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Prevalence, Antimicrobial Susceptibility, and Distribution of Virulence Genes Involved in Biofilm Formation in Multidrug-Resistant *Acinetobacter baumannii* Isolated from Shahrekord Medical Centers, Chaharmahal and Bakhtiari, Iran

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ABSTRACT

Background and Aim: The ability to form biofilms is an effective way for *Acinetobacter baumannii* to survive in stressful conditions. The aim of this study was to investigate the prevalence, antimicrobial susceptibility, and distribution of virulence genes involved in biofilm formation in multidrug-resistant *Acinetobacter baumannii* isolated from Shahrekord medical centers in Chaharmahal and Bakhtiari Province, Iran.

Materials and Methods: In this study, 150 samples from Shahrekord medical centers in Chaharmahal and Bakhtiari Province were isolated and identified using biochemical tests. Then, the antimicrobial susceptibility of *A. baumannii* isolates was determined using these antibiotics, Ampicillin/Sulbactam, Doxycycline, Ceftazidime, Ciprofloxacin, Erythromycin, Trimethoprim/ Sulfamethoxazole, Gentamicin, Colistin, Imipenem, and Amikacin. Finally, the rate of biofilm formation and the frequency of virulence genes associated with biofilm formation (*bap, ompA, csuA, csuE, epsA, bfmS, bfmR, pgaA, pgaD, and surA*) were evaluated.

Results: Out of 150 samples, 90 were identified as *A. baumannii*. The results of antimicrobial susceptibility testing showed that there was the highest resistance rate to Ciprofloxacin and Imipenem (100%), followed by Ceftazidime (90%) and Ampicillin/ Sulbactam (77.77%). The highest frequency of virulence genes associated with biofilm formation was related to *bap* (100%), *ompA* (100%), and *pgaA/ pgaD* (98%).

Conclusion: Biofilm formation significantly reduces susceptibility to antibiotic agents. Evaluation of biofilm formation showed that all isolates could produce biofilm; hence, they are very important for public health. Therefore, it is necessary to determine antibiotic susceptibility, biofilm formation capacity and the frequency of biofilm-related virulence genes in the clinical setting.

Keywords: *Acinetobacter baumannii*, Antibiotic resistance, Biofilm, Virulence genes

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1. Introduction

Antimicrobial or antibiotic resistance is an important worldwide phenomenon that has increased health costs. In recent years, mortality among patients infected with this bacterium has

increased significantly due to the longer hospitalization and treatment with complications **(1)**. Although new antimicrobial agents have been used against this microorganism in the past decades, resistance to these agents seems to be a growing problem worldwide. Numerous pieces of research in recent decades have shown that community-acquired and hospital-acquired resistance is growing along with an increasing number of older patients with primary or secondary immunodeficiency **(1, 2)**. *Acinetobacter baumannii* is a critical opportunistic pathogen responsible for a relatively high rate of healthcare-related infections. Infections caused by this bacterium are diverse and include acquired and ventilator-associated pneumonia, urinary tract infections, meningitis, bacteremia, gastrointestinal or skin infections, and ulcers **(3)**. *A. baumannii* strains usually show incredible antimicrobial resistance. Many isolated samples are often resistant to a wide range of clinically effective antibiotics. Accordingly, they are classified into two categories: multidrug-resistant strains (MDR) or extensively drug-resistant bacteria strains (XDR) **(4)**. Due to the vital effect of multidrug-resistant *A. baumannii* on public health, the World Health Organization has classified this organism as the priority pathogen among antibiotic-resistant microorganisms **(5)**. Recent reports have shown that the environment is the principal source of multidrug-resistant *A. baumannii* in health facility settings **(6)**. This in turn has contributed to the wide spread of this pathogen worldwide **(7)**.

A biofilm can be defined as a collection of microorganisms that are trapped in a matrix. This matrix acts as a protector and increases microorganisms' ability to resist environmental stressors and various antibiotics **(8)**. The ability to form biofilms plays a crucial role in the survival of this bacterium **(9)**. It also enables bacteria to transport easily in hospital environments and contaminate various biological and non-biological surfaces such as vascular catheters, urinary catheters, and cerebrospinal fluid shunts **(10)**.

Despite the increasing importance of *A. baumannii* as a multidrug-resistant bacterium in nosocomial infections, the function, and mechanism of virulence elements related to the pathogenesis of this bacterium in human infections stay in large part obscure. Recently, animal models of disorder and cellular infection have supplied valuable data on the pathogenesis mechanisms of this bacterium. Some factors associated with this bacterium seem like very essential in causing disease, including outer membrane purines, surface structures such as capsules and lipopolysaccharides, various enzymes, iron-acquisition systems, and regulatory proteins. These virulence factors are involved in the infection process, including transmission, attachment to host structures, cell damage, and invasion **(11)**. The relevance between biofilm formation, antibiotic resistance, and the presence of virulence factors have been of excellent interest to researchers. The nature of this correlation has been controversial and changing over the past two decades. Some studies have suggested that the resistance factors obtained by *A. baumannii* determine its capacity for biofilm formation. Further, MDR or XDR strains of *A. baumannii* have shown a better capability to form biofilms as compared to susceptible strains **(4, 12)**. The aim of this study was to investigate the prevalence, antimicrobial susceptibility, and distribution of virulence genes involved in biofilm formation in multidrug-resistant samples of *A. baumannii* isolated from Shahrekord medical centers in Chaharmahal and Bakhtiari Province.

2. Materials and Methods

Isolation and Identification of Strains

In this cross-sectional descriptive study, 150 samples were collected randomly from Shahrekord medical centers in Chaharmahal and Bakhtiari Province between 2020 and 2021. The samples included blood, urine, wounds, trachea, and respiratory samples. Based on microbiological and biochemical tests such as gram staining, oxidase, catalase, aerobic and anaerobic OF, TSI, indole, motility (SIM), Simon citrate, MR-VP, and lactose fermentation in McConkey agar (Merck, Germany) medium, *A. baumannii* isolates were identified and included in the study. Samples that did not have these characteristics were excluded.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility of *A. baumannii* isolates was tested using the standard disk diffusion method (Kirby-Bauer) according to CLSI 2019 standard using these antibiotics, Ampicillin / Sulbactam (10/10 µg), Doxycycline (30 µg), Ceftazidime (30 µg), Ciprofloxacin (5 µg), Erythromycin (15 µg), Trimethoprim / Sulfamethoxazole (1.25 / 23/75 µg), Gentamicin (10 µg), Amikacin (30 µg), Imipenem (10 µg), and Colistin (10 µg) (MAST, Merseyside, U.K). Multidrug resistance (MDR) was defined as resistance to at least one agent in three or more classes of antibiotics **(13)**.

Biofilm Formation Assay

The biofilm formation potential of *A. baumannii* isolates was determined by the microtiter plate method. In brief, *A. baumannii* isolates were grown overnight in tryptic soy broth (TSB) medium at 37°C. Free cells were eliminated, and the biofilm was washed three times with phosphate-buffered saline

(PBS) and fixed with methanol. Wells were stained with 1% (w/v) crystal violet for 20 min at room temperature. Crystal violet was dissolved in 33% ethanol/acetone solution (80, 20, v/v) for 20 minutes, and the absorbance was measured at 595 nm. Biofilm formation severity was defined in Table 1 **(14)**.

Table 1. Classification of bacteria based on the strength of biofilm formation

Absorbance	Biofilm score
A <1	non-biofilm production
1<A≤2	weak
2<A≤3	moderate
A≥3	strong

DNA Extraction

Genomic DNA extraction was done from an overnight culture of *Acinetobacter baumannii* isolates using a DNA extraction kit (Sinaclon, Iran) according to the kit instructions.

Detection of Virulence Genes Associated with Biofilm Formation

The presence of genes related to biofilm formation, including *bap, ompA, csuA, csuE, epsA, bfmS, bfmR, pgaA, pgaD,* and *surA* was assessed by PCR. The sequence of primers is given in Table 2. PCR was performed using Master mix 2x (SinaClon, Iran). Each PCR tube contained 25 µL of the reaction mixture, which includes 12.5 µL of Mastermix, 1 µL of each forward and reverse primers, 1 µL of DNA at a concentration of 200 ng/µL and nuclease-free water to complete the volume. PCR program included initial denaturation at 94°C for 5 minutes, then 30 cycles of denaturation (94°C, 1 min), annealing (annealing temperature was considered to be 59°C for all genes) for 1 minute, extension at 72°C for 1 min, and then final extension at 72°C for 10 min. PCR products were electrophoresed on 1% agarose gel containing ethidium bromide and were observed by gel documentation.

Table 2. Primers Sequences used in this study

Target Genes	Primers sequences (5' to 3')	amplicon Size (bp)	Reference
bap	F: GAGGGAACTTCTGCAAAACTTTC	108	(15)
	R: CAGACGTATGACTGCATTGGT		
ompA	F: GAGTCGTATTGCACTTGCTAC	594	(15)
	R: GCAGGCTTCAAGTGACCACC		
csuA	F: TGGTGAAGCTACCACAGGTT	322	(16)
	R: ACGACTACCATCATGGGCTG		
csuE	F: ACCAATGCTCAGACCGGAG	751	(15)
	R: CTTGTACCGTGACCGTATCTTG		
epsA	F: AAACATTACCAGCGATACAACC	602	(15)
	R: CTGGTTTTCTCGTGTGCTGAC		
bfmS	F: CATTAGTGAAGGAGTCGCTCG	990	(15)
	R: GGTGTACCCTGCTCTAGTTTT		
bfmR	F: GAAGTTGGTGTAGAAACCGATG	557	(15)
	R: GGATTTTCAGGATCATCGCC		
pgaA	F: ATTCAAAAGTCAGTTGATGGGC	460	(15)
	R: TTTTTTGTCCTTGCTCCAGC		
pgaD	F: CCCCTGCTCATCATAATGTAAG	353	(15)
	R: GGTTTTGTTTAATGTGGCTGC		
surA	F: GATGCGATTGCACCTGGAAC	822	(16)
	R: TTGACGTGCCATACGCTCTT		

Statistical Analysis

Statistical analysis was performed using SPSS software version 22 (SPSS Inc., Chicago, IL., USA), and the correlation between antibiotic resistance and frequency of virulence genes was investigated by T-test.

3. Results

Identification of *A. baumannii*

Microbiological and biochemical tests were performed to determine the definitive strains of *A. baumannii*. For this purpose, all 150 samples were cultured on MacConkey Agar and Blood Agar medium and incubated at 37°C for 24 hours. Gram staining was performed to confirm the presence of gram-negative coccobacilli using the microscopic method. Biochemical tests, including IMVIC, urease, TSI, OF, MR-VP, SIM, catalase, oxidase, and growth at 37 and 42°C were then performed. Finally, isolates that were lactose-negative, immobilized, oxidase-negative, catalase-positive, indole-negative, pigment-negative, urease-positive, citrate-positive, H2S-negative, MR, and VP-negative (90 samples) were confirmed as *A. baumannii*. After that, all samples were stored in peptone water containing 20% glycerol at -80°C.

Antimicrobial Susceptibility

The antimicrobial susceptibility pattern of the isolates is presented in Tables 3 and 4. In general, all isolates were resistant to one or more antimicrobial agents, with the highest resistance to Ciprofloxacin and Imipenem (100%), followed by Ceftazidime (90%) and Ampicillin/Sulbactam (77.77%). All isolates were resistant to at least one agent from three or more antimicrobial classes and were considered MDR. The most common patterns of MDR were resistance to Ampicillin/Sulbactam, Doxycycline, Ceftazidime, Ciprofloxacin, Erythromycin, Trimethoprim /Sulfamethoxazole, Gentamicin, Amikacin, Imipenem with a frequency of 30% (Table 3).

Table 3. Antibiotic resistance assessment of *A. baumannii* isolates

Antibiotic	Resistant N (%)	Intermediate N (%)	Susceptible N (%)
Ampicillin-Sulbactam	70 (77.77)	1 (1.11)	19 (21.11)
Doxycycline	55 (61.11)	7 (7.77)	28 (31.11)
Ceftazidime	81 (90)	0 (0)	9 (10)
Ciprofloxacin	90 (100)	0 (0)	0 (0)
Erythromycin	51 (56.67)	30 (33.33)	9 (10)
Trimethoprim-Sulfamethoxazole	53 (58.89)	24 (26.66)	13 (14.45)
Gentamicin	59 (65.55)	3 (3.33)	28 (31.12)
Amikacin	42 (46.66)	29 (32.23)	19 (21.11)
Imipenem	90 (100)	0 (0)	0 (0)
Colistin	0 (0)	0 (0)	90 (100)

Table 4. Antibiotic resistance patterns of *A. baumannii* isolates

No. of antimicrobial agents	Antibiotic resistance patterns	Percent (%) of all isolates
4	Ciprofloxacin, Gentamicin, Imipenem, Ampicillin-Sulbactam	10
5	Ceftazidime, Imipenem, Ciprofloxacin, Erythromycin, Gentamicin	18
7	Ceftazidime, Imipenem, Doxycycline, Ciprofloxacin, Gentamicin, Trimethoprim-Sulfamethoxazole, Amikacin	22
8	Ampicillin-Sulbactam, Ceftazidime, Ciprofloxacin, Erythromycin, Trimethoprim-Sulfamethoxazole, Gentamicin, Amikacin, Imipenem	20
9	Ampicillin-Sulbactam, Doxycycline, Ceftazidime, Ciprofloxacin, Erythromycin, Trimethoprim- Sulfamethoxazole, Gentamicin, Amikacin, Imipenem	30

Biofilm Formation Assay

All isolates of *A. baumannii* were capable of forming biofilms. A total of 12 isolates were weak, 28 isolates were moderate, and 50 isolates showed a solid ability to biofilm formation.

Distribution of Biofilm-related Genes

All identified samples of *A. baumannii* were tested for biofilm-forming virulence genes such as *bap, ompA, csuA, csuE, epsA, bfmS, bfmR, pgaA, pgaD* and *surA*. The results showed that the highest frequency of virulence genes associated with biofilm formation was related to *bap* (100%), *ompA* (100%), and *pgaA / pgaD* (98%). In this study, *epsA* gene was not observed in any isolates. The frequency of other genes studied in this study was *bfmR / bfmS* (96%), *surA* (94%), and *csuA / csuE* (90%).

4. Discussion

As an emerging pathogen, *A. baumannii* is responsible for causing a wide range of life-threatening infections **(17)**. Increased regulation of intrinsic resistance mechanisms, including overexpression of efflux pumps and the acquisition of external genetic determinants such as plasmids, are vital features for the survival of this bacterium in harsh environments such as hospitals **(18)**. The growing worldwide emergence of *A. baumannii* strains resistant to all antimicrobial agents highlights the ability of this microorganism for fast adaptation to selective environmental stressors. Multidrug-resistant *A. baumannii* (MDR) is one of the most difficult-to-treat antibiotic-resistant gram-negative bacilli **(19)**.

In our study, 100% of *A. baumannii* isolates were resistant to one or more antimicrobial agents. The highest resistance was observed against Ciprofloxacin and Imipenem (100%), and Ceftazidime (90%). Sadeghi *et al.* reported the highest antibiotic resistance to Ceftazidime, Aztreonam, and Ciprofloxacin during a study in Shahrekord, which is very similar to the present study **(20)**. Recently, it has been shown that resistance to Ampicillin / Sulbactam is growing among *A. baumannii* isolates **(21)**. In this study, there is a significant amount of Ampicillin / Sulbactam-resistant *A. baumannii* (77%), which poses another challenge in treating infections. The high incidence of antibiotic resistance is most likely due to improper use of antimicrobial agents. In addition, the loss and acquisition of resistance genes by mobile genetic elements (MGEs) is an important mechanism in the development of multidrug-resistant isolates. The study of antibiotic susceptibility of *A. baumannii* isolates shows high resistance of this bacterium to a wide range of antibiotics **(22, 23)**. Based on previous studies in Iran, the frequency of multidrug-resistant *A. baumannii* isolates was between 32.7% and 93% **(21)**. The outcomes of our study confirmed that 100% of *A. baumannii* isolates were MDR. The high incidence of MDR isolates has been reported in some research in Iran and different countries **(24-28)**. Iran seems to be a hotbed for the emergence of MDR isolates of *A. baumannii*, which raises significant concerns in the healthcare sector.

The ability of colonization and biofilm formation on living and non-living surfaces by *A. baumannii* is an important factor. According to our results, all isolates of *A. baumannii* were capable of producing biofilms, and 55% of the isolates had the robust capability to shape biofilms. Our results are constant with previous studies showing that more than 75% of *A. baumannii* isolates constitute biofilms **(29, 30)**. Numerous pieces of research have shown that biofilm-related genes such as *csuE*, *ompA*, *bap*, *epsA*, *bfmS,* and *pgaABCD* are responsible for biofilm formation and antibiotic resistance **(31)**. According to our results, the most common genes were *bap* and *ompA* (100%). The results of the frequency percentage of genes in the present study are very similar to the study of Mozafari *et al.,* in which the frequency of *bap* gene was reported to be 98% **(16)**. Fallah *et al.* reported that the frequency of this gene was 92% **(32)**. *ompA* is an outer membrane protein encoded by the *ompA* gene. This protein is important for binding to human epithelial cells, biofilm development, and antimicrobial resistance (29). The frequency of *ompA* was high in our study (100%). Bardbari *et al.* reported a 100% abundance of this gene in respiratory samples, which is very similar to the present study **(33)**.

The *pgaABCD* gene locus plays a major role in polysaccharide synthesis and biofilm formation. Poly-N-acetylglucosamine is a major component of biofilms in microbial-host interactions, virulence, immune evasion, and protection against antibiotics. The *pga* gene locus encodes proteins involved in the synthesis and transport of poly-N-acetylglucosamine to the bacterial surface **(34)**. In the present study, the frequency of *pgaA* and *pgaD* genes was 98%, which is very similar to the study of Al-Shamiri *et al.* **(15)**.

In this study, the frequency of *csuA* and *csuE* genes was 90%, which is very similar to Mozafari *et al.* **(16)**. The csu A/BABCDE family of proteins is involved in the assembly of pili and forming a biofilm on surfaces. The expression of these proteins is controlled by the *bfmR*/*bfmS* two-component regulatory system. This system is used to produce biofilm on plastic surfaces **(35)**. Previous studies have shown that inactivation of the *bfmR* gene causes loss of ability to pili production, bind, and biofilm formation on plastic surfaces **(36)**. In the present

study, the frequency of *bfmR* and *bfmS* genes was 96%, which is very similar to the study of Mozafari *et al.* **(16)**. The frequency of these genes has been reported to be excessive in clinical isolates. Thummeepak *et al.* reported a frequency of 84% for these genes **(29)**.

surA is a serum resistance factor in *Acinetobacter baumannii* **(8)**. In the present study, the frequency of *surA* gene was 94%, which is very similar to the study of Liu *et al.* and Mozafari *et al.* **(11, 16)**.

In a few research, the similarity of the results with the outcomes of this study turned very low, which can be because of the kind of samples, study time, and the type of antibiotic discs used. In this study, all isolates of *A. baumannii* forming strong biofilms carried the *bap* and *ompA* genes simultaneously. However, these biofilm-related genes were additionally recognized in a few isolates of *Acinetobacter baumannii,* forming a medium biofilm, which was predicted to form a strong biofilm because of the excessive abundance of associated genes.

5. Conclusion

Our study showed a high occurrence of multidrug-resistant *A. baumannii,* which forms biofilm with an excessive prevalence of biofilm-associated genes, which include *bap*, *ompA*, *pgaA,* and *pgaD*. According to these results, appropriate regulatory and control measures are needed to prevent the transmission of resistant strains.

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Conflict of Interest

The authors declared no conflict of interest.

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