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Biofilm Formation Ability of Carbapenem-Resistant *Acinetobacter baumannii* Isolates from Intensive Care Unit Patients and Genetic Factors Contributing to Biofilm Formation

Yoğun Bakım Ünitesi Hastalarından İzole Edilen Karbapenem-Dirençli *Acinetobacter baumannii* İzolatlarının Biyofilm Oluşturma Yeteneği ve Biyofilm Oluşumuna Katkıda Bulunan Genetik Faktörler

Nada Muhamed SAID(İD), Aylin ÜSKÜDAR GÜÇLÜ(İD)

Department of Medical Microbiology, Başkent University Faculty of Medicine, Ankara, Türkiye

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ABSTRACT

Introduction: *Acinetobacter baumannii is a significant pathogen commonly associated with nosocomial infections. Its ability to form biofilm enables A. baumannii to survive in hospital settings and makes it difficult to eradicate. This study aimed to determine the structural characteristics of biofilm formation in carbapenem-resistant A. baumannii isolates from intensive care units and to investigate the genetic factors contributing to biofilm development.*

Materials and Methods: *A total of 52 clinical A. baumannii isolates were included in the study. The antibiotic susceptibilities were evaluated, and the biofilm formation ability of these isolates was determined and visualized under scanning electron microscopy. Biofilm viability was measured with the MTT assay, and the presence of biofilm-related genes, including csuE, ompA, abaI, pgaA, bap, and* bla_{PER-1}*, was detected by polymerase chain reaction.*

Results: *Among 52 A. baumannii isolates, 96.1% formed biofilm at different levels. The biofilm-related bap and abaI genes were found in 98% of the isolates. The ompA and csuE genes were carried by 96.1% and 94.0% of isolates, respectively. The presence of pgaA and* bla_{PER-1} *was detected in 50.0% and 40.3% of isolates, respectively. MTT assay demonstrated variable levels of viability in biofilms formed by A. baumannii.*

Conclusion: *Understanding the presence of these factors and their interrelationships is essential for developing novel strategies to prevent and manage A. baumannii infections-particularly by targeting genes involved in biofilm formation.*

Key Words: *Acinetobacter baumannii; Biofilm; Intensive care units; Multidrug-resistant*

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ÖZ

Yoğun Bakım Ünitesi Hastalarından İzole Edilen Karbapenem-Dirençli *Acinetobacter baumannii* İzolatlarının Biyofilm Oluşturma Yeteneği ve Biyofilm Oluşumuna Katkıda Bulunan Genetik Faktörler

Nada Muhamed SAID, Aylin ÜSKÜDAR GÜÇLÜ

Başkent Üniversitesi Tıp Fakültesi, Tıbbi Mikrobiyoloji Anabilim Dalı, Ankara, Türkiye

Giriş: *Acinetobacter baumannii önemli bir patojen olup genellikle hastane infeksiyonlarıyla ilişkilidir. Biyofilm oluşturma yeteneği A. baumannii'nin hastane ortamlarında hayatta kalmasını sağlamakta ve eradikasyonunu zorlaştırmaktadır. Bu çalışmada, yoğun bakım ünitelerindeki hastalardan izole edilen karbapeneme-dirençli A. baumannii izolatlarının biyofilm oluşumunun yapısal özelliklerinin belirlenmesi ve biyofilm oluşumuna katkıda bulunan genetik faktörlerin tespit edilmesi amaçlanmıştır.*

Materyal ve Metod: *Çalışmaya toplam 52 klinik A. baumannii izolatı dahil edilmiştir. Bu izolatların antibiyotik duyarlılıkları değerlendirilmiş ve biyofilm oluşturma yetenekleri belirlenerek taramalı elektron mikroskobu ile görüntülenmiştir. Biyofilm canlılığı MTT testi ile ölçülmüş ve csuE, ompA, abaI, pgaA, bap ve bla_{PER-1} gibi biyofilmle ilişkili genlerin varlığı polimeraz zincir reaksiyonu yöntemiyle tespit edilmiştir.*

Bulgular: *Toplam 52 izolatın %96.1'i farklı seviyelerde biyofilm oluşturmuştur. Biyofilmle ilişkili bap ve abaI genleri izolatların %98'inde bulunmuştur. ompA ve csuE genlerinin sırasıyla izolatların %96.1'i ve %94.0'ı tarafından taşındığı görülmüştür. pgaA ve bla_{PER-1} sıklığı sırasıyla %50.0 ve %40.3'tür. MTT testi, A. baumannii izolatlarının oluşturduğu biyofilmin farklı seviyelerdeki canlılık düzeyini göstermiştir.*

Sonuç: *Bu faktörlerin varlığını ve aralarındaki ilişkiyi anlamak, örneğin, dikkati biyofilm oluşumunda rol oynayan genlere yönelterek, A. baumannii infeksiyonlarını önlemeye ve kontrol etmeye yönelik yeni yaklaşımlar geliştirmekte kritik önem taşımaktadır.*

Anahtar Kelimeler: *Acinetobacter baumannii; Biyofilm; Yoğun bakım ünitesi; Çok ilaca direnç*

INTRODUCTION

Acinetobacter baumannii is an ESKAPE pathogen (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) associated with hospital-acquired antibiotic-resistant infections. *A. baumannii* is responsible for almost 10% of all hospital-acquired infections caused by gram-negatives[1]. World Health Organization declared carbapenem-resistant *A. baumannii* (CRAB) as "critical" in this priority 1 list of bacterial pathogens that pose the greatest threat to human health. CRAB has emerged as a leading cause of healthcare-associated infections, such as pneumonia, bloodstream infections, and urinary tract infections, particularly in intensive care units (ICUs)[2]. The pathogen's ability to rapidly generate resistance factors, as well as to tolerate harsh environmental conditions, particularly by forming biofilm, makes it endemic in ICUs[1]. The ability of *A. baumannii* to colonize and produce biofilm on biotic and abiotic surfaces contributes to chronic and persistent infections, antibiotic resistance, as well as survival in hospital environments and transfer[1].

Biofilms are complex communities of bacteria that adhere to various surfaces, forming a tertiary structure in which bacterial cells contact with each other and are surrounded by a protective matrix of extracellular polymeric substances (EPSs)[3]. The ability of *A. baumannii* to form biofilms is associated with the presence of various biofilm-related factors including pili formation for the ability of adhesion to surfaces, Poly-b-(1–6)-N-acetylglucosamine (PNAG) for the integrity of the biofilm, quorum sensing (QS) system for the biosynthesis of the signal molecules, cell surface proteins for the maturation such as Bap involved in biofilm maturation and three-dimensional structure development, outer membrane proteins (*omp*A) for nutrient and other small molecule uptake and also PER-1 type beta-lactamase for conferring resistance to antibiotics[4-10]. These factors are encoded by specific biofilm-re-

lated genes, and the distribution of these genes among isolates with different biofilm-forming abilities provides insight into the potential predictors of strong biofilm formation. Understanding the biofilm-forming characteristics of this pathogen and the genetic factors contributing to biofilm formation is crucial for combating this opportunistic pathogen in ICUs, as biofilms play a significant role in the persistence and spread of infections. Consequently, conducting in-depth biofilm-related studies is imperative for public health to effectively manage and prevent the transmission of this pathogen in healthcare settings. The aim of this study was to comprehensively characterize the structural aspects of biofilm formation in CRAB isolates from ICU patients and to elucidate the underlying genetic determinants contributing to this phenotype.

MATERIALS and METHODS

This study was approved by Başkent University Institutional Review Board (project no: KA22/411) and supported by Başkent University Research Fund.

Isolates Profile

Clinical *A. baumannii* strains (n= 52) isolated from patients in ICUs of Ankara Hospital of Başkent University between 01.09.2019 and 30.12.2020 were included in this study. Only one isolate from each patient (non-repetitive) was included in the study. Isolate identification and antimicrobial susceptibility testing against imipenem, meropenem, amikacin, gentamicin, levofloxacin, ciprofloxacin, and trimethoprim/sulfamethoxazole were performed by the Phoenix automated identification and susceptibility testing system according to manufacturer instructions. Commercially available Sensititre broth microdilution method (Thermo Fisher, UK) was applied for colistin, and all antimicrobial susceptibility testing results were interpreted according to the breakpoint tables for interpretation of MICs and zone diameters of European Committee on Antimicrobial Susceptibility Testing criteria[9-11]. Isolate identification was confirmed by polymerase chain reaction through detection of the chromosomally encoded $bla_{\text{OXA-51}}$ gene, as described in a previous study[12].

Genetic relatedness among clinical carbapenem-resistant *A. baumannii* isolates was determined by PFGE, as described in a previous study[13]. Genomic DNA was lysed with lysis solutions and digested with the ApaI restriction enzyme (Takara, Japan). Electrophoresis was performed in a pulsed-field electrophoresis system (CHEF-DR III System; Bio-Rad, USA). Cluster analysis was performed using BioNumerics software. Isolates were defined as closely related if they differ by ≤3 bands and possibly related if they differ by 4–6 bands according to Tenover criteria[14].

Biofilm Formation Assay

Biofilm formation was assessed using the crystal violet method, as previously described, with slight modifications[15,16]. Each plate included the following controls: media alone, *A. baumannii* ATCC 19606 and *S. aureus* ATCC 6538 (positive control), *S. aureus* ATCC 29213 (negative control). The formula that was used to determine the optical density cut-off value (ODc) was as follows: (ODc) is calculated by taking the mean (OD) of the negative control and adding three times its standard deviation. According to the optical densities of the results, we were able to classify them as: a) strong-biofilm-producer ($4 \times ODc < OD$); b) medium-biofilm-producer ($2 \times ODc < OD \leq 4 \times ODc$); c) weak-biofilm-producer ($ODc < OD \leq 2 \times ODc$); and d) non-biofilm producer ($OD \leq ODc$).

MTT Assay

The MTT colorimetric method based on the reduction of a tetrazolium salt was used to determine the viability of microbial cells inhabiting the biofilm population, as in previous research[17]. Colony forming unit (CFU) counts were calculated based on the MTT values obtained from the biofilm viability assay, and these CFU values corresponded to the OD measurements of different isolates. A regression curve was generated using the OD values of these isolates. The percentage of viable cells within the biofilm was also calculated by using the OD values from MTT assays.

Detection of Biofilm-Related Genes

DNA isolation was performed by boiling bacterial suspensions at 95 °C for 10 min and

immediately cooling on ice. After centrifugation at 14.000 rpm for 10 min, DNA was recovered from the supernatant[18]. The *csu*E, *omp*A, *aba*I, *pga*A, *bap*, and bla_{PER-1} genes were screened using a set of primers giving the 751 base pair (bp), 594bp, 435bp, 460bp, 108bp, and 925bp product sizes, respectively (Table 1)[19-21].

SEM Analysis of Biofilm Formation Ability

The scanning electron microscope (SEM) was used to visualize the biofilm of *A. baumannii* isolates. Polystyrene squares measuring 1 × 1 cm were prepared as surfaces for biofilm formation. They were placed in 6-well cell culture plates, and 200 µL of *A. baumannii* (0.5 McFarland) suspensions were inoculated into 1.8 ml of TSB in each well, and then incubated at 37 °C. After 36 hour of incubation, the materials were washed twice with phosphate-buffered saline to remove any unattached and floating cells, then 2.5% glutaraldehyde in 0.1 M cacodylic acid (pH 7.2) was used for fixation at 4 °C for 24 hours. After that, 0.1 M cacodylic acid was added for about 10 min at room temperature, and then the plates were washed twice with distilled water for 15 min, followed by gradual dehydration with ethanol, and air-dried for a minimum of 24 hours. The fixed biofilms were then coated with a layer of gold-palladium (7 nm thick) and examined by SEM (QUANTA 400F Field Emission SEM)[22].

Statistical Analysis

Biofilm formation rates in the presence and absence of biofilm-related genes, as well as the distribution of biofilm formation level (non-biofilm forming, weak, moderate, and strong) in relation to the presence of specific genes (*csu*E, *omp*A, *aba*I, *pga*A, *bap*, and bla_{PER-1}), were statistically analyzed using Fisher's exact and Chi-square (χ^2) tests. These analyses were conducted to evaluate the association between the presence of a given gene and the ability to form biofilm, as well as the differences in biofilm formation levels according to gene presence. IBM SPSS Statistics version 25.0 was used for the statistical analysis, and $p< 0.05$ was considered statistically significant.

RESULTS

Isolate Profile

The study comprised 52 CRAB isolates of patients hospitalized in ICUs of Başkent University Ankara Hospital between July 2019 to December 2020. Among the isolates, 42.3%

Table 1. The primer sequences, amplicon sizes, and the amplification conditions

Genes	Primer sequence (5 →3)	Amplicon Size	Amplifications Conditions
*csu*E	F- 5′- ACC AAT GCT CAG ACC GGA G-3′ R- 5′-CTT GTA CCG TGA CCG TAT CTT G-3′	751 bp	95 °C 10 min, 95 °C 30 sec, 59 °C 30 sec, 72 °C 60 sec, 35 cycles, 72 °C 10 min[19]
*omp*A	F-5′- GAG TCG TAT TGC ACT TGC TAC-3′ R-5′- GCA GGC TTC AAG TGA CCA CC-3′	594 bp	94 °C 5 min, 94 °C 60 sec, 57 °C 60 sec, 72 °C 45 sec, 35 cycles, 72 °C 5 min[19]
*aba*I	F: 5′- AAA GTT ACC GCT ACA GGG-3′ R: 5′- CAC GAT GGG CAC GAA A-3′	435 bp	94 °C 5 min, 94 °C 60 sec, 52 °C 30 sec, 72 °C 1:30 sec, 30 cycles, 72 °C 5 min[21]
*pga*A	F- 5′- ATT CAA AAG TCA GTT GAT GGG C-3′ R- 5′- TTT TTT GTC CTT GCT CCA GC-3′	460 bp	95 °C 5 min, 95 °C 30 sec, 55 °C 30 sec, 72 °C 60 sec, 30 cycles, 72 °C 5 min[19]
bap	F-5′- GAG GGA ACT TCT GCA AAA CTT TC-3′ R- 5′- CAG ACG TAT GAC TGC ATT GGT-3′	108 bp	95 °C 5 min, 95 °C 30 sec, 58 °C 30 sec, 72 °C 60 sec, 30 cycles, 72 °C 5 min[19]
bla_{PER-1}	F- 5′- AAT TTG GGC TTA GGG CAG AA-3′ R- 5′- ATG AAT GTC ATT ATA AAA GC-3′	925 bp	94 °C 4 min, 94 °C 60 sec, 50 °C 60 sec, 72 °C 45 sec, 35 cycles, 72 °C 7 min[20]

Bp: Base pair.

(n= 22) of the strains were obtained from transtracheal aspirate and respiratory secretions, 26.9% (n= 14) from blood cultures, 11.5% (n= 6) from wounds and abscesses, 7.6% (n= 4) from urine cultures, 7.6% from drain cultures, and 3.8% from catheter cultures. Antimicrobial susceptibility testing showed that all isolates were resistant to gentamicin, imipenem, meropenem, levofloxacin, ciprofloxacin, tigecycline, trimethoprim/sulfamethoxazole, and amikacin. Colistin was the most effective antibiotic against the isolates, with an 80.77% (n= 42) susceptibility rate.

Based on PFGE analysis, 16 pulsogroups were identified, applying an 80% similarity cutoff. Within these pulsogroups, eight subgroups were detected where isolates shared greater than 85% similarity, indicating closer genetic relatedness within these subgroups (Supplementary Figure 1). Some pulsogroups contained multiple closely related isolates, while others were represented as unique.

Biofilm Formation Ability

Among 52 *A. baumannii* isolates, 96.1% formed biofilm. The biofilm formation at 24h, 36h, 48h, and 72h was also compared to establish the best incubation period for biofilm-related assays. Figure 1 shows the *A. baumannii* biofilm formation rate and biofilm profile over time. Thirty-six hours was the best incubation time for biofilm formation and preventing harmful chemicals from metabolic activities. Some samples developed stronger biofilm than *A. baumannii* ATCC19606, and they were classified as: strong 46.1% (n= 24), moderate 42.3% (n= 22), weak 7.6% (n= 4), non-biofilm 3.8% (n= 2). The majority of colistin-susceptible strains (n= 19; 44.2%) were weak biofilm formers, followed by non-biofilm formers with 34.9% (n= 15). A total of nine isolates were resistant to colistin, of which 55.5% (n= 5) were weak biofilm formers, followed by non-formers with 33.3% (n= 3). None of the colistin-resistant isolates were strong biofilm formers.

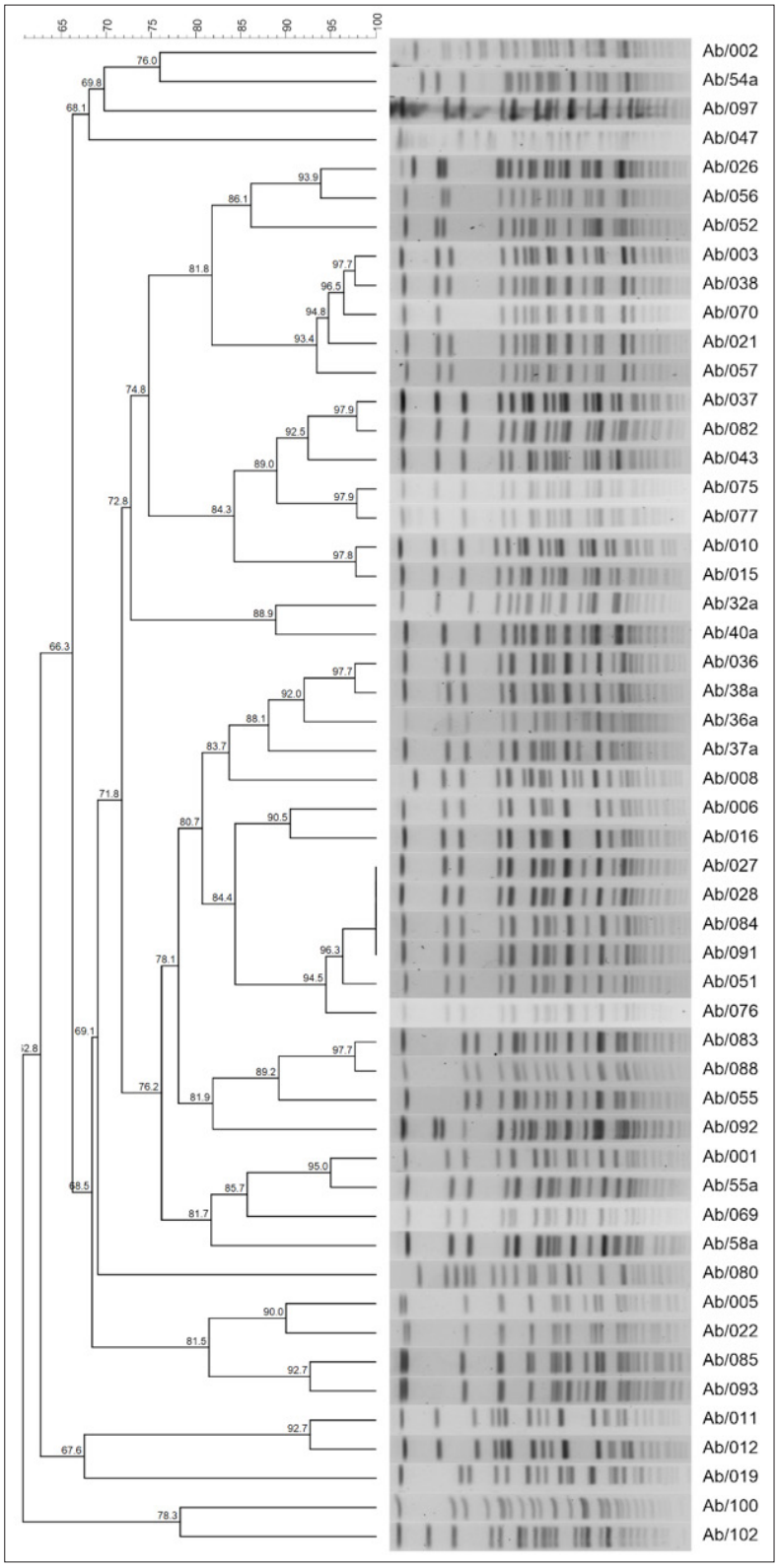
Biofilm Viability

CFU counts were calculated based on the MTT values obtained from the biofilm viability assay, and the corresponding CFU values for each OD measurement are presented in Table 2. Using these values, a regression curve was generated, and the resulting equation was found to be Y= 1.441X + 8.582, as shown in Table 2 (R^2= 0.9468). Using this formula, the OD450 nm values obtained from the MTT assay were used to calculate the CFU for each isolate. The percentage of viable cells within the biofilm was also calculated. The viable cell rates of biofilms formed by the isolates, along with their corresponding percentages, are presented in Table 3.

By using the Y= 1441X + 8582 formula, the OD values from MTT assays were used to calculate the viable cells within the biofilm for each isolate (Table 3). The viability percentages were between 13.5% to 112.4%. The results showed that both the number and percentage of viable cells within the biofilm were high. The number of CFUs ranged from $8.0x10^8$ to $5.2x10^{12}$.

The Biofilm-related Genes

To further explore the relationship between the ability to form biofilms and the presence of genes, six genes were revealed that are believed to be associated with biofilm formation and the ability of bacteria to resist. Electrophoresis revealed that the presence of the genes varied among the bacterial isolates, with the corresponding percentages presented in Table 4. The highest gene prevalence among the samples was observed for *bap*, *aba*I (98%), followed by *omp*A (96.1%) and c*su*E (94%). The genes *pga*A and bla_{PER-1} were detected in 50% and 40.3% of the isolates, respectively. The co-existence of multiple biofilm-associated genes was observed in the majority of the isolates. No statistically significant correlation was found between individual genes and biofilm strength ($p> 0.05$). There were no statistically significant differences between these genes in terms of their association with biofilm-forming ability ($p> 0.05$). Only samples that carried biofilm-associated genes and exhibited biofilm formation were included in Table 5, alongside a comparison with non-biofilm-forming isolates to highlight the differences.



Supplementary Figure 1. PFGE dendrogram for 52 CRAB isolates included in the study.

Table 2. CFU values for each OD value obtained from the MTT assay and colony counting technique

Isolates	MTT	CFU/mL	CFU/mL (log_{10})
MTT	0.14	0	0
1	0.55	8E+08	8.90309
2	0.69	7.79E+09	9.891537
3	0.81	1.04E+10	10.0187
4	1.17	1.7E+10	10.23045
5	2.00	3.9E+11	11.59106
6	2.15	4.21E+11	11.62428
7	2.55	7E+11	11.8451
8	2.871	1E+13	13

Table 3. Viable cell rates of biofilms formed by isolates

Biofilm Structures	MTT (OD)	CFU/mL	MTT (%)
Weak	1.23-2.5	5.707E+09 - 1.183E+12	45.1% - 97.2%
Moderate	0.47-2.73	1.791E+09 - 3.277E+12	13.5% - 106.6%
Strong	0.83-2.87	6.018E+09 - 5.237E+12	35.8% - 112.4%

Table 4. Prevalence of biofilm-related genes in isolates and corresponding biofilm formation patterns

	Biofilm Structure, n (%)			
Genes (n)	Non	Weak	Moderate	Strong
All isolate (52)	2 (3.8)	4 (7.6)	22 (42.3)	24 (46.1)
*csu*E (49)	2 (4.1)	4 (8.1)	21(42.8)	22 (44.8)
*omp*A (50)	2 (4.0)	4 (8.0)	21(42.0)	23 (46)
*aba*I (51)	2 (3.9)	4 (7.8)	22 (43.1)	23 (45.1)
*pga*A (26)	0 (0)	2 (7.6)	14 (53.8)	10 (38.4)
bap (51)	2 (3.9)	4 (7.8)	22 (43.1)	23 (45.1)
bla_{PER-1} (21)	2 (9.5)	1 (4.7)	7 (33.3)	11(52.3)

Table 5. Biofilm formation rates in the presence and absence of the biofilm-related genes

		Biofilm Formation		
Genes	PCR	Positive, n (%)	Negative, n (%)	p
*csu*E	Positive	47 (95.9)	2 (4.0)	1.000
	Negative	3 (100)	0 (0)	
*omp*A	Positive	48 (96)	2 (4)	1.000
	Negative	2 (100)	0(0)	
*aba*I	Positive	49 (96)	2 (3.9)	1.000
	Negative	1 (100)	0(0)	
*pga*A	Positive	26 (100)	0 (0)	0.490
	Negative	24 (92.3)	2 (7.6)	
bap	Positive	49 (96)	2 (3.9)	1.000
	Negative	1 (100)	0 (0)	
bla_{PER-1}	Positive	19 (90.4)	2 (9.5)	0.158
	Negative	31 (100)	0 (0)	

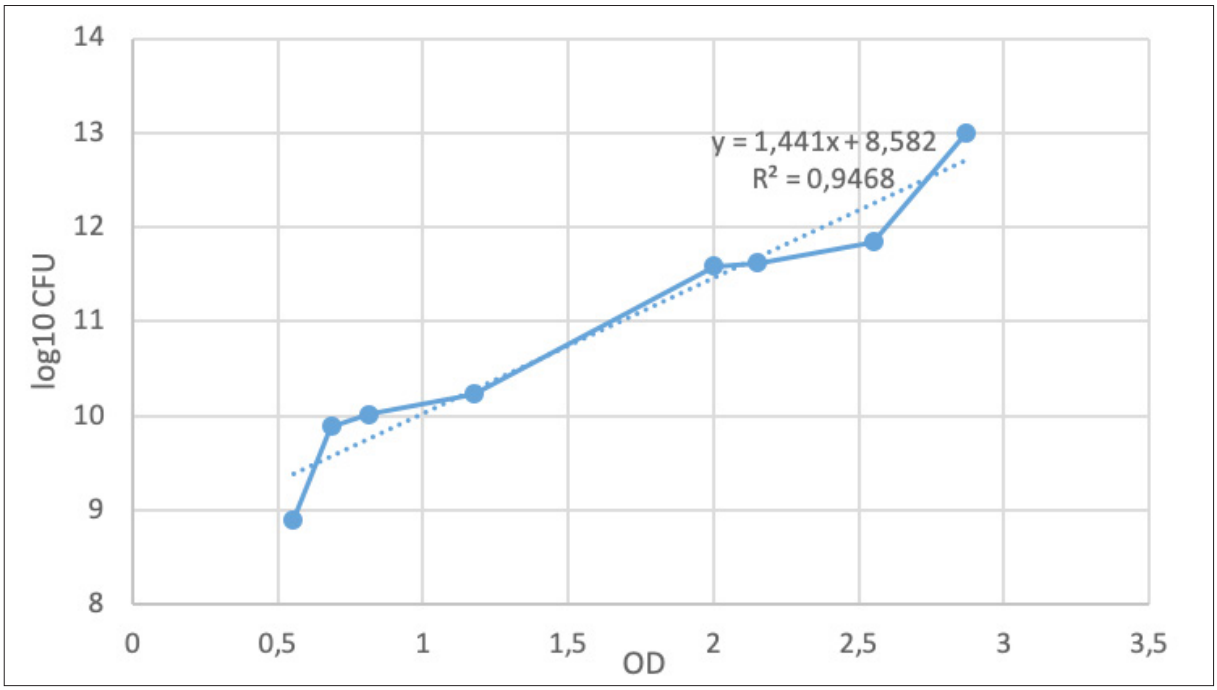


Figure 2. Regression curve. OD450nm of MTT assays to $\log_{10}$ CFU.

SEM Analysis

Microscopic examination revealed four stages of biofilm development, as illustrated in Figure 3: attachment/colonization (Figure 3a-c), irreversible adhesion (Figure 3d-f), biofilm maturation (Figure 3g–i), and detachment and dispersal (Figure 3j–l). This mechanism facilitates the dispersion of the biofilm and initiates a new cycle.

The SEM analysis of *A. baumannii* isolates demonstrated that strains capable of producing strong biofilm formed large clusters of cells; in contrast, strains producing moderate biofilm showed fewer clusters, which were almost insignificant in weak biofilm producers. No cell clustering was observed in strains that did not produce biofilm (Figure 4).

DISCUSSION

A. baumannii is a gram-negative, opportunistic pathogen that is associated with hospital-acquired infections. *A. baumannii*'s ability to acquire resistance to multiple antibiotics has led to a significant increase in multidrug-resistant strains, with the prevalence of extensively drug-resistant strains exceeding 60%, and reaching approximately 90% in some hospital settings[23]. Due to its ability to form biofilm, the eradication of *A. baumannii* from medical environments is challenging. The National Institutes of Health and the Centers for Disease Control and Prevention have reported that approximately 65% to 80% of human infections are caused by biofilm-forming bacteria[24]. Its ability to acquire antibiotic resistance and form biofilms has made it a major cause of infections in intensive care units.

The current study evaluated the biofilm-forming capacity of 52 non-duplicate clinical *A. baumannii* isolates obtained from ICUs. PFGE highlighted the presence of both clonal dissemination and particularly genetic diversity among the *A. baumannii* isolates included in the study. It was found that 50 of 52 (96.1%) *A. baumannii* ICU isolates were able to form biofilm. Among the isolates, 24 (46.1%) were strong biofilm formers, 22 (42.3%) moderate, four (7.6%) weak, and two (3.8%) were non-biofilm formers. These results are consistent with a previous study that reported 99% of *A. baumannii* isolates were capable of forming biofilms, with half classified as strong biofilm producers[19]. For the investigation of the biofilm formation ability of isolates, the plates were inoculated with

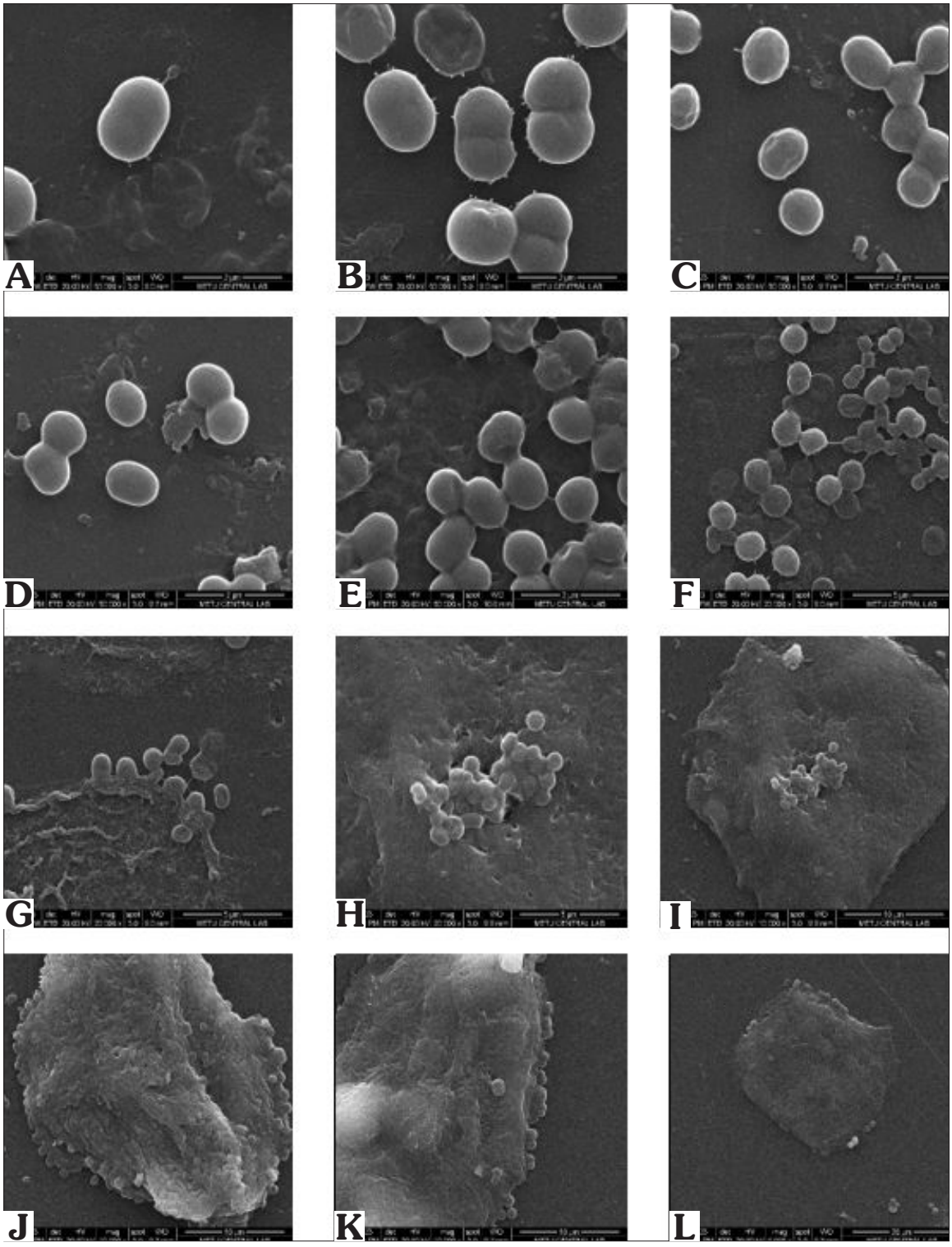


Figure 3. Biofilm stages. **A-C**. attachment/colonization, **D-F**. irreversible attachment, **G-I**. maturation, **J-L**. detachment.

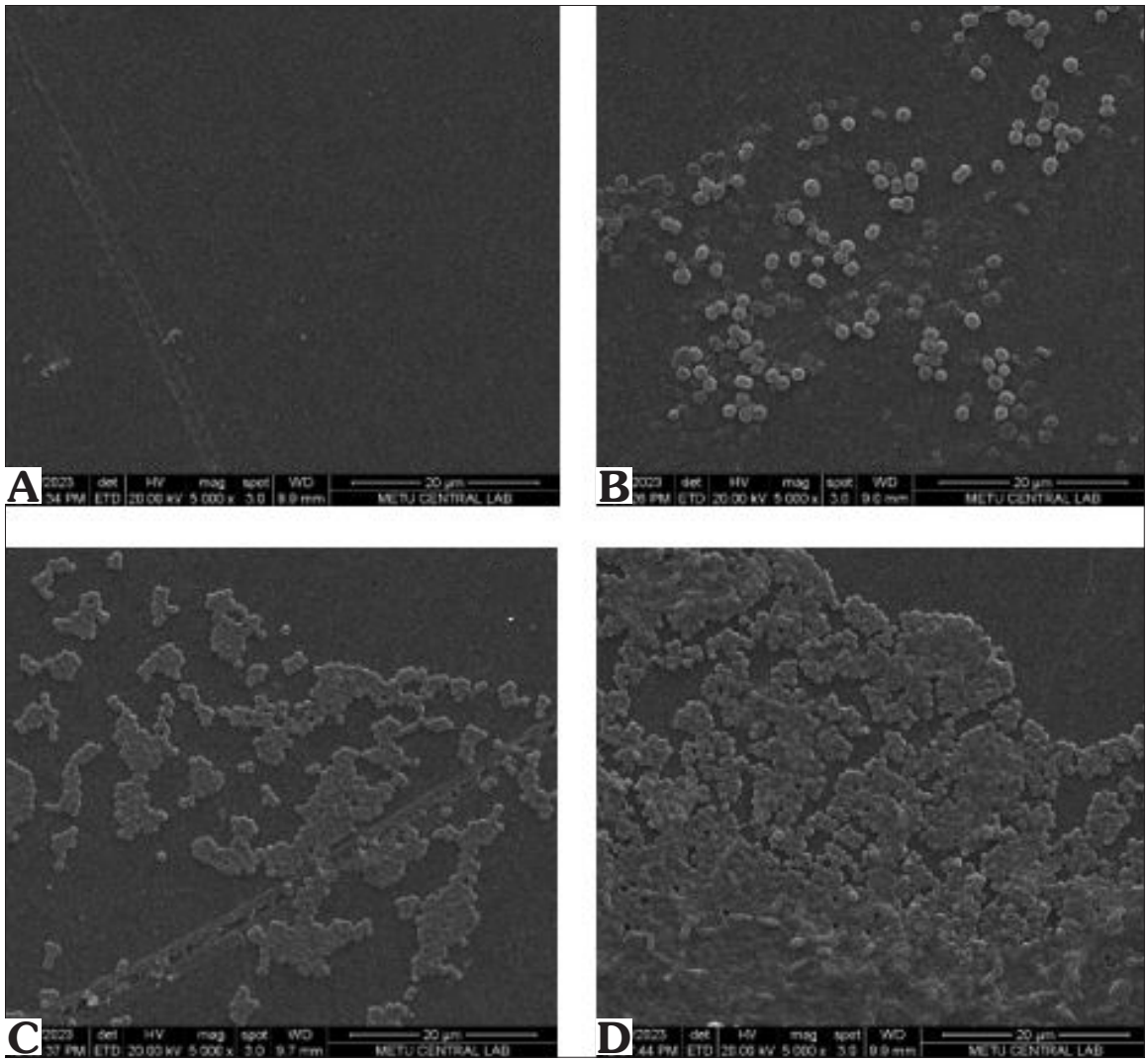


Figure 4. Biofilm patterns by SEM Analysis. **A.** non-biofilm formation; **B.** weak biofilm formation; **C.** moderate biofilm formation, strong biofilm formation; **D.** strong biofilm formation.

A. baumannii isolates and incubated for 36 hours. Since the medium must be changed every 24 hours to maintain bacterial growth, which may cause the loss of the original bacterial colonies, the 36 hours of incubation was chosen for biofilm formation in all experiments instead of 48 hours and 72 hours of incubation.

The presence of *csu*E, *omp*A, *aba*I, *pga*A, *bap*, and bla_{PER-1} genes, which have an impact on biofilm development, was screened. The *csu*E gene, which is part of the chaperone-usher assembly system, facilitates adhesion to surfaces and contributes to biofilm formation. A genetic group that encodes and secretes bacterial sites, capillary production, and capillary assembly functions has been linked to early biofilm development[1,25]. In the current study, 94% of the isolates carried the *csu*E gene. A study reporting that 100% of *A. baumannii* isolates carried the *csu*E gene supports the role of this gene in biofilm formation[26]. *A. baumannii*'s outer membrane contains *omp*A, which supports bacterial survival and proliferation, facilitates the evasion of the host immune system, and contributes to antimicrobial resistance. It enables epithelial cell attachment and invasion, biofilm growth, serum resistance, surface motility, host cell apoptosis, and cytotoxicity[1,27,28]. In this study, 96.1% of

the isolates carried the *omp*A gene, which is in concordance with a study by Mona et al., who reported that 98.4% of the isolates carried the *omp*A gene[19]. The *aba*I gene in *A. baumannii* produces an auto-inducer molecule, a tiny chemical that alerts other bacteria to high population density. The auto-inducer molecule starts a biofilm-forming cascade at a particular concentration[1]. It was found that 98.0% of the clinical *A. baumannii* isolates carried *aba*I, similar to a previous study that showed 100% of isolates had *aba*I[29]. The *pga*A is another biofilm-associated gene in *A. baumannii*. It produces PNAG, a key biofilm matrix component. PNAG sticks bacteria together and protects them from immune evasion. It was found that 50% of the isolates carried *pga*A. Biofilm production begins with *A. baumannii* cells attaching to surfaces with *bap*. The *bap* produces EPS, which hold the biofilm together and protect it from the environment. The *bap* gene helps biofilm mature into a more complex, structured entity. The *bap* gene is involved in *A. baumannii* pathogenicity and biofilm development. A total of 98% of the isolates were found to have the *bap* gene. Finally, the bla_{PER-1} gene, a member of the extended-spectrum β-lactamase (ESBL) family, has been associated with biofilm development. Only 40% of isolates had bla_{PER-1}. This is in line with the findings of a previous study that was carried out on this gene, which found that it produced the lowest percentage in comparison to the other genes in this study[28-26].

The correlation between biofilm and the biofilm-related genes was also determined. Biofilm formation abilities of the isolates were evaluated according to the presence of various genes known to be associated with biofilm formation. There was no statistically significant correlation between the individual presence of these genes and biofilm formation levels ($p > 0.05$). This suggests that no single gene had a dominant influence on biofilm-forming capacity among the isolates. The high prevalence of multiple gene co-occurrence across the isolates may have contributed to this lack of differentiation, reflecting the complex, multifactorial nature of biofilm formation. Rather than acting in isolation, these genes likely operate synergistically or in overlapping pathways. Future research should therefore explore combinatorial gene expression patterns. Biofilm production is shaped not only by the presence of a particular gene, but also by its expression level, regulatory mechanisms, environmental factors, and interactions with the genetic background. Moreover, the fact that the same biofilm phenotype can occur with different gene combinations supports that biofilm formation is a polygenic trait. Consequently, the evaluation of the genetic determinants of biofilm formation should be supported by more comprehensive analyses (e.g., gene co-expression, transcriptomic studies, or genetic network analyses), taking into account coexistence patterns and functional relationships of genes beyond their individual effects. In this context, biofilm-associated genes are thought to be effective when they work in combination rather than in isolation.

While our study employed crystal violet staining and MTT assays, which are well-established and widely accepted for quantifying biofilm biomass and assessing metabolic activity, respectively, we acknowledge that Confocal Laser Scanning Microscopy (CLSM) and Live/Dead staining could have offered more detailed spatial and structural insights into biofilm architecture. This is a limitation and suggests that future studies should integrate CLSM to provide a three-dimensional perspective on biofilm formation. However, this limitation was partially addressed through the inclusion of SEM, which allowed us to visualize the biofilm architecture, stages of development, and differences in structural integrity across isolates. SEM images clearly demonstrated morphological distinctions between strong, moderate, weak, and non-biofilm-producing isolates, thereby offering valuable spatial insights into biofilm formation. We also employed the MTT assay, which offers a key advantage by assessing the metabolic activity of cells within the biofilm matrix, thus reflecting the viability of the biofilm-forming population. As a result, after a 36-hour incubation, the bacteria had a high survival and growth rate. Together with SEM analysis, which provided morphological visualization, and crystal violet staining, which quantified total

biofilm mass, the MTT assay served as a critical viability indicator that strengthened our phenotypic assessment of biofilm-forming capacity.

In the current study, we investigated the genetic determinants and structural characteristics associated with biofilm formation in *A. baumannii* isolates obtained from clinical specimens of ICU patients at Başkent University Ankara Hospital. Our findings demonstrated that a substantial proportion of the isolates (96.1%) exhibited the capacity to form biofilms, underscoring the clinical relevance of this phenotype in critical care settings. The high prevalence of biofilm-associated genes among these isolates suggests a pivotal role for these genetic elements in mediating biofilm development. Although individual gene presence was not significantly correlated with biofilm strength, their widespread occurrence points toward a polygenic and multifactorial mechanism. The elucidation of these genetic contributors enhances our understanding of *A. baumannii* pathogenicity and highlights potential targets for therapeutic intervention. The notably high incidence of biofilm-producing strains in the ICU setting is of particular concern, as biofilm formation not only facilitates environmental persistence but also confers enhanced resistance to antimicrobial agents. These findings reinforce the need for integrative approaches—combining genomic, phenotypic, and clinical data-to develop more effective strategies for the prevention and management of A. baumannii infections in healthcare environments.

A. baumannii poses a considerable challenge in healthcare settings due to its extensive antibiotic resistance and biofilm-forming abilities. Understanding the presence and the relationship between these factors is vital for devising novel approaches to avert and manage *A. baumannii* infections, for instance, by directing attention towards the genes implicated in the formation of biofilms. These findings also highlight the importance of biofilm surveillance in ICU settings, where device-related infections by biofilm-forming organisms such as *A. baumannii* may require more aggressive decontamination protocols and tailored antimicrobial strategies.

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ETHICS COMMITTEE APPROVAL

This study was approved by the Başkent University Medical and Healthy Sciences Research Committee (Decision no: KA22/411, Date: 12.10.2022).

CONFLICT of INTEREST

The authors have no conflicts of interest to declare that are relevant to the content of this article.

AUTHORSHIP CONTRIBUTIONS

Concept and Design: AÜG

Analysis/Interpretation: AÜG

Data Collection or Processing: NMS, AÜG

Writing: NMS, AÜG

Review and Correction: NMS, AÜG

Final Approval: NMS, AÜG

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Address for Correspondence/Yazışma Adresi

Dr. Aylin ÜSKÜDAR GÜÇLÜ

Department of Medical Microbiology,
Başkent University Faculty of Medicine,
Ankara, Türkiye

E-posta: uskudaraylin@gmail.com