BASKENT UNIVERSITY
INSTITUTE OF HEALTH SCIENCES
DEPARTMENT OF MEDICAL MICROBIOLOGY
MASTER'S PROGRAM

PHENOTYPIC AND GENOTYPIC BIOFILM FORMATION CHARACTERISTIC OF ACINETOBACTER BAUMANNII ISOLATED FROM INTENSIVE CARE UNITS

MASTER'S THESIS

BY

NADA MOHAMED MOHAMED SAID

ANKARA - 2023

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This study, which was prepared by Nada Mohamed Mohamed Said within the framework of the Department of Medical Microbiology Master's Program, was accepted as the Master's Thesis by the following jury.

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Thesis Jury Members (Title, Name - Surname, Institution) **Signature**

APPROVAL

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ABSTRACT

SAİD M M N. Phenotypic and Genotypic Biofilm Formation Characteristic of *Acinetobacter baumannii* Isolated from Intensive Care Units. Baskent University, Institute of Health Sciences, Department Of Medical Microbiology, Medical Microbiology Master's Program with Thesis, Ankara, 2023.

Acinetobacter baumannii is a significant pathogen commonly associated with nosocomial infections. Its ability to form biofilms enables it to survive in hospital settings and poses challenges for eradication. This study aimed to investigate the structural characteristics of biofilm formation in imipenem, meropenem, amikacin, gentamicin, levofloxacin, ciprofloxacin, trimethoprim/sulfamethoxazole, tigecycline and colistin resistant *A. baumannii* isolates from intensive care units (ICUs) and identify the genetic backgrounds contributing to biofilm formation. A total of 52 clinical *A. baumannii* isolates obtained from ICUs at Baskent University Ankara Hospital were included in the study. Antibiotic susceptibilities were tested, and biofilm formation ability was assessed using the crystal violet staining method. Additionally, biofilms were visualized under scanning electron microscope (SEM). Biofilm viability was measured using the MTT assay, and the presence of biofilm-related genes, including bla_{CsuE}, bla_{OmpA}, bla_{abaI}, bla_{pgaA}, bla_{bap}, and bla_{Per1}, was detected using PCR. Among the 52 *A. baumannii* isolates, 96.1% demonstrated biofilm formation ability. The biofilm-related genes bla_{bap} and bla_{abaI} were detected in 98% of the isolates. Following these, bla_{OmpA} and bla_{CsuE} were present in 96.1% and 94.0% of the isolates, respectively. The presence of bla_{pgaA} and bla_{Per1} was observed in 50.0% and 40.3% of the isolates, respectively. The MTT assay revealed varying viability percentages ranging from 13.5% to 112.4%. Understanding the presence and relationships between these factors is crucial for developing novel approaches to prevent and manage *A. baumannii* infections. Attention should be directed towards the genes involved in biofilm formation to devise effective strategies for combating this pathogen.

Keywords: *A.baumannii*, Biofilm, Intensive Care Units, Multi Drug Resistance

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

ÖZET

SAİD M M N. Yoğun Bakım Ünitelerinden izole edilen *Acinetobacter baumannii* izolatlarının Fenotipik ve Genotipik Biofilm Formasyon Özellikleri. Başkent Üniversitesi, Sağlık Bilimleri Enstitüsü Tıbbi Mikrobiyoloji Yüksek Lisans Tezi, Ankara, 2023.

Acinetobacter baumannii, nozokomiyal enfeksiyonlarla yaygın olarak ilişkili önemli bir patojendir. Biyofilm oluşturma yeteneği, hastane ortamlarında hayatta kalmasını sağlar ve yok edilmesi için zorluklar yaratır. Bu çalışmada, yoğun bakım ünitelerinden (YBÜ) izole edilen imipenem, meropenem, amikasin, gentamisin, levofloksasin, siprofloksasin, trimetoprim/sülfametoksazol, tigesiklin ve kolistin dirençli *A. baumannii* izolatlarında biyofilm oluşumunun yapısal özelliklerinin araştırılması ve buna katkıda bulunan genetik altyapının belirlenmesi amaçlanmıştır. Başkent Üniversitesi Ankara Hastanesi yoğun bakım ünitelerinden elde edilen toplam 52 klinik *A. baumannii* izolatı çalışmaya dahil edildi. Antibiyotik duyarlılıkları test edildi ve kristal viyole boyama yöntemi kullanılarak biyofilm oluşturma yetenekleri değerlendirildi. Ek olarak, biyofilmler taramalı elektron mikroskobu (SEM) altında görselleştirildi. Biyofilm canlılığı, MTT yöntemi kullanılarak belirlendi ve *bla*$_{\text{CsuE}}$, *bla*$_{\text{OmpA}}$, *bla*$_{\text{abaI}}$, *bla*$_{\text{pgaA}}$, *bla*$_{\text{bap}}$ ve *bla*$_{\text{Per1}}$ dahil olmak üzere biyofilmle ilgili genlerin varlığı, PCR kullanılarak tespit edildi. 52 *A. baumannii* izolatının %96,1'i biyofilm oluşturma yeteneği göstermiştir. Biyofilm ilişkili genler *bla*$_{\text{bap}}$ ve *bla*$_{\text{abaI}}$ izolatların %98'inde tespit edilmiştir. Bunları takiben izolatların sırasıyla %96,1'inde *bla*$_{\text{OmpA}}$ ve %94,0'ında *bla*$_{\text{CsuE}}$ saptanmıştır. İzolatların sırasıyla %50,0 ve %40,3'ünde *bla*$_{\text{pgaA}}$ ve *bla*$_{\text{Per1}}$ genleri bulunmuştur. MTT analizi ile izolatların biyofilmdeki canlılıkları %13,5 ile %112,4 arasında belirlenmiştir. Bu faktörlerin varlığını ve aralarındaki ilişkileri anlamak, *A. baumannii* enfeksiyonlarını önlemek ve yönetmek için yeni yaklaşımlar geliştirmek için çok önemlidir. Bu patojenle mücadelede etkili stratejiler geliştirmek için biyofilm oluşumunda yer alan genlere dikkat edilmelidir.

Anahtar Kelimeler: *A.baumannii*, Biyofilm, Yoğun Bakım Üniteleri, Çoklu İlaç Direnci

Bu çalışma Başkent Üniversitesi Tıp ve Sağlık Bilimleri Araştırma Kurulu tarafından onaylanmış (Proje no: KA22/411) ve Başkent Üniversitesi Araştırma Fonunca desteklenmiştir.

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LIST OF SYMBOLS AND ABBREVIATIONS

WHO	World health organization
DNA	Deoxyribonucleic Acid
PLC	Phospholipase C
EPS	Exopolymeric substance
QS	Quorum sensing
MIC	Minimum inhibitory concentration
PIA	Polysaccharide intercellular adhesion
AHL	Acyl-homoserine-lactone
MTT	Thiazolyl blue tetrazolium bromide (MTT) bioChemical.
TBE	Tris-borate-EDTA
ESBL	Extended-spectrum beta-lactamases
TEM	Transmition electron microscope
SEM	Scanning electron microscope
CFU	Colony Forming Unit
XDR	Extensively drug resistance
MDR	Multi drug resistance
ECDC	European center for disease prevention and control.
NIH	National Institutes of Health

1. INTRODUCTION

Acinetobacter baumannii is an opportunistic pathogen that poses significant challenges in hospital settings. *A.baumannii* is responsible for various hospital-acquired infections, mostly in intensive care units (ICUs). The ability of *A. baumannii* to develop antibiotic resistance and form biofilms contributes to its survival in harsh environmental hospital conditions, making it difficult to treat and control. Because of the overuse of antibiotics with wide spectrum activity, multi-drug and extensively-drug resistant strains of *A.baumannii* has emerged as a clinical threat worldwide and in Turkey. According to The Central Asian and European Surveillance of Antimicrobial Resistance network (CAESAR), carbapenem, as a last resort, resistance rate of *A.baumannii* is 91% in 2019. Antimicrobial resistance mechanisms including enzymatic inactivation-degradation, efflux pumps, drug target modification and decreased permeability have been well defined in *A.baumannii* and one of the resistance mechanism is biofilm formation. The ability of *A.baumannii* to form biofilm and antimicrobial resistance profile may be involved in hospital survival. Biofilm forming ability on both abiotic and biotic surfaces is an efficient way to augment the survival of bacterium under harsh hospital conditions such as ICUs. Also, biofilm formation is an important factor in the occurrence of the persistent and recurrent infections, and biofilm-associated infections typically persist longer time and lead the usage of elevated dosages of antibiotics. Investigation of biofilm forming characteristics of this pathogen and the virulence factors contributing biofilm formation is necessary to cope with this opportunistic pathogen of ICUs. The ability of *A. baumannii* to form biofilms is associated with the presence of various biofilm-related genes, such as *bap, pga, csuE,* and *ompA*. The distribution of these genes among isolates with different biofilm-forming abilities may provide insight into the potential predictors of strong biofilm formation. The aim of this study is to evaluate the biofilm forming ability, the structural characteristics of biofilm formation, the presence of the biofilm related genes, comparison of these characteristics in studied *A. baumannii* isolates of ICUs, and the relationship between antibiotic resistance and biofilm formation.

2. GENERAL INFORMATIONS

2.1. The Genus of *Acinetobacter*

The genus *Acinetobacter* has undergone considerable taxonomic changes over the years and the current taxonomic data shows that the genus *Acinetobacter* is classified in the family *Moraxellaceae* that belong to *Pseudomonadales* order, the class *Gammaproteobacteria. Moraxella, Acinetobacter,* and *Psychrobacter* are the three genera of the family *Moraxellaceae* (1). There is a current increased interest in this genus *Acinetobacter* because of the emergence of multi-drug resistant strains, and also some of *Acinetobacter* isolates are reported as pan-resistant to antibiotics (2). In 1911, the Dutch microbiologist Beijerinck was the first person to successfully isolate the bacterium from soil. *Micrococcus calco-aceticus* was the original name given to *Acinetobacter* when it was discovered. Brisou and Prevot gave the idea of creating the *Acinetobacter* genus to distinguish it from the motile organisms that are included in the genus *Achromobacter. Acinetos* is derived from the Greek word "*akinetos*," which can be translated as "*non-motile*" (3). At least 59 species belong to the *Acinetobacte*r genus, and many members of the genus are found in various environments including healthcare settings (4). *Acinetobacte*r species are ubiquitous saprophytes, recovered in nature and in the hospital. This genus contain non-motile, non-fermenting, Gram-negative, non-fastidious, oxidase-negative, catalase-positive, bacteria with 39-47% DNA G+C content (5). They have the ability to survive on both moist surfaces and on dry surfaces including mechanical ventilation equipment and human skin.

The genus *Acinetobacter* is subdivided into two groups as glucose-oxidizing species and glucose nonoxidizing species. Most human infections are caused by *A. baumannii* (6). There are already more than one thousand references to "infections and resistant *Acinetobacter*" in the scientific literature that is published on a global scale (7). *Acinetobacter* was widely disregarded in the 1960s when it was identified from clinical samples because it was thought of as a commensal, an opportunist, and a relatively low-grade pathogen (8,9). However, due to the enormous advancements in laboratory techniques, the sorts of infections that are caused by *Acinetobacter* have shifted. Infections caused by *Acinetobacter* can be acquired through the community in warm and humid regions, such as tropical countries. These infections typically present themselves as bacteremia or lung infections (10). During the late 1970s*, Acinetobacter* emerged as a serious nosocomial

pathogen. This was most likely a consequence, at least in part, of the increasing utilization of broad-spectrum antibiotics in healthcare facilities.

2.2. *Acinetobacter baumannii*

The majority of clinically important isolates are from the species *A. baumannii*. It is non-motile, oxidase-negative, strictly aerobic Gram-negative bacterium that it typically appears as short rods or cocci under a microscope (figure 1). The majority of infections are observed in patients in ICUs which treat patients who are critically ill. In these kinds of facilities, large outbreaks are possible, and some epidemic strains of *A. baumannii* may infect or colonize a large number of patients (11). *A. baumannii* is an ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) pathogens which cause of nosocomial infections throughout the world and they are mostly multi-drug resistant. This property leads the greatest challenges in clinical practice. WHO declared a list of antibiotic-resistant "priority pathogens" of 12 families of bacteria that threaten human health the most. Carbapenem resistant *A.baumannii* is indicated as "critical" in the Priority 1 list. Carbapenem resistant *A.baumannii* has been placed in this list since they cause deadly infections, and long hospital stays, they are resistant to widely used antibiotics, they spread between animals and humans, and they can be transmitted from person to person.

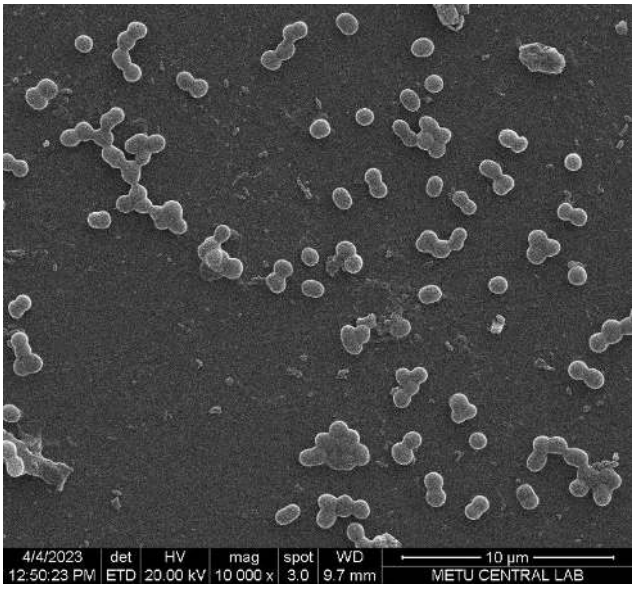

Figure 1. *A.baumannii* cells under scanning electron microscopy

2.2.1. The virulence factors of *A. baumannii*

When compared to other Gram-negative bacteria, the number of virulence factors that have been reported for *A. baumannii* is relatively low. Some of them include outer membrane proteins (OMPs), lipopolysaccharides, efflux pumps, hemolytic factors, iron acquisition and systems. They can cause the immune system of the host to react or cause bacteria to stick to epithelial cells (12). Phospholipases C (PLCs) and D are the major phospholipase classes found in *A. baumannii*. These phospholipases hydrolyze phospholipids and, as a result, facilitate the spread of infection within hosts. Elastase is another major virulence factor that breaks down elastin and destroys the host's defenses or tissues (13).

A. baumannii has the ability to infect both human and animal hosts due to its capacity to express various genes that enhance its virulence. Some factors, such as surface antigen protein 1, K1 capsular polysaccharides, outer membrane porins, acinetobactin transporters, and iron acquisition systems, along with resistance to the vast majority of antimicrobials, have contributed to *A. baumannii's* evolution into a pathogen that is difficult to treat. *A. baumannii* has emerged as critical problem worldwide (14).

Another essential trait of *A. baumannii* that induces pathogenicity and is associated with the extended survival of bacteria in hospital settings is the production of biofilms by various factors (15,16). There is a possibility that the in vivo virulence of *A. baumannii* could be reduced if the genes associated with the virulence factor were deleted or rendered dysfunctional. Also, virulence factors are what make *A. baumannii* strains stick to surfaces, take over environments, and invade other organisms (14,17). During the formation of a biofilm, cellular communication and surface-organized attachments are essential microbial functions, in addition to the secretion of macromolecules (18,19). *A. baumannii* has the capability to attach to and create biofilms on both living and non-living surfaces due to its possession of a range of virulence genes and proteins related to biofilm formation. The capacity of the organism to perform such function is attributed to the involvement of specific proteins and genes. (20). Because these genes are present, *A. baumannii* is able to survive in a wide variety of environments and is able to put up a formidable fight against antimicrobials and immune cells (21). The biofilm formation ability of this bacterium contributes both the pathogenesis and antimicrobial resistance.

2.3. The Biofilm

Biofilms refer to clusters of microbial cells that are bound together by their exopolysaccharide matrices. Biofilms develop on biotic or abiotic substrates. According to research, biofilms exhibit greater resilience to antibiotics, host immune defenses, and unfavorable environmental conditions in comparison to their free-living counterparts (22). The Center for Disease and Prevention and the National Institutes of Health (NIH) indicates that almost 80% of the infections are caused by microorganisms with biofilm formation ability in human (23).

2.3.1. The Biofilm-Associated Infections

The extracellular biofilm consists of a microbial polymeric block that is usually composed of proteins, polysaccharides and extracellular nucleic acid, which helps these bacteria to survive in the host(22). Biofilms are a form of microbial growth that can be characterized as a safeguarded mode of growth, which serves to shield microorganisms from unfavorable environmental conditions. The development of biofilms by pathogenic microorganisms of medical significance is a primary factor in the occurrence of numerous persistent and recurring infections. In comparison to acute infections caused by planktonic cells, biofilm-associated infections typically persist over a longer duration and thus necessitate elevated dosages of antibiotics. Infections associated with biofilms typically exhibit a gradual onset of prominent symptoms and seldom result in mortality. Nonetheless, these microorganisms can persist in anatomical regions of the human physique for extended periods of time, ranging from months to years, or even indefinitely, thereby diminishing the overall standard of living. (24).

Both one microbial species and mixture of different species may produce biofilm-associated infections, and it is known that the interactions between more species increase their persistence. There are two types of biofilm-associated infections;

- The first type is associated with indwelling medical devices including central venous catheters, peritoneal dialysis catheters, shunts, endotracheal tubes, mechanical heart valves, urinary catheters, joint prostheses, cardiac pacemakers, intrauterine devices and dental unit waterlines. Biofilms are firstly formed on the surfaces of such medical devices and then may cause infections such as bloodstream and urinary tract infections. In such circumstances, causative agents are generally the members of the

epithelial flora of patients, healthcare workers or other sources in the hospital environment. Related pathogens can be transferred to tissues and organs through indwelling medical devices inserted into the human body.

- In the second type, biofilm-associated infections may occur in the sterile body side, and this type is known as native biofilm infections. This type is mostly chronic, opportunistic infection occur in host tissue. chronic osteomyelitis, chronic wounds, dental caries, endocarditis, urinary tract infection can be given as examples (24).

2.3.2. Biofilm-associated antibiotic resistance

The most clinically significant feature of microbial biofilms is its high antibiotic resistance, which poses serious problems in the treatment of biofilm-associated infections. Comparing to planktonic bacteria the minimum inhibitory concentration (MIC) value of antibiotics for biofilm-forming bacteria might be up to 1000-fold higher. Antimicrobial resistance mechanisms has been well described in *A.baumannii* (figure 2), and multiple biofilm-specific factors have been identified that contribute to the development of enhanced antibiotic resistance in the biofilm phenotype, and these mechanisms are distinct from the antibiotic resistance mechanisms of planktonic cells.

a) The diffusion of antibiotics into biofilms can be hindered due to the presence of a biofilm matrix which acts as a physical barrier. This can lead to a delay in the antibiotic diffusion process. In some cases, antibiotics may chemically react with the components of the biofilm matrix or attach to anionic polysaccharides, further impeding their penetration. Prolonged exposure to antibiotics due to delayed penetration can result in antibiotic resistance.

b) Biofilms are comprised of persister cells, which constitute a minor and reversible subset. The persister cells exhibit an ability to acclimate to a lifestyle characterized by slow or negligible growth, thereby generating small colony variants that demonstrate resilience to extracellular stressors, including antibiotics. Due to the growth-specific factors that antibiotics typically target, slow- or non-growing cells within the biofilm are frequently less impacted by antibiotic treatment in comparison to their fast-growing counterparts. Biofilm-associated cells display reduced growth rates, which make them less vulnerable to antibiotics.

c) Biofilms experience nutrient depletion as a result of nutrient consumption by peripheral cells and decreased diffusion of oxygen and nutrients within the biofilm. As a

consequence, the activation of stress responses that are induced by starvation, such as the stringent response, is observed. The stringent response is typified by the inhibition of bacterial growth and division, along with the induction of amino acid biosynthesis to enhance bacterial viability.

d) Glucan is a cyclic polysaccharide which is facilitated by a glucan synthase complex on the plasma membrane. Following the process of synthesis, it is released from the periplasmic space and covalently attached to diverse constituents of the cell wall. It is noteworthy that glucan may also be incorporated as a component of the biofilm matrix. In addition to its function in the structure of the cell wall, glucan plays a pivotal role in cellular adhesion, virulence, and the formation of biofilms.

e) Extracellular DNA is a vital component in the process of biofilm formation as it aids in the attachment and aggregation of microcolonies. In addition to its role as a structural support for biofilm architecture, extracellular DNA has the ability to bind to cations present in the biofilm matrix. This binding leads to a limited availability of cations, which in turn activates two-component regulatory systems such as PhoP/Q and PmrA/B. The utilization of these systems is imperative for the manifestation of various antibiotic resistance.

f) Efflux pumps are frequently upregulated in biofilms and have a role in antibiotic resistance. It is widely believed that the intrinsic resistance of biofilms to antibiotics is largely attributed to the upregulation of efflux pump systems in bacteria that form biofilms. They work to remove of antimicrobial agents from the biofilm matrix, thus they avoid to access to the targets.

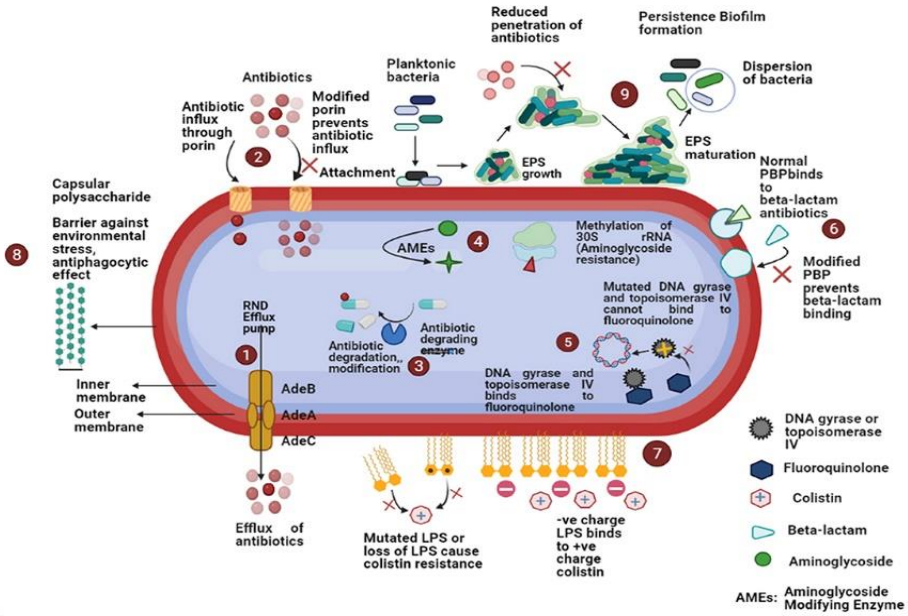

Figure 2. Schematic diagram of different antimicrobial resistance mechanisms in *A. baumannii*. The picture was adapted from Roy et al. (25).

2.4. The Biofilm formation

Biofilm formation is a complex process and environmental conditions, the location of the strain, and the ecological niche have on the spread of bacterial strains and their capacity to create biofilm may influence the biofilm formation. Although biofilm-producing bacteria can be found in a wide variety of habitats and settings, biofilm-forming *A. baumannii* are mainly seen on clinical settings. It is possible that bacteria at one place in the hospital develop more biofilm than ones at another site in the same hospital (26). Biofilm formation process is the end result of many physical, chemical, and biological processes (27–29). The formation and growth of a biofilm can be seen as a series of steps (27). The following steps explain the biofilm formation process;

I. Single cells adhere to moist surfaces and produce a layer to form a biofilm (30). Once bacterial cell stick to the surface, they can transport more free-floating microorganisms, which leads to co-aggregation and many layers of biofilm (31). This layer is an organic monolayer that grows on surfaces, docks the first cells that are permanently attached, but the strength of the biofilm depends on how well it sticks together (28,31). After only a few minutes, this layer can grow for hours in a liquid like water (30). Some bacterial cell's components including pili and fimbriae play a role in reaching the bulk lattice of the layer. This starts chemical processes and makes

the bacteria-surface interaction stronger (28,32). The availability of nutrients also has an effect on adhesion (33). Electrostatic forces make things move away from each other because germs and inanimate surfaces are negatively charged (34).

II. Irreversible Attachment: In the irreversible attachment stage, microorganisms become permanently attached to the surface. This stage is characterized by the production of EPS, which forms a protective matrix around the bacterial community (33,35). The production of EPS strengthens the bond between the microorganisms to each other and to the surface, providing stability and protection within the biofilm structure forming aggregates on the substratum (34,36).

III. Development of Biofilm Architecture: A sharp increase in population can be noticed after a lag period, which is dependent on the physical and chemical characteristics of the surrounding environment (37). The cells begin growing to form micro colonies and create the more EPS. This mechanism anchors the cells to the surface and fixes them (38). During this phase, both physical and chemical mechanisms contribute to the termination of the first adhesion. Biological activities then begin to dominate, and stronger bonds were obtained by the interaction of divalent cations and the polysaccharide intercellular adhesion polymers (37).

IV. Maturation: In this step, interconnected tiny colonies evolve into a mature biofilm by multiplying and accumulating debris and new planktonic bacteria (36,38). EPS is required for cell-cell and cell-substratum adhesion at this stage (36). Biofilms use quorum sensing (QS) to monitor cell density and regulate collective behavior at high cell densities(28,36,39). Despite popular belief, quorum sensing also mediates interspecies communication (40). Gram-negative bacteria employ different QS systems to produce, detect, and respond to external signaling molecules called auto inducers, acylated homoserine lactones for gram-negative bacteria (41). Using auto inducers, bacteria regulate gene expression (39). Following maturation biofilm contains three layers: a connecting film, a base film of dense bacteria, and a surface film. The free floating bacteria can spread from the surface film layer (42). When bacterial cells send quorum sensing (QS) signals, the phenotypic and genotypic traits are activated. QS signals help cells to communicate each other when environmental factors change such as oxygen level, temperature, pH value and the growth medium (29).

V. Dispersal: It is the last step in the process of making a biofilm. During this step, cells that were stuck together separate and move to a new place to live (27). Biofilm cells

can be spread out by the release of actively dividing cells, or by detachment as a results of some factors like lack of nutrients (27,36). The sloughing, erosion, and abrasion processes are all part of the detachment stage (30). Erosion is the process of removing single cells or small pieces of biofilm over time (43). In sloughing a large pieces of biofilm biomass are lost (43). This loss is caused by a lack of nutrients and dissolved oxygen at the bottom of the biofilm (30). Abrasion refers to the process by which biofilm is eroded due to the impact of airborne particles (43). It is possible to relocate any liberated cells to novel locations, thereby initiating the biofilm development cycle anew (27). The dissemination of pathogens is attributed to their detachment from biofilms (36).

The biofilm formation is a multifaceted and evolving process that act as a long-lasting source of infections. It is widely recognized that alterations in the environment can induce a shift in the phenotype from the planktonic to the sessile form. These environmental factors, like the amount of nutrients, temperature, pH, and ionic strength, can affect the formation of biofilm (27).

2.4.1. Biofilm related genes in *A.baumannii*

Pili formation in Gram-negative bacteria is a crucial factor in their ability of adhesion to surfaces, establish biofilms, and invasion to host cells. Pili possess the capacity to impact the motility of Gram-negative bacteria. (44). The chaperone-usher (CU) family of pili has been extensively studied. Most clinically significant *Acinetobacter* species have a chaperone-usher (CU) pathway involved in the formation of the pilus. (45). The CU pathway involves genes such as *Csu*-type genes. The aforementioned genes are contained within a singular operon consisting of six genes, commonly known as *Csu* (46). The polymerization of *CsuA and CsuB* commences in the presence of the *CsuC*, leading to the production of the principal subunit of the pilus (47). Further experimental and bioinformatics investigations have yielded proof that *CsuD* operates as the usher, and *CsuE* acts as the tip adhesion. *CsuA* and *CsuB*, on the other hand, may have minor pilin subunit functions. Although conserved in several sequenced strains of *A. baumannii*, the functionality of the *Csu* gene has only been described in the *A.baumannii* ATCC 19606 strain. This particular strain is commonly employed in various laboratory settings(45,46). As per the findings of genetic investigations carried out on this particular strain, the absence of *CsuC* or *CsuE* mutation leads to the complete cessation of *Csu* pili expression and impedes the development of biofilm (45).

Furthermore, the BfmRS two-component regulatory system is implicated in the modulation of *Csu* expression. The aforementioned assertion is supported by the observation that a mutant lacking bfmR exhibited a lack of expression of *CsuA/B* and a consequent inability to generate biofilms(46).

Poly-b-(1–6)-N-acetylglucosamine (PNAG) is one of the primary components of biofilm and it is involved in immune evasion as well as the interaction between the host and the microbes. Four different genes: *pgaA, pgaB, pgaC,* and *pgaD* of the *pga* locus are involved in the synthesis and translocation of PNAG. *A. baumannii pga A* consists of a porin domain and a super helical periplasmic domain. The porin domain assists PNAG translocation through the outer membrane, and the super helical periplasmic domain play an important part in protein-protein interaction. Along with *pgaA*, the protein *pgaB* possesses a putative polysaccharide deacetylase domain involved in the process of PNAG export. The *pgaD* and *pgaC* gene are involved in PNAG synthesis. The *pgaC* gene is part of the glycosyltransferase 2 family. PNAG is important in the integrity of the biofilm that *A. baumannii* forms (48).

The QS system controls how genes are expressed by reacting to changes in the number of bacteria in the area (49,50). Autoinducers, which are molecules that send signals, are needed for this system to work. In *A. baumannii*, QS system is based on acyl-homoserine-lactone (AHL). This system is made up of a LuxI-type *AbaI* and a LuxR-type *AbaR* (51,52). The gene *abaI* encodes for the production of an autoinducer synthase protein that facilitates the biosynthesis of the signal molecule N-hydroxy dodecanoyl-L-homoserine lactone (53). The *abaR* gene encodes a receptor protein that facilitates the binding of autoinducers and regulates the transcriptional process. The complex formation between *AbaR* and AHL is instrumental in binding to a distinct DNA sequence known as "lux box" and regulating the expression of target genes (52,53). The biofilm formation, pellicle formation, and motility in *A. baumannii* are regulated by the QS system (52,54). The mutant *abaR* strain exhibits significant deficiencies in both biofilm formation and cellular motility, key factors that facilitate bacterial colonization of abiotic surfaces (51).

In biofilm, the three-dimensional structure tower and water channel are formed by a cell surface protein called Bap (57,58). Bap is released by type I secretion system and helps *A. baumannii* form and mature biofilms (19). It helps cells stick to each other and is needed for higher-order structures to form on materials like polystyrene and titanium that are used

in medicine (56). Most strains of *A. baumannii* have a *bap* gene. This could be because the highly repetitive parts of *bap* coding sequences are prone to recombination or sequence alignment errors (20). *bap* has been shown to produce an 854 kDa protein that undergoes significant change. *bap* encoding gene is a large, 16 kb in size, and repetitive gene (20). *bap* could be found because it had a structure that kept repeating that was the same as a bacterial cell surface adhesion protein. Many researches on the *bap* -positive strain showed that *bap* is expressed at the cell surface, is linked to the formation of biofilms (20).

Proteins known as porins are responsible for the formation of channels through the outer membrane of certain types of bacteria. These channels act as a form of defense for the bacterium by preventing the entry of larger molecules that could be harmful to it. They permit the passage of smaller molecules into the bacterial cell, such as nutrients, but they do not permit the passage of larger molecules. *OmpA* is a porin protein that is encoded by the *OmpA* gene in *A.baumannii*. *OmpA* plays a role in the import of various nutrients and other small molecules into the cell. It is also believed to play a part in the bacterium's ability to colonize host tissues and cause infection. The *OmpA* protein is a trimer, and each of its monomers is composed of a beta barrel structure. This structure, which forms the channel through which small molecules can pass, makes up the *OmpA* protein. The channel is selective, allowing only molecules that meet certain requirements regarding their size and charge to pass through it. This protein is essential to the bacterium's ability to survive and grow in various environments. In addition to its function in the process of nutrient absorption, the *OmpA* protein has been shown to play a part in the ability of the bacterium to escape from host's immune system and in the bacterium's resistance to specific antimicrobial agents.(48). The presence of *OmpA* is necessary for the complete virulence of *A. baumannii*. Furthermore, it has been discovered that *OmpA* plays a role in the bacterium's ability to withstand specific antimicrobial agents, including beta-lactams and aminoglycosides (59,60,61). It is believed that the resistance is partially facilitated by *OmpA's* capacity to prevent the entry of said agents into the cell. The *OmpA* protein is a versatile protein that serves as a crucial component in the biology and pathogenicity of *A. baumannii*.

PER-1-type Extended-spectrum lactamase (ESBL) was first discovered for in 1993 in a *Pseudomonas aeruginosa* in France (77). It is responsible for conferring resistance to penicillins, cefotaxime, ceftibuten, ceftazidime, and the monobactam aztreonam, but it does

not contribute to resistance to carbapenems or cephamycins. Clavulanic acid has an inhibitory effect on its activity (59,60). In Turkey, the *bla*$_{\text{PER-1}}$ gene has been found in widespread populations of *Acinetobacter spp* (60,61). It has been reported that it location can be found either on the chromosome or on the plasmid (60,62). The production of *bla*$_{\text{PER-1}}$ by Gram-negative bacilli of clinical significance reduces the viability of antimicrobial chemotherapy as a treatment option.

3. MATERIALS AND METHODS

3.1. Equipment and Materials

- Autoclave (Hirayama, Japan)
- Biosafety cabinet (Heraeus, Germany)
- Vortex (Daihan, South Korea)
- Deep freezer (Arcelik, Turkiye)
- Thermal Cycler (Labnet, Finland)
- Automated pipettes (Eppendorf, Germany)
- Filtered pipette tip (Eppendorf, Germany)
- Petri dish (Firatmed, Turkiye)
- Sterile plastic core (Firatmed, Turkiye)
- Sterile cotton-tipped swab (Citoswab, China)
- Glass tube 16x100 mm (GenLab, Turkiye)
- Mc-Farland reader Bd phoenixspec nephelometer (phoenixspec, USA)
- 96 wells microplates (NUNC, Denmark)
- Sterile plastic microcentrifuge tubes (Genaxy, India)
- Elisa reader (USA)
- CHEF-DR III System Cihazı (Bio-RAD, ABD)
- Dry block heating thermostat (Biosan, TDB-100, Latvia)
- Centrifuge (Hettich, mikro 200R, Germany)
- Microwave (Arcelik, Turkiye)
- Sensitive electronic balance (Kern ABJ, Germany)
- Electrophoresis (Themo Scientirfic, USA)
- Quanta 400 environmental scanning electron microscope (ESEM) (USA)
- 6 wells (TPP, Switzerland)
- Electronic balance (Scoltec, ABD)
- Ultrasonic cleaner set (Daihan, WUC-D06h, Korea)
- Ph meter (Inolab, Germany)
- Power supply (Cleaver Scientific, United Kingdom)

3.2. Media and chemical

- 5% Sheep Blood Agar (Or-Bak, Turkiye)
- Triptych Soy Broth (CondaLab, Spain)
- EtBr (AppliChem, Germany)
- PCR Master mix (Thermos scientific, USA)
- Gel loading solution/ Bromophenol (SNP Biotechnology, Turkiye)
- Primers (Eurofine genomics ATGmbH, Oligonucleotide, Austria)
- EMB agar (CondaLab, Spain)
- PBS (wwr,international,llc,USA)
- Methanol 96% (Merck. Germany)
- Crystal violet 2%(Merck. Germany)
- Ethanol (Alkokim, Pakistan)
- Mueller Hinton Agar (CondaLab, madrid, Spain)
- Agarose (Sigma, USA)
- TBE (Sigma, USA)
- DNA ladder (Thermos scientific, USA)
- DMSO (CDH,sigma, USA)
- Distilled water (Polifarma, Turkiye)
- Glutaraldehyde (Merck, Germany)
- Cacodylic acid
- Glycine (Glisin, biorad medical, China)
- MTT thiazolyl blue tetrazolium bromide (Panreac, applichem, Germany)

3.3. Bacterial Isolates Identifications

Clinical *A. baumannii* strains (n=52) isolated from ICUS of Ankara Hospital of Baskent University between 2019-2021 were included in this study. They were cultured on 5% sheep blood agar and incubated for 24 hours at 37 °C (figure 3). A single streaking was performed of the bacteria twice for the same sample to activate the samples after extracting them from storage at -4 °C. Identification and antimicrobial susceptibility testing of the isolates against imipenem, meropenem, amikacin, gentamicin, levofloxacin, ciprofloxacin,

trimethoprim/sulfamethoxazole, tigecycline were performed by Phoenix automated identification and susceptibility testing system according to manufacturer instructions. Commercially available sensititer broth microdilution method was applied for colistin and all the 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 (EUCAST) criteria routinely (63). Isolate identification was confirmed by PCR by screening chromosomally encoded bla$_{OXA\text{-}51}$ *gene* in previous study.

Figure 3. Growing bacteria in the 5% sheep blood agar. Single-colony streaking method was applied and the plate was incubated at a temperature of 37 °C for 24 hours.

3.4. Biofilm Formation Assay

Biofilm imaging and quantification were performed by the crystal violet method as previously described with slight modifications (64,65). Each well of a flat-bottom 96-well polystyrene microplate was filled with 180 µL of tryptic soy broth (TSB) and 20 µL of *A. baumannii* suspension at ~1.0×10^7 CFU/mL. After incubation at 37°C for 24, 36,48 and 72 h (figure 4,a) (64), the plates were gently washed three times with 200 µL phosphate-buffered saline (PBS), after that 200 µL of methanol was added for 20 minutes for fixation. For the staining step, 200 µL of 0.2% crystal violet was applied for 15 minutes and plates were left at room temperature. After the plates were gently washed three times with PBS again, and 99% ethanol was used for the solubilization of the wells and the Optical Density (OD) of the supernatants in each well was measured using a microplate reader at 450 nm (figure 4, b). The average optical density of the negative control was measured and used for

the identification of biofilm-producing isolates, which were defined as the optical density of solubilized crystal violet in 99% ethanol was higher than the negative control. Each plate contained positive control and the OD value of positive control was used for the calculation of the average optical density of solubilized crystal violet for *A. baumannii* ATCC 19606. All experiments were performed in triplicate. In each plate, media alone, *A. baumannii* ATCC 19606 and *Staphylococcus aureus* ATCC 6538 (positive control), *Staphylococcus epidermidis* ATCC 29312 (negative control) were included (66,67).

The formula that was used to determine the optical density cut-off value (ODc) was as follows: The (ODc) value was determined to be three standard deviations (SD) above the mean of the optical density (OD) of the negative control: (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 follows:

I. strong biofilm producer ($4 \times ODc < OD$);
II. medium biofilm producer ($2 \times ODc < OD \leq 4 \times ODc$);
III. weak biofilm producer ($ODc < OD \leq 2 \times ODc$); and
IV. non-biofilm producer ($OD \leq ODc$)(68)

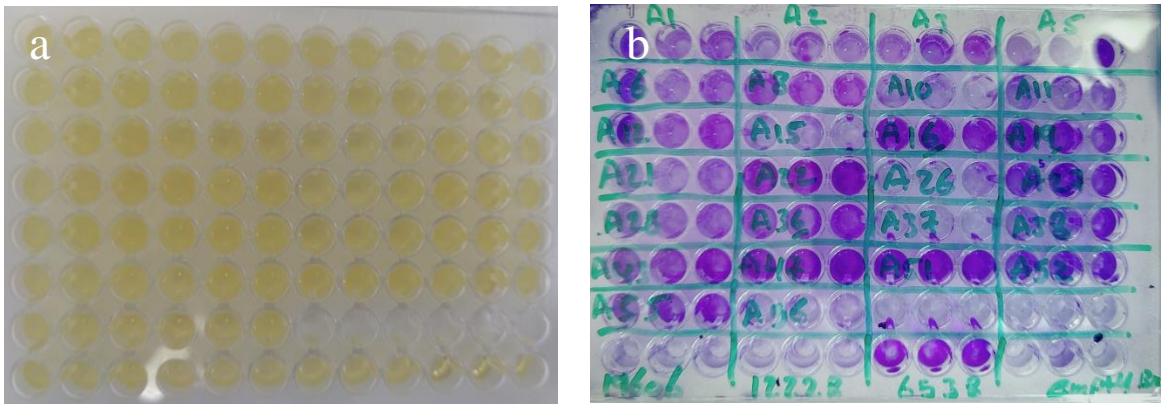

Figure 4. Biofilm formation assay in flat-bottom 96-well polystyrene microplate. The growth of bacteria and the formation of a biofilm in tryptic soy broth incubation for 36 hours at a temperature of 37°C **a.** before staining **b.** after staining with 0.2% crystal violet.

3.5. Biofilm Viability Assay

To determine the viability of microbial cells residing in the biofilm population, MTT method was used. MTT is a colorimetric method that can measure the reduction of a tetrazolium salt. Biofilms were formed in each well for 36 h and the wells were emptied. In

each well, 150 µL of PBS, and 50 µL of MTT solution (0.3% in PBS) were added and incubated for 2 h at 37°C. Then the wells were emptied again and MTT was replaced with 150 µL of DMSO and 25 µL of glycine buffer (0.1 M, pH 10.2). For the complete dissolving of formed purple formazan crystals, the plates were incubated for 15 min. at room temperature, with gentle agitation. The optical density of the wells containing biofilms was measured by a spectrophotometer (figure 5) (69).

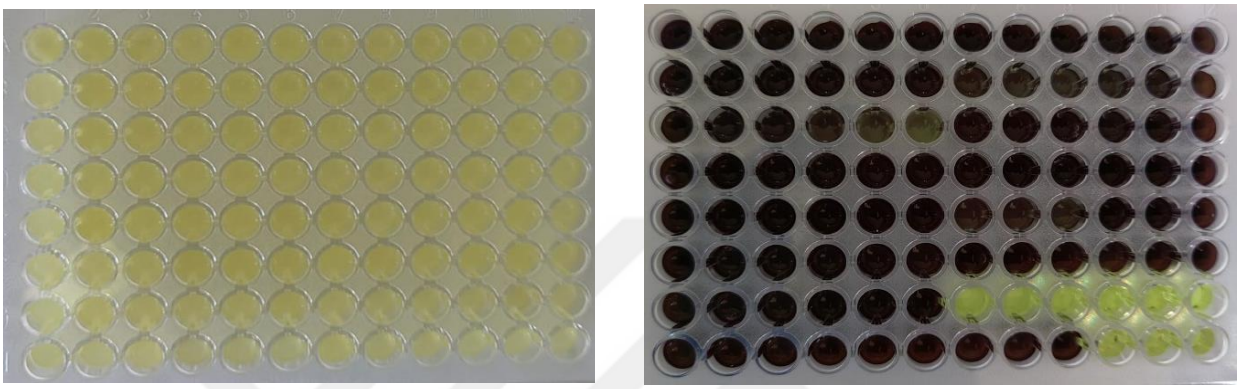

Figure 5. MTT assay. a. before staining, b. after staining with MTT the yellow wells had MTT without bacteria growth.

To obtain CFU, inoculate a pure culture of *A. baumannii* in tryptic soy broth and incubate it at 37°C for 36 hours. A series of dilutions were prepared for each isolate from 10^{-2} to 10^{-14} in 990 µL of TSB add 3.3 µL from each well for the same isolate it will be10^{-2}, then take 10 µL for the next tube that contained within 990 µL TSB it will be 10^{-4}. In the same way 10^{-6},10^{-8} was performed in this step 100 µL were taken from 10^{-8} to the next tube contained with 900 µL TSB to make10^{-9}, the same way was repeated for 10^{-10}, 10^{-12}, 10^{-14}, by taking 100 µL from each dilution and plotting it in tryptic soy agar plates by covering the entire surface of the agar with bacteria by streaking with a lube and incubated the plates in 37°C for 24 hours. The result can be obtained by counting the single colonies (figure 6).

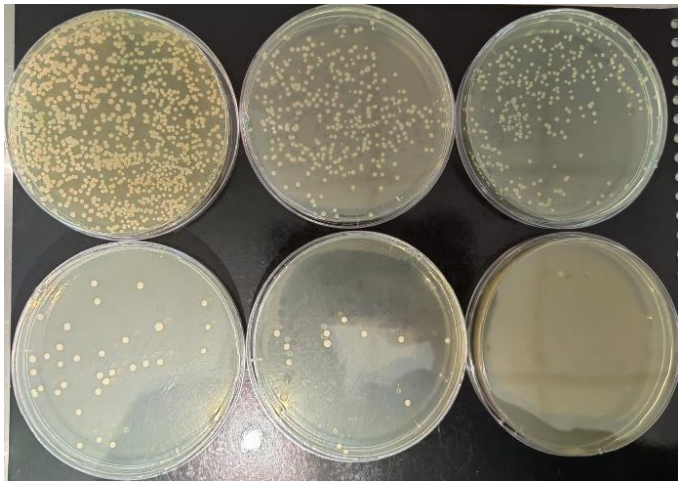

Figure 6. Colony counting to calculate the viable cells. The number of colony forming units on the countable plate were obtained by serial dilutions.

3.6. DNA Isolation

In a biosafety cabinet, fresh overnight cultures of *A. baumannii* isolates were used for DNA isolation. First, bacteria that had been stored in skim milk at a temperature of -20^{0}C were allowed to defrost, and then a single colony was cultured on 5% blood sheep agar. Following a period of 24 h of incubation at 37 ^{0}C, a single colony was extracted using a sterile disposable needle loop and then suspended in one milliliter of deionized water. After that, it was heated to 95 ^{0}C and boiled for 10 minutes. After that, it was cooled on ice for 10 minutes. In order to eliminate any debris left behind by the cells, the tubes were centrifuged at 14.000g for 10 minutes. After that, the supernatant was placed in a clean tube and kept at a temperature of -20 ^{0}C until the Polymerase Chain Reaction (PCR) study.

3.7. PCR-Based detection of Virulence Genes

PCR assays for detection of *bla*$_{\text{Csu E}}$, *bla*$_{\text{Omp A}}$, *bla*$_{\text{aba I}}$, *bla*$_{\text{pga A}}$, *bla*$_{\text{bap}}$ and *bla*$_{\text{Per 1}}$ genes were performed by a set of primers giving the 751bp, 594bp, 435bp, 460bp, 108bp, and 925bp product sizes respectively(52,70,71). PCR assays were performed using PCR Master Mix in a thermal cycler. PCRs were carried out in 25 µL reaction volume and consisted of 5µL of genomic DNA, 12.5 µL PCR Master Mix, Primers 2 µL and 5.5 µL H_2O.

The primers' sequences and the conditions for the PCR were:

***bla*$_{\text{Csu E}}$:**

Primer sequence (5 →3)

- CsuE-Forward; 5'- ACCAATGCTCAGACCGGAG-3'
- CsuE-Reverse; 5'- CTTGTACCGTGACCGTATCTTG-3'

***bla*$_{\text{Omp A}}$:**

Primer sequence (5 →3)

- OmpA-Forward; 5'- GAGTCGTATTGCACTTGCTAC-3'
- OmpA-Reverse; 5'- GCAGGCTTCAAGTGACCACC-3'

***bla*$_{\text{aba I}}$:**

Primer sequence (5 →3)

- abaI-Forward; 5'- AAAGTTACCGCTACAGGG-3'
- abaI-Reverse; 5'- CACGATGGGCACGAAA-3'

***bla*$_{\text{pga A}}$:**

Primer sequence (5 →3)

- pgaA-Forward; 5'- ATTCAAAAGTCAGTTGATGGGC-3'
- pgaA-Reverse; 5'- TTTTTTGTCCTTGCTCCAGC-3'

***bla*$_{\text{bap}}$:**

Primer sequence (5 →3)

- Bap-Forward; 5'- GAGGGAACTTCTGCAAAACTTTC-3'
- Bap-Reverse; 5'- CAGACGTATGACTGCATTGGT-3'

***bla*$_{\text{Per 1}}$:**

Primer sequence (5 →3)

- Per1-Forward; 5'- AATTTGGGCTTAGGGCAGAA-3'
- Per1-Reverse; 5'- ATGAATGTCATTATAAAAGC-3'

3.7.1. Amplifications Conditions

***bla*Csu E:**

İnitial denaturation: 95°C 10 minutes

35 cycles: 95°C 30 seconds, 59°C 30 seconds, 72°C 60 seconds,

Final elongation: 72°C 10 minutes.

***bla*Omp A:**

İnitial denaturation: 94°C 5 minutes,

35 cycles: 94°C 60 seconds, 57°C 60 seconds, 72°C 45 seconds

Final elongation: 72C° °C 5 minutes

***bla*aba I:**

İnitial denaturation: 94°C 5 minutes

30 cycles: 94°C 60 seconds, 52°C 30 seconds, 72°C 1:30 seconds

Final elongation: 72°C 5 minutes.

***bla*pga A:**

İnitial denaturation: 95°C 5 minutes,

30 cycles: 95°C 30 seconds, 55°C 30 seconds, 72°C 60 seconds

Final elongation: 72°C 5 minutes.

***bla*bap:**

İnitial denaturation: 95°C 5 minutes,

30 cycles: 95°C 30 seconds, 58°C 30 seconds, 72°C 60 seconds

Final elongation: 72°C 5 minutes.

***bla*Per 1:**

İnitial denaturation: 94°C 4 minutes,

35 cycles 94°C 60 seconds, 50°C 60 seconds, 72°C 45 seconds

Final elongation: 72°C 7 minutes.

Table 1. PCR reaction mix

PCR reaction mix	Csu E	PER 1	Omp A	Aba I	pga A	Bap
PCR master Mix (µl)	12.5	12.5	12.5	12.5	12.5	12.5
Primer (F+R) (10pmol)	2	2	2	2	2	2
DNA template (µl)	5	5	5	5	5	5
Water H_2O (µl)	5.5	5.5	5.5	5.5	5.5	5.5
Total Volume (µl)	25	25	25	25	25	25

3.8. Microscopic Analysis of Biofilm Formation Ability

The biofilm formation ability of *A. baumannii* isolates were visualized by scanning electron microscope (SEM). 1x1 cm sized square polystyrene materials were prepared to form biofilms on it. They were placed in 6-well cell culture plates and 200 µL of *A. baumannii* (0.5 McFarland) suspensions was inoculated into 1.8 ml of TSB into each well, and then incubated at 37°C for 36h. To remove any unattached and floating cells, the materials were washed twice with PBS, then 2.5% glutaraldehyde in 0.1 M cacodylic acid (pH 7.2) was used for fixation at 4 °C for 24 h. After that 0.1 M cacodylic acid was added for about 10 min at room temperature, and then plates were washed twice with distilled water for 15 min, followed by gradual dehydration with ethanol, and air dry for at least 24 h (figure 7). The fixed biofilms on square polystyrene materials were then coated with a layer of gold–palladium (7 nm thick) and visualized under the SEM (QUANTA 400F Field Emission SEM) in Middle East Technical University Center Laboratory (72–74).

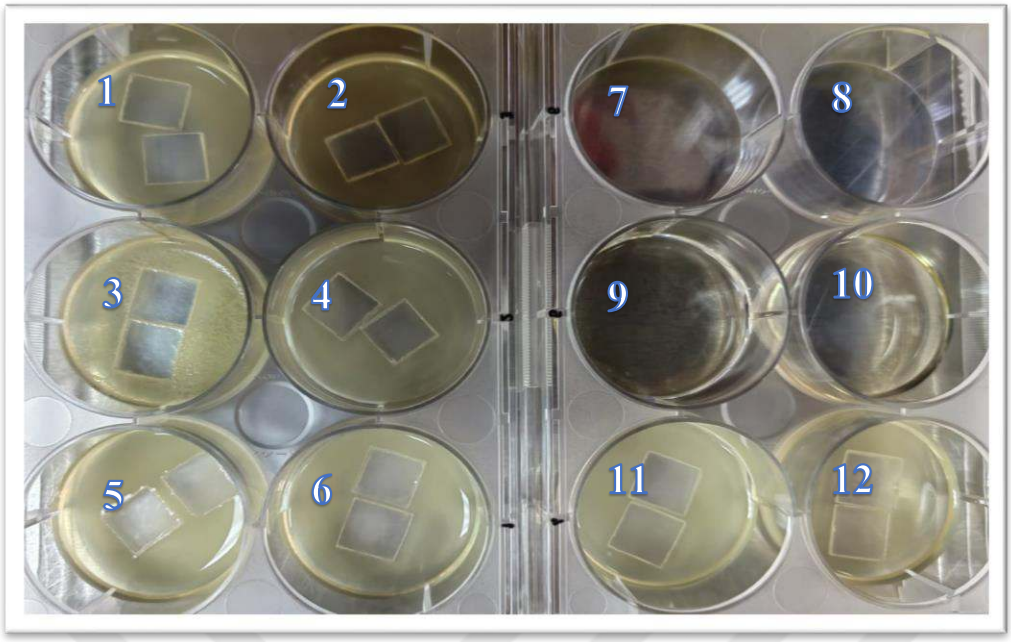

Figure 7. Preparing biofilm on 1x1 cm sized polystyrene materials.

4. RESULTS

4.1. Isolate Profile

Totally 52 *A. baumannii* isolated from the patients who were hospitalized in ICUs of Baskent University Ankara Hospital between 2019-2021 were included in the study. The 42.3% (n=22) of the tested strains were isolated from transtracheal aspirate and respiratory secretion, 26.9% (n=14) of them from blood culture, 11.5% (n=6) of them from wound and abscess, 7.6% (n=4) of them from urine culture, 7.6% (n=4) from drain culture, and 3.8% (n=2) from catheter culture.

According to antimicrobial susceptibility testing results, 100% (n=52) of the isolates were resistant to gentamicin, imipenem, meropenem, and amikacin. Colistin was the most effective antibiotic against the isolates that a 19.2% (n=10) resistance rate was observed. According to European Centre for Disease Prevention and Control (ECDC), all the isolates were defined as extensively drug-resistant (XDR) since they were non-susceptible to ≥1 agent in all but ≤2 categories (75).

4.2. Biofilm Formation Assay

The 96.1% of the total samples of *A. baumannii* produce a biofilm. For the other time points, it was described also that the biofilm formations for different time including 24h, 36h, 48h, and 72h it was compared to determine the optimal time of incubation for biofilm-related assays**.** Biofilm formation patterns of *A. baumannii* in different time points were given in figure 8. The optimal time was determined as 36 h. Compared to the other periods of incubation, the most appropriate time for biofilm formation was thirty-six hours for the biofilm to form completely, as well as to prevent the accumulation of toxic compounds produced by metabolic activities and consumption of nutrients. In forty-eight hours, as well as seventy-two hours, there is a need to change the medium every twenty-four hours to ensure the continuation of bacteria growth, but changing the media may cause a risk of losing the original colonies of bacteria, so thirty-six hours were selected as the appropriate incubation time for biofilm formation all experiments for all samples. When compared with *A. baumannii* ATCC19606, some samples produce stronger than this strain, as it was indicated in Table 2, the results of bacterial biofilm production, range from strong 46.1% (n=24), moderate 42.3 (n=22), weak 7.6% (n=4), non-biofilm produce 3.8% (n=2) (figure 9).

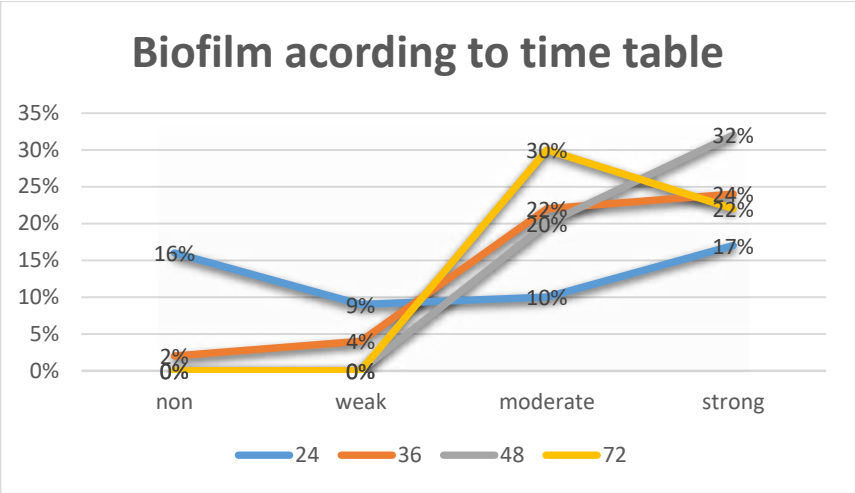

Figure 8. Biofilm formation pattern of *A. baumannii* in different time points

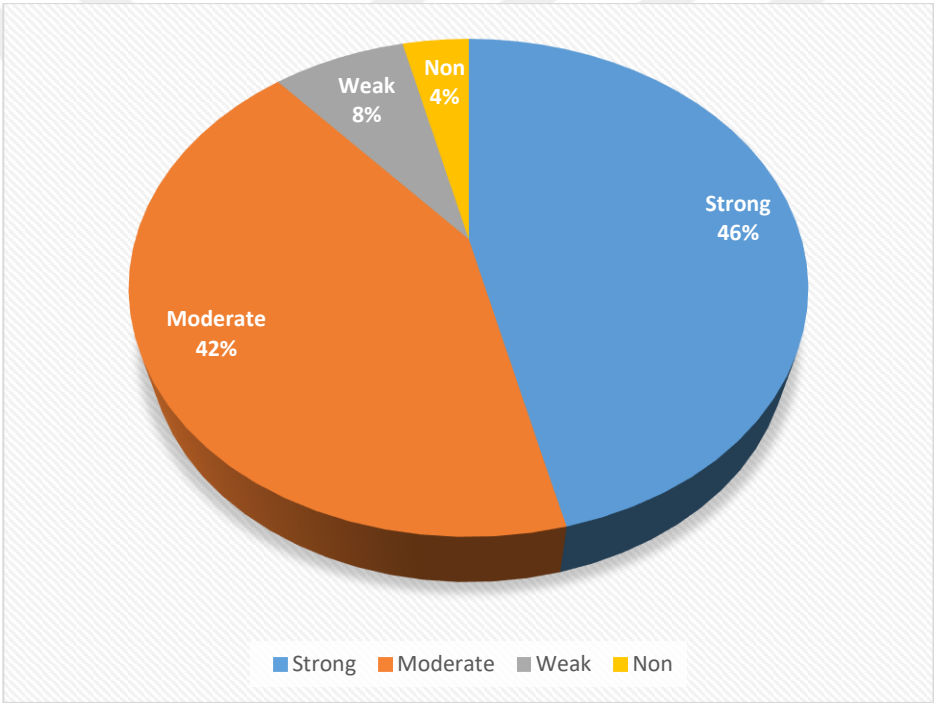

Figure 9. The biofilm formation results for 36 hours

4.3. 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. It was found that the genes are available in bacteria in varying proportions after examining the genes using electrophoresis, which gave percentages as shown in the table 2. The highest percentage of genes availability in samples

was for genes *bla*$_{bap,}$ *bla*$_{aba\ I}$ 98%. It is followed by the gene *bla*$_{Omp\ A}$ where it is 96.1%. *bla*$_{Csu\ E}$, where it is 94%. It is followed by gene *bla*$_{pga\ A}$ and *bla*$_{Per\ 1}$ in percentages of 50% and 40.3% respectively.

Table 2. Biofilm related gene presence rate in isolates and biofilm formation patterns

Genes (n)	Biofilm Structure n (%)			
	Non	Weak	Moderate	Strong
All isolate (52)	2 (3.8)	4 (7.6)	22 (42.3)	24 (46.1)
***bla*$_{Csu\ E}$** (49)	2 (4.1)	4 (8.1)	21(42.8)	22 (44.8)
***bla*$_{Omp\ A}$** (50)	2 (4.0)	4 (8.0)	21(42.0)	23 (46)
***bla*$_{aba\ I}$** (51)	2 (3.9)	4 (7.8)	22 (43.1)	23 (45.1)
***bla*$_{pga\ A}$** (26)	0 (0)	2 (7.6)	14 (53.8)	10 (38.4)
***bla*$_{bap}$** (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)

Only samples that carry genes and make biofilm were taken and placed in table 3 with a comparison with those that do not make biofilm to show the difference in proportions between them without looking at its thickness.

Table 3. Biofilm formation rates in the presence and absence of the biofilm related genes

Genes	PCR	Biofilm Formation	
		positive n (%)	negative n (%)
***bla*$_{Csu\ E}$**	positive	47(95.9)	2 (4.0)
	negative	3 (100)	0 (0)
***bla*$_{Omp\ A}$**	positive	48(96)	2(4)
	negative	2(100)	0(0)
***bla*$_{aba\ I}$**	positive	49(96)	2(3.9)
	negative	1(100)	0(0)
***bla*$_{pga\ A}$**	positive	26(100)	0(0)
	negative	24(92.3)	2(7.6)
***bla*$_{bap}$**	positive	49(96)	2(3.9)
	negative	1(100)	0(0)
***bla*$_{Per\ 1}$**	positive	19(90.4)	2(9.5)
	negative	31(100)	0(0)

Through genetic examination of bacteria and by tracking the genetic sequences of each gene as shown in the pictures figures 10,11,12,13,14,15.

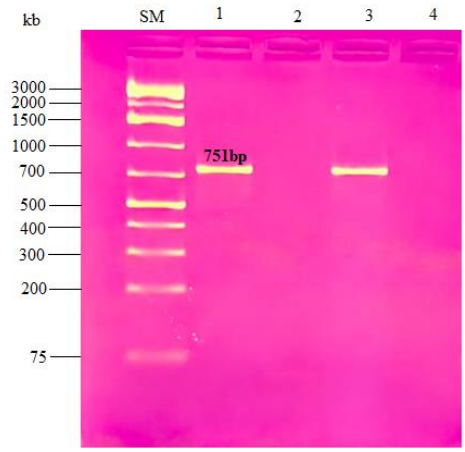

Figure 10. Agarose gel electrophoresis for ***bla***$_{\text{Csu E}}$ gene *SM =DNA ladder 1kb, 1. positive control, 2. negative control, 3. positive sample, 4. negative sample.

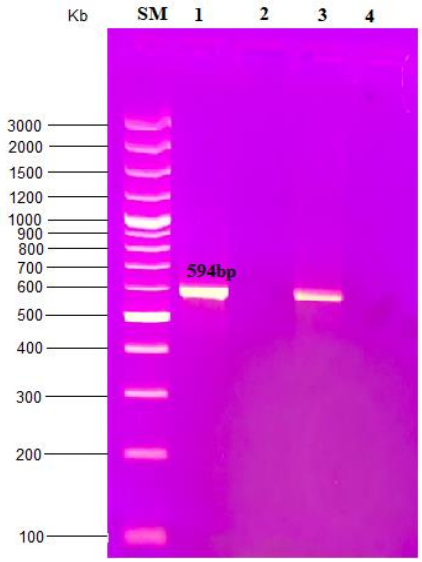

Figure 11. Agarose gel electrophoresis for ***bla***$_{\text{Omp A}}$ gene *SM =DNA ladder 100kb, 1. positive control, 2. negative control, 3. positive sample, 4. negative sample.

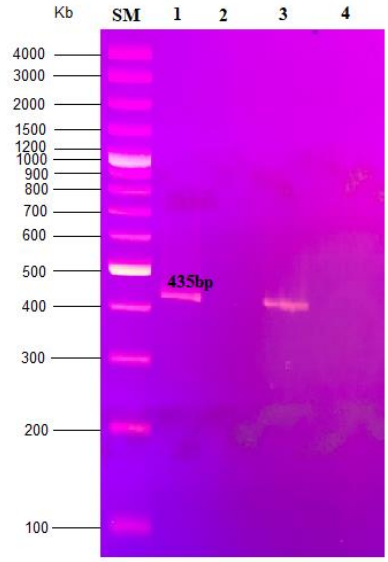

Figure 12. Agarose gel electrophoresis for ***bla*$_{\text{aba I}}$** gene *SM =DNA ladder 100kb, 1. positive control, 2. negative control, 3. positive sample, 4. negative sample.

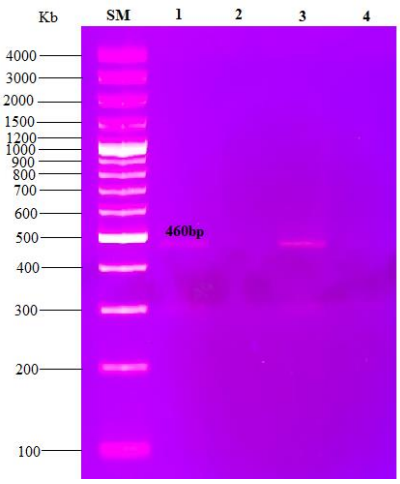

Figure 13. Agarose gel electrophoresis for ***bla*$_{\text{pga A}}$** gene *SM =DNA ladder 100kb, 1. positive control, 2. negative control, 3. positive sample, 4. negative sample.

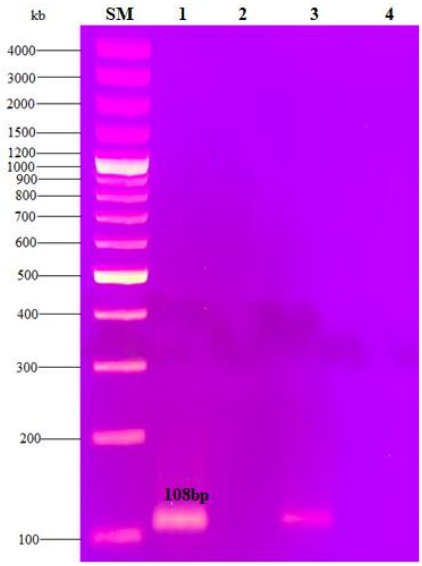

Figure 14. Agarose gel electrophoresis for ***bla***$_{\mathbf{bap}}$ gene *SM =DNA ladder 100kb, 1. positive control, 2. negative control, 3. positive sample, 4. negative sample.

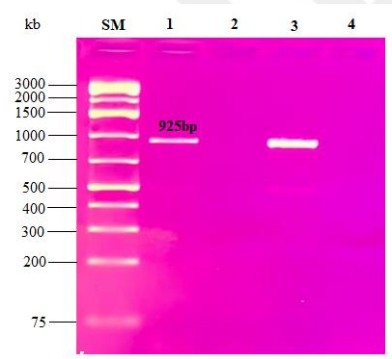

Figure 15. Agarose gel electrophoresis for ***bla***$_{\mathbf{Per\ 1}}$ gene *SM =DNA ladder 1kb, 1. positive control, 2. negative control, 3. positive sample, 4. negative sample.

4.4. Biofilm Viability Assay

The number of CFU was calculated for different MTT values obtained from biofilm viability assay ant the CFU values representing the ODs were given in table 4. By using these values, the regression curve was drawn and the formulation were found as Y = 1,441X + 8,582 as shown in figure 16 (R^2=0.9468). From this formula, the OD_{450nm} value of each isolates from MTT assay the CFU was calculated for each isolate. The percentage for viable cells within the biofilm was also calculated (table 5).

Table 4. CFU values for each OD values obtained from MTT assay and colony counting technique.

Isolate n	MTT	CFU	log10
EMPTY 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

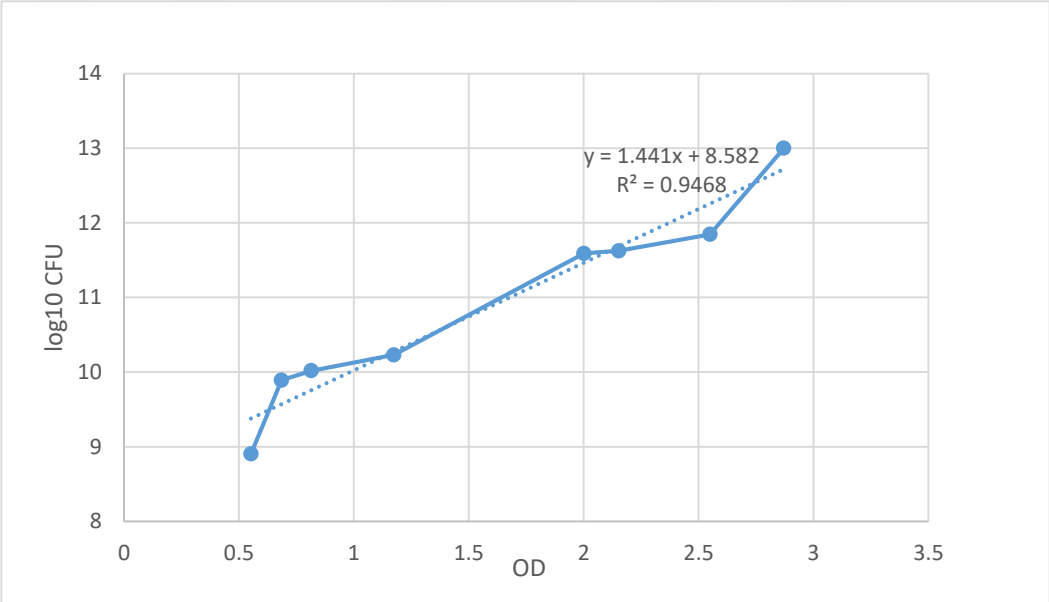

Figure 16. Regression curve. OD_{450nm} of MTT assays to Log_{10} CFU

By using Y = 1,441X + 8,582 formula the OD values from MTT assays were used to calculate the viable cells within the biofilm for each isolate table 5. The viability percentages were between 13.5% to 112.4%. It was shown that the number of viable cell and the percentage of viable cell of biofilm were high. The number of CFU were found between $8.0x10^8$ to $5.2x10^{12}$.

Table5. MTT assay results. CFU values and viability rates of each isolate.

isolate n	OD MTT	CFU	Viability(%)
A1	2.01	3.016E+11	77.1
A2	0.83	5.978E+09	28.5
A3	1.13	1.639E+10	41.0
A5	2.15	4.831E+11	82.9
A6	1.94	2.372E+11	74.1
A8	1.80	1.509E+11	68.5
A10	Non biofilm former		
A11	1.23	2.284E+10	45.1
A12	1.03	1.157E+10	36.7
A15	Non biofilm former		
A16	1.65	9.042E+10	62.2
A19	1.93	2.323E+11	73.8
A21	2.73	3.277E+12	106.6
A22	2.31	8.232E+11	89.5
A26	2.42	1.183E+12	94.0
A27	2.38	1.020E+12	92.2
A28	2.39	1.077E+12	92.8
A36	2.50	1.529E+12	97.2
A37	0.82	5.707E+09	27.9
A38	2.05	3.444E+11	78.7
A43	2.22	6.080E+11	85.8
A47	2.03	3.244E+11	78.0
A51	2.22	6.120E+11	85.8
A52	1.54	6.333E+10	57.7
A55	1.17	1.882E+10	42.7
A56	2.41	1.142E+12	93.6
A57	2.00	2.924E+11	76.7
A69	1.29	2.797E+10	47.6
A70	1.24	2.374E+10	45.6
A75	2.55	1.803E+12	99.2
A76	1.48	5.253E+10	55.4
A77	1.24	2.374E+10	45.6
A80	2.87	5.237E+12	112.4
A82	1.00	1.063E+10	35.6
A83	2.47	1.400E+12	96.1
A84	1.07	1.327E+10	38.4
A85	1.15	1.727E+10	41.7
A88	2.80	4.138E+12	109.5
A91	1.22	2.169E+10	44.5
A92	1.82	1.586E+11	69.1

A93	1.03	1.175E+10	36.9
A97	1.01	1.073E+10	35.8
A100	2.03	3.201E+11	77.8
A102	1.11	1.509E+10	40.0
AU32	1.86	1.850E+11	71.0
AU36	1.62	8.168E+10	60.9
AU37	2.56	1.842E+12	99.5
AU38	2.62	2.255E+12	102.0
AU40	2.43	1.220E+12	94.4
AU54	0.83	6.018E+09	56.7
AU55	2.49	1.477E+12	96.7
AU58	0.47	1.791E+09	13.5

4.5. Microscopic Analysis of Biofilms Formation Ability

Through microscopic examination, it can be said that the formation of biofilms and the resistance of bacteria to external conditions due to this feature. The bacteria pass through four stages until they reach the formation of biofilm capable of protecting bacteria from any external influence. It was indicated that these steps include attachment/colonization, irreversible adhesion, maturation of biofilm and detachment and dispersal of biofilm cells. During the initial stage, referred to as attachment/colonization (figure 17a-c), planktonic microorganisms encounter a conditioned surface via either random movement or chemotaxis. The process of reversible attachment is subject to the influence of various physicochemical variables. During the second stage, there is an irreversible adhesion process where bacteria express adhesion proteins and generate a polymeric matrix called extracellular polymeric substances (EPS) (figure 17d-f). The matrix serves as a protective barrier and promotes the development of micro-colonies. The process of biofilm maturation entails a rise in both density and intricacy, as surface-adhered bacteria undergo replication and engage with their surroundings to generate a glycocalyx (figure 17g-i). Biofilm survival and spreading are facilitated by a range of processes, including quorum sensing, gene transfer, and persister development. Over time, the biofilm experiences detachment and dispersal as aging cells separate and establish themselves in fresh regions. Detachment can be influenced by various factors, including fluid dynamics and shear effects (figure 17j-l). This mechanism facilitates the dispersion of the biofilm and initiates a fresh cycle (76)

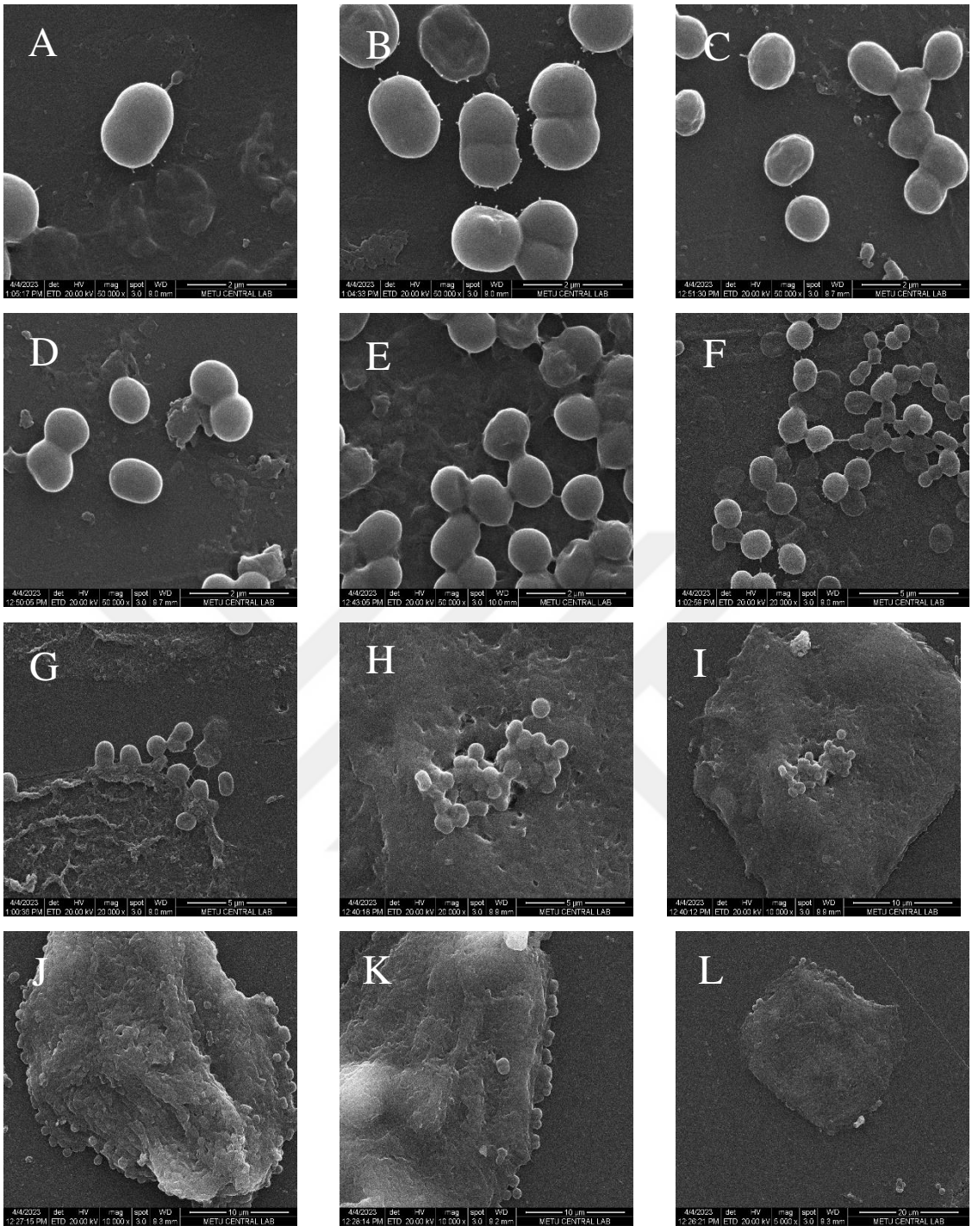

Figure 17. Biofilm stages**,** A-C.attachment/colonization, D-F.arriversible attachment, G-I.maturation, J-L.Detachment

Furthermore, through detection by using the scanning electron microscope (SEM), it was found that the strains that contain the strong biofilm have large clusters of cells, but in the strains that produce a moderate biofilm, the clusters are less and are almost insignificant

in the strains that produce the biofilm weakly and the lack of cell clustering in Strains that do not produce a biofilm, according to the pictures in figure 18.

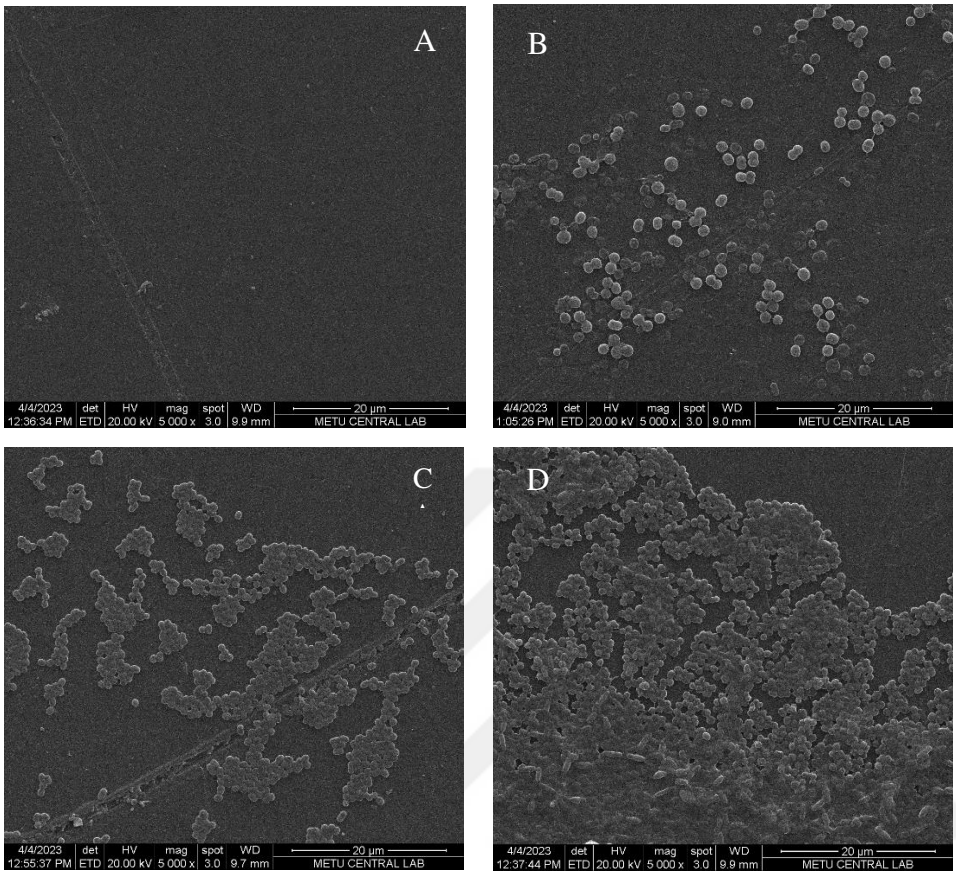

Figure 18. Biofilm patters by SEM Analysis. A. non-biofilm formation; B. weak biofilm formation; C. moderate biofilm formation, strong biofilm formation; D. strong biofilm formation

5. DISCUSSION

Acinetobacter baumannii is a Gram-negative, opportunistic pathogenic bacterium belonging to the genus *Acinetobacter*. It is associated with hospital-acquired infections and is known for its multidrug-resistant profile making it a significant concern in hospital settings (3). Due to the association of this bacteria with hospital infections, it has become a common pathogen and is increasing daily due to its ability to survive in difficult conditions within these environments. This resistance made it one of the most important causes of diseases in intensive care units. The antimicrobial resistance mechanisms of *A.baumannii* have been studied widely and they have reported the contribution of the spread of antimicrobial resistance genes. On the other hand, the ability of surviving of *A.baumannii* on many surfaces is an important factor that prevents the bacterium from being eradicated from hospital settling. At this point, the biofilm-forming ability and the biofilm characteristics of *A. baumannii* need to be widely determined since biofilm formation is one of the most important factors in both antimicrobial resistance and survival of the bacterium in hospitals (77).

A. baumannii can cause a variety of infections, including pneumonia, meningitis, and sepsis. One of the key factors that contribute to its pathogenicity is its ability to form biofilms. Biofilms are structured communities of bacteria that are attached to a surface. They are difficult to treat with antibiotics because they are protected from the immune system and from the effects of antibiotics. According to this study 50 of the 52 isolates of *A.baumannii* from ICUs have the ability to form biofilm. Among them 24 (46.1%) of the isolates produced strong biofilm, 22 (42.3%) produces intermediate, 4 (7.6%) weak, and 2 (3.8%) are non-biofilm former which makes the total percentage of samples forming biofilms 96.1% (figure 9). This result closely aligned with the percentage studied in a previous research (72) on biofilm formation with a score range of 15-45%. However, our study differs in that we incubated samples for a period of thirty-six hours in all steps, and the number of isolates varied from the previous study. Compared to the other periods of incubation in which the biofilm was compared, the most suitable time for biofilm formation was thirty-six hours for the biofilm to form completely, as well as to prevent the accumulation of toxic compounds produced by metabolic activities and consumption of nutrients. In forty-eight hours, as well as seventy-two hours, there is a need to change the medium every twenty-four hours to ensure the continuation of bacteria growth, but changing the media may cause a risk of losing

the original colonies of bacteria, so thirty-six hours were selected as the appropriate incubation time for biofilm formation all experiments for all samples (figure 8).

Biofilms are formed by passing through three stages, first stage the bacteria attach to a surface using a variety of extracellular organelles such as flagella, pili, fimbriae and proteins (OMP). The genes chaperon-usher pilus *bla*$_{\text{Csu E}}$**,** and the outer membrane proteins *bla*$_{\text{Omp A}}$ play important roles in this step, this stage called reversible attachment. The next stage the irreversible attachment, in this stage the bacteria aggregate to form micro colonies and produce EPS. EPS serves as an adhesive matrix that facilitates bacterial aggregation and shields them from external factors. The genetic element known as the quorum sensing gene, *bla*$_{\text{aba I}}$, which encodes proteins, is known to exert significant influence in this particular stage. The final phase entails the maturation of the biofilm. During this phase, the biofilm undergoes maturation and develops heightened resistance against both the immune system and antibiotics. The gene *bla*$_{\textbf{bap}}$, which is associated with biofilm, is a crucial factor in this particular stage. The co-occurrence of genes associated with biofilm formation in bacterial isolates that exhibit both biofilm-forming and non-biofilm-forming phenotypes suggests that these genes may be implicated in other physiological pathways beyond the formation of biofilms. The gene *bla*$_{\textbf{pga A}}$ is responsible for encoding genes that participate in the synthesis of PNAG, a crucial constituent of bacterial cell walls. The process of biofilm formation is a multifaceted phenomenon that is subject to a range of factors, encompassing bacterial taxonomy, substrate characteristics, and ambient conditions. The objective of this investigation was to assess the existence of genes associated with biofilm formation and the genetic basis of biofilm formation in *A. baumannii* isolates obtained from ICUs.

Understanding the things that control biofilm formation is important for coming up with new ways to prevent and treat infections that are caused by biofilms (70). In this study, the link between the presence of *bla*$_{\text{Csu E}}$, *bla*$_{\text{OmpA}}$, *bla*$_{\text{aba I}}$, *bla*$_{\textbf{pga A}}$, *bla*$_{\textbf{bap}}$, and *bla*$_{\text{Per 1}}$ genes and the formation of biofilms was looked into. The goal was to find out about the genetic background of biofilm formation in *A. baumannii* from ICUs and the structural characteristics of formed biofilm. Previous studies looked at the link between genes and the way bacteria form biofilms. The most important genes include *bla*$_{\text{Csu E}}$, *bla*$_{\text{OmpA}}$, *bla*$_{\text{aba I}}$, *bla*$_{\textbf{pga A}}$, *bla*$_{\textbf{bap}}$, and *bla*$_{\text{Per 1}}$.(78).

The *bla*$_{\text{Csu E}}$ gene, chaperone–usher assembly system expression, is responsible for the ability to stick, bind, and form biofilms on surfaces under the influence of different

conditions and cellular morphology. Studies have also shown a link with a genetic group that encodes and secretes bacterial sites, capillary production, and capillary assembly functions that contribute to the formation of the early stage of biofilms (78,79). The 94% of the studied isolates carried *bla*$_{\text{Csu E}}$ gene. The was a correlation with other studies such as Habib et al., who reported 100% of the *A. baumannii* isolate carrying the same gene in which the isolates were more susceptible (80). Hui liu et al. reported only 59.8% positive results. However, they studied 122 isolates 54.9% only from ICUs (81).

Porins are proteins that form channels through the outer membrane of bacteria. OmpA is a porin protein in *A. baumannii* that is involved in nutrient uptake, it has a selective channel, allowing the passage of molecules with a certain size and charge. OmpA is located in the outer membrane of *A. baumannii*, where it plays a critical role in the bacterium's ability to survive and grow in different environments. OmpA has been shown to be involved in the bacterium's ability to evade the host immune system, as well as its resistance to certain antimicrobial agents. Several studies have investigated the role of OmpA in the biology and pathogenicity of *A. baumannii*. The OmpA is required for the full virulence of *A. baumannii* and also give the ability to adhesion of bacterial cells to epithelial cell and invasion, biofilm development, serum resistance, surface motility, and induction of apoptosis in host cells or induces cytotoxicity (48,78,82). The 96.1% from the studied isolates carried *bla*$_{\text{OmpA}}$ gene. Our result is close to Mona et al. reported in their study totally 70 samples carried the *bla*$_{\text{OmpA}}$ gene was 98.4% (70). It can be concluded that *bla*$_{\text{OmpA}}$ gene occurrence is high in *A.baumannii* isolates.

The *bla*$_{\text{aba I}}$ gene is a quorum-sensing gene in *A. baumannii*. Quorum sensing is a process by which bacteria communicate with each other to coordinate their activities. In the case of *A. baumannii*, the *bla*$_{\text{aba I}}$ gene is responsible for the production of an autoinducer molecule, which is a small molecule that signals to other bacteria that the population density is high. When the autoinducer molecule reaches a certain concentration, it triggers a cascade of events that lead to biofilm formation (78). In this study, the result of detecting the gene was 98.0%, which makes it close to a previous study conducted for the same gene. It was available in all samples 100% (83) which was referring to the high percentage of the gene in samples that form biofilms.

The *bla*$_{\text{pga A}}$ gene is a biofilm-associated gene in *Acinetobacter baumannii*. It is responsible for the production of PNAG, a major component of the biofilm matrix. PNAG

is a sticky substance that helps to hold the bacteria together and to protect them from the environment. PNAG is involved in immune evasion and host-microbe interactions. PNGA has a role in the formation of biofilm, cell-cell adherence as well as protection against innate host defenses (48). The 50% of the isolates contain this gene, This result is not consistent with the other study it was 97.1% (70) because the samples for which the detection of the gene was conducted were all highly sensitive to antibiotics, as well as their high ability to produce a biofilm. The reason is also due to the different places where the samples were collected, as the samples were from different countries.

The *bla*$_{bap}$ gene is a multidomain protein that has been shown to play a role in a variety of biofilm-related processes, including attachment to surfaces: *bla*$_{bap}$ helps *A. baumannii* cells to attach to surfaces, which is a necessary first step in biofilm formation. Cell-cell adhesion: *bla*$_{bap}$ helps *A. baumannii* cells to adhere to each other, which helps to form the three-dimensional structure of the biofilm. EPS production: *bla*$_{bap}$ is involved in the production of extracellular polymeric substances (EPS), which is a sticky substance that helps to hold the biofilm together and to protect it from the environment. The process of biofilm maturation entails the development of a more intricate and structured entity, wherein *bla*$_{bap}$ plays a significant role. Apart from its function in the formation of biofilms, the bap gene has demonstrated its involvement in the pathogenicity of *A. baumannii*. The aforementioned proposition implies that directing interventions towards the bap gene could potentially serve as a viable approach in the prevention and management of *A. baumannii* infections (56,78). The result for the *bla*$_{bap}$ gene in our isolates was 98%. This does not bring our results anywhere near those of a study that was carried out in Iran that showed 48.43%. That study focused on collecting samples from people who were less than fifty years old. In addition, the detection of the gene was only limited to samples that had high biofilm formation (84).

The *bla*$_{Per\ 1}$ gene is a member of the extended-spectrum class of A β-lactamases Numerous studies have been done on it, leading researchers to believe that it is connected to the development of biofilms in bacterial populations(72). When compared to the other genes, the isolating process resulted in the lowest percentage of detectable copies of this gene due to the fact that it was only present in forty percent (40%) of the total number of samples. 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 only 2% carried the gene in the total number of 100 isolates (80).

The present investigation employed the MTT assay to assess bacterial viability. The results indicate that the bacteria exhibited robust survival and growth, as evidenced by the high percentage of bacterial availability following a 36-hour incubation period (figure 16). This finding is in line with a prior investigation, wherein it was observed that in the absence of any growth inhibitor, the bacteria would persist in their growth and viability(85).

By observing the formation of biofilms under an electron microscope, it was found that the manifestation of bacterial-associated elements during the process of biofilm formation is contingent upon the accessibility of nutrients, the detection of environmental cues facilitated by sensor kinases such as BfmS, intercommunication with other kinases, and the phosphorylation of response regulators such as BfmR at the substrate level. It is plausible that surface proteins, such as a homolog of Bap, could potentially serve as a stabilizing factor for biofilms on diverse surfaces. The adhesion and biofilm formation of *A. baumannii* can be augmented by various factors such as the existence of metal cations and the ability to resist broad-spectrum antibiotics. Nevertheless, the precise mechanisms underlying adhesion to diverse surfaces and host cells remain ambiguous (figure 17). This is consistent with a previous study conducted to detect biofilm formation stages using the electron microscopy technique (15)

The microscopic examination also showed us something that is consistent with the results of the biofilm formation of the samples. The result of the examination showed the extent of the thickness of each sample as strong, moderate, weak, or non-biofilm-forming, as well as the extent of the connection between the surface and the cells to each other. This examination also showed us something that is consistent with the results of the biofilm formation of the samples (figure 18).

In this thesis, the focus has been on the structural characteristics and genetic background for formation of biofilms of *A.baumanni* isolates from ICUs.

6. CONCLUSION

The present investigation aimed to examine the genetic background and structural characteristics of *A.baumannii* isolates isolated from clinical samples of patients hospitalized in Intensive Care Units (ICUs) of Baskent University Ankara Hospital with respect to their biofilm formation potential. The findings indicate that a significant proportion of the isolates, specifically 96.1%, exhibited the ability to generate biofilms. The genetic markers *bla*$_{\text{Csu E}}$, *bla*$_{\text{OmpA}}$, *bla*$_{\text{aba I}}$, *bla*$_{\text{pga A}}$, *bla*$_{\text{bap}}$, and *bla*$_{\text{Per 1}}$ were identified as being linked to the process of biofilm formation. The related genes are implicated in diverse biofilm-associated mechanisms, encompassing adhesion to surfaces, intercellular adhesion, extracellular polymeric substance synthesis, and biofilm development. The identification of these genes in *A. baumannii* isolates implies their significant involvement in the bacterium's biofilm formation capability. Biofilms pose a significant challenge in clinical environments, as they can enhance bacterial resistance to antibiotics and impede their eradication. The determination of the genetic components implicated in the formation of biofilms in *A. baumannii* constitutes a significant advancement in the pursuit of novel approaches to mitigate and manage *A. baumannii* infections. Moreover, an elevated incidence of biofilm production in *A. baumannii* isolates obtained from ICUs is a matter of apprehension, given that biofilms can augment bacterial resistance to antibiotics and impede their eradication. The evaluation of these genes implicated in the formation of biofilm in *A. baumannii* isolated represents a significant advancement in the pursuit of novel approaches to the prevention and management of *A. baumannii* infections.

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.

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