and its relationship with multidrug resistance. Asian Pac J Trop Biomed. 2016;6(6):528–33.
33. Dehbalaei MA, Najar-Peerayeh S, Taherikalani M, Behmanesh M. Clinical isolates of *Acinetobacter baumannii* from tehran hospitals: pulsed-field gel electrophoresis characterization, clonal lineages, antibiotic susceptibility, and biofilm-forming ability. Jundishapur J Microbiol 2017, 10(7).
34. Rao RS, Karthika RU, Singh S, Shashikala P, Kanungo R, Jayachandran S, Prashanth K. Correlation between biofilm production and multiple drug resistance in imipenem resistant clinical isolates of *Acinetobacter baumannii*. Ind J Med Microbiol. 2008;26(4):333–7.
35. Emami S, Eftekhar F. The correlation between biofilm formation and drug resistance in nosocomial isolates of *Acinetobacter baumannii*. Avicenna J Clin Microbiol Infect. 2015;2(2):23954–23954.
36. Yu K, Zeng W, Xu Y, Liao W, Xu W, Zhou T, Cao J, Chen L. Bloodstream infections caused by ST2 *Acinetobacter baumannii*: risk factors, antibiotic regimens, and virulence over 6 years period in China. Antimicrob Resist Infect Control. 2021;10:1–9.
37. Saadati M, Rahbarnia L, Farajnia S, Naghili B, Mohammadzadeh R. The prevalence of biofilm encoding genes in multidrug-resistant *Acinetobacter baumannii* isolates. Gene Rep. 2021;23:101094.
38. Rouhi V, Safarkar R, Habibi S. Phonotypic Investigation of Biofilm formation and determination of Presence of bap and blaOXA-51 genes in *Acinetobacter baumannii* from clinical specimens in Tehran. Iran J Med Microbiol. 2020;14(6):566–83.
39. Mahmoudi Monfared A, 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.
40. Mozafari H, Mirkalantari S, Sadeghi Kalani B, Amirmozafari N. Prevalence determination of virulence related and Biofilm formation genes in *Acinetobacter baumannii* isolates from clinical respiratory samples in Imam Khomeini Hospital, Tehran, Iran in 2018. Iran J Med Microbiol. 2021;15(3):266–80.
41. 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 2020:1547–58.
42. Vijayakumar S, Rajenderan S, Laishram S, Anandan S, Balaji V, Biswas I. Biofilm formation and motility depend on the nature of the *Acinetobacter baumannii* clinical isolates. Front Public Health. 2016;4:105.
43. Goh HS, Beatson SA, Totsika M, Moriel DG, Phan M-D, Szubert J, Runnegar N, Sidjabat HE, Paterson DL, Nimmo GR. Molecular analysis of the *Acinetobacter baumannii* biofilm-associated protein. Appl Environ Microbiol. 2013;79(21):6535–43.

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