NEAR EAST UNIVERSITY

INSTITUTE OF GRADUATE STUDIES

DEPARTMENT OF MEDICAL MICROBIOLOGY AND CLINICAL MICROBIOLOGY

EVALUATION OF BIOFILM FORMATION AND DETECTION OF EXOPOLYSACCHARIDE ENCODING GENES AMONG *PSEUDOMONAS AERUGINOSA* CLINICAL ISOLATES AT NEAR EAST UNIVERSITY HOSPITAL

M.Sc. THESIS

Batur ÖZLER

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Advisor

Assoc. Prof. Buket BADDAL

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Approval

We certify that we have read the thesis submitted by Batur ÖZLER titled "**Evaluation of Biofilm Formation and Detection of Exopolysaccharide Encoding Genes Among *Pseudomonas aeruginosa* Clinical Isolates At Near East University Hospital**" and that in our combinedopinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Educational Sciences.

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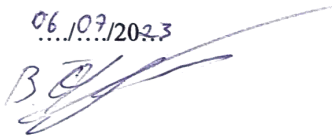
Declaration

I hereby declare that all information, documents, analysis and results in this thesis have been collected and presented according to the academic rules and ethical guidelines of Institute of Graduate Studies, Near East University. I also declare that as required by these rules and conduct, I have fully cited and referenced information and data that are not original to this study.

Batur ÖZLER

06/07/2023



Acknowledgments

It’s a relief and also cause of huge happiness to make my first step into academic field. I’m quite lucky to have Dr. Buket by my side for this journey. She’s a great mentor and a real teacher who enjoys hardworking and letting her students to enjoy science as far as I see. Thank you so much for everything.

I’d also like to thank my lab partner Afnan. We handled many difficulties together as much as we had fun together. Thank you for being there for me when I needed. I’m also very thankful to my family and friends for being supportive to me all this time.

Abstract

Evaluation of Biofilm Formation and Detection of Exopolysaccharide Encoding Genes Among *Pseudomonas aeruginosa* Clinical Isolates At Near East University Hospital

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M.Sc., Medical Microbiology and Clinical Microbiology Department

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May, 2023, 40 pages

Pseudomonas aeruginosa is a common pathogen with an increasing multi-drug resistance phenotype. Major virulence factors of the bacterium include antimicrobial resistance and biofilm formation which correlate with disease severity. The aim of this study was to investigate biofilm formation capacity and and antibiotic resistance rates of *P. aeruginosa* clinical strains as well as to investigate the frequency and distribution of exopolysaccharide-encoding gene *algD* among the strains. A total of 100 clinical *P. aeruginosa* isolates were obtained from clinical specimens and identified using standard microbiological techniques. Antimicrobial susceptibility was assessed using the VITEK-2 system and biofilm quantification was measured using the quantitative crystal violet assay. The presence of the *algD* was evaluated using conventional polymerase chain reaction. Among isolates, 35% were strong-biofilm former, 28% was moderate-biofilm former, 19% was weak-biofilm former and 18% was non-biofilm former. The rates of multi-drug resistance (MDR) and extensive drug-resistance (XDR) were 27% and 1%, respectively. PCR analysis indicated that 93% of the isolates were *algD* positive. The *algD*-positive genotype was associated with moderate/high biofilm formation ($p<0.05$). This study reports the first data-set on the molecular profile of *P. aeruginosa* in Cyprus. Our results showed that most of the strains have the biofilm forming capacity with an *algD*-positive genotype.

Key Words: *Pseudomonas aeruginosa,* biofilm, virulence, antibiotic resistance

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List of Abbreviations

AL: Acute leukemia

AST: Antimicrobial susceptibility testing

BSI: Bloodsteam infections

CAP: Community-acquired pneumonia

CF: Cystic Fibrosis

CO_2: Carbon dioxide

DNA: Deoxyribonucleic acid

EDTA: Ethylenediaminetetraacetic acid

ELISA: Enzyme-linked immunosorbent assay

EMB: Eosine methylene blue

EPS: Extracellular polymeric substences

GDP: Guanosine diphosphate

HAI: Healthcare associated infections

HSCT: Hemapoetic stem cell transplantation

HTH: Helix-turn-helix

ICU: Intensive care unit

MDR: Multi-drug resistant

OD: Optical density

PA: *Pseudomonas aeruginosa*

PCR: Polymerase chain reaction

RNA: Ribonucleic acid

RT-PCR: Real time polymerase chain reaction

TBE: Tris-borate EDTA

TCS: Two component system

UV: Ultraviolet

WHO: World Health Organization

XDR: Extensively-drug resistant

CHAPTER I

Introduction and Aims

1. Aims and Scope

Pseudomonas aeruginosa is a gram-negative, aerobic, non-spore forming rod shaped bacterium that is capable of causing a variety of infections in both immunocompetent and immunocompromised hosts. Its predilection to cause infections among immunocompromised hosts, extreme versatility, antibiotic resistance and a wide range of dynamic defenses makes it an extremely challenging organism to treat in modern-day medicine (Wilson & Pandey, 2022).

P. aeruginosa is a major threat to human health and was identified as one of the most urgent priority pathogens requiring research and development (R&D) of novel antibiotics by the World Health Organization in 2017. The organism's high intrinsic antibiotic resistance and ability to adapt to antimicrobial agents have led to limited efficacy of common antimicrobial agents, resulting in increased mortality rates. Additionally, the capacity of *P. aeruginosa* to form biofilms contributes to its ability to evade host defenses, promote colonization, and persist long-term, making treatment of infections difficult. This biofilm formation is facilitated by quorum sensing, an effective cell-to-cell communication system within *P. aeruginosa* microbial communities (Thi, et al., 2020).

Most bacteria in nature have the ability to adhere to different surfaces and create biofilms. These biofilms are complex colonies of bacteria enclosed within a self-produced extracellular polymeric substance (EPS) matrix, and they are one of the main tactics that species use to endure changes in conditions such as temperature fluctuations and nutrient availability. Within a biofilm, bacteria are capable of evading host immune responses and can be up to 1000 times more resistant to antimicrobial treatments than their planktonic counterparts. *P. aeruginosa* is a well-known organism for forming biofilms, which makes it a good model for studying biofilm formation. A sturdy biofilm is a crucial tool for *P. aeruginosa* to succeed, survive and dominate in the polymicrobial niche of particularly the cystic fibrosis (CF) lung. Additionally, *P.*

aeruginosa effectively colonizes a range of surfaces including medical devices (urinary catheters, implants, contact lenses) and equipment used in the food industry (vats, mixing tanks and tubing) (Wilson & Pandey, 2022).

P. aeruginosa biofilms consist of three main exopolysaccharides: alginate, Psl, and Pel. Among these, alginate is mainly produced by clinical isolates of *P. aeruginosa*. Alginate is a linear polymer composed of β-d-mannuronic acid and α-l-guluronic acid and plays a vital role in the structural stability and protection of the biofilm. The algACD operon regulates alginate synthesis in *P. aeruginosa*. AlgD, which is encoded by the *algD* gene, is a GDP-mannose dehydrogenase that facilitates the production of GDP-mannuronic acid from GDPmannose. The *algD* gene is responsible for controlling alginate biosynthesis and the transcription of Alg proteins, as well as the final production of the precursor GDP-mannuronic acid, which is the main structure for polymerization and synthesis of alginate (Kamali, et al., 2020).

The purpose of this study is to evaluate the biofilm formation capacity and antimicrobial susceptibility of *P. aeruginosa* collected from patients attending Near East University Hospital and determine the molecular prevalence of the *algD* gene among clinical isolates.

CHAPTER II

Literature Review

2. General Information

Pseudomonas aeruginosa is a prevalent bacterium found in the environment that can cause infections in humans, particularly when their immune system is weakened. Due to its ability to adapt to different environments, this bacterium possesses numerous metabolic pathways and regulatory genes. Its capacity to utilize various nutrients, extensive virulence factors and strong resistance to antibiotics make it a formidable foe to eliminate from infected individuals, especially those with CF who suffer from chronic lung infections (Wu, et al., 2015).

2.1 Morphology

P. aeruginosa is a Gram-negative bacterium that has a rod-like shape and is asporogenous. It possesses a single flagellum and has a pearlescent sheen with a soapy odor. This microbe thrives at temperatures between 25°C to 37°C. Its ability to grow at 42°C distinguishes it from other Pseudomonas species. It is an omnipresent microorganism that can survive under diverse environmental conditions (Wu, et al., 2015).

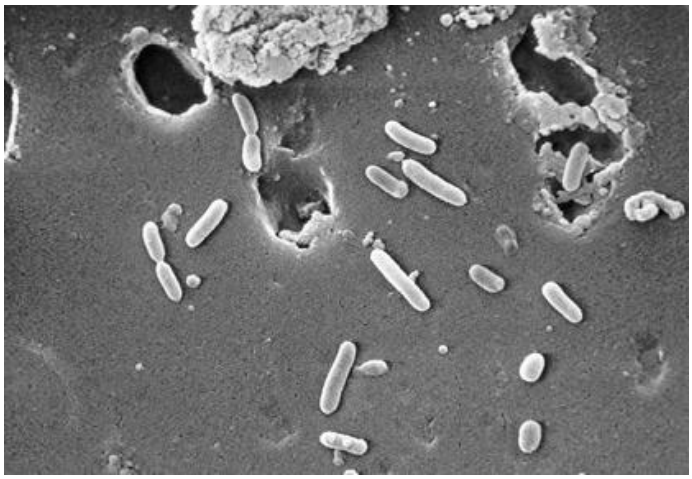


Figure 1. Scanning electron micrograph of *P. aeruginosa* (https://phil.cdc.gov/)

2.2 Laboratory Identification

P. aeruginosa is currently one of the most problematic multidrug-resistant bacterial agents responsible for nosocomial infections. Infection with *P. aeruginosa* can result in long-term chronic ailments, especially affecting immunocompromised individuals, including those who are neutropenic or admitted to the ICU. Researchers have been striving to develop rapid and sensitive methods to detect *P. aeruginosa*. Various contemporary detection techniques have been devised, such as flow cytometry (Rüger et al., 2014), immunological assays, and molecular biology-based approaches. Nevertheless, conventional culture methods continue to be the most frequently utilized diagnostic methods in clinical practice (Tang, et al., 2017).

Conventional methods for detecting *P. aeruginosa* rely on the biological characteristics of the bacterium with specific culture conditions. These methods include assessing the Gram (-) or Gram (+) status, evaluating the metabolites and secreted molecules such as oxidase, acetamidase, arginine dihydrolase, and pyocyanin. Lowbury et al. (1955) introduced a selective medium for *P. aeruginosa*, supplemented with 0.03% cetrimide. This medium demonstrated high selectivity and subsequently became a commonly used selective medium for detecting *P. aeruginosa*. Brown et al. (1965) improved the selective medium by incorporating cetrimide, based on earlier studies, and showed that the Lemco-based selective medium (CTA1) produced more pyocyanin. Growth on the selective medium with King's medium B as the base (CTA2) resulted in stronger fluorescence at a temperature of 42°C, while other *P.* species did not generate fluorescence at this temperature. As a result, the CTA2 medium can serve as a specific diagnostic medium for detecting *P. aeruginosa* (Tang, et al., 2017).

P. aeruginosa possesses the unique ability to utilize acetamide to produce ammonia as a nutrient, a characteristic not found in other bacteria. This mechanism has been utilized by Szita et al. (1990) to develop a liquid synthetic medium that offers enhanced detection selectivity, sensitivity, and speed compared to culture medium supplemented with cetrimide. Moreover, many other researchers have explored the potential of using cetrimide as a contribution to identify *P. aeruginosa* (Tang, et al., 2017).

Automated systems have revolutionized the diagnostic process by providing shorter turnaround times for results and are widely utilized in clinical laboratories for

identifying bacterial species and conducting antimicrobial susceptibility testing (AST). These systems offer numerous benefits, such as high levels of automation with a user-friendly operating procedure, enhanced specimen handling, excellent reproducibility and accuracy, and more. In China, common automated identification systems include Vitek-2 (BioMérieux, France), Phoenix 100 (BD Biosciences, USA), and MicroScan WalkAway (Dade Behring, Inc., USA). Among these, Vitek-2 is one of the earliest and most extensively employed automated identification systems (Tang, et al., 2017).

Furthermore, more advanced detection techniques have been used to identify *P. aeruginosa* such as enzyme-linked immunosorbent assays (ELISAs), immunoflourecent methods, conventional and real-time polymerase chain reaction (PCR, RT-PCR), electrochemical assays.

2.3. Epidemiology

P. aeruginosa is a usual causative agent of healthcare-associated infections, which include pneumonia, urinary tract infections, surgical site infections, and bacteremia. It is estimated that *P. aeruginosa* accounts for 7.1%–7.3% of all healthcare-associated infections. Pneumonia is the most common site of *P. aeruginosa* infection and is the most prevalent Gram-negative organism identified in nosocomial pneumonia. In recent years, the prevalence of *P. aeruginosa* infections has been on the rise. In critically ill patients in the intensive care unit (ICU), the bacterium is responsible for an even greater proportion of healthcare-associated infections. A large international study of infections in ICU patients found that *P. aeruginosa* was responsible for 16.2% of patient infections and caused 23% of all ICU-acquired infections, with respiratory sources being the most common site of *P. aeruginosa* infection (Reynolds & Kollef, 2021).

There is an alarming increase in antibiotic-resistant Gram-negative infections. *P. aeruginosa* is a significant public health concern due to its ability to develop resistance to multiple antibiotics, including carbapenems. The WHO has identified carbapenem-resistant *P. aeruginosa* as a critical priority pathogen in need of new treatment options. The increasing prevalence of MDR *P. aeruginosa* infections in healthcare settings is associated with poorer outcomes and increased healthcare costs (Raman, et al., 2018).

	Levofloxacin %R	Imipenem %R	Meropenem %R	Piperacil-lin-tazobactam %R	Ceftazidim %R	Amikacin %R
2017	79.8%	56.6%	62.3%	24.5%	54.8%	48.7%
2018	72.7%	50.4%	50.9%	26.6%	50.1%	30.7%
2019	74.7%	53.9%	53.1%	33.7%	50.8%	50.0%
2020	61.8%	52.4%	38.2%	54.0%	58.4%	45.9%
2021	55.2%	47.2%	39.7%	54.6%	56.8%	42.3%
2022	56.3%	52.6%	42.2%	47.5%	51.1%	46.0%

Figure 2. Heatmap presenting the resistance profile of *P. aeruginosa* regarding the main antibiotics used in treatment (Cos, et al., 2022)

2.4. Risk Factors

As an opportunistic pathogen, *P. aeruginosa* may cause severe infections in immunocompromised patients and hospitalized patients. Also as a common biofilm former, *P. aeruginosa* infections are extremely difficult to treat.

2.4.1. *Burn Wounds*

Approximately 60% of burn patient deaths are due to infection, with *P. aeruginosa* having the highest mortality rate among all bacteremia-causing agents. Burn-related infections caused by *P. aeruginosa* typically worsen quickly, resulting in the spread of the infection throughout the body and death occurring within days or weeks (Gonzales, et al., 2016).

2.4.2. *Cystic Fibrosis*

Chronic infection of the lower airways with *P. aeruginosa* is a significant factor in the morbidity and mortality of people with the genetic condition CF. Although it was previously believed that each patient acquired distinctive strains of *P. aeruginosa* that were present in their environment, several studies have now shown that individuals with CF share strains of *P. aeruginosa* (Parkins, et al., 2018).

2.4.3. *Acute Leukemia*

In patients with hematological malignancies, *P. aeruginosa* bloodstream infection (BSI) is a common complication with high mortality rates. The prevalence of antibiotic-resistant strains has made this even more serious, leading to increased difficulty in managing these patients. Patients with acute leukemia (AL) are at heightened risk for serious nosocomial infections due to distinct disease characteristics, extended hospital stays, long periods of severe neutropenia, and treatment methods like high-dose combined chemotherapy, corticosteroids, and hematopoietic stem cell transplantation (HSCT) that can impair the immune system, hematopoietic function, and protective skin and mucosal barriers (Zhao, et al., 2020).

2.4.4. *Overextended Antimicrobial Therapy*

Infections caused by *P. aeruginosa* can be severe, life-threatening, and challenging to treat due to their limited susceptibility to antibiotics and the frequent emergence of antibiotic resistance during therapy, leading to severe adverse outcomes. The issue of antibiotic resistance in *P. aeruginosa* is on the rise, and the increased level of drug resistance can result from both the *de novo* emergence of resistance in a specific organism after exposure to antimicrobials and the spread of resistant organisms between patients. The accumulation of resistance after exposure and cross-resistance between agents can lead to the development of multidrug-resistant (MDR) *P. aeruginosa* (Aloush, et al., 2006).

2.5. Infections caused by *P. aeruginosa*

P. aeruginosa commonly causes nosocomial infections, particularly pneumonia in immunocompromised patients and those with structural lung disease such as CF. Recent epidemiological research indicates a rising incidence of antimicrobial resistance, particularly multi-drug resistant (MDR) strains. *P. aeruginosa* employs various virulence strategies, such as quorum sensing, the secretion of toxins, and the formation of biofilms, to increase its capacity to cause severe infections. In managing *P. aeruginosa* infections, prevention is prioritized when feasible, and obtaining cultures and promptly initiating antimicrobial treatment,

sometimes in conjunction with combination therapy depending on the clinical scenario, are essential to sustain effectiveness against *P. aeruginosa.* (Reynolds & Kollef, 2021).

2.5.1. *Cystic Fibrosis*

Early and aggressive antibiotic prophylaxis or therapy can prevent chronic lung infections caused by biofilm-growing mucoid strains of *P. aeruginosa* in patients with CF. In addition, chronic suppressive therapy may be necessary. A recent study indicates that oral ciprofloxacin, when added to inhaled tobramycin, could potentially reduce lung inflammation. New formulations of old antibiotics, such as aztreonam lysine, for inhalation therapy have produced positive outcomes in clinical trials against chronic *P. aeruginosa* infection. These outcomes include improvements in patient-reported symptoms, lung function, time to acute exacerbations, and sputum density of *P. aeruginosa* (Høiby, 2021).

2.5.2. *Malignant otitis externa (MOE)*

Malignant otitis externa is a serious, highly debilitating, and rarely life-threatening infection affecting the soft tissues of the outer ear and its surrounding structures, with potential spread to the periosteum and skull base bone. The first documented case was published in 1838 by Toulmouche, and the term "malignant otitis externa" was coined by Chandler in 1968 due to its high morbidity and mortality rates before the advent of effective antibiotic therapies. This infection primarily affects immunocompromised individuals over 65 years of age, especially those with conditions such as diabetes, hematologic disorders (such as leukemia or granulocytopenia), or arteriosclerosis (Treviño González, et al., 2021).

2.5.3. *PA endophthalmitis*

Endophthalmitis is a potential complication of penetrating injuries to the eye, intraocular surgery, hematogenous spread from other sites of Pseudomonas infection, or posterior perforation of corneoscleral ulcer. This condition is typically rapidly progressive and sight-threatening, and requires immediate therapeutic intervention (Sridhar, et al., 2015).

2.5.4. *Pneumonia*

The clinicians are required to be aware of several prominent syndromes of *P. aeruginosa* respiratory tract infection. The main infection among them is community-acquired pneumonia (CAP), in which *P. aeruginosa* colonized in the upper respiratory tract leads to the lung infection. This is believed to be the mechanism for pneumonia in non-adults with cystic fibrosis and patients with chronic lower respiratory tract disease. *P. aeruginosa* pneumonia associated with bronchiectasis has become uncommon. Instead, a different syndrome, which is more prevalent, is now linked to ICU pneumonia caused by the aspiration of contaminated nebulizer fluid or mechanical ventilation. It can also be linked to hospital-acquired pneumonia in patients with preexisting colonization and chronic lower respiratory tract disease (Fujitani, et al., 2011).

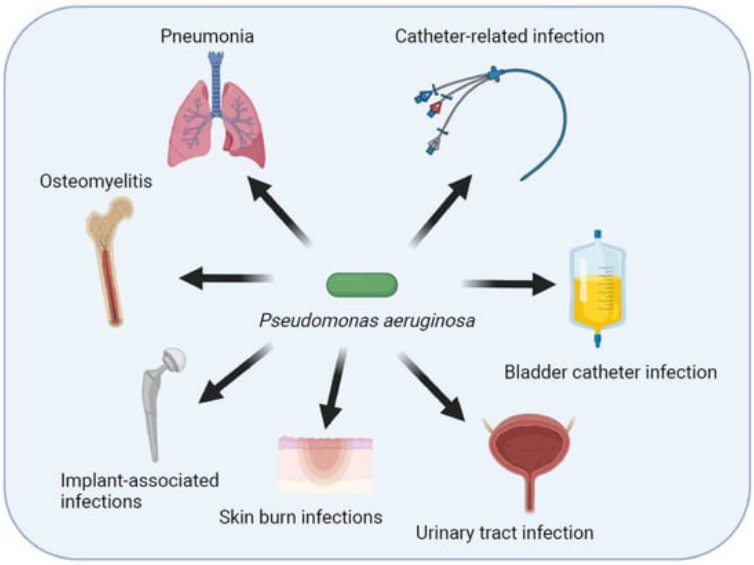


Figure 3. Schematic representation of main infections caused by *P. aeruginosa* (Tuon, et al., 2022)

2.6. Virulence Factors

P. aeruginosa possesses various virulence factors that enable pathogenicity by promoting adhesion and disrupting host cell signaling pathways while targeting the extracellular matrix. This bacterium can cause several diseases by invading the organism and compromising the immune system, making infections almost impossible to treat completely. The virulence factors that contribute to its pathogenicity include lipopolysaccharide, flagellum, type IV Pili, type III secretion system, exotoxin A, proteases, quorum sensing and biofilm formation. These factors act differently in the immune system but are all major contributors to the bacterium's pathogenicity (Rocha, et al., 2019).

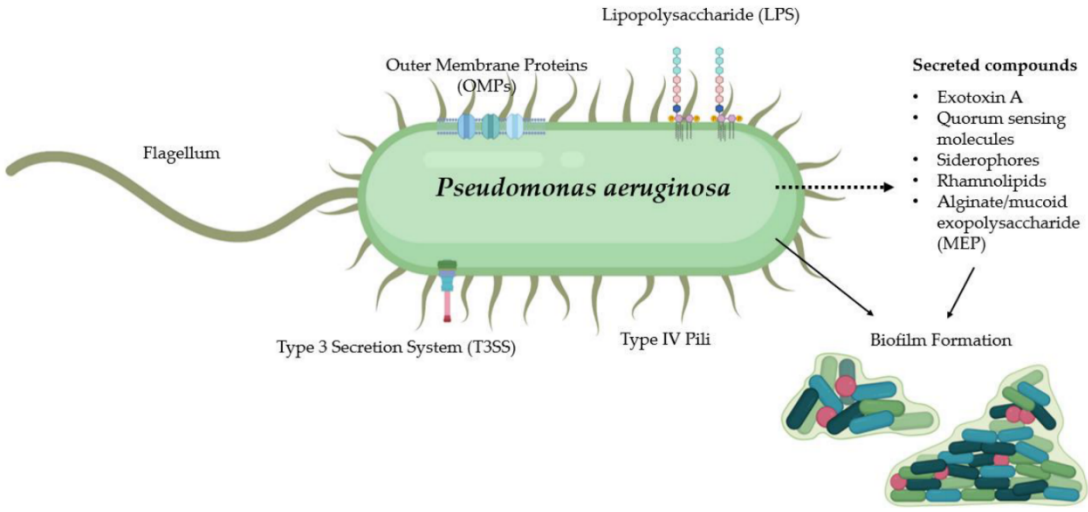


Figure 4. Key virulence factors important in the pathogenesis of *P. aeruginosa* infections (Killough, et al., 2022)

2.6.1. *Type III Secretion System*

The type III secretion system is a major virulence factor of *P. aeruginosa* and plays a crucial role in the pathogenesis of infections caused by this bacterium. This system allows *P. aeruginosa* to deliver effector proteins directly into the cytoplasm of host cells, which can then manipulate host cell signaling pathways, induce apoptosis, and modulate the immune response. The type III secretion system is involved in a range of infections caused by *P. aeruginosa*, including pneumonia, sepsis, and urinary tract infections, and is therefore a target for new therapeutic interventions (Galle, et al., 2012).

2.6.2. *Pyocyanin Production*

Pyocyanin is a blue-green pigment produced by *P. aeruginosa* that is known to generate reactive oxygen species (ROS), leading to oxidative stress in host cells. Pyocyanin has been shown to have various effects on different organ systems, including respiratory, cardiovascular, urological, and central nervous systems. These effects include apoptosis, inflammation, and damage to host tissues. The ability of *P. aeruginosa* to produce pyocyanin may contribute to its pathogenicity and resistance to host immune defenses (Hall, et al., 2016).

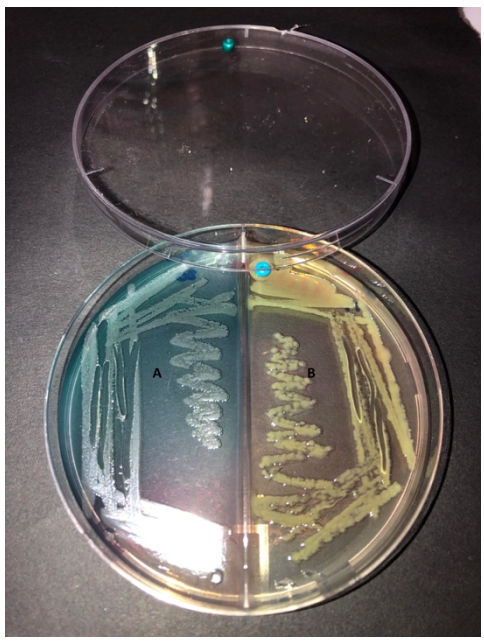


Figure 5. Pigmentation of *P. aeruginosa* in mueller-hinton agar A: Pyocyanin producing *P. aeruginosa* strain, B: Pyoverdine producing *P. aeruginosa* strain

2.6.3. *Exotoxin A (ETA)*

Exotoxin A produced by *P. aeruginosa* and diphtheria toxin produced by *Corynebacterium diphtheriae* have similar mechanisms of action. Both toxins are ADP-ribosyltransferases that inactivate elongation factor 2 by transferring an ADP-ribose group to it, thereby inhibiting protein synthesis and leading to cell death. The receptor for Exotoxin A is different from the receptor for diphtheria toxin, but both toxins enter cells in a similar manner (Strateva & Mitov, 2011).

2.6.4. *Type IV Pili*

Type IV pili are crucial for the attachment and movement of bacteria, but they also play a role in sensing and responding to their environment. When *P. aeruginosa* encounters a surface, the pili initiate a signaling cascade that results in the expression of genes associated with pathogenicity and twitching motility, which allows the bacteria to colonize the surface and spread. This highlights the importance of understanding bacterial behavior and adaptation to new environments, which can have important implications for the development of new treatments and preventative strategies (Persat, et al., 2015).

2.6.5. *Biofilm Formation*

Biofilms play a crucial role in *P. aeruginosa* infections, particularly in chronic infections that are difficult to treat. In a biofilm, bacteria are protected from the host immune system and antibiotics, making them more resistant to treatment. The extrapolymeric substance (EPS) of biofilms provides a physical barrier that prevents the penetration of antibiotics and antibodies, and also sequesters nutrients from the surrounding environment, allowing the bacteria to survive in nutrient-limited conditions. Additionally, the EPS can act as a reservoir for virulence factors, contributing to the pathogenicity of the bacteria. Overall, the ability of *P. aeruginosa* to form biofilms is a significant factor in its pathogenicity and contributes to its persistence in chronic infections (Bjarnsholt, et al., 2010).

2.7. *P. aeruginosa* Biofilms

The EPS matrix of biofilms not only provides mechanical stability and adhesion to surfaces but also plays a critical role in mediating the exchange of nutrients, signaling molecules, and waste products among the biofilm cells. The matrix also acts as an external digestive system, allowing the biofilm to efficiently acquire nutrients from the surrounding environment and to recycle waste products. The EPS matrix also protects biofilm cells from external stresses, including antibiotics and host immune defenses. Therefore, understanding the composition and function of the biofilm matrix is important for developing effective strategies to prevent and treat biofilm-associated infections (Flemming & Wingender, 2010).

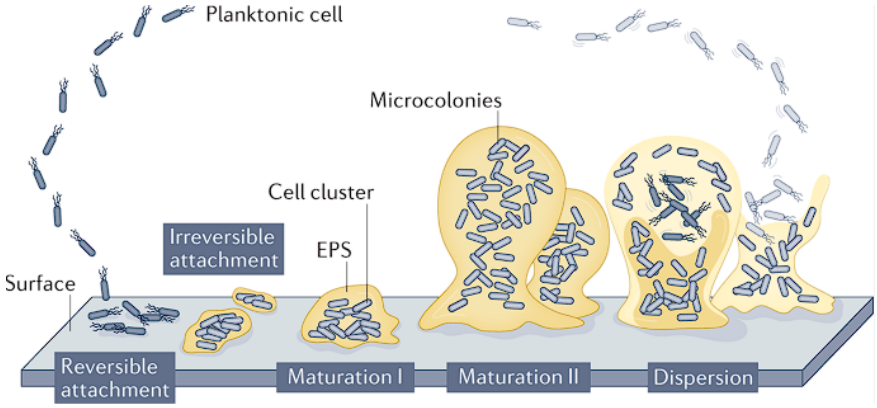


Figure 6. The five step model of biofilm developement (Sauer, et al., 2022)

2.7.1. *EPS and Biofilm Architecture*

EPS surround cells within a biofilm and make up their immediate environment. Certain EPS, such as those forming capsules, are more closely linked to cell surfaces than others. Production and quantity of EPS is vital for the creation and maintenance of organized multicellular microbial communities. The concentration, charge, cohesion, specificity, and nature of EPS components, along with the 3D matrix architecture, dictate the biofilm's mode of life. Biofilm morphology can be smooth, rough, fluffy, or filamentous, and its porosity can range from having mushroom-like macrocolonies surrounded by water-filled voids. Regardless of morphology, all

biofilms transiently immobilize cells, fostering mixed-species microconsortia with long-term interactions and gradients, creating diverse microhabitats that support biodiversity on a small scale (Golovlev, 2022).

2.7.2. *Quorum Sensing*

The coordination of individual cells within bacterial communities is crucial for the development of *P. aeruginosa* biofilms. This process is facilitated by quorum sensing (QS), a network of interconnected signal transduction pathways that allow the bacteria to communicate and coordinate collective behavior in response to changes in cell density and environmental stresses. QS involves the production, secretion, and accumulation of autoinducers (AI) molecules, which are sensed by transcriptional regulators and lead to the expression of specific genes on a population-wide scale. QS is involved in various physiological processes, including biofilm development, virulence-factor production, metabolic adjustment, stress tolerance and host-pathogen interactions. Understanding and manipulating these chemical communication systems could offer alternative treatments to conventional antimicrobials and antibiotics.

P. aeruginosa employs four distinct QS pathways, namely Las, Rhl, PQS, and IQS. Each pathway produces its cognate AI molecule, namely N-3-oxo-dodecanoyl-L-homoserine lactone (3O-C12-HSL), N-butyryl-L-homoserine lactone (C4-HSL), 2-heptyl-3-hydroxy-4-quinolone (PQS), and 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde (IQS), respectively. These pathways are organized hierarchically, with the Las system at the top of the cascade. The Las and Rhl systems are triggered by increased cell density during the preliminary exponential growth phase, while the PQS and IQS systems are activated during the late exponential growth phase, particularly under iron limitation and phosphate starvation conditions, respectively. The AI molecules undergo membrane trafficking, which is presumably mediated by free diffusion, efflux pumps, or outer membrane vesicles. The trafficked 3O-C12-HSL molecule binds to the regulator protein LasR, which activates the lasI synthase gene, leading to the autoinduction feed-forward loop. The LasR–3O-C12-HSL complex also induces the expression of rhlR and rhlI genes, as well as the pqsR and pqsABCDH genes, which encode the Rhl and PQS systems, respectively. Similarly, the RhlR–C4-HSL complex activates the rhlI gene, which leads to the second autoinduction feed-

forward loop. In the PQS system, the PqsR–PQS complex activates the pqsABCDH genes and feeds back to induce rhlRI gene expression. The expression of both pqsR and pqsABCDH genes can be inhibited by RhlR, which controls the correct ratio between 3-oxo-C12-HSL and C4-HSL and thus the activation of the PQS pathway. The identity of the transcriptional regulator in the IQS system is still unknown, although its binding to IQS activates the pqsR gene. The IQS molecule was previously thought to be enzymatically produced by the ambBCDE genes, but it was later found to be a byproduct of the siderophore pyochelin biosynthesis. The ambBCDE genes code for proteins involved in the biosynthesis of the anti-metabolite L-2-amino-4-methoxy-trans-3-butenoic acid (AMB) (Lee & Zhang, 2015).

2.7.3. *Antimicrobial Resistance*

The growth of bacteria as structured aggregates termed biofilms leads to their protection from challenging environmental conditions such as physical and chemical stresses, shearing forces, and limited nutrient availability. This adaptation allows biofilm-forming bacteria to survive and persist, making them resistant to antibiotic therapies and immune clearance. This poses a significant problem in hospital settings where biofilms often cause chronic and device-related infections, resulting in a substantial burden on the healthcare system. While antibiotics are the primary therapeutic approach to infections, biofilms' adaptive resistance often renders them inadequate for clearing biofilm infections (Taylor, et al., 2014).

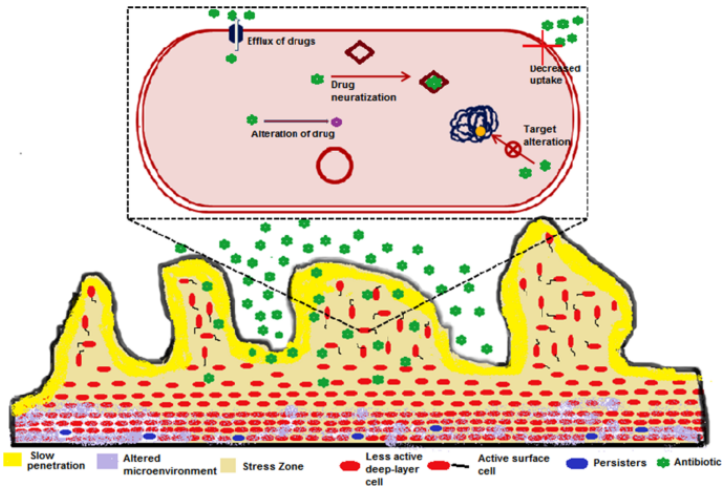


Figure 7. Diagrammatic representation of the potential mechanisms of antibiotic resistance in biofilm communities (Sharma, et al., 2019)

2.7.4. *Regulation of Biofilm Formation in P. aerugionsa*

When bacteria transition to a sessile lifestyle, their physiology changes significantly. Many factors contribute to the different stages of biofilm formation, including extracellular appendages and polysaccharides like Pel and Psl. However, regulatory systems which control the production of aforementioned components in response to environmental stimuli and cause the bacterium to switch between lifestyles are even more important. In *P. aeruginosa*, many of these regulators belong to the two-component systems (TCSs).

TCSs are composed of a sensor kinase and a response regulator and are the predominant signalling mechanism in most bacteria. Sensor kinases can be membrane-bound or cytosolic and monitor environmental conditions such as nutrients, temperature, pH, osmolarity, or toxic substances. When an environmental stimulus is detected, an appropriate response is elicited to ensure the bacterium's survival in a given niche. The prototypical sensor kinase has a variable N-terminal input domain, often located in the periplasm for membrane-bound sensors, and a conserved C-terminal transmitter domain in the cytosol. Response regulators have a conserved N-terminal receiver domain, followed by a C-terminal output domain with a helix-turn-helix (HTH) motif that binds DNA. Upon signal detection, the sensor kinase autophosphorylates on a conserved histidine residue in the transmitter domain. The phosphoryl group is then transferred to a conserved aspartate residue in the response regulator's receiver domain, activating it as a transcription factor (Golovlev, 2002).

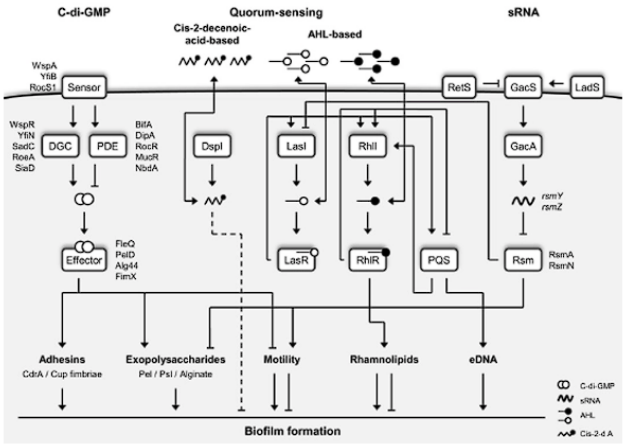


Figure 8. Schematic overview of the regulation of biofilm formation in *P. aeruginosa*. (Fazli, et al., 2014)

2.7.5. *Alginate Biosynthesis and algD Gene Regulation*

P. aeruginosa is protected from the host's immune response by alginate, which impedes complement activation and reduces phagocytosis by macrophages and neutrophils, while also trapping the free radicals released by these cells. Alginate also helps *P. aeruginosa* to withstand different environmental stressors. Moreover, the overproduction of alginate has an impact on the expression of other virulence factors, including flagellum and T3SS. It has been recently discovered that AlgT/U plays a critical and central role in regulating virulence factors in a coordinated manner.

The algD operon comprises twelve genes that encode the fundamental alginate biosynthesis machinery. The gene products of this operon are responsible for the four primary stages of alginate biosynthesis in P. aeruginosa: (i) synthesis of the precursor; (ii) polymerization and transfer across the cytoplasmic membrane; (iii) transfer and modification in the periplasmic space; and (iv) export through the outer membrane. The regulation of alginate synthesis is a complicated process, involving numerous regulatory genes that respond to various environmental stimuli (Wu, et al., 2015).

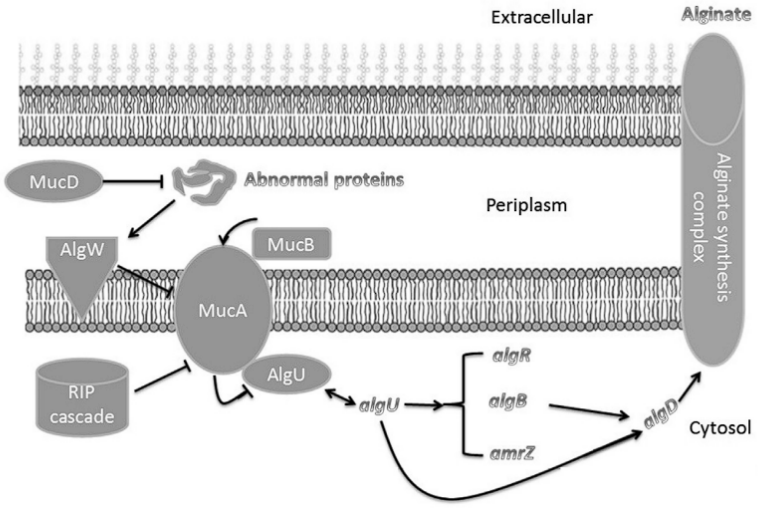


Figure 9. Regulation of alginate biosynthesis in *P. aeruginosa* (Wu, et al., 2015)

CHAPTER III

Materials and Methods

3. Methodology

This study was conducted between January 2020 and December 2022. A total of 100 non-duplicate *P. aeruginosa* isolates were recovered from clinical samples collected from patients admitted to Near East University Hospital in Cyprus. Isolates were cultured on sheep blood agar and EMB agar at 35ºC and 5% CO_2. Bacterial identification of the isolates was performed by VITEK-2® automated system (BioMérieux, Marcy l'Etoile, France). Isolates were stocked in glycerol and kept in -80 ºC for further use. Patient demographic data including age, gender and clinical information including the admission status, hospital ward, sample type was collected and electronically stored.

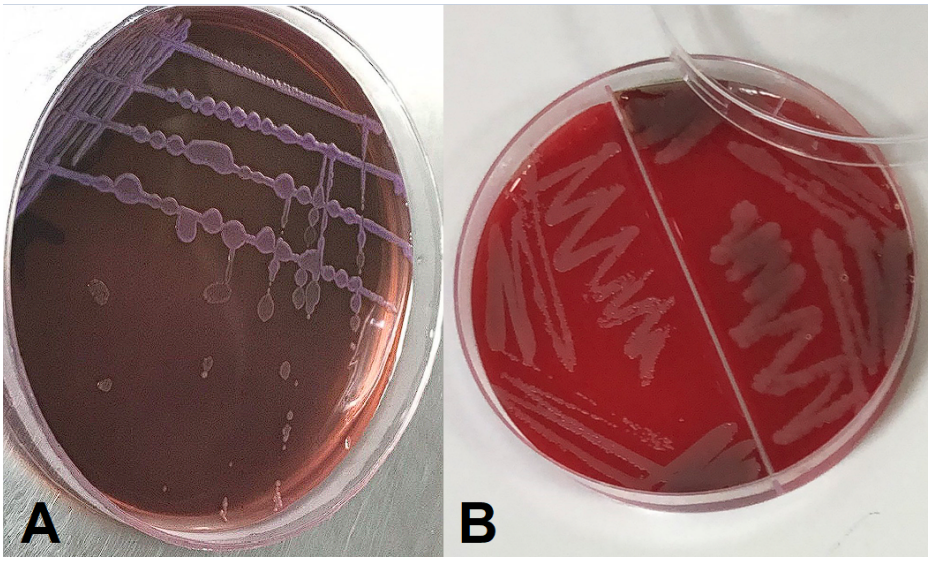


Figure 10. *P. aeruginosa* inoculums on A: EMB Agar, B: Sheep blood agar

3.1. Antibiotic susceptibility testing

Antibiotic susceptibility testing (AST) of the isolates was performed using VITEK® 2 system. The standard MicroScan and VITEK-2 inoculation protocols were performed using bacterial isolates which were cultured on blood agar plates after

overnight incubation at 35ºC with 5% CO_2. Using a sterile swab, a sufficient number of morphologically comparable colonies were transferred to the saline polystyrene test tube. A homogeneous suspension was generated with a density equivalent to the relevant 0.5 McFarland standard. For AST, Gram negative bacillus identification cards were used, and 145 µl of the suspension was transferred to a second tube containing 3.0 ml of saline. The tube was then inserted into the cassette along with a susceptibility card. Fourteen antibiotics were tested: piperacillin/tazobactam (TZP), ceftazidime (CAZ), aztreonam (ATM), gentamicin (GEN), cefepime (CFPM), colistin (CST), ciprofloxacin (CIP), levofloxacin (LEV), meropenem (MEM), netilmicin (NTL), imipenem (IPM), amikacin (AK), piperacillin (PIP), tobramycin (TOB).

Multidrug-resistant *P. aeruginosa* (MDR-PA) was defined as nonsusceptibility to at least one antibiotic in at least three classes for which *P. aeruginosa* susceptibility is generally expected (i.e., antipseudomonal penicillins [e.g., piperacillin–tazobactam], cephalosporins [ceftazidime, cefepime], fluoroquinolones, aminoglycosides, and carbapenems [meropenem, imipenem]). Extensively drug-resistant *P. aeruginosa* (XDR-PA) was defined as nonsusceptibility to at least one agent in all but two or fewer classes of antibiotics.

3.2. Biofilm formation assay

All samples were inoculated on Mueller-Hinton agar from the glycerol stock tubes and incubated overnight at 35ºC with 5% CO_2. A few colonies were inoculated into 10 mL of Tryptic Soy Broth (TSB) and incubated at 35ºC with 5% CO_2 for 24 hours. A volume of 95 µL of TSB and 5 µL of bacterial suspension for each sample were added into wells of flat bottomed 96 well-plate in triplicates and incubated at 35ºC for 24 hours. 100 µL of TSB without the bacterial suspension as the control group. Subsequently, all wells were washed with 200 µL of phosphate buffered saline (PBS), then each well was stained with 150 µL of 0.1% crystal violet solution in water for 10-15 minutes. Wells were washed again with 200 µL PBS for three times. Each well was decolorized with 150 µL of 30% acetic acid in water. The absorbance of the wells was measured at 570 nm using a microplate spectrophotometer (VersaMax, Molecular Device, Sunnyvale, ABD). The average optical density (OD) of the negative control was used to define the cutoff OD (ODc). The isolates were categorized into

four levels by ODc: Non-Biofilm Former (OD ≤ ODc), Weak-Biofilm Former (ODc < OD ≤ 2x ODc), Moderate Biofilm Former (2 × ODc < OD ≤ 4 × ODc) and Strong Biofilm Former (OD > 4 × ODc).

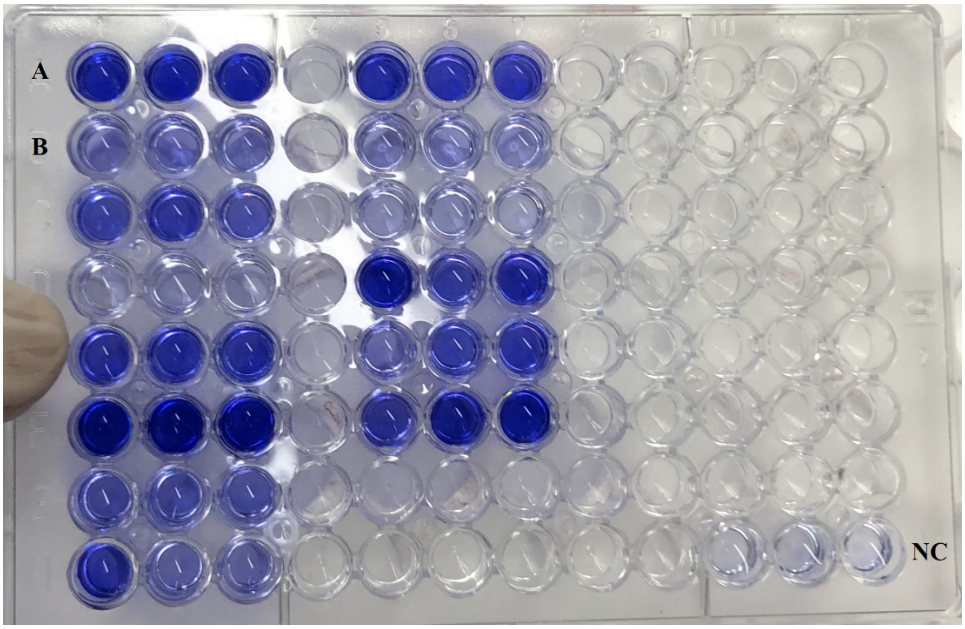


Figure 11. 96-well plate after crystal violet staining. A: Strong biofilm producer, B: Weak biofilm producer, NC: Negative control

3.3. DNA extraction

Samples were inoculated on Mueller-Hinton agar media from the glycerol stock tubes and incubated overnight at 35°C with 5% CO_2. A loop of fresh culture from agar plates for each isolate was inoculated into a volume of 500 µL sterile deionized water into sterile 1.5 mL eppendorf tubes. Boiling method was used for DNA extraction. Briefly, samples were heated up to 100°C for 10 minutes on a heat block. After heating, samples were centrifuged for 10 minutes at 14,000 rpm to sediment the debris. A volume of 300 µL DNA-containing supernatant was transferred into sterilized eppendorf tubes and stored at -20°C until use.

3.4. Amplification of *algD* gene

The amplification of *algD* gene was performed by using conventional polymerase chain reaction (PCR). Each 25 µl PCR reaction contained: 12.5µl of 2x PCR master mix (Thermo Scientific); 1 µl of both forward and reverse primers (at a final concentration of 10pmol/µl) for the target genes; 6.5 µl of nuclease free PCR grade water (Thermo Scientific) and 4 µl of DNA template. The cycling parameters, primer sequence, and amplicon size for PCR amplification are given in Table 1 and 2.

Table 1. The primer sequences and amplicon size for the PCR amplification used in the study

Gene	Primer sequence (5′ to 3′)	Product Size (bp)
algD-F	CTACATCGAGACCGTCTGCC	593 bp
algD-R	GCATCAACGAACCGAGCATC	

Table 2. PCR cycling parameters used in the study

Gene	Steps	Temperature	Time	Cycles
algD	Initial denaturation	95°C	10 min	1
	Denaturation	95 °C	30 sec	30
	Annealing	60 °C	40 sec	
	Extension	72 °C	45 sec	
	Final Extension	72 °C	7 sec	1
	Hold	4 °C	-	1

3.5. Agarose gel electrophoresis

The amplified products (7µl) were mixed with 1 x loading dye (Thermo Fischer Scientific) and were electrophoresed in 2% agarose gel (W/V) prepared with 1x Tris Borate EDTA (TBE) buffer; ethidium bromide was used as a fluorescent tag at a concentration of 0.5µg/ml. As a DNA molecular weight marker, a 50 bp and a 100 bp DNA ladders (Hibrigen, Turkey) were utilized. The isolated DNA fragments were visualized and imaged using a UV transilluminator (DNA MiniBIS Pro Gel Imaging System.)

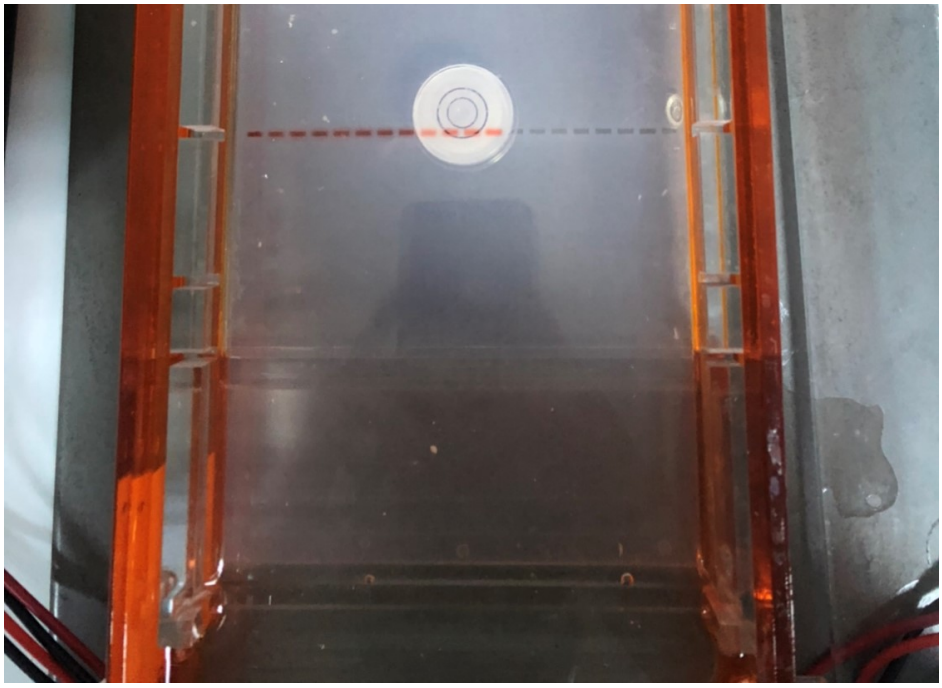


Figure 12. Amplified DNA suspensions loaded to agarose gel with loading dye ready to run with gel electrophoresis

3.6. Statistical analysis

All collected data was imported into SPSS Software version 23.0 (IBM Corp., Armonk N.Y., USA). Statistical analysis was performed using Pearson Chi Square, Fisher's Exact Test and One-Way ANOVA test. A value of p <0.05 was considered statistically significant.

CHAPTER IV

Results

4.1. Patient characteristics

A total of 100 *P. aeruginosa* isolates recovered from patients admitted to different hospital services and clinical sample types were included in this study. Among the patients, 61% were inpatients and 39% were outpatients. There was a similar distribution of gender in which 51% of the patients were female and 49% were male. Patient age ranged from 1 to 95 years (mean: 69.01, median: 73.50, standard deviation: 21.40), and the majority of patients with *P. aeruginosa* were over the age of 65 (68.0%)

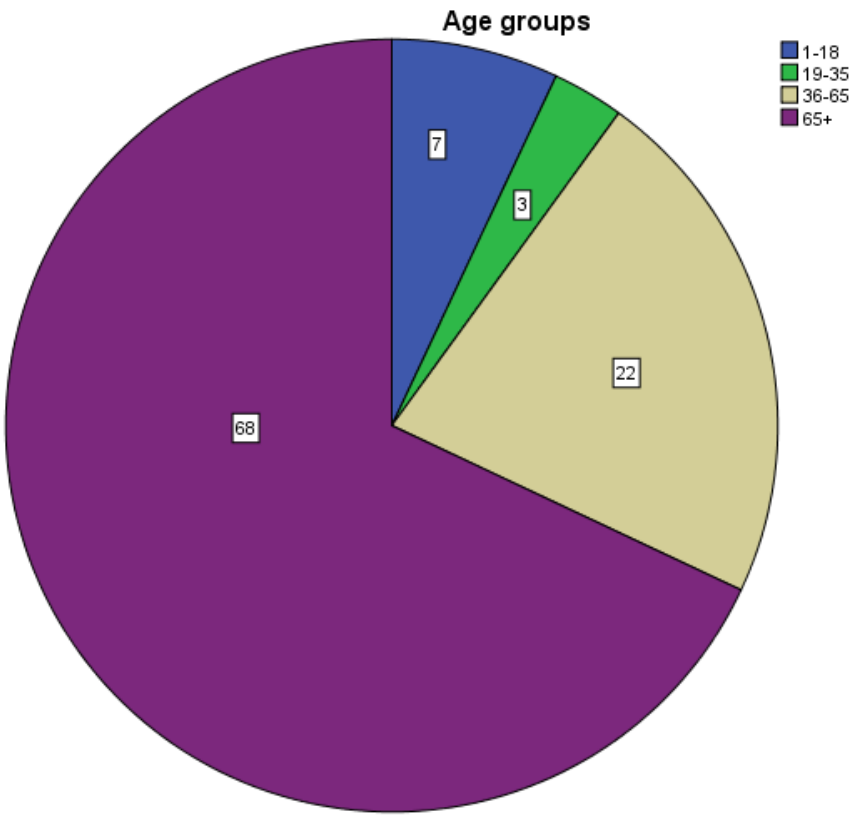


Figure 13. Diagrammatic distribution of isolates according to age groups

4.2. Sample characteristics

A vast majority of the *P. aeruginosa* obtained in this study were isolated from patients admitted to the anesthesiology (n=28, 28.0%), cardiology (n=14, 14.0%), respiratory diseases and allergy (n=11, 11.0%), radiology (n=9, 9.0%) and geriatrics (n=9, 9.0%) departments followed by pediatrics (n=6, 6.0%), internal medicine (n=5, 5.0%) and other departments including dermatology, neurology, orthopedics, otolaryngology, obstetrics and gynecology, oncology, general surgery and nephrology. A high proportion of the clinical isolates were recovered from urine (30.0%), aspirate (27.0%), sputum (17.0%), wound and pus (16.0%) samples, and the remaining isolates were from blood culture, catheter tip, ear discharge, cerebrospinal fluid and rectal samples.

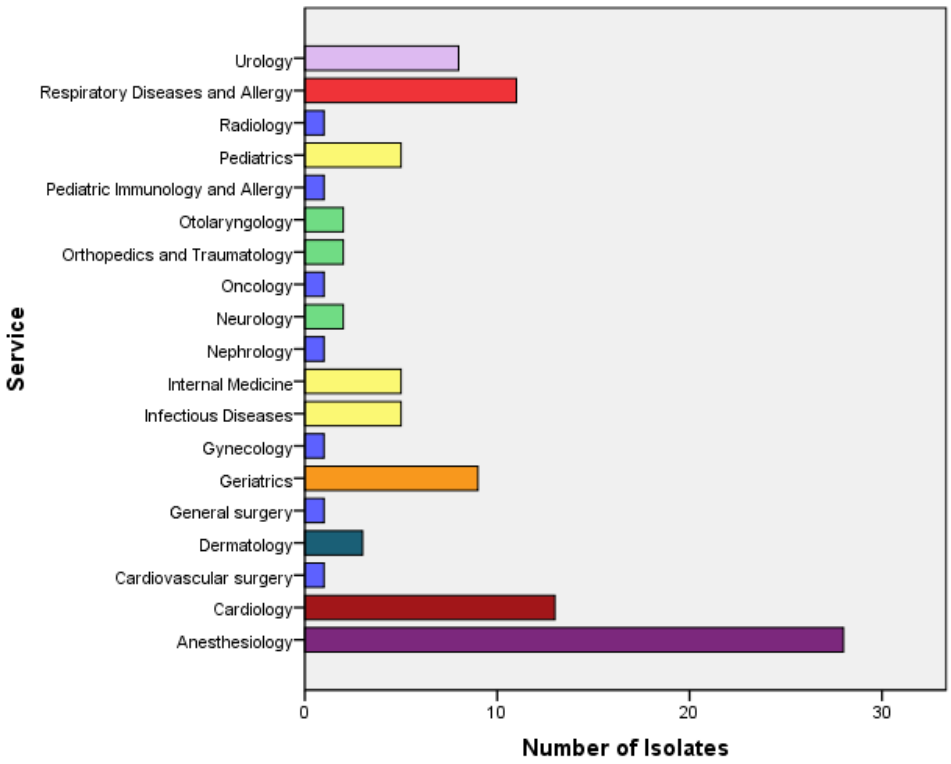


Figure 14. Distribution of isolates according to hospital services

4.3. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing revealed that isolates were mainly non-susceptible to aztreonam (65/94=69.1%), piperacillin (32/88=36.4%), cefepime (31/97=32.0%) and ceftazidime (27/100=27.0%) among beta-lactams (piperacillin/tazobactam, 33/99=33.3%), imipenem (29/96=30.2%) and meropenem (24/98=24.5%) among carbapenems, levofloxacin (30/81=37.0%) and ciprofloxacin (22/99=22.2%) among fluoroquinolones, netilmicin (28/88=31.8%) and gentamicin (13/81=16.0%) among aminoglycosides. Highest antimicrobial susceptibility among the isolates was observed to colistin (94/99=94.9%) and amikacin (90/99=90.9%). According to phenotypical classification, 27 isolates (27.0%) were classified as MDR and only 1 isolate (1%) was detected to be XDR.

Table 3. Antimicrobial susceptibility patterns of *P. aeruginosa* isolates

Antibiotic	**%**
Aztreonam	69.1
Levofloxacin	37.0
Piperacillin/Tazobactam	33.3
Cefepime	32.0
Netilmicin	31.8
Imipenem	30.2
Ceftazidime	27.0
Meropenem	24.5
Ciprofloxacin	22.2
Gentamicin	16.0
Amikacin	9.1
Tobramycin	7.3
Colistin	5.1

4.4. Biofilm production

The biofilm formation assays indicated that 35%, 28% and 19% of the *P. aeruginosa* isolates were strong, moderate and weak-biofilm formers, respectively. Of the 100 isolates studied, 18% were observed not to form any biofilm. No statistical association between biofilm formation and admission status of patients (inpatient/outpatient) (p=0.482), patient age (p=0.410), gender (p=0.313) or sample type (p=0.095) was observed. The correlation of biofilm formation and antibiotic resistance phenotype in the isolates was also investigated. Among the strong/moderate biofilm forming *P. aeruginosa* isolates (n=63), 20 (31.7%) of them were found to be MDR-PA, while 43 (68.3%) isolates were non-MDR-PA phenotype. No significant association between the MDR phenotype and strong/moderate-biofilm forming capacity of the isolates was observed (p=0.243).

Table 4. Distribution of *algD* among MDR and non-MDR *P. aeruginosa* isolates

Phenotype	N	*algD* (%)
MDR	27	24 (88.9)
Non-MDR	73	69 (94.5)

Table 5. Distribution of *algD* genes according to the biofilm formation category

Related Function	Gene	Strong-biofilm former	Moderate-biofilm former	Weak-biofilm former	Non-biofilm former
Alginate production	*algD*	34	26	16	17

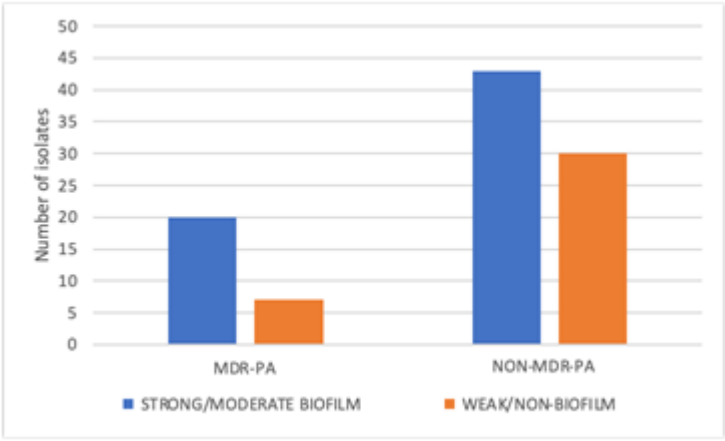


Figure 15. Biofilm formation of isolates according to MDR phenotype

4.5. Virulence profiling

PCR analysis was used for the screening of the gene *algD* among the *P. aeruginosa* isolates. The results highlighted that the 93% of the isolates were *algD* positive. The *algD*-positive genotype was associated with moderate or high biofilm formation (p<0.05). The PCR detection of the *algD* gene are given in Figure 15. There was no statistically significant association between *algD* positivity and MDR-PA phenotype of the isolates (p=0.384, respectively).

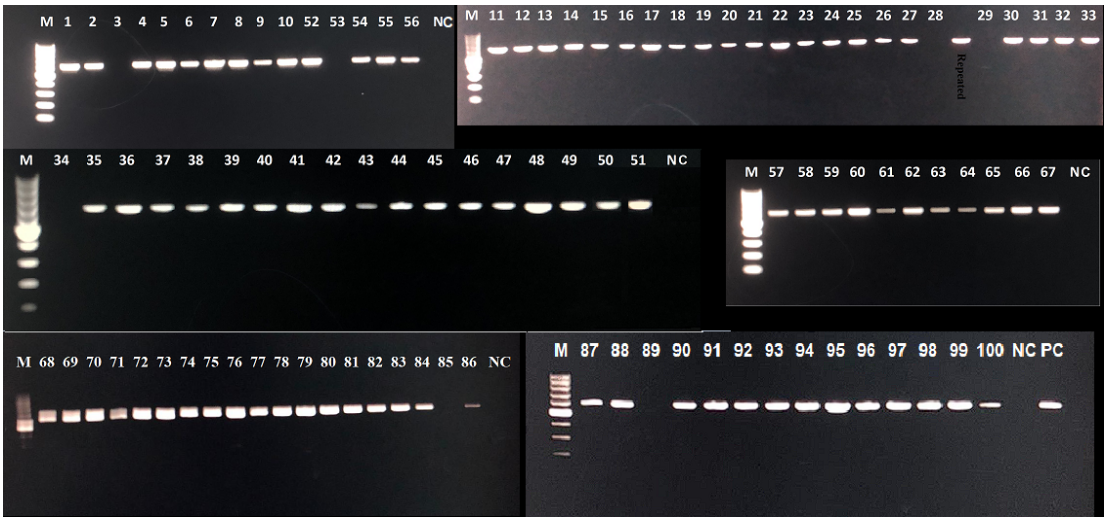


Figure 16. PCR detection of *algD* gene (593 bp) in *P. aeruginosa* isolates. M: Marker, 100 bp DNA ladder; NC: negative control; PC: positive control

CHAPTER V

Discussion

Pseudomonas aeruginosa is a common opportunistic pathogen. The presence of several virulence factors, such as exotoxins and biofilm production contribute to its pathogenicity. *P. aeruginosa* strains cause a wide range of human infections, the majority of which occur in hospitalized patients. Consequently, *P. aeruginosa* are among the most frequently isolated bacteria from clinical specimens. Their increasing multi-drug resistance and role in nosocomial outbreaks of various localizations are highlighted by their involvement in severe respiratory and urinary tract infections, as well as skin and soft tissue infections or bacteremia. Due to a variety of mechanisms for adaptation, survival, and resistance to a variety of antimicrobial drugs, this has become a public health issue.

In the current study, patients' demographic information such as age and gender, as well as relevant clinical information such as hospital service and sample origin were obtained from the hospital medical record database. Both subgroups are almost equivalent in terms of gender, accounting for 51 % (n=51) females and 49 % (n=49) males, and the age group “above 65” years had the highest number of patients (n=68; 68 %). In terms of the distribution of *P. aeruginosa* infections among patients, the data analysis revealed a significant increase among inpatients, regardless of gender, versus outpatients. Patients who stay in the hospital for extended periods of time are more prone to be colonized by this pathogen and acquire an infection. These unforeseen infections occur during inpatient care and result in significant morbidity and mortality; prolong hospital stays; and necessitate additional diagnostic and therapeutic interventions, all of which add to the costs already incurred by the patient's underlying disease. Similar findings of patient distribution by gender and inpatients/outpatient subgroup were described in a previous investigation carried out in Iran (Kunwar, et al., 2021).

P. aeruginosa is a common hospital contaminant, which results in life-threatening infections worldwide. According to the findings in this study, the majority of *P. aeruginosa* infections were observed in the Anesthesiology (n=28; 28%), Cardiology (n=13; 13%), Respiratory Diseases and Allergy (n=11; 11%) and Urology

(n=9; 9 %) departments. This could be due to the use of contaminated anesthetic equipment, which can lead to pulmonary infections. The presence of *P. aeruginosa* in individuals with chronic airway inflammation has significant clinical and prognostic implications, as well as therapeutic implications. *P. aeruginosa*, as an opportunistic pathogen, causes urinary tract infections by forming biofilms on the surface of urinary catheters (Cole, et al., 2014). It can also cause poor antibiotic penetration into the biofilm, which is a major cause of morbidity and mortality in patients with cystic fibrosis (Bragirath, et al., 2016). Therefore, it is critical to identify the groups of individuals who would benefit the most from studies focusing on the early detection of *P. aeruginosa*.

P. aeruginosa strains had the highest resistance to ATM, IPM, NTL, TZP, CFPM, CAZ, LEV, and PIP, while the strains had the lowest resistance to CST and AK. The most effective antibiotics against *P. aeruginosa* clinical isolates were CST, GEN, and AK. These findings are in line with those of Ghanbarzadeh et al., (2015) who found a significant rate of antibiotic resistance to CIP, ATM, CAZ, PIP, AK, and IPM. Similar results were obtained in a study conducted by Bahador and colleagues in Iran, in which the most efficient antibiotics against *P. aeruginosa* isolates were reported as CST (98.6 %), AK (84.3 %), and GEN (82.9 %), whereas *P. aeruginosa* isolates showed the highest resistance rate to CAZ (17.5%) (Bahador, et al., 2019). Another study that contradicted our findings, indicating the highest levels of antibiotic resistance to ceftazidime and the lowest resistance to amikacin in *P. aeruginosa* isolates from clinical and hospital environments (Fazeli & Momtaz, 2014). *P. aeruginosa* has several mechanisms of intrinsic antibiotic resistance, including restricted outer-membrane permeability, efflux systems that pump drugs out of cells, and the production of antibiotic-inactivating enzymes. Quinolone antibiotics, such as ciprofloxacin and levofloxacin, prevent DNA replication by inhibiting DNA gyrase and topoisomerase IV. The molecular structures of β-lactam antibiotics such as penicillin, cephalosporin, carbapenem, and monobactam all contain a β-lactam ring (Fazeli & Momtaz 2014).

The formation of biofilm has been identified as a significant factor of pathogenicity in *P. aeruginosa* infections (Bhagirath, el al., 2016) According to our results, 82% of *P. aeruginosa* isolates produced biofilms. In a research published by Jabalameli et al., (2021) 96.9 % of the isolates produced biofilms. Our findings vary from those of Kunwar et al., (2012) who observed that only about 25% of *P.*

aeruginosa isolates produced biofilms. There was a relatively high rate of inpatients among the strong biofilm forming isolates as expected.

Alginate is an important member of biofilm architecture which enhances the pathogenicity and survivability of the bacterium. Gene *algD* is a founding regulation factor for alginate production. Our study shows huge prevalence of *algD*+ among the isolates and our findings are consistent with some other studies such as (Bazghandi, et al., 2021; Ghadaksaz, et al., 2015; Jabalameli, et al., 2012). Prevalence of *algD* genes shown as 95.51%, 87.5% and 78.6% in those studies with the same order.

This study also presents a significant correlation between *algD* gene expression (93%) and biofilm production (82%).

CHAPTER VI

Conclusions

For decades, *P. aeruginosa* has been used as a model organism and has received considerable attention from the scientific community in order to explore bacterial mechanisms and pathogenesis. It has always been of particular importance as it is responsible for persistent infections in CF and immunocompromised patients. In this study, a high circulation of biofilm formation among *P. aeruginosa* isolates in the hospital setting was demonstrated for the first time in Cyprus. The isolates were found to habour *algD* virulence genes. Although the rate of resistance to multiple antibiotics among *P. aeruginosa* isolates was relatively low in the current study, prudent antimicrobial use and high infection prevention and control standards are required to avoid the occurrence of resistant strains in the future. The current study also highlighted several well-known molecular processes that enable the pathogen to survive in a variety of hostile environments and enhance bacterial pathogenicity. Further studies are required to explore the mechanisms of host-pathogen interactions and molecular mechanisms of antibacterial resistance in the bacterial strains.

Limitations

This study has a number of limitations. The clinical samples were collected from a single hospital. Further studies should cover a wider area for the sample collection. In addition, only one gene of the alginate biosynthesis operon was investigated due to financial and time constraints. *Pel* and *Psl* genes should be investigated in further studies.

REFERENCES

Aloush, V., Navon-Venezia, S., Seigman-Igra, Y., Cabili, S., & Carmeli, Y. (2006). Multidrug-Resistant Pseudomonas aeruginosa: Risk Factors and Clinical Impact. *Antimicrobial Agents and Chemotherapy*, *50*(1), 43–48. https://doi.org/10.1128/AAC.50.1.43-48.2006

Bahador, N., Shoja, S., Faridi, F., Dozandeh-Mobarrez, B., Qeshmi, F. I., Javadpour, S., & Mokhtary, S. (2019). Molecular detection of virulence factors and biofilm formation in Pseudomonas aeruginosa obtained from different clinical specimens in Bandar Abbas. *Iranian Journal of Microbiology*, *11*(1), 25–30.

Bazghandi, S. A., Arzanlou, M., Peeridogaheh, H., Vaez, H., Sahebkar, A., & Khademi, F. (2021). Prevalence of Virulence Genes and Drug Resistance Profiles of Pseudomonas aeruginosa Isolated from Clinical Specimens. *Jundishapur Journal of Microbiology*, *14*(8), Article 8. https://doi.org/10.5812/jjm.118452

Bhagirath, A. Y., Li, Y., Somayajula, D., Dadashi, M., Badr, S., & Duan, K. (2016). Cystic fibrosis lung environment and Pseudomonas aeruginosa infection. *BMC Pulmonary Medicine*, *16*(1), 174. https://doi.org/10.1186/s12890-016-0339-5

Bjarnsholt, T., Tolker-Nielsen, T., Høiby, N., & Givskov, M. (2010). Interference of Pseudomonas aeruginosa signalling and biofilm formation for infection control. *Expert Reviews in Molecular Medicine*, *12*, e11. https://doi.org/10.1017/S1462399410001420

Cole, S. J., Records, A. R., Orr, M. W., Linden, S. B., & Lee, V. T. (2014). Catheter-Associated Urinary Tract Infection by Pseudomonas aeruginosa Is Mediated by Exopolysaccharide-Independent Biofilms. *Infection and Immunity*, *82*(5), 2048–2058. https://doi.org/10.1128/IAI.01652-14

Coșeriu, R. L., Vintilă, C., Mare, A. D., Ciurea, C. N., Togănel, R. O., Cighir, A., Simion, A., & Man, A. (2022). Epidemiology, Evolution of Antimicrobial Profile and Genomic Fingerprints of Pseudomonas aeruginosa before and during COVID-19: Transition from Resistance to Susceptibility. *Life*, *12*(12), Article 12. https://doi.org/10.3390/life12122049

Fazeli, N., & Momtaz, H. (2014). Virulence Gene Profiles of Multidrug-Resistant Pseudomonas aeruginosa Isolated From Iranian Hospital Infections. *Iranian Red Crescent Medical Journal*, *16*(10), e15722. https://doi.org/10.5812/ircmj.15722

Fazli, M., Almblad, H., Rybtke, M. L., Givskov, M., Eberl, L., & Tolker-Nielsen, T. (2014). Regulation of biofilm formation in Pseudomonas and Burkholderia species. *Environmental Microbiology*, *16*(7), 1961–1981. https://doi.org/10.1111/1462-2920.12448

Flemming, H.-C., & Wingender, J. (2010). The biofilm matrix. *Nature Reviews. Microbiology*, *8*(9), 623–633. https://doi.org/10.1038/nrmicro2415

Fujitani, S., Sun, H.-Y., Yu, V. L., & Weingarten, J. A. (2011). Pneumonia Due to Pseudomonas aeruginosa: Part I: Epidemiology, Clinical Diagnosis, and Source. *Chest*, *139*(4), 909–919. https://doi.org/10.1378/chest.10-0166

Galle, M., Carpentier, I., & Beyaert, R. (2012). Structure and Function of the Type III Secretion System of Pseudomonas aeruginosa. *Current Protein and Peptide Science*, *13*(8), 831–842. https://doi.org/10.2174/138920312804871210

Ghadaksaz, A., Imani Fooladi, A. A., Hosseini, H. M., & Amin, M. (2015). The prevalence of some Pseudomonas virulence genes related to biofilm formation and alginate production among clinical isolates. *Journal of Applied Biomedicine*, *13*(1), 61–68. https://doi.org/10.1016/j.jab.2014.05.002

Ghanbarzadeh Corehtash, Z., Khorshidi, A., Firoozeh, F., Akbari, H., & Mahmoudi Aznaveh, A. (2015). Biofilm Formation and Virulence Factors Among Pseudomonas aeruginosa Isolated From Burn Patients. *Jundishapur Journal of Microbiology*, *8*(10), e22345. https://doi.org/10.5812/jjm.22345

Golovlev, E. L. (2002). The Mechanism of Formation of Pseudomonas aeruginosa Biofilm, a Type of Structured Population. *Microbiology*, *71*(3), 249–254. https://doi.org/10.1023/A:1015866123848

Gonzalez, M. R., Fleuchot, B., Lauciello, L., Jafari, P., Applegate, L. A., Raffoul, W., Que, Y.-A., & Perron, K. (2016). Effect of Human Burn Wound Exudate on Pseudomonas aeruginosa Virulence. *MSphere*, *1*(2), e00111-15. https://doi.org/10.1128/mSphere.00111-15

Hall, S., McDermott, C., Anoopkumar-Dukie, S., McFarland, A. J., Forbes, A., Perkins, A. V., Davey, A. K., Chess-Williams, R., Kiefel, M. J., Arora, D., & Grant, G. D. (2016). Cellular Effects of Pyocyanin, a Secreted Virulence Factor of Pseudomonas aeruginosa. *Toxins*, *8*(8), 236. https://doi.org/10.3390/toxins8080236

Høiby, N. (2011). Recent advances in the treatment of Pseudomonas aeruginosa infections in cystic fibrosis. *BMC Medicine*, *9*, 32. https://doi.org/10.1186/1741-7015-9-32

Jabalameli, F., Mirsalehian, A., Khoramian, B., Aligholi, M., Khoramrooz, S. S., Asadollahi, P., Taherikalani, M., & Emaneini, M. (2012). Evaluation of biofilm production and characterization of genes encoding type III secretion system among Pseudomonas aeruginosa isolated from burn patients. *Burns*, *38*(8), 1192–1197. https://doi.org/10.1016/j.burns.2012.07.030

Kamali, E., Jamali, A., Ardebili, A., Ezadi, F., & Mohebbi, A. (2020). Evaluation of antimicrobial resistance, biofilm forming potential, and the presence of biofilm-related genes among clinical isolates of Pseudomonas aeruginosa. *BMC Research Notes*, *13*(1), 27. https://doi.org/10.1186/s13104-020-4890-z

Killough, M., Rodgers, A. M., & Ingram, R. J. (2022). Pseudomonas aeruginosa: Recent Advances in Vaccine Development. *Vaccines*, *10*(7), 1100. https://doi.org/10.3390/vaccines10071100

Kunwar, A., Shrestha, P., Shrestha, S., Thapa, S., Shrestha, S., & Amatya, N. M. (2021). Detection of biofilm formation among Pseudomonas aeruginosa isolated from burn patients. *Burns Open*, *5*(3), 125–129. https://doi.org/10.1016/j.burnso.2021.04.001

Lee, J., & Zhang, L. (2015). The hierarchy quorum sensing network in Pseudomonas aeruginosa. *Protein & Cell*, *6*(1), 26–41. https://doi.org/10.1007/s13238-014-0100-x

Parkins, M. D., Somayaji, R., & Waters, V. J. (2018). Epidemiology, Biology, and Impact of Clonal Pseudomonas aeruginosa Infections in Cystic Fibrosis. *Clinical Microbiology Reviews*, *31*(4), e00019-18. https://doi.org/10.1128/CMR.00019-18

Persat, A., Inclan, Y. F., Engel, J. N., Stone, H. A., & Gitai, Z. (2015). Type IV pili mechanochemically regulate virulence factors in Pseudomonas aeruginosa. *Proceedings of the National Academy of Sciences*, *112*(24), 7563–7568. https://doi.org/10.1073/pnas.1502025112

Rajabi, H., Salimizand, H., Khodabandehloo, M., Fayyazi, A., & Ramazanzadeh, R. (2022). Prevalence of algD, pslD, pelF, Ppgl, and PAPI-1 Genes Involved in Biofilm Formation in Clinical Pseudomonas aeruginosa Strains. *BioMed Research International*, *2022*, 1716087. https://doi.org/10.1155/2022/1716087

Raman, G., Avendano, E. E., Chan, J., Merchant, S., & Puzniak, L. (2018). Risk factors for hospitalized patients with resistant or multidrug-resistant Pseudomonas aeruginosa infections: A systematic review and meta-analysis. *Antimicrobial Resistance and Infection Control*, *7*, 79. https://doi.org/10.1186/s13756-018-0370-9

Reynolds, D., & Kollef, M. (2021). The Epidemiology and Pathogenesis and Treatment of Pseudomonas aeruginosa Infections: An Update. *Drugs*, *81*(18), 2117–2131. https://doi.org/10.1007/s40265-021-01635-6

Rocha, A. J., Barsottini, M. R. de O., Rocha, R. R., Laurindo, M. V., Moraes, F. L. L. de, & Rocha, S. L. da. (2019). *P. Aeruginosa*: Virulence Factors and Antibiotic Resistance Genes. *Brazilian Archives of Biology and Technology*, *62*. https://doi.org/10.1590/1678-4324-2019180503

Sauer, K., Stoodley, P., Goeres, D. M., Hall-Stoodley, L., Burmølle, M., Stewart, P. S., & Bjarnsholt, T. (2022). The biofilm life cycle: Expanding the conceptual model of biofilm formation. *Nature Reviews Microbiology*, *20*(10), Article 10. https://doi.org/10.1038/s41579-022-00767-0

Sharma, D., Misba, L., & Khan, A. U. (2019). Antibiotics versus biofilm: An emerging battleground in microbial communities. *Antimicrobial Resistance & Infection Control*, *8*(1), 76. https://doi.org/10.1186/s13756-019-0533-3

Sridhar, J., Kuriyan, A. E., Flynn, H. W., & Miller, D. (2015). ENDOPHTHALMITIS CAUSED BY PSEUDOMONAS AERUGINOSA: Clinical Features, Antibiotic Susceptibilities, and Treatment Outcomes. *Retina (Philadelphia, Pa.)*, *35*(6), 1101–1106. https://doi.org/10.1097/IAE.0000000000000469

Strateva, T., & Mitov, I. (2011). Contribution of an arsenal of virulence factors to pathogenesis of Pseudomonas aeruginosa infections. *Annals of Microbiology*, *61*(4), Article 4. https://doi.org/10.1007/s13213-011-0273-y

Tang, Y., Ali, Z., Zou, J., Jin, G., Zhu, J., Yang, J., & Dai, J. (2017). Detection methods for Pseudomonas aeruginosa: History and future perspective. *RSC Advances*, *7*(82), 51789–51800. https://doi.org/10.1039/C7RA09064A

Taylor, P. K., Yeung, A. T. Y., & Hancock, R. E. W. (2014). Antibiotic resistance in Pseudomonas aeruginosa biofilms: Towards the development of novel anti-biofilm therapies. *Journal of Biotechnology*, *191*, 121–130. https://doi.org/10.1016/j.jbiotec.2014.09.003

Thi, M. T. T., Wibowo, D., & Rehm, B. H. A. (2020). Pseudomonas aeruginosa Biofilms. *International Journal of Molecular Sciences*, *21*(22), Article 22. https://doi.org/10.3390/ijms21228671

Treviño González, J. L., Reyes Suárez, L. L., & Hernández de León, J. E. (2021). Malignant otitis externa: An updated review. *American Journal of Otolaryngology*, *42*(2), 102894. https://doi.org/10.1016/j.amjoto.2020.102894

Tuon, F. F., Dantas, L. R., Suss, P. H., & Tasca Ribeiro, V. S. (2022). Pathogenesis of the Pseudomonas aeruginosa Biofilm: A Review. *Pathogens*, *11*(3), Article 3. https://doi.org/10.3390/pathogens11030300

Wilson, M. G., & Pandey, S. (2022). Pseudomonas Aeruginosa. In *StatPearls [Internet]*. StatPearls Publishing. https://www.ncbi.nlm.nih.gov/books/NBK557831/

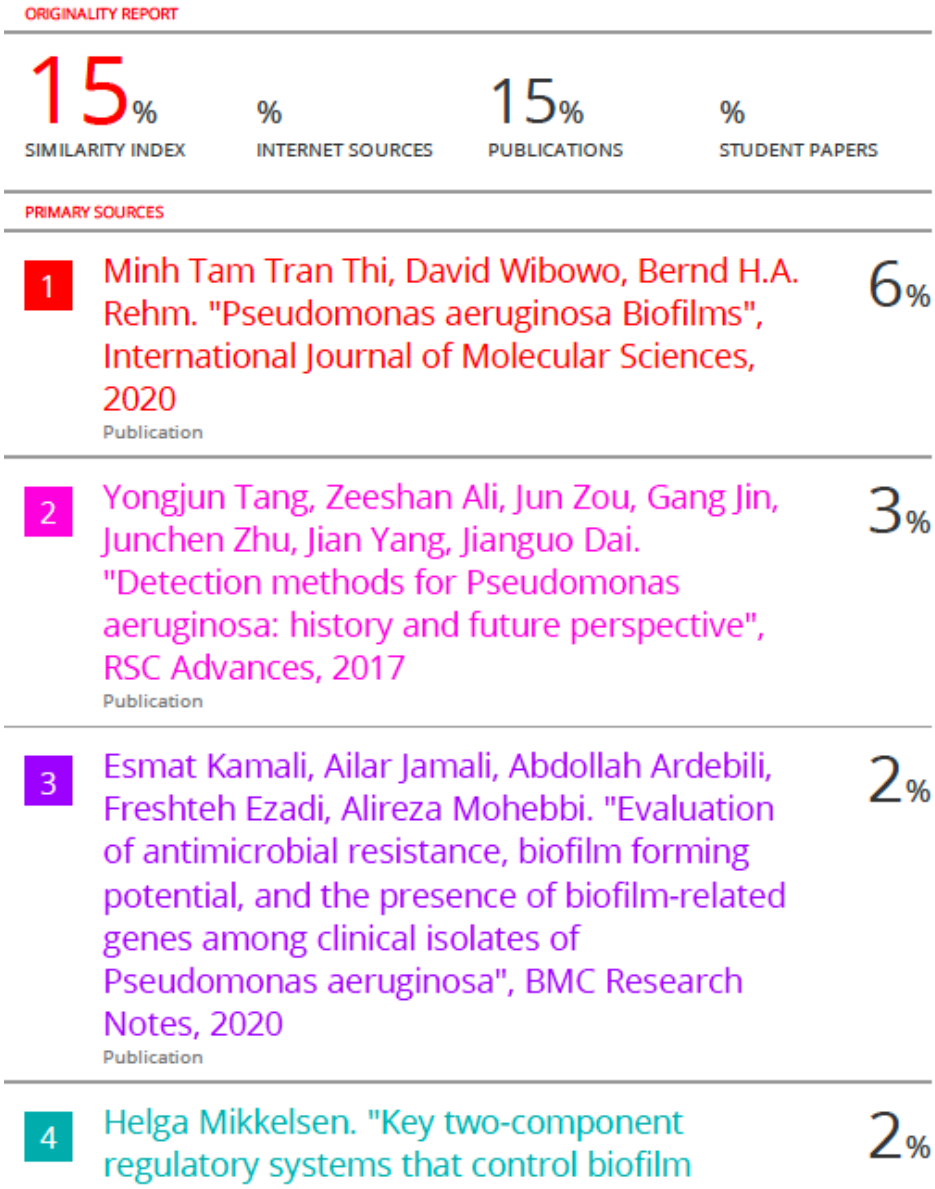
Wu, W., Jin, Y., Bai, F., & Jin, S. (2015). Chapter 41—Pseudomonas aeruginosa. In Y.-W. Tang, M. Sussman, D. Liu, I. Poxton, & J. Schwartzman (Eds.), *Molecular Medical Microbiology (Second Edition)* (pp. 753–767). Academic Press. https://doi.org/10.1016/B978-0-12-397169-2.00041-X

Zhao, Y., Lin, Q., Liu, L., Ma, R., Chen, J., Shen, Y., Zhu, G., Jiang, E., Mi, Y., Han, M., Wang, J., & Feng, S. (2020). Risk Factors and Outcomes of Antibiotic-resistant Pseudomonas aeruginosa Bloodstream Infection in Adult Patients With Acute Leukemia. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America*, *71*(Suppl 4), S386–S393. https://doi.org/10.1093/cid/ciaa1522

APPENDICES

Appendix A

Turnitin Similarity Report



ORIGINALITY REPORT

15%	%	15%	%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	Minh Tam Tran Thi, David Wibowo, Bernd H.A. Rehm. "Pseudomonas aeruginosa Biofilms", International Journal of Molecular Sciences, 2020 Publication	6%
2	Yongjun Tang, Zeeshan Ali, Jun Zou, Gang Jin, Junchen Zhu, Jian Yang, Jianguo Dai. "Detection methods for Pseudomonas aeruginosa: history and future perspective", RSC Advances, 2017 Publication	3%
3	Esmat Kamali, Ailar Jamali, Abdollah Ardebili, Freshteh Ezadi, Alireza Mohebbi. "Evaluation of antimicrobial resistance, biofilm forming potential, and the presence of biofilm-related genes among clinical isolates of Pseudomonas aeruginosa", BMC Research Notes, 2020 Publication	2%
4	Helga Mikkelsen. "Key two-component regulatory systems that control biofilm	2%

Appendix B

CURRICULUM VITAE

1. PERSONAL INFORMATION

NAME, SURNAME:	Batur Özler
DATE of BIRTH and PLACE:	09/08/1993 Bostancı/TRNC
CURRENT OCCUPATION: MSc Student ADDRESS of CORRESPONDENCE: Gaziveren/Lefke - TRNC TELEPHONE: 05488731525 E-MAIL: baturozler@gmail.com	

2. EDUCATION

YEAR	GRADE	UNIVERSITY	FIELD
2011-2019	2.83/4.00	Ankara University	Biology

3. ACADEMIC EXPERIENCE

PERIOD	TITLE	DEPARTMENT	UNIVERSITY
N/A			

4. FIELD OF INTERESTS

FIELDS OF INTERESTS	KEY WORDS
Clinical Microbiology, Molecular Microbiology	biofilms, probiotics, microbiology, molecular biology, biotechnology, genetics

Appendix C

Ethical Approval

YAKIN DOĞU ÜNİVERSİTESİ
BİLİMSEL ARAŞTIRMALAR ETİK KURULU

ARAŞTIRMA PROJESİ DEĞERLENDİRME RAPORU

Toplantı Tarihi :24.02.2022

Toplantı No :2022/100

Proje No :1493

Yakın Doğu Üniversitesi Tıp Fakültesi öğretim üyelerinden Doç. Dr. Buket Baddal'ın sorumlu araştırmacısı olduğu, YDU/2022/100-1493 proje numaralı ve **"Investigation of Pseudomonas aeruginosa biofilm formation and toxin synthesis in clinical isolates at Near East University Hospital"** başlıklı proje önerisi kurulumuzca değerlendirilmiş olup, etik olarak uygun bulunmuştur.

Prof. Dr. Şanda Çalı

Yakın Doğu Üniversitesi

Bilimsel Araştırmalar Etik Kurulu Başkanı

Kurul Üyesi	**Toplantıya Katılım**	**Karar**
	Katıldı(✓)/ Katılmadı(X)	*Onay(✓)/ Ret(X)*
Prof. Dr. Tamer Yılmaz	✓	✓
Prof. Dr. Şahan Saygı	✓	✓