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Research Article

Assessment of Antibiotic resistance pattern in clinical *Acinetobacter baumannii* carrying biofilm formation genes

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ABSTRACT

Acinetobacter baumannii is recognized as a predominant cause of hospital-acquired infections on a global scale. The persistent nature of these infections can be attributed to antimicrobial resistance and biofilm production. Therefore, the objective of this study was to investigate the antimicrobial susceptibility pattern, as well as the characteristics of *A. baumannii* biofilms. This study was conducted on 150 patients hospitalized in medical diagnostic and healthcare service centers. The Isolates were identified using standard laboratory methods. The antibiotic resistance pattern was determined by disk diffusion susceptibility test. The ability to biofilm formation was assessed using the microplate method. Detection of *bap*, *pbpG*, *csuA*, *plD and surA* genes in the isolates was carried out by polymerase chain reaction (PCR). Out of the total 150 clinical isolates, 40 isolates were identified as *A. baumannii*. 67.5% (27/40) of the isolates were multidrug-resistant patterns. There was a significant relationship between resistance and the phenotypic frequency of biofilm production ability. 4 isolates (10%), 13 isolates (32.5%) and 23 isolates (57.5%) showed weak, intermediate and strong biofilm formation respectively. The frequency of the *bap*, *pbpG, csuA*, *surA*, and *plD* genes among the isolates was 77.5%, 42.5%, 77.5%, 65%, and 100%, respectively. It is crucial to emphasize the importance of prudent antimicrobial utilization and maintaining rigorous infection prevention and control measures to prevent the escalation of antimicrobial resistance. Additionally, employing combination strategies that involve appropriate anti-pseudomonal antibiotics in conjunction with anti-biofilm agents can be a viable approach for effectively eradicating infections associated with biofilm formation.

1. Introduction

Acinetobacter is a genus of Gram-negative, oxidase-negative and non-motile bacteria and is an important hospital pathogen that is considered a major problem of medical science in the field of microbial resistance (Elham & Fawzia, 2019). The low nutritional requirements of this bacterium have led to its abundance in different parts of hospitals and the surrounding environment (Malta et al., 2020). This bacterium colonizes the healthy individuals without causing infection but becomes a pathogenic opportunistic organism in individuals with immune deficiencies and hospitalized patients, causing various infections related to several compilations (Roy et al., 2022). As a global problem, the increasing rate of multidrug-resistance (MDR) strains has resulted in the medical therapy against *A. baumannii* be complicated. The pathogenicity of this organism is associated with the production of virulence

factors such as adhesins, pili, flagella, proteases, endotoxins, and biofilms (Wong et al., 2017). Furthermore, the capacity of *A. baumannii* to form biofilm is considered a significant contributing factor in the development of chronic infections. Biofilms are intricate communities of microbial cells encased within an extracellular matrix consisting of proteins, extracellular DNA, and exopolysaccharides. This matrix provides a protective environment for bacteria, rendering them highly resilient and difficult to eliminate using conventional antimicrobial agents (Yang et al., 2019). As a result, the treatment of biofilm-associated infections poses considerable challenges and often incurs substantial costs. *A. baumannii* is renowned for its capacity to form biofilms. The biofilms confer advantages to bacteria, rendering them more resilient against antibiotics and the immune system. Within *Acinetobacter* species, numerous genes associated with biofilm formation have been identified. One pivotal gene is *bap*, responsible for encoding biofilm-associated protein, a large protein that plays a critical role in biofilm formation. This protein facilitates bacterial adhesion to surfaces and contributes to the structural integrity of the biofilm matrix. Another notable gene is *pbpG*, encoding penicillin-binding protein G, which participates in cell wall synthesis. In *Acinetobacter*, *pbpG* is associated with both biofilm formation and antibiotic resistance, believed to contribute to the stability and architecture of the biofilm structure (Islam et al., 2022). The *csuA* gene is part of the chaperone-usher pathway, which orchestrates the assembly and secretion of adhesive organelles called pili or fimbriae. *CsuA* specifically contributes to the development of *Csu* pili, which are indispensable for biofilm formation in *A. baumannii* (Mozafari et al., 2021). Additionally, the *pld* gene encodes phospholipase D, an enzyme that hydrolyzes phospholipids. This enzymatic activity aids in breaking down host cell membranes, facilitating the release of nutrients and promoting heightened biofilm development and persistence in *A. baumannii* biofilms (Stahl et al., 2015). Lastly, the *surA* gene encodes surfactant synthesis protein A, a protein involved in the synthesis of surfactants. These surface-active molecules enhance bacterial adhesion and facilitate biofilm formation. *SurA* plays a crucial role in the initial stages of biofilm development by facilitating cell attachment to surfaces (Birkle et al., 2022). The identification of biofilm-producing strains by *A. baumannii* is of great importance due to the negative effects that biofilms can have on various industries and human health. The most significant problem faced by healthcare systems and hospitals regarding this bacterium is the emergence of multidrug-resistant (MDR) strains of *A. baumannii*. Therefore, for addressing these issues, the identification of biofilm-producing strains by *A. baumannii* and the investigation of the mechanisms of biofilm formation and factors influencing their establishment and persistence are highly significant (Liu et al., 2023). Therefore, the main aims of this project were to isolate and identify *A. baumannii* strains from clinical samples of patients, determine the antibiotic resistance pattern of the isolated bacteria using antibiotic susceptibility testing, and investigate the presence and frequency of biofilm-associated genes (*bap, pbpG, csuA, plD* and *surA*).

2. Materials and Methods

2.1. Sample Collection and bacterial isolation

Bacterial isolates were obtained from clinical samples of patients, including urine, wound, and burn, between September and December 2023. The collection took place in three university-affiliated hospitals located in Tehran, Iran. To identify the *A. baumannii* isolates, standard microbiological and biochemical techniques were employed in the laboratory (Simo Tchuinte et al., 2019). These methods included oxidase and catalase tests, reactions on triple sugar iron (TSI) agar, sulfide, indole, motility (SIM) tests, methyl red (MR), Voges Proskauer (VP), citrate and oxidative-fermentative (OF) media (Merck, Germany).

2.2. Antibiotic Resistance Profile of A. baumannii isolates

The susceptibility of the bacterial isolates to various antibiotics was determined using the disk diffusion agar method on Mueller-Hinton agar (Merck, Germany), following the recommendations of the Clinical and Laboratory Standards Institute (Rai, *et al.,* 2023). After the inoculation of Mueller-Hinton agar with 0.5-McFarland microbial suspension, the antibiotic

disks (Padtan Teb. Co., Iran) tested included Cefepime (30 µg), Ceftriaxone (30 µg), Amikacin (30 µg), Imipenem (10 µg), Piperacillin/Tazobactam (30 µg), Meropenem (10 µg), Gentamicin (10 µg), Tetracycline (30 µg), Ceftazidime (30 µg), Aztreonam (30 µg), Ciprofloxacin (5 µg) and Levofloxacin (5 µg).Multidrug-resistant (MDR) *A. baumannii* was defined as an isolate that showed resistance to more than one antimicrobial agent in three or more antimicrobial categories. After 24 hours of incubation at 37°C, the inhibition zone around each disk was determined and recorded with the results being reported as sensitive (S), intermediate (I), or resistant (R) according to the CLSI guidelines.

2.3. Evaluation of Biofilm Production

Biofilm formation was quantitatively assessed using the colorimetric microtiter plate assay (Stepanović et al., 2007) with some modifications. An overnight culture of *A. baumannii* was adjusted to the turbidity of a 1 McFarland standard. The suspensions were then diluted 1:100 in 200 µL of nutrient broth (Merck, Germany) and transferred to sterile flat-bottomed 96-well polystyrene microplates (JET Biofil, Guangzhou, China). After 24 hours of incubation at 37 °C, the wells were gently washed three times with sterile phosphate-buffered saline (PBS, pH 7.1). Adherent biofilms were fixed by treating them with 99% methanol for 15 minutes. The methanol solution was then removed, and the plate was air-dried. The biofilms were stained with 100 µL of 0.1% crystal violet (Sigma Chemical Co., St. Louis, MO, USA) for 5 minutes at room temperature. After staining, the wells were rinsed with water and allowed to dry. Each well's biofilm was destained by treating it with 200 µL of 95% ethanol for 30 minutes. The optical density (OD) of the destained solution was measured at 570 nm using a microtiter plate reader (BioTek, Bad Friedrichshall, Germany). All experiments were performed in triplicate and repeated three times for accuracy. A cut-off value (ODc) was established, defined as three standard deviations (SD) above the mean OD of the negative control: ODC = average OD of negative control + (3 × SD of negative control). Based on the OD values, the isolates were classified into the following four categories: 1. OD ≤ OD control (ODc) = Negative, 2. ODc < OD ≤ 2ODc = Weak positive, 3. 2ODc < OD ≤ 4ODc = Positive, and 4. 4OD < ODc = Strong positive.

2.4. DNA extraction and PCR of biofilm genes

All *A. baumannii* isolates were evaluated for five biofilm-encoding genes, *bap, pbpG, csuA, plD* and *surA* by polymerase chain reaction (PCR) method. DNA extraction was performed from bacterial colonies by a commercial DNA extraction kit (Karmania Pars Gene, Iran) according to the manufacture`s instruction. Primer sequences to amplify biofilm genes have been shown in table 1. The PCR reaction for each gene was carried out in a volume of 20 µL, and the PCR temperature program for all genes was as follows: 95 ºC for 5 minutes, and 40 cycles for 95 ºC for 40 seconds, annealing at 58 ºC for 1 minute, and extension at 72 ºC for 2 minutes, and final extension at 72 ºC for 5 minutes. The amplified gene fragments using this technique were visualized using 1% agarose gel electrophoresis alongside a 50 bp ladder and examined for the presence of the target genes using DNA Green viewer for confirmation of the amplified fragments. A chi-squared test was conducted using SPSS software, version 18.0 (SPSS Inc., Chicago, IL, USA), to assess the association between categorical variables, specifically biofilm characteristics and antimicrobial resistance. A p-value of less than 0.05 was considered statistically significant in determining the presence of a significant relationship (Mozafari et al., 2021).

3. Results

3.1. Distribution of the isolates

A total of 40 distinct *A. baumannii* isolates were obtained from 150 clinical specimens. Analysis of *A. baumannii* distribution in clinical specimens indicated that the most isolates (n□=□11, 27.5%) were originated from urine and, followed by burn (n□=□24, 60%), wound (n□=□5, 12.5%). Based on the CLSI interpretive criteria, resistance rate among *A. baumannii* isolates to antibiotics tested was as follow: Cefepime 97.4% Ceftriaxone 96.5%, Amikacin 95.4%, Imipenem 98.5%, Piperacillin-Tazobactam 95.7%, Meropenem 96.4%, Gentamicin 49%, Tetracycline 89.6%, Ceftazidime 96.5%, Aztreonam 95.6%,

Ciprofloxacin 74.6% and Levofloxacin 64.6%. The prevalence of MDR *A. baumannii* in case of urine, burn and wound specimens was 81.81%, 66.66% and 40 % respectively (Table 2).

3.2. Results of biofilm formation assay

Biofilm phenotypes accounted for 40 isolates, being distributed in the following categories: 57.5% (n□=□23) produced strong biofilm; 32.5% (n□=□13) produced moderate biofilm; 10% (n□=□4) produced weak biofilm (Table 1). A high occurrence of biofilm-encoding genes was found (Table. 1): 17.5% (n□=□7) of the isolates presented all 5 *bap, pbpG, csuA, plD* and *surA* genes. In addition, the frequency of *the bap, pbpG, csuA, surA,* and *plD* genes among the strains was 77.5%, 42.5%, 77.5%, 65%, and 100%, respectively (Table 3).

Furthermore, there was a strong positive correlation between *plD* and both *bap* and *csuA* genes (rs = 0.745 and 0.604, respectively, FE test $P < 0.05$). Similarly, *bap* exhibited a positive correlation with *surA* (rs = 0.599, FE test = 0.00047). It is noteworthy that the simultaneous presence of *bap* or *csuA* and *plD* had an equivalent predictive value for strong biofilm formation (100%) as the simultaneous detection of the three genes *plD*, *bap*, and *csuA*.

Table 1. Genes and primer sequences (Mozafari et al., 2021)

Genes	Sequences (5'-3')	Length size (bp)
plD	GCTGTGGCTTTGACAGGTTG TAGCGCAAACGGTGTTGTTG	695
bap	ATAACTCGGCTGTTTACGG ACTGATGGTGTTGGAAGTG	223
pbpG	TGGATGCGCAAACAGGTGAG CTGCTAATAGTTCTGCGCATC	467
csuA	AGACATGAGTAGCTTTACG CTTCCCCATCGGTCATTC	322
surA	GATGCGATTGCACCTGGAAC TTGACGTGCCATACGCTCTT	822

Table 2. Results of Antibiotic resistance pattern

Antibiotics	Intermediate (%)	Sensitive (%)	Resistance (%)
Cefepime	٠	2.6	97.4
Ceftriaxone	٠	3.5	96.5
Amikacin	٠	4.6	95.4
Imipenem	٠	1.5	98.5
Piperacillin-tazobactam	٠	4.3	95.7
Meropenem	٠	3.6	96.4
Gentamicin	4.1	46.9	49
Tetracycline	7.8	2.6	89.6
Ceftazidime	٠	3.5	96.5
Aztreonam	٠	4.4	95.6
Ciprofloxacin	13.3	12.1	74.6
Levofloxacin	19.2	16.2	64.6

Table 3. Phenotypic and genotypic profile biofilm formation of A. baumannii isolates

Isolates No. / Genes	*bap*	*pbpG*	*csuA*	*surA*	*plD*	Biofilm formation
1	-	-	-	+	+	Weak
2	-	-	+	+	+	Moderate
3	+	-	-	+	+	Moderate
4	-	-	-	+	+	Moderate
5	+	+	+	+	+	Strong
6	+	-	+	+	+	Strong
7	+	-	+	+	+	Moderate
8	+	-	-	+	+	Moderate
9	+	-	-	+	+	Weak
10	+	+	+	+	+	Strong
11	-	-	+	+	+	Moderate
12	-	-	+	+	+	Moderate
13	+	+	+	+	+	Strong
14	-	-	+	-	+	Weak
15	+	+	-	-	+	Moderate
16	+	+	+	-	+	Strong
17	+	-	+	-	+	Strong
18	+	+	+	-	+	Strong
19	+	+	+	+	+	Strong
20	+	+	+	-	+	Strong
21	-	-	-	-	+	Weak
22	-	+	+	-	+	Strong
23	+	+	+	-	+	Strong
24	+	+	+	-	+	Strong
25	+	-	-	+	+	Moderate
26	+	+	-	-	+	Moderate
27	-	+	+	-	+	Moderate
28	+	-	+	-	+	Moderate
29	+	-	+	-	+	Moderate
30	+	-	+	+	+	Strong
31	+	-	+	+	+	Strong
32	+	-	+	+	+	Strong
33	+	+	+	+	+	Strong
34	+	-	+	+	+	Strong
35	+	-	+	+	+	Strong
36	+	+	+	+	+	Strong
37	+	-	+	+	+	Strong
38	+	-	+	+	+	Strong
39	+	+	+	+	+	Strong
40	+	+	+	+	+	Strong

The results demonstrated that 23, 13, and 4 isolates can make biofilm strongly, moderately, and weakly, respectively. The occurrence of the *bap, pbpG, csuA, surA,* and *plD* genes among the strains was 77.5%, 42.5%, 77.5%, 65%, and 100%, respectively.

4. Discussion

Infections caused by *A. baumannii* pose a serious concern due to high rates of multidrug resistance (Eze et al., 2018). This problem is exacerbated by its ability to form biofilms (Yang et al., 2019). It is estimated that antibiotic resistance in biofilms can be over 1000 times higher than planktonic cells, limiting the effectiveness of available antimicrobial treatments. In the present study, 67.5% (27/40) of *A. baumannii* isolates were MDR (multidrug-resistant) patterns, consistent with previous reports (Araujo Lima et al., 2020; Sherif et al.,

2021; Simo Tchuinte et al., 2019). Our findings indicated higher levels of resistance more than 90% resistance 9 antibiotics. This significant increase in resistance is concerning as these antibiotics are considered the first-line treatment for severe infections caused by resistant *A. baumannii*. In addition, the isolates demonstrated a much higher frequency (100%) of biofilm production. Several studies have identified a high frequency of biofilm formation in *A. baumannii*, which is associated with long-term survival and resistance to external stresses such as limited nutrients and water scarcity. The nature of the relationship between resistance profiles and biofilm formation in *A. baumannii* is somewhat controversial (Al-Shamiri et al., 2021). According to some studies, biofilm formation is strongly associated with MDR strains compared to susceptible strains (Bardbari et al., 2017). The present study investigated the potential association between phenotypic-genotypic resistance profiles and the ability to form biofilm in clinical strains of *A. baumannii*. Accordingly, our results highlight a significant association between MDR and biofilm formation. Relevant findings supporting this association have been reported by researchers from Egypt (Asaad et al., 2021), India (Badave & Kulkarni, 2015), Bangladesh (Nahar et al., 2013) and Iran (Ranjbar & Farahani, 2019). The increased synthesis of exopolysaccharides in *A. baumannii* biofilm formation likely creates a barrier to antibiotic penetration and, therefore, contributes to resistance. Slow growth rate, cellular physiological changes, and increased horizontal gene transfer within biofilm communities may provide additional explanations for the associated high resistance (Roy et al., 2022; Yang et al., 2019). Although not all resistance traits of our isolates were associated with stronger biofilm formation, there was a statistically significant correlation with specific antibiotic sensitivities. On one hand, biofilm formation was associated with resistance to most of antibiotics. This may be explained by the upregulation of inducible genes that provide a fitness advantage for such resistant strains in biofilm formation (Amin et al., 2019; Qi et al., 2016; Shenkutie et al., 2020). Additionally, based on the data from this study, a significant association between biofilm formation and resistance to the used antibiotics can be reported in agreement with other studies that reported overexpression of the efflux pumps, leading to high-level resistance to beta lactam and aminoglycoside antibiotics (He et al., 2015). Another aim of this study was to investigate the relationship between biofilm formation and the presence of biofilm-associated genes and antibiotic resistance in isolates. For this purpose, we found a significant association between biofilm formation and the presence of the *bap* or *csuA*, *Sura,* and *plD* genes. Some researches previously reported that *A. baumannii* strains carrying D OXA carbapenemase genes have strong biofilm-forming capabilities (Azizi et al., 2015). Regarding biofilm-associated genes, our results are consistent with the findings of another study where the most identified genes were *csuE* (90%), followed by bap (83.8%), *ompA* (76.7%), and blaPER-1 (46.7%) (Zeighami et al., 2019). In *A. baumannii* isolates, *bap, csuA,* and *plD* play a role in resistance to antimicrobial agents required for attachment to eukaryotic cells and contribute to biofilm formation to some extent (Yang et al., 2019). This study confirmed a significant association between the intensity of biofilm formation and the presence of the *bap* gene ($p=0.0125$). Importantly, we observed a strong positive correlation between *bap* and both *csuA* and *plD* genes, which are involved in the expression of biofilm-related proteins on bacterial surfaces, and *csuA*, which mediates adhesion and biofilm formation. Furthermore, a significant statistical relationship between these genes and the ability to form biofilm was observed. Actually, these gene pairs have a high diagnostic value for identifying strong biofilm-forming phenotypes. Interestingly, three weak biofilm-forming isolates also possessed some biofilm-associated genes, emphasizing the role of factors such as stress, nutrition, environment, and the pathogen itself in inducing and developing biofilms (Al-Shamiri et al., 2021). In total, the presence of *bap* and both other related genes *csuA* and *plD*, along with the biofilm-related genes *surA* and *bpbG* affect the intensity of the biofilms formed. Investigating the relationship between antibiotic resistance and biofilm formation may solve the complex dilemma of managing MDR pathogens, in general, and *A. baumannii*, in particular. Since *A. baumannii*, is known today as one of the opportunistic pathogenic bacteria in causing hospital infections, it is recommended to pay

special attention to the identification and awareness of the presence of this bacterium in hospitals. Determining sources of infection in hospitals using molecular methods. The importance of detecting and tracking antibiotic-resistant pathogens and reporting resistant *A. baumannii* species in medical centers and study on other effective factors in the pathogenicity of this bacterium, such as types of secretory systems that increase resistance or significantly change it are very important.

Conclusion

This study has provided clarity on the complex relationship between biofilm formation and resistance in clinical isolates of *A. baumannii*. The relationship between antibiotic resistance and biofilm formation is a complex and significant issue in clinical settings. Biofilm formation plays a crucial role in antibiotic resistance for several reasons. The biofilm matrix acts as a physical barrier that protects bacteria from the direct contact of antibiotics. Antibiotics may struggle to penetrate the biofilm structure effectively, leading to reduced efficacy. This barrier effect contributes to the persistence of bacteria within biofilms, allowing them to survive even in the presence of high antibiotic concentrations. In addition, bacteria within biofilms undergo physiological changes that can enhance antibiotic resistance. These changes include alterations in gene expression, upregulation of efflux pumps that actively pump out antibiotics, and the formation of persister cells. Furthermore, Biofilms facilitate horizontal gene transfer, allowing bacteria within the biofilm to exchange genetic material, including antibiotic resistance genes. This genetic exchange can contribute to the spread of resistance determinants among the bacterial population within the biofilm, leading to increased antibiotic resistance.

Conflict of interest

None

Refereces

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