NEAR EAST UNIVERSITY

INSTITUTE OF GRADUATE STUDIES

DEPARTMENT OF MEDICAL MICROBIOLOGY AND CLINICAL MICROBIOLOGY

INVESTIGATION OF *PSEUDOMONAS AERUGINOSA* BIOFILM FORMATION AND TOXIN SYNTHESIS IN CLINICAL ISOLATES AT NEAR EAST UNIVERSITY HOSPITAL

M.Sc. THESIS

Afnan Saadeldin Bashir Elhag ALI

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Advisor
Assoc. Prof. Buket BADDAL

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Approval

We certify that we have read the thesis submitted by Afnan Ali titled **"Investigation of *Pseudomonas aeruginosa* Biofilm Formation and Toxin Synthesis in Clinical Isolates at Near East University Hospital"** and that in our combined opinion 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.

Afnan Ali

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"There is no value in anything until it is finished"

- Genghis Khan

Acknowledgments

What an incredible journey! Completing a master thesis necessitates a combination of perseverance, luck, and faith in others. Fortunately, I had a supervisor who made things much easier for me than they could have been. Dr. Buket, you have been an excellent mentor who has continued to feed my enthusiasm by providing me with numerous possibilities to enhance my profession. You have always been supportive and ready to chat, whether it was giving me larger role within the research, motivating me to assist on other projects, or even encouraging me to find my own way in developing and confidently working in the lab. I've learned a lot thanks to your guidance and instruction. Thank you for your time and attention to my journey; I will apply many of the techniques I learned directly from you in the future.

I'd also like to thank my colleagues for putting up with me during my time there. Batur and Tchamou, in particular, made the difficult times bearable while also making the easy times interesting.

Words cannot express how wonderful my parents, Saad and Magda, and the rest of my family have been. You've always believed in me, encouraged me, and pushed me to achieve my goals. This is as much your achievement as it is mine, because without you, none of this would have been possible. You've earned it as a reward for how wonderful you've been.

Afnan Ali

Abstract

Investigation of *Pseudomonas aeruginosa* Biofilm Formation and Toxin Synthesis in Clinical Isolates at Near East University Hospital

Afnan Saadeldin Bashir Elhag Ali

M.Sc., Medical Microbiology and Clinical Microbiology Department

Supervisor: Assoc. Prof. Buket Baddal

May, 2022, 69 pages

Pseudomonas aeruginosa is a common bacterium that causes nosocomial infections in hospitals worldwide. It is an opportunistic infection that infects immunocompromised individuals, particularly those with severe burns, cystic fibrosis, cancer, or AIDS. *P. aeruginosa* infections are notoriously difficult to treat since the organism is innately resistant to numerous antibiotic classes and can develop resistance to all available drugs. Antibiotic resistance strains have emerged and spread quickly, posing a threat that must be addressed immediately. The virulence factor involved in the pathogenesis determines the severity of an infection. This bacterium produces a wide range of pathogenic virulence factors, including: Biofilm formation, phospholipase C, pigment production, exotoxins and cytotoxins, elastase, protease A, flagella and pili, and QS regulatory system proteins. The purpose of this study is to investigate the ability of *P. aeruginosa* to produce biofilm as well as identify the prevalence of exotoxin A and Type III secretory proteins exoT, exoS and exoU in clinical isolates at Near East University Hospital. Sixty-seven isolates were identified by standard microbiological methods including colony morphology, fruity odor, pigment production, Gram staining, and gene amplification by polymerase chain reaction (PCR). Type III effector toxins, Exotoxin T gene, Exotoxin S gene, and Exotoxin U gene were detected in 86.6 %, 59.7 % and 32.8 % of the isolates respectively. This study demonstrates the first report to investigate how intracellularly produced T3SS effectors and exotoxin A contribute to pathogenesis in Northern Cyprus, and it has paved the way for more in-depth research.

Keywords: *Pseudomonas aeruginosa*, biofilm, virulence factors, exotoxin A, type III secretion

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List of Abbreviations

T3SS: Type III secretion system

AIDS: Acquired immunodeficiency syndrome

CF: Cystic fibrosis

QS: Quorum Sensing System

IQS: Integrated Quorum Sensing

PQS: The *Pseudomonas* Quinolone Signal

TCSs: Two-Component Regulatory Systems

ADPRT: ADP ribosyl transferase

HK: Histidine kinase

DHp: Histidine phosphotransfer domain

eDNA: Extracellular DNA

ECM: Non-crystalline extracellular matrix

HAI: Healthcare-associated infections

UTIs: Urinary tract infections

CBD: chaperone binding domain

ECDC: European Center for Disease Prevention and Control

EUCAST: European Committee on Antimicrobial Susceptibility Testing

MLD: Membrane localization domain

GAP: GTPase activating protein

CoNS: Coagulase negative staphylococci

ADP: Adenosine diphosphate

NAD+: Nicotinamide adenine dinucleotide cation

PE: *Pseudomonas* exotoxin A

Exotoxin A: *exoA*

Exotoxin S: *exoS*

Exotoxin U: *exoU*

Exotoxin T: *exoT*

Exotoxin Y: *exoY*

TCS: Two-component system

ICU: Intensive care unit

MDR: Multi-drug resistant

TRNC: Turkish Republic of Northern Cyprus

WHO: World Health Organization

CHAPTER I

Introduction and Aims

1. Aims and Scope

Pseudomonas aeruginosa clinical outcome and illness etiology are influenced by antibiotic resistance patterns, biofilm production, and virulence characteristics in bacterial strains. The infections caused by this bacterium can be life threatening due to a variety of mechanisms for adaptability, survival, and resistance to various antibiotics. These factors have combined to create a major public health concern that must be discussed.

Pseudomonas is derived from the Greek words pseuds (false) and monas (unit). Earlier in the history of microbiology, the stem word mona was used to refer to germs. The name *aeruginosa* is derived from the Latin word verdigris, which means "copper rust" and refers to the blue-green color of the species' bacterial cultures. This blue-green pigment is composed of two *P. aeruginosa* metabolites, pyocyanin (blue) and pyoverdine (green), which give cultures their distinctive blue-green color (“Etymologia,” 2012; Lister et al., 2009). Carle Gessard, a chemist and bacteriologist from Paris, France, was the first to discover *P. aeruginosa* in 1882, during an experiment in which the bacterium was distinguished by its water-soluble pigments when exposed to ultra-violet light and turned blue-green (Lister et al., 2009). *P. aeruginosa* has a distinct odor that has been described as smelling like corn tortillas, grapes, or Pear Drops on agar plates.

Pseudomonas aeruginosa is the leading cause of nosocomial infections. It is one of the most commonly isolated pathogens that cause both acute and chronic infections. This organism is widely distributed in nature and commonly occurs among hospitalized patients. It is an opportunistic pathogen which often infects immunocompromised individuals, such as those suffering from severe burns, acquired immunodeficiency syndrome (AIDS), cystic fibrosis, or cancer. *P. aeruginosa* significance in severe respiratory and urinary tract infections, skin and soft tissue infections, and bacteremia underlines the organism's antibiotic resistance and its presence in nosocomial epidemics around the world (Moradali et al., 2017).

Although it rarely causes illness in healthy people outside of a healthcare setting, it may cause a significant risk to hospitalized patients, particularly those with severe underlying diseases. The increase fatality rate associated with these infections are attributed to bacterial antibiotic resistance, the combination of compromised host defenses, and the development of extracellular bacterial enzymes and toxins. Therefore, the pathogenicity of *P. aeruginosa* is influenced by a number of factors that will be discussed in this research (Uddin et al., 2021). The bacterium produces exotoxin A (ETA), one of its major virulence components. Most of the isolates harbors the *exoA* gene, which encodes ETA, that is crucial for inhibiting cellular protein synthesis and inducing tissue necrosis in *P. aeruginosa* infections (Nicas & Iglewski, 1985). The bacterium's ability to produce biofilm is another important factor that enables it to develop severe infections, making it extremely resistant to the immune system of the host as well as existing antibiotic therapy (Pericolini et al., 2018). A further key virulence determinant in *P. aeruginosa* is the type III secretion system (T3SS), also known as injectisome, which is a needle-like structure that allows the bacterium to inject bacterial toxins into the cytoplasm of the host cell. Many other gram-negative bacteria share this complex bacterial mechanism (Hauser, 2009).

The purpose of this study is to determine the molecular prevalence of type III secretion system effector toxins (ExoT, ExoS, and ExoU) *in P. aeruginosa* isolates from patients admitted to the Near East University Hospital in Northern Cyprus, as well as to investigate biofilm formation in the clinical strains.

CHAPTER II

Literature Review

2. General Information

2.1. The genus *Pseudomonas*

Pseudomonas is one of the most diverse bacterial genera, and it is currently the genus of Gram-negative bacteria with the largest number of species (Gomila et al., 2015). Members of the *Pseudomonas* are gram negative, rod shaped, motile, aerobic, non-spore forming, and encapsulated. They test negative for Voges Proskauer, indole, and methyl red but positive for catalase. Although some species react negatively in the oxidase test, most species, including *P. fluorescens*, respond positively (Meyer, 1987).

Another defining feature of *Pseudomonas* is the secretion of pyoverdin, a fluorescent yellow-green pigment (siderophore) that is secreted under iron-limiting conditions. Other pigments produced by *Pseudomonas* species include pyocyanin (blue pigment, a siderophore) secreted by *P. aeruginosa*, quinolobactin (yellow, dark green in presence of iron, a siderophore) secreted by *P. fluorescens*, pyorubrin (reddish pigment), and pyomelanin (brown pigment). A hemolytic reaction can be seen on blood agar (Alavi et al., 2013).

Pseudomonas has 144 species, making it the most diverse genus of Gram-negative bacteria (Gomila et al., 2015). They are non-motile and non-spore forming. They are halotolerant (growth in the presence of 10% sodium chloride) and most of the species are facultative anaerobe such as *P. fluorescens* and *P. denitrificans* by using nitrate or nitrite as a terminal electron acceptor. *P. aeruginosa* was thought to be an obligately aerobic bacterium, but it is now known to be highly adapted to anaerobic conditions. The bacterium can adapt and proliferate in conditions of partial or total oxygen deficiency (Arai, 2011; Kampers et al., 2021). To date, the genus *Pseudomonas* contains over 140 species, the majority of which are saprophytic. Humans are associated with over 25 species.

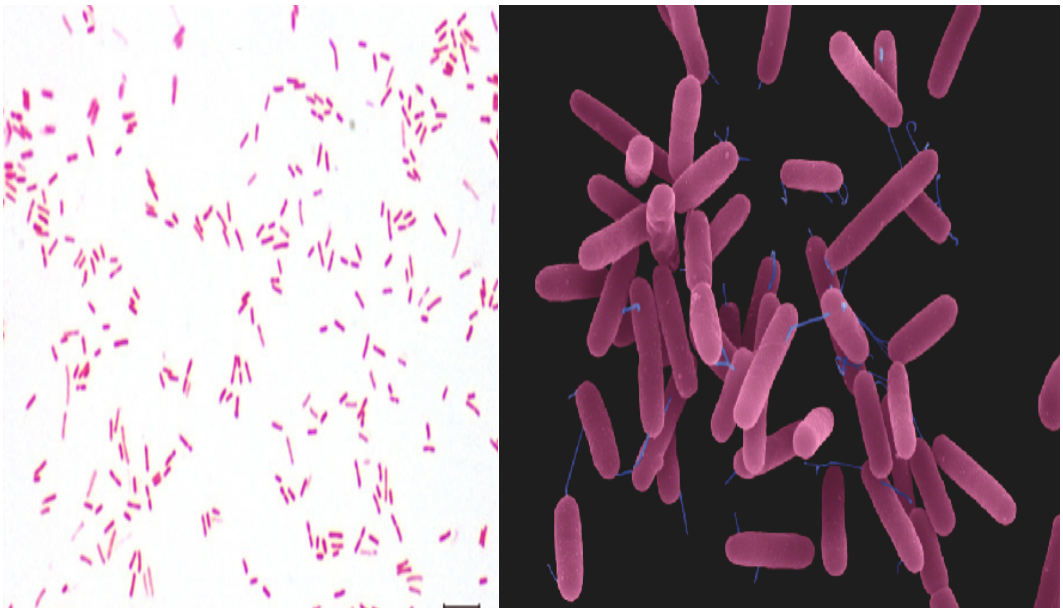


Figure 1. *P. aeruginosa* micrographs by Gram-staining and electron microscopy imaging Contributed by The Centers for Disease Control and Prevention (CDC)

2.2. Pseudomonas aeruginosa

P. aeruginosa is one of the most common opportunistic pathogens that causes nosocomial infections. The infections are difficult to treat since the bacterium is capable of developing resistance to a wide range of antibiotics. One of the most important aspects in its pathogenicity is its inherent resistance to antibiotics and disinfectants (Pang et al., 2019).

2.3 Metabolic evolution of *Pseudomonas aeruginosa*

The ability of the bacterium to obtain the nutrients required for growth and survival is critical for pathogenesis. Survival of bacteria in a new environment is dependent on their ability to detect and respond to available nutrients, including the activation of certain metabolic pathways that enhance growth efficacy (Brown et al., 2008). *P. aeruginosa* typically colonizes the airways of cystic fibrosis (CF) patients as an opportunistic pathogen, where the nutritional content and environmental factors vary enormously from its typical natural habitat. In response to available nutrition and physicochemical

conditions, *P. aeruginosa* migration from the environment to the airways of CF patients demands reprogramming of regulatory and metabolic networks. As a result of their survival and adaptability, environmental isolates of *P. aeruginosa* have significant growth rates and a diverse metabolism, as opposed to airway adapted isolates (Yang et al., 2008).

2.4 The chromosome

Strain PAO1, originating from an Australian wound isolation from the 1950s, was the first to have its entire genome sequenced (Stover et al., 2000). The PAO1 strain has been and continues to be the most widely used genetic and functional reference for *P. aeruginosa* research. The PAO1 genome is a circular chromosome with a 6.264-Mbp size that encodes 5,570 estimated protein coding sequences (Klockgether et al., 2011).

2.4.1 The core genome

P. aeruginosa genome is large over 6 Mbp in size, and encodes around 6000 open reading frames. It has a mosaic structure, with a large core genome into which accessory genes are introduced at specified sites, referred to as the region of genomic plasticity (RGP) (Valot et al., 2015). While the diversity of genomic islands is well established, doubts remain about the size and composition of the core genome (Mathee et al., 2008). The genome encodes a large number of regulatory enzymes that are necessary for organic molecule metabolism, transportation, and efflux. The higher coding capability of the *P. aeruginosa* genome allows for greater metabolic diversity and environmental adaptability (Stover et al., 2000).

2.5 Laboratory diagnosis

The isolation and laboratory identification of *P. aeruginosa* are required for diagnosis. It grows well on a variety of laboratory media and is frequently isolated on blood agar plates or eosin-methylthionine blue agar, but Mueller-Hinton agar is most commonly used to stimulate the production of its blue-green pigments (pyocyanin and pyoverdin). The bacterium is characterized by its morphology, inability to ferment lactose,

positive oxidase reaction, fruity odor, and capacity to grow at 42 °C. Fluorescence under UV light aids in the early identification of its colonies and can also be used to detect the bacteria in wounds. It also tends to form mucoid smooth colonies with flat edges, due to the synthesis of slime polysaccharide, which is commonly derived from respiratory and urinary tract secretions The bacteria are surrounded by a zone of beta hemolysis when grown on blood agar. The Kirby-Bauer disk diffusion test is the most extensively used method for antimicrobial susceptibility testing because it allows for simultaneous testing of bacterial sensitivity to several antibiotics.

2.6 Key virulence factors of *Pseudomonas aeruginosa*

A significant number of cell-associated and extracellular factors have a role in the pathogenicity of the bacterium. Several cell-based virulence factors such as pili, lectins, alginate, and lipopolysaccharide, as well as secreted virulence factors like pyocyanin, cytotoxin, proteases, hemolysins, siderophores, exotoxin A, type III secretion systems exoenzymes, allow *P. aeruginosa* to cause infections (Strateva & Mitov, 2011). The virulence factors play an important pathogenic role in the colonization, survival, and bacterial tissue invasion. Virulence factors come in two varieties: (1) acute infection factors: these factors are found on the surface of *P. aeruginosa* or are released in the form of enzymes. The pili help the bacterium to adhere to the epithelium. *P. aeruginosa* effector exoenzymes from the Type III secretion system are antiphagocytic and cytotoxic, and can aid in epithelial cell adhesion. Exoenzyme S plays a pathogenic function by disrupting normal cytoskeletal structure, destroying immunoglobulin G and A, causing actin filament depolymerization, and contributing to macrophage resistance (Kroken et al., 2022). At least four proteolytic enzymes produced by *P. aeruginosa* cause tissue necrosis and hemorrhage. Exotoxin A is responsible for tissue necrosis. Phospholipase C are a diverse category of enzymes with the ability to hydrolyze one or more molecules in glycerophospholipids (Hamood et al., 1996). (2) virulence factors involved in chronic infection: siderophores (pyoverdin and pyochelin) enable the bacteria to proliferate in the absence of ferrous ions (Ben Haj Khalifa et al., 2011). A capsule made of alginate protects *P. aeruginosa* isolated from cystic fibrosis patients against phagocytosis, dehydration, and

antibiotics. The bacteria also promotes biofilm formation by attaching to epithelial cells, which serves as a barrier between them and the external environment, as well as making them resistant antimicrobial agents (Costerton et al., 1995). The majority of these virulence determinants are regulated by the quorum sensing system and the two-component transcriptional regulatory system. The survival and proliferation of the pathogen in the host is dependent on these two mechanisms.

2.7 The quorum sensing system (QS)

Quorum sensing is a bacterial cell-to-cell communication system that modify gene expression and controls specialized processes only when the impact on the environment or on a host is enhanced. One of the unique effects of quorum sensing is that certain products are delayed until the population load is increased. *P. aeruginosa* employs quorum sensing (QS) in order to modulate gene transcription in response to cell density. (Ding et al., 2018). Las, rhl, pqs, and integrated QS (IQS) are the four types of QS systems that have been addressed in *P. aeruginosa* to date (Vetrivel et al., 2021).

In the las system, LasI synthase induces the synthesis of the signaling molecule N-(3-oxododecanoyl)-L-homoserine lactones (3-oxo-C12-HSL), which is identified by its LuxR-type receptor protein LasR and regulates transcription of target genes. In the rhl system, the signaling molecule N-butanoyl-L-homoserine lactone (C4-HSL) developed via RhlI synthase is identified by its signal receptor RhlR in the rhl system, causing targeted gene expression to be regulated (M & Ep, 2006). Both the las and rhl systems are involved in biofilm formation as well as the regulation of numerous gene expressions required for the synthesis of virulence factors (K. Lee & Yoon, 2017).

The third QS system, pqs, produces the signaling component 2-heptyl-3-hydroxy-4-quinolone (PQS), which identifies and regulates eDNA release during biofilm formation via its cognate receptor PqsR (Pamp & Tolker-Nielsen, 2007). Other metabolic processes modulated by the pqs system in *P. aeruginosa* include the production of elastase, rhamnolipid, and membrane formation (Senturk et al., 2012). Many researchers have shown that pqs is significant for virulence, and it is becoming more common in *P. aeruginosa*-infected cystic fibrosis patients.

In the QS system, the autoinducer component 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde is detected by IqsR, las, and PhoB, a phosphate stress regulatory response (Déziel et al., 2004). *P. aeruginosa* has a highly complex quorum sensing system with many signals and receptors. RpoS and RpoN receptors, global transcription regulators like AlgQ, MvaT, DksA, and VfR, and mainly two LuxR homologs, QscR and VqsR, are all known to regulate the QS signaling circuit (J. Lee & Zhang, 2015). QscR is a critical component of the *P. aeruginosa* QS system because it acts as a down regulation of the QS system by repressing both the las and rhl systems (M & Ep, 2006). The GacS/GacA are two component systems regarded as a super-regulator of the QS network that modulates the synthesis of virulence-associated factors as well as biofilm formation (Rodrigue et al., 2000). The integrated QS network in *P. aeruginosa* is illustrated in Figure 2.

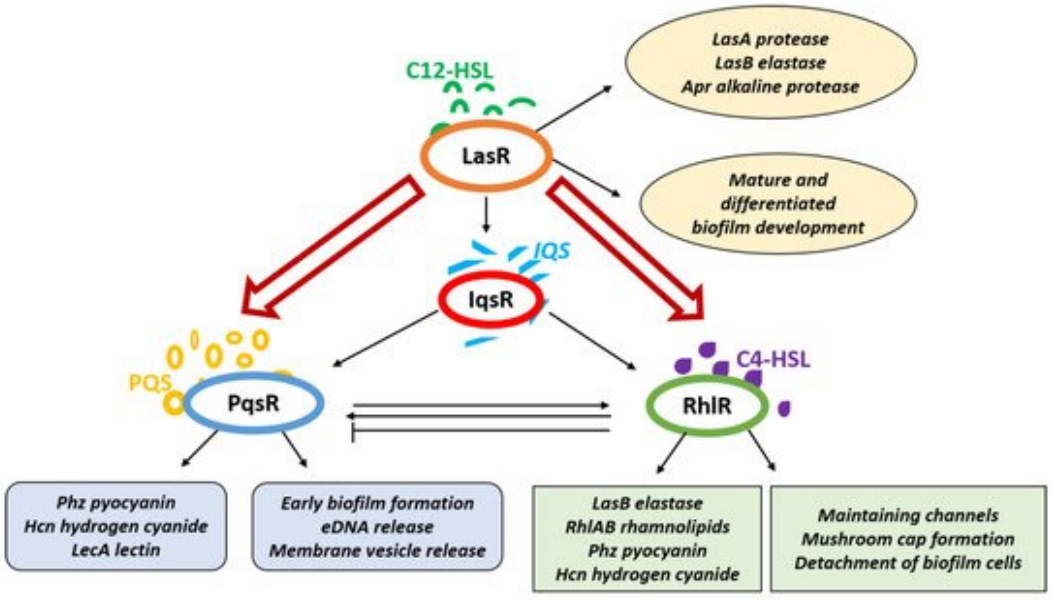


Figure 2. Quorum sensing (QS) mechanism of *Pseudomonas aeruginosa* (Vetrivel et al., 2021).

2.8 Two-Component Regulatory System (TCSs)

Two-Component Regulatory Systems (TCSs) in *P. aeruginosa* are significant for modulating virulence determinants in both endogenous and exogenous promoters. Several TCSs and numerous regulatory genes make up its genome (Rodrigue et al., 2000). It

consists of both resistance and virulence characteristics, and its adaptability is due to its vast genome and core-essential genes (Stover et al., 2000). TCSs are important signal transduction mediators in bacteria (Mitrophanov & Groisman, 2008). They are also vital for detecting numerous external stimuli and adapting to environmental modification. A histidine kinase (HK) as well as its cognate response regulator (RR) make up the majority of these TCSs, which tends to regulate multiple signaling pathways (Mitrophanov & Groisman, 2008).

The major domains of the sensor histidine kinases include the cytoplasmic sensor domain, signal transduction domain, dimerization histidine phosphotransfer domain (DHp), and ATP catalytic domain. The HK sensor is essential for autophosphorylating the conserved histidine in the HK domain and transferring the phosphate from such a residue to the aspartate residue of its cognate RR. The cognate response regulator controls intracellular activities, and its protein interaction domain undergoes conformational modifications that enabling it to attach to DNA, resulting in changes in gene expression (Stock et al., 2000; Figure 3).

Generally, TCSs are found in bacterial genomes in various amounts, and the overall number changes from bacterium to another. Many bacterial species require a wide range of TCSs due to the various input detecting domains of HK, that enable bacteria to accept a number of environmental stimuli. The number of TCSs associated with pathogenicity of *P. aeruginosa* is quickly increasing, due to the improvement of whole genome-based approaches (Bhagirath et al., 2019). Therefore, acknowledging the serious illnesses caused by *P. aeruginosa* infection and developing innovative medications to treat this pathogenic bacterium require understanding the mechanisms by which TCSs receive and respond to environmental signals, as well as regulating virulence component synthesis throughout the infection.

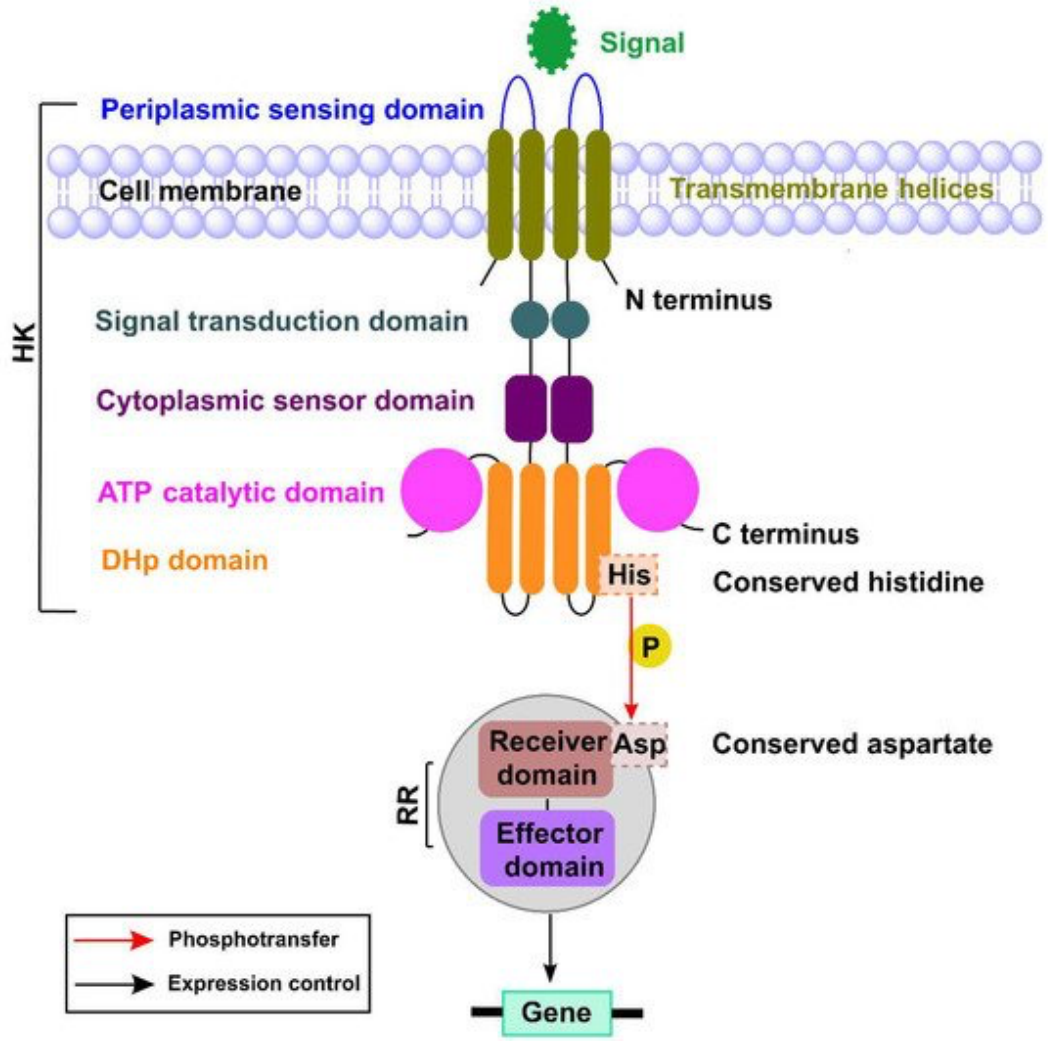


Figure 3. A two-component signaling systems fundamental mechanisms. The sensing domain, which is linked to the signal transduction domain, as well as an ATP catalytic domain, a cytoplasmic sensor domain, and a dimerization histidine phosphotransfer domain (DHp), all work together to detect environmental signals. The phosphate is transferred by the conserved histidine residue of HK to the conserved aspartate residue of the recipient domain of its RR. The effector domain of a phosphorylated RR interacts with its intended target and helps regulate gene expression. (Sultan et al., 2021).

2.9 Biofilm: overview

Microorganisms are rarely observed as planktonic species, as the most prevalent preferred growth state is the formation of a biofilm, which is present in 90% of bacteria, especially in chronic infections (Li & Lee, 2017). Microorganisms regulates many biological processes during pathogenicity and antimicrobial therapy, and they use evolved genotypic events to promote diverse molecular pathways and phenotypes that are essential for survival in a changing environment (Moradali et al., 2017). Biofilms are multicellular colonies made up of bacteria enclosed in a non-crystalline extracellular matrix (ECM) that is persistently attached to a surface and difficult to remove even with mild cleaning. Anton Van Leeuwenhoek first discovered the notion of biofilm in 1684 while using a primitive microscope to examine the surface of a tooth (Donlan & Costerton, 2002). Biofilms can be found on a wide range of surfaces, including living tissues, hotels, industrial settings, laboratories, wastewater pipelines, restrooms, and catheterization medical equipment, and are most commonly found on hard surfaces immersed in or exposed to an aqueous media (Costerton et al., 1999).

Biofilms are heterogeneous, with microcolonies having 15% of the cells and 85% of the polymeric extracellular material. The biofilm matrix varies by species, but it often comprises proteins, polysaccharides, and nucleic acids. The matrix is also engaged in a number of other functions, including cell-to-cell communication, surface adherence, quorum sensing (QS), and even resistance (Flemming & Wingender, 2010).

2.9.1 The role of biofilm in health-care-associated infections

Biofilm growth in unwanted places may lead to medical and industrial concerns since they show resistance to cellular immunity in the host, antibacterial, and biocide treatments, while biofilm formation on man-made surfaces confers its relevance in relation to pathogenicity (Harmsen et al., 2010). As a result, it plays a significant role in healthcare-associated infections (HAI), particularly in those associated with the implantation of medical devices such as urine catheters, orthopedic implants, and intravascular catheters. According to the European Center for Disease Prevention and Control 2008, HAI affect around 4,000,000 patients in European hospitals each year, resulting in around 40,000

deaths (Francolini & Donelli, 2010). Every year, approximately 200,000 cases of bloodstream infections are reported in the United States as a result of the implantation of central venous catheters (Mermel et al., 2001). According to current reports, catheter-associated urinary tract infections impact almost 500,000 patients in US hospitals each year (UTIs) (Klevens, 2007).

The most common bacterial infections affecting people are urinary tract infections (UTIs), which represent a public health problem among biofilm-associated infections. Despite this, biofilm-related bloodstream infections were the 12th leading cause of mortality in 2017, with a fatality rate of up to 30 percent (Pinto et al., 2021).

Biofilms account for over 80% of all microbial infections today, with biofilms on medical equipment accounting for 60–70% of nosocomial infections (Wenzel, 2001). Biofilm solutions are still in demand in a range of industries, including biomedical and environmental fields, nearly decades after the first publication on the subject (Maukonen et al., 2003).

2.9.2 Pseudomonas aeruginosa biofilms

Given the prevalence of antimicrobial-resistant strains that cause life-threatening illnesses, *P. aeruginosa* is one of the "top ten" most common hospital "superbugs." It is the most commonly isolated hospitalized pathogen from chronic wounds and is regarded as a strong biofilm producer because it acts as a barrier in wound repair and has high antibiotic resistance (Klrissa & Mohammad, 2016).

P. aeruginosa was ranked as the sixth most frequently occurring organism responsible for nosocomial infections, the second most common pathogen responsible for ventilator-related pneumonia, and the seventh main causative pathogen of catheter-linked bloodstream infections, accounting for a high death rate in individuals with cystic fibrosis, AIDS and burn wounds, according to the US National Healthcare Safety (Valderrey et al., 2010).

P. aeruginosa biofilm-forming mechanism begins with a free-floating bacterium that is reversibly adhered to a conditioned surface, then adherent bacteria are irreversibly

attached to such a surface via adhesins, followed by the formation of an extracellular matrix to form a fully matured biofilm. Microorganisms eventually disperse from the matrix and colonize other surfaces (Figure 4).

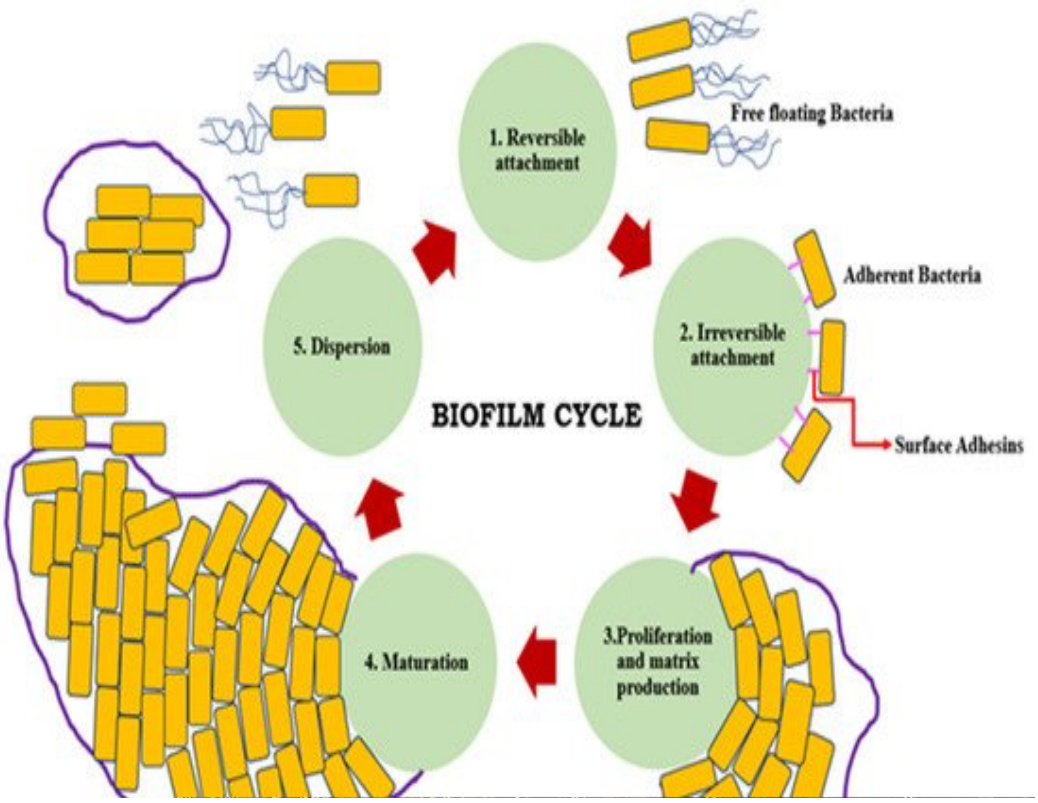


Figure 4. The process of the biofilm cycle (Costerton et al., 1999)

Several polysaccharides, such as alginate, pel, and psl, determine the stability of *P. aeruginosa* biofilm structure (Ghafoor et al., 2011). Alginate is a polymer chain made up of D-mannuronic acid and L-glucuronic acid that runs in a linear fashion. The biofilm structure requires this polymer for protection and stability. Alginate also enables the matrix's contents, such as nutrients and water, to last longer (Rasamiravaka et al., 2015). Pel polysaccharide is a glucose-rich matrix substance whose composition remains unknown, and psl is a pentasaccharide made up of repeating D-mannose, L-rhamnose, and D-glucose residues. Each of these polysaccharides contributes to the initial stages of biofilm formation whilst also serving as a main structural substrate. (Tacconelli et al., 2018). Extracellular DNA (eDNA), which is considered to be a source of nutrition for

embedded bacterium and takes part in cell-to-cell interaction, is another important component of the *P. aeruginosa* biofilm. The synthesis of alginate, pel, and psl polysaccharides is regulated by bis-(3-5)-cyclic dimeric guanosine monophosphate (c-di-GMP), an intercellular widespread second agent found in bacteria. Excessive amounts of c-di-GMP stimulate the formation of alginate and pel polysaccharides, while lower concentrations of c-di-GMP promote bacterial motility, however the exact mechanism that helps regulate the polymerization of these polysaccharides substances remains uncertain (V. T. Lee et al., 2007).

2.9.3 Biofilm challenges in *P. aeruginosa* to antimicrobial agents

Planktonic cells are more susceptible to antibiotics and antimicrobial agents, but bacteria in a biofilm matrix are resistant to the host immune system and antimicrobials due to their high tolerance and resistance. The majority of microorganism mechanisms of resistance are transferable and do not require antibiotic interaction with the target (Olivares et al., 2020). The biofilm composition of *P. aeruginosa* have a higher level of antibiotic resistance for a variety of reasons, including mild or insufficient antibiotic penetration, a modified chemical conditions within the biofilm, and cellular proliferation in a biofilm (Mah & O'Toole, 2001). Every one of these mechanisms occur as a consequence of the multicellular nature of biofilm communities, resulting in antibiotic resistance of biofilm matrix and treatment approach failure (Kragh et al., 2016). Treatment of *P. aeruginosa* infections has become extremely difficult due to high levels of resistance to the majority of available antibiotics. The World Health Organization (WHO) recently identified *P. aeruginosa* as a potentially fatal pathogen in which novel antibiotics must be developed to prevent infection (Tacconelli et al., 2018). Empirical antibiotic therapy has been utilized to treat *P. aeruginosa* infections to date, but growing antibiotic use could lead to multidrug-resistant strains of *P. aeruginosa* and the failure of empirical antibiotic therapy against this type of bacteria (Park et al., 2012).

P. aeruginosa confers antibiotic resistance through a variety of mechanisms, including intrinsic, acquired, and adaptive resistance (Hancock & Speert, 2000). The intrinsic type of resistance is characterized by a significant reduction in outer membrane

permeability, efflux pump activity, and the production of antibiotic-inactivating enzymes while the, mutational changes or horizontal resistance gene transport are examples of acquired resistance, and the final adaptive resistance is linked to the production of biofilms in infected individuals' lungs, which might act as a diffusion barrier, preventing antibiotics from reaching the bacteria (Breidenstein et al., 2011).

Apart from the above-mentioned resistance mechanisms, multidrug-tolerant persister cells may grow in biofilm communities, surviving anti-bacterial attacks and infecting cystic fibrosis patients for lengthy periods of time. Persisters are a bacterial group with multidrug tolerance as a phenotypic trait instead of genetic differences (Mulcahy et al., 2010). Mulachy et al. discovered that cystic fibrosis patients have higher levels of persister cells than wild-type *P. aeruginosa* strains, implying that they are extremely antibiotic-resistant and multidrug-tolerant (Mlynarcik & Kolar, 2017; Mulcahy et al., 2010). Over time, the majority of biofilm cells enter the stationary phase, and persister cells become abundant (Amato et al., 2014; Keren et al., 2011).

Biofilm formation occurs on medical implants such as cardiac and urine catheters, heart valve replacements, and joint implants, antibiotic-resistant bacterial cells that can result in human infections. They pose a serious risk to humans when it comes to their pathogenicity and are linked to a vast number of pathogenic infections (Kaur et al., 2017; Singh et al., 2000). Based on several findings, Sharma et al indicates that the multicellular formation process of biofilms could be addressed for the development of novel targets and techniques to treat antibiotic-resistant microorganisms (Sharma et al., 2019).

3. Type III secretion system structure of *Pseudomonas aeruginosa*

The Type III secretion system (T3SS) is related to the flagellar system in terms of evolution, but it is unclear which mostly originated first. (Saier, 2004). It is a complex macromolecular mechanism with highly conservative structural and functional properties. The five major T3SS families are the Inv-Mxi-Spa family, the Ssa-Esc family, and the Ysc-family of animal pathogens, as well as two distinct Hrp T3SS of plant pathogens (Troisfontaines & Cornelis, 2005). The Asc system of *Aeromonas salmonicida*, the Yop system of *Yersinia* spp., and the Psc system of *P. aeruginosa* are all members of the Ysc-

family of T3SS. The Inv-Mxi-Spa group consists the Salmonella enterica (SPI-1) and Shigella processes, whereas the Ssa-Esc family includes enteropathogenic E. coli (EPEC), Salmonella enterica (SPI-2), and enterohaemorrhagic E. coli (EHEC) systems. The resemblances between these systems are due to their functions rather than their sequences, proving pathogenic bacteria's ability to adapt to their environment in particular. In 1996, the presence of T3SS in *P. aeruginosa* was discovered (Yahr, Barbieri, et al., 1996), but only till 2005 the T3SS was structurally visualized (Pastor et al., 2005). The T3SS of *P. aeruginosa* is a macromolecular complex made up of various proteins that spans the inner bacterial membrane, periplasmic space, peptidoglycan layer, outer bacterial membrane, extracellular space, and host cell membrane (Figure 5). The secretion apparatus or needle complex, the translocation or targeting apparatus, and the secreted toxins (effector proteins) and cognate chaperones are all functionally defined (Moraes et al., 2008).

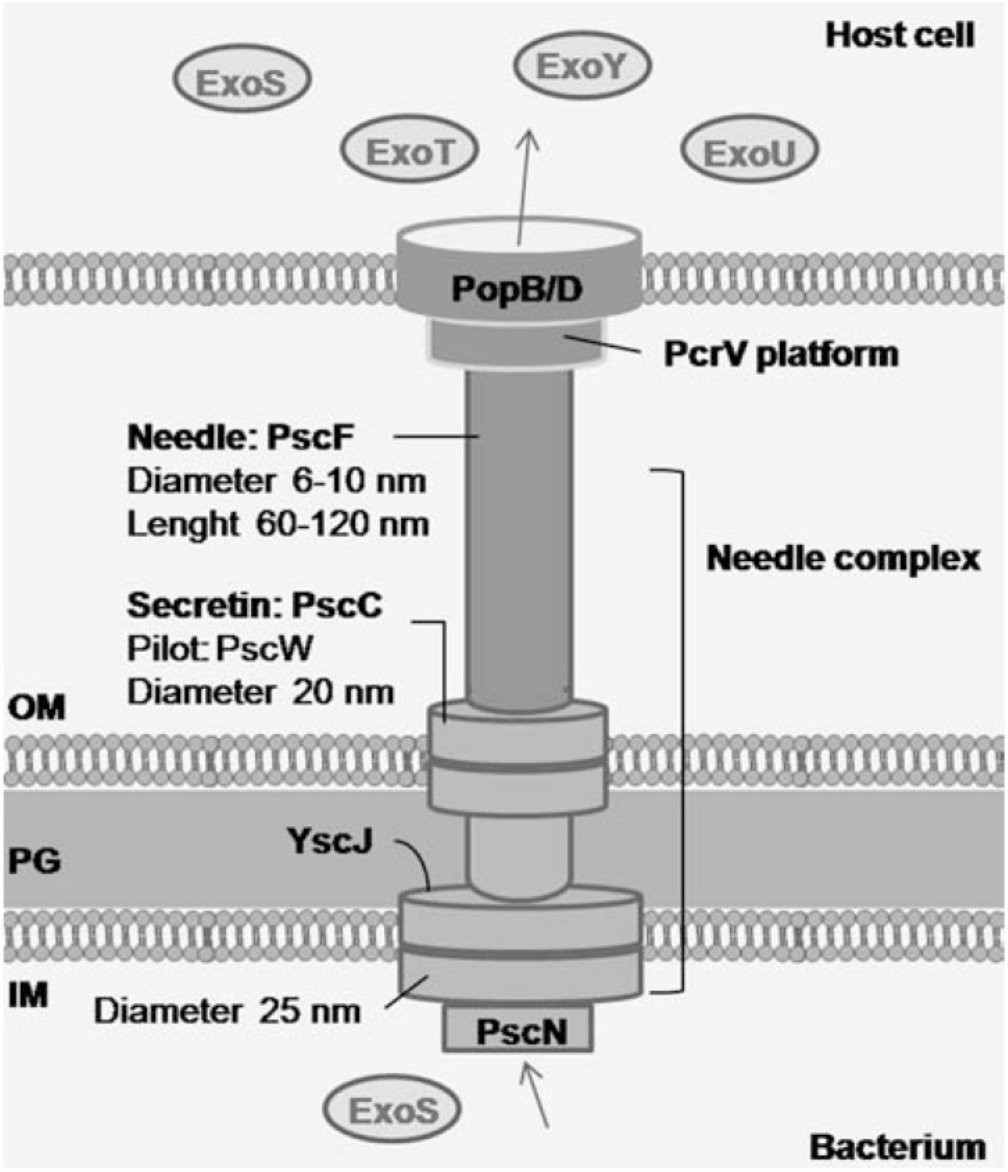


Figure 5. Type 3 Secretion system (T3SS) structure of *P. aeruginosa.* The T3SS is mainly composed of a needle complex, a translocating apparatus, and effector toxins that are directly injected into the host cell cytoplasm from the bacterium. Chaperones and regulatory proteins help in facilitating this process (Galle et al., 2012).

3.1 The type III secretion system needle complex

The secretion apparatus of *P. aeruginosa* is composed of a basal body and an injectisome, both of which have a syringe-like shape. The T3SS basal body, which

supports the syringe-like shape, is the T3SS's only endothelial constituent, extending the internal bacterial membrane, the peptidoglycan layer, and the external bacterial outermost part (Cornelis, 2006). The basal body carries bacterial cytosol secreted toxins to the syringe-like structure which is made up of an inner and an outer membrane. While PscJ is thought to be present in the T3SS's inner membrane, the secretin family's PscC protein acts as the outer membrane component of a variety of multicomponent export systems, such as Type II secretion and Type IV pilus formation. Secretins enlarge pores by combining 12-14 units to produce a homomultimeric cyclic structure that extends the outer membrane and emerges the periplasm (Moraes et al., 2008). PscC oligomerizes with the secretin chaperone PscW to form an outer membrane channel. (Burghout et al., 2004).

Additional proteins hypothesized to be part of the *P. aeruginosa* T3SS basal body include lipoprotein PscJ and ATPase PscN, however this has yet to be verified. PscF proteins are helical polymerized to form the needle-like structure, or injectisome (Burns et al., 2008; Sorg et al., 2006). The effector toxins are transported from the basal body towards the translocator apparatus via a hollow molecular needle. It is widely assumed that it plays a role in the detection of host cells, which is required for T3SS activation.

3.2 The apparatus for translocation

The translocation apparatus transports the effector toxins from the needle-like structure throughout the host cell membrane and into the cytoplasm. In non-secreting conditions, the T3SS forms a needle-like structure with a decreased release of effector toxins into the environment, though this is inadequate for cell intoxication. It forms an active injectisome when it interacts with a host cell, that is accompanied by a functioning translocation system anchored in the plasma membrane of the host cell, leading in the formation of a functional translocation pore. PopB, PopD, and PcrV, also commonly known as translocators, are three proteins required for *P. aeruginosa* T3SS translocation apparatus (Mueller et al., 2008). The T3SS secretes these proteins, which bind with one another and are important to produce a translocation pore. The translocation pore, which is made up of hydrophobic domains, is formed when PopB and PopD oligomerize. Despite

the fact that PcrV is hydrophilic and does not belong to the pore component, is therefore needed for the assembly of a functional translocation pore (Fields et al., 1999).

3.3 The secreted effector toxins

Among the many virulence determinants, T3SS is regarded as a crucial factor in the poor clinical outcome of *P. aeruginosa* infections. This system has so far been identified for injecting at least four effector proteins in *P. aeruginosa*, including Exoenzyme S, Exoenzyme T, Exoenzyme Y, and Exoenzyme U (Feltman et al., 2001). These effector toxins are relatively infrequent to be present simultaneously, so each strain either carry the ExoU or the ExoS gene. The presence of effector toxins characterizes a strain's phenotype, and the expression of T3SS effector toxin is associated to clinical illness prognosis. The toxin ExoS is responsible for cell death via apoptosis (Kaufman et al., 2000), whereas ExoU is essential for cell lysis. ExoT and ExoS are extremely distinct despite sharing 76 percent protein sequences and multiple resemblance. (Barbieri & Sun, 2005; Yahr, Goranson, et al., 1996). ExoS is a 453-amino acid-long enzyme. The secretion domain is made up of the initial 15 N-terminal regions, the chaperone binding range of regions 15-51, and the membrane localization area of residues 51-72. A secretion signal directs these effector toxins toward the apparatus. SpcS is bound by the chaperone binding domain, the one required during ExoS secretion, most likely to keep ExoS in like a secretion-competent form. The membrane localization region directs the exoenzymes towards the host cell's membrane, which is required for effective variation of host proteins (Shen et al., 2008). Among residues 96 and 233 lies the GAP domain together with Rho GAP process, immediately preceding the ADPRT domain with ADP ribosylating activity, that lies between residues 233 and 453. Residues 418-429 in the ADPRT domain form the binding site for cofactor FAS, a 14-3-3 protein that is essential for the activity of ADPRT. ADPRT activity is also dependent on Glu379 and Glu381 (Radke et al., 1999). GAP activity necessitates the presence of Arg146 within the GAP domain.

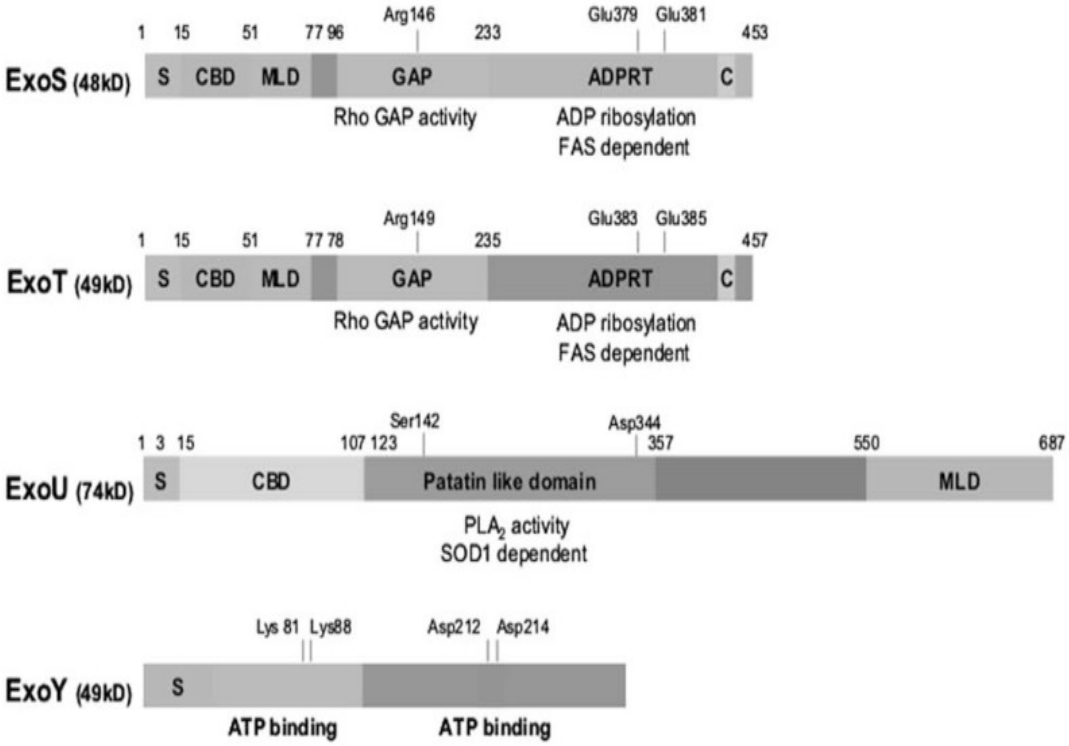


Figure 6. *P. aeruginosa* ExoS, ExoT, ExoY, and ExoU structure. *Pseudomonas aeruginosa* ExoS and ExoT are Type III–secreted bifunctional cytotoxin. They both share an S-domain, ADPRT activity and GAP, as well as C-domains, MLD, and CBD. For GAP activity, Arg146 is needed by exoenzyme S, whereas ADPRT involves both Glu379 and Glu381. ExoT's GAP activity is completely dependent on Arg149, while its ADPRT activity is reliant on Glu383 and Glu385. ExoU has a patatin-like domain that seems to be essential for the activity of phospholipase A2. This process requires both the amino acids asp344 and ser142. ExoY is an adenylyl cyclase with 378 amino acids. This exoenzyme activity necessitates the presence of Asp212, Asp214, Lys81, and Lys88, which are considered to be essential for ExoY-ATP interactions. S: secretion signal, CBD: chaperone binding domain, MLD: membrane localization domain, GAP: GTPase activating protein, ADPRT: ADP ribosyl transferase; C: cofactor binding site (Galle et al., 2012).

ExoT is a 457 amino acid enzyme that is structurally similar to ExoS. It has a chaperone binding domain, a membrane localization domain, and an N-terminal secretion domain all within the first 50 residues. The GAP domain is found on residues 78-235, and the ADPRT domain is observed on residues 235-457. Between residues 422-433 is the cofactor binding site. Arg149, like ExoS, is necessary for ExoT's GAP activity, and the ADP ribosylation activity involves both Glu383 and Glu385. (Garrity-Ryan et al., 2000).

ExoU has 687-amino acid phospholipase A2 activities that involve the cofactors Asp344 and Ser142 (Rabin & Hauser, 2005). The chaperone binding domain is expected to be between residues 3 and 123, while the secretion domain at the N-terminus is considered to be in the initial 15 residues of the protein. A patatin-like domain containing the phospholipase A2 action appears to be present among 107 and 357 residues (Phillips et al., 2003). There is no distinguishable motif between 357 and 550, however a membrane localization domain exists between residues 550–687 (Rabin et al., 2006).

ExoY is a natural adenylyl cyclase that contains 378 amino acids. It is made up of two domains which interact to bind ATP. Their activity is dependent on the presence of the residues Lys81, Lys88, Asp212, and Asp214, that are considered to display the binding site of ATP. T3SS effectors lack secretion and translocation signals, making novel virulence factors difficult to identify. To identify possible T3SS effector proteins, researchers employed bioinformatics to analyze a vast number of sequenced genomes of plant and animal diseases (Rabin et al., 2006).

3.4 Chaperons of the T3SS apparatus

The apparatus of T3SS chaperones are bacterial cytoplasmic molecule complexes which help in the assembly and functionality of the T3SS. Despite the fact that they aid in the production of exoenzyme toxins, the T3SS needle rarely transports them. The following chaperones are classified according to the type of protein they help: class I chaperones help pore-forming proteins, class II chaperones help needle-like structure subunits, and class III chaperones help secretory toxins. T3SS chaperones are considered to have a variety of roles and modes of action (Ghosh, 2004). Class I chaperones maintain the injectisome's helical components monomeric when combining to subunits and

concealing the polymerization domains that prevent the subunits from interacting with one another. They may aid in delivering protein to the export route. The needle component PscF is kept in a 1:1:1 configuration in the cytoplasm by the chaperones PscE and PscG (Quinaud et al., 2005). Class II chaperones reduce toxicity in hydrophobic translocators. Level of free translocator-chaperones may indicate to the bacteria the pore formed and the fully operational injectisome (Page & Parsot, 2002).

PcrH acts as a chaperone for the translocator proteins PopB and PopD (Schoehn, 2003). The effector proteins have specialized chaperones of class I that interact to their adjacent partner via the chaperone binding domain. SpcS chaperones interacts with ExoS and ExoT toxins, which is considered necessary for their maximum secretion, while SpcU chaperones ExoU, no chaperone has been described for ExoY. Each of these chaperones can aid to keep their associates in the bacterial cytoplasm to avoid incorrect secretion, but they can also assist in delivering their corresponding collaborator for secretion (Parsot, 2003). Some other line of evidence implies that chaperones are required for directing these collaborating molecules to the ATPase enzyme and thus enhancing one's unfolding. (Akeda & Galán, 2005).

3.5 Regulation of the T3SS effector toxins

Direct interaction with the host cell recognized via T3SS needle is the signal that stimulates the production of T3SS proteins, however the specific signaling process is unclear. *P. aeruginosa* ability to secrete proteins is affected by metabolic stress, which lowers the proportion of bacterial cells capable of assembling effective secretion mechanisms (Rietsch et al., 2004). The secretion is modulated by two different stages. The T3SS genes transcription occurs first, preceded by protein secretion. When secretion activators or suppressors that control T3SS gene transcription are removed, T3SS transcription proteins are coupled to the actual initiation of secretion (McCaw et al., 2002). The transcriptional activator ExsA regulates T3SS gene transcription in *P. aeruginosa* by binding with ExsA common region present in T3SS genes promoter as well as the promoter of its own (Brutinel et al., 2008). There are three proteins, ExsC, ExsD, and ExsE, involved with the secretion activity of ExsA-dependent transcription (McCaw et

al., 2002). ExsC, the anti-activator, may adhere to ExsD that can adversely modulate it, though when not secreting it is coupled with ExsE, which has a higher affinity (McCaw et al., 2002). Once the injectisome of T3SS has initiated after coming into contact with the host cell, ExsE ejection into the host cell cytoplasm, the link between transcription and secretion is maintained (Urbanowski et al., 2007). ExsE levels are lowered, allowing the ExsC chaperone to attach ExsD. ExsD then frees ExsA, allowing transcription to begin.

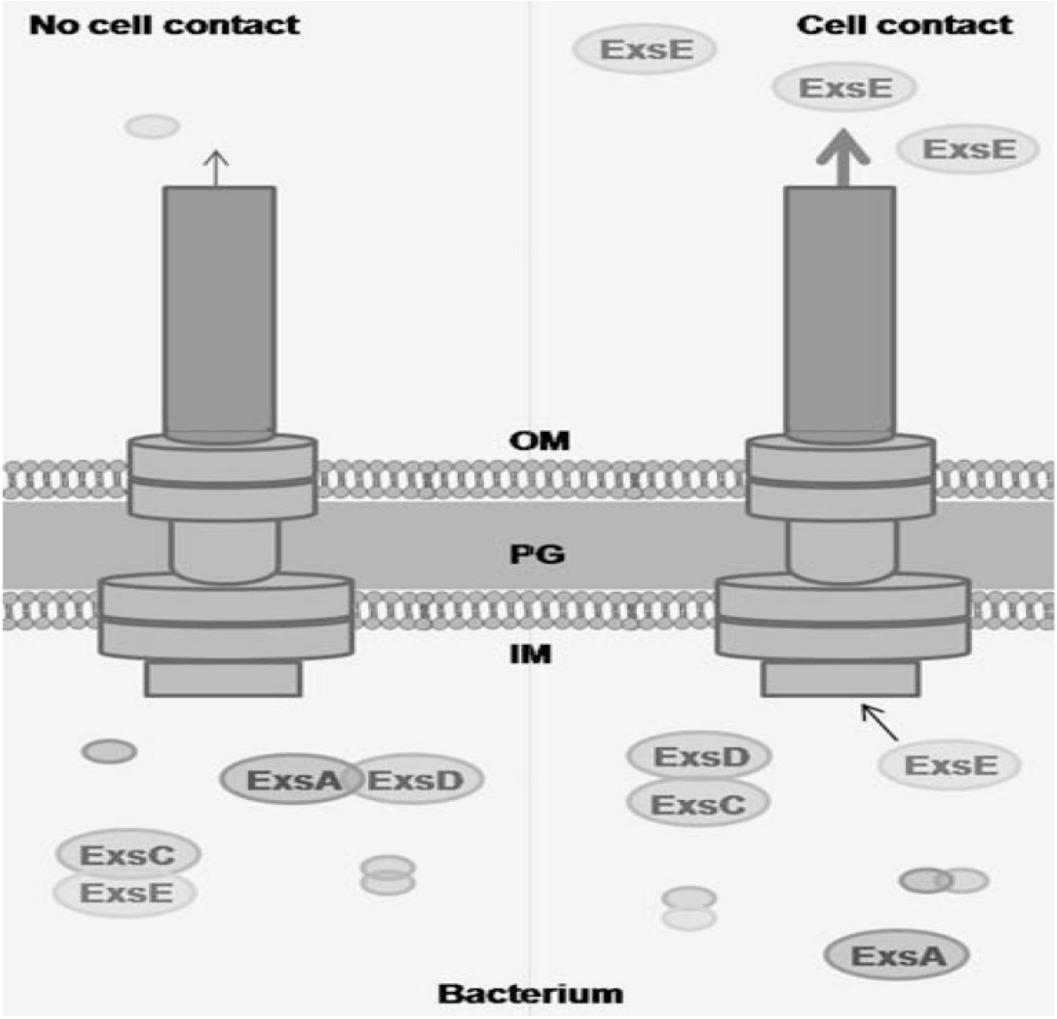


Figure 7. T3SS transcription and secretion coupling process. The interaction of four proteins, ExsA, ExsD, ExsC, and ExsE, is responsible for the coupling of transcription and secretion (Galle et al., 2012).

3.6 Exotoxin A (ETA)

One of the most important virulence factors of *P. aeruginosa* is Exotoxin A. ETA is a type of secreted bacterial toxin that transfers the adenosine diphosphate (ADP)-ribose moiety of the nicotinamide adenine dinucleotide cation (NAD+) to particular target proteins in eukaryotic cells (West, 2000).

Similar to many of relevant toxins, it follows a straightforward A-B structure-function model, during which the A domain exhibits enzymatic activity and the B domain interacts to a particular receptor on target cells' membranes (Michalska & Wolf, 2015). Iglewski and Kabat both validated ETA's capability to block protein synthesis (1975). They showed that in a rabbit-reticulocyte cell-free lysate, ETA stimulates the transfer of the NAD+ ADP-ribose moiety to elongation factor 2. Elongation factor 2 is in charge of eukaryotic ribosome translocation during protein synthesis, which is controlled by guanosine triphosphate hydrolysis. Diphtheria toxin catalyzes a similar enzymatic process (Iglewski & Kabat, 1975).

The toxins affect diphthamide, a post-translationally modified histidine residue (Van Ness et al., 1980). Inactivation of diphthamide blocks protein synthesis, which results in apoptosis. In the absence of elongation factor 2, ETA has both ADP-ribosyltransferase and NAD+-glycohydrolase activity (Chung & Collier, 1977) ETA is more resistant to mouse cells than diphtheria toxin. This shows that they bind to separate receptors and that their binding domains are different (Carroll & Collier, 1988). The study of toxin-domain structure in relation to certain functional sections of the molecule has been made possible by exotoxin.

3.6.1 *Pseudomonas* Exotoxin A (PE)

The PE gene has been identified in *P. aeruginosa* strain PA 103, and study among its 5′ and 3′ promoter region revealed their translation due to monocystronic expression. (Gray et al., 1984). PE has 638-amino-acid protein that are classified with various domain characteristics as shown in Figure 6A (Wedekind et al., 2001). PE belongs to the two-component AB toxin members, consisting of an enzymatically active A domain and a cell-binding B domain (Odumosu et al., 2010).

PE is made up of a hydrophobic starting peptide of 25 amino acids being at N-terminus, which is excreted upon secretion. The binding affinity domain Ia (aa 1–252), that are mostly made up from antiparallel ß-sheets, appears after the leader sequence. Domain II (aa 253–364), which contains six consecutive α-helices, enables the PE to pass through cellular membranes. The catalytic component of the toxin with ADP-ribosyltransferase activity is synthesized from the last four residues (aa 400–404) of domain Ib (aa 365–404) and domain III (aa 405–613) (Siegall et al., 1989).

3.6.2 PE intoxication molecular pathways

PE expression modulation is a complicated phenomenon that has yet to be fully understood. PE expression and iron metabolism have been linked in a number of studies. One essential characteristic that allows *P. aeruginosa* to colonize the host is its ability to absorb iron efficiently. Siderophores, such as pyoverdine, are released substances with a high affinity for chelating *P. aeruginosa* reactive iron molecules. In the presence of high concentrations of iron ions, pyoverdine stimulate signaling pathway for the proliferation of PE expression (Hunt et al., 2002; Lamont et al., 2002). Studies also revealed a relationship to bacterial glucose metabolism. The bacterium favors respiration as metabolism as a facultative aerobic organism (Daddaoua et al., 2012). It obtains its energy when transferring electrons out of a lowered substrate, glucose, towards the ultimate terminal electron acceptor, oxygen. The metabolism of glucose early phases take place throughout the periplasmic space, with glucose being converted to 2-ketogluconate, that is subsequently transported to the cytosol and thus further metabolized. PtxS, a transcriptional repressor protein, can bind to 2-ketogluconate. In the absence of 2-ketogluconate, two PtxS molecules become connected to a monomer of the PtxR regulator, which attaches to the PE promotor's –35 domain and suppressing their transcription. PtxS disintegrates from the PtxR/DNA complex after 2-ketogluconate binding, allowing PtxR to engage RNA polymerase to proceed in toxin transcription as shown in Figure 6B (Daddaoua et al., 2012).

Pseudomonas exotoxin A penetrates the extracellular medium via the general secretory pathway, a two-step process that is highly consistent across gram-negative

bacteria (Gérard-Vincent et al., 2002). PE is primarily carried towards the cytosol by the translocase following cytoplasmic production like an unfolded precursor protein (Douzi et al., 2012). Short peptides at the N- terminal are cleaved and ETA is then secreted inside the periplasmic region during translocation via the inner membrane. Within hydrophilic periplasm, PE folds to a full protein complex detected by (T2SS), classified Xcp of the bacteria, allowing toxin secretion into extracellular space. (Voulhoux et al., 2000). Mutagenesis studies show that two N-terminal glutamic acid residues at domain Ia's positions +2 and +3, along with PE region II, seem to be essential in extracellular secretion and folding. (Lu et al., 1993). As a result, it's possible that the relevant residues are part of a yet-to-be discovered conformational secretion signal for PE that T2SS recognizes, or that they're crucial for the proper presentation of this kind of signal (Lu et al., 1993; Voulhoux et al., 2000). The protease enzyme of the periplasmic host cleaves ETA terminal lysine (aa 613) by toxins in the extracellular surroundings. As a result this C-terminal motif develop between REDLK (aa 609–613) and REDL (aa 609–612), which enable the toxin adhere to receptors of KDEL across the Golgi apparatus upon trafficking of further intracellular vesicles (Hessler & Kreitman, 1997). PE interacts with CD91 on the host surface via domain Ia, which is also known as alpha2-macroglobulin receptor/low-density lipoprotein receptor-related protein (α2MR/LRP) (Kounnas et al., 1992).

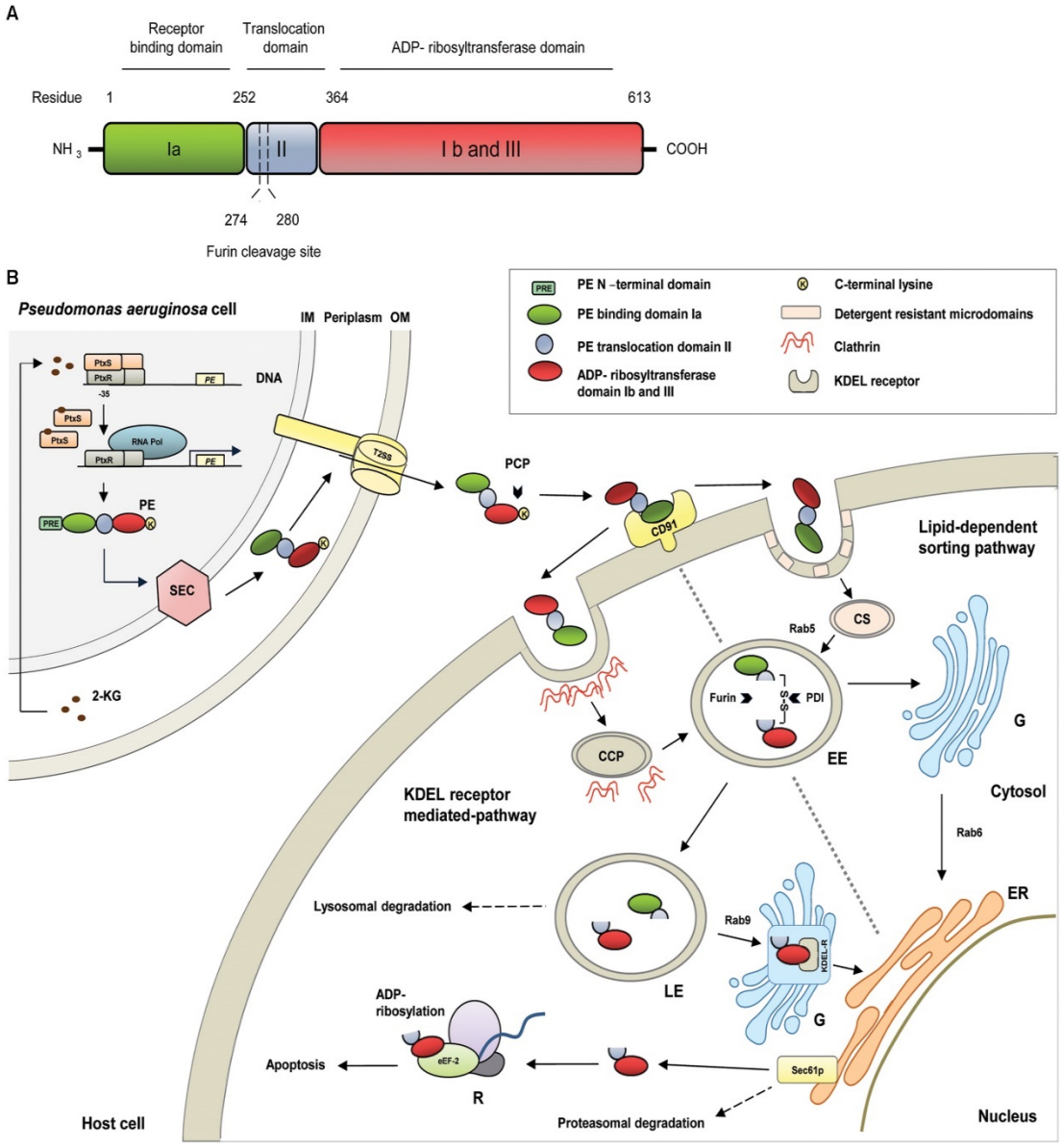


Figure 8. (A) Illustration of the structural and functional domains of *Pseudomonas* exotoxin A (PE). **(B)** Molecular pathways of PE. 2-KG, 2-ketogluconate; CCP, clathrin coated pit; CD91, CD91 receptor; CS, caveosome; EE, early endosome; eEF-2, eukaryotic elongation factor-2; ER, endoplasmatic reticulum; G, Golgi apparatus; KDEL-R, KDEL-receptor; PCP, plasma carboxypeptidases; PDI, protein disulfide isomerase; PtxR, PtxS, transcription regulators; R, ribosome; Rab, Rab-GTPase; RNA Pol, RNA polymerase; Sec61p, Sec61p translocon; T2SS, type II secretion system (Michalska & Wolf, 2015).

CHAPTER III

Materials and Methods

3.1 Bacterial isolates

This project was approved by the Institutional Review Board at Near East University (Project No: YDU/ 2022/100-1493). A total of 67 isolates of *P. aeruginosa* were obtained from patients admitted to the Near East University Hospital in the Turkish Republic of North Cyprus between July 2018 and March 2022. Standard bacteriological tests such colony morphology, Gram staining, and pyocyanin pigment formation, as well as the VITEK 2 system for rapid, accurate bacterial detection and antibiotic susceptibility testing were utilized to identify the isolates. All isolates were acquired from wound/pus, urine, rectal swab, ear discharge, cerebrospinal fluid (CSF), catheter tip, aspirate, and sputum. Samples from both inpatient and outpatient groups were included in the study, and they were subcultured onto Mueller Hinton agar plates at the Near East University Hospital Microbiology Laboratory to obtain pure cultures.

3.2 Antibiotic susceptibility testing

Antibiotic susceptibility testing 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 agar plates after overnight incubation.

Using a sterile swab, a sufficient number of morphologically comparable colonies were transferred to the saline polystyrene test tube. A homogeneous microbial suspension was generated with a density equivalent to the relevant 0.5 McFarland standard. For AST, Gram negative bacillus identification cards was 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. Nineteen 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), tigecycline,

tobramycin (TOB), norfloxacin (NOR), Trimethoprim/Sulfur (SXT), tetracycline (TET), and ertapenem (ETP).

3.3 Biofilm formation assay

The ability of the *P. aeruginosa* isolates to produce biofilm was investigated using a microtiter dish biofilm formation assay. In this method, *P. aeruginosa* isolates were grown overnight at 37˚C in tryptic soy broth (TSB). The cultures were diluted 1:100 in TSB medium. The bacterial suspension was inoculated into sterile 96-well polystyrene microtiter plates and incubated at 37°C for 24 hours without agitation. Planktonic cells were removed after 24 hours of growth, and wells were washed twice with 200 µL phosphate buffered saline (PBS) water before being stained for 10 - 15 minutes with 150 µL of 0.1 % crystal violet solution in water. After staining, the wells were washed three times with PBS. The wells were destained with 150 µL of 30 % acetic acid in water. The absorbance of the destaining solution was measured at 570 nm using a microtiter plate reader. Each test was performed in triplicate. Uninoculated medium served as a negative control.

The average optical density (OD) of the negative control was used to estimate the cutoff OD (ODc). The isolates were divided into four categories by ODc: Non biofilm former ($OD \leq ODc$), Weak biofilm former ($ODc < OD \leq 2 \times ODc$), Moderate biofilm former ($2 \times ODc < OD \leq 4 \times ODc$), and Strong biofilm former ($OD > 4 \times ODc$).

3.4 DNA extraction for molecular tests

All isolates were grown on Mueller Hinton agar and incubated at 37°C overnight. Boiling method was used for DNA isolation from the bacterial strains. Specifically, few colonies were suspended in 500 µl of sterile water in eppendorf tubes. In a heat block, the cell suspensions were incubated at 100°C for 15 minutes. The tubes were centrifuged at 14000 rpm for 10 minutes to sediment the debris. The bacterial DNA-containing supernatant was transferred to sterile tubes and kept at -20°C until PCR amplification was performed.

3.5 Detection of T3SS genes

The presence of *exoT*, *exoS*, and *exoU* genes were screened by PCR method. 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. Table 1 and 2 lists the cycling conditions, primer sequences, and amplicon sizes used in this study for each PCR amplification

Table 1: Primers used in this study

Gene	Primer sequence (5′ to 3′)	Product size (bp)
exoT-F	AATCGCCGTCCAACTGCATGCG	152 bp
exoT-R	TGTTCGCCGAGGTACTGCTC	
exoS-F	TCAGGTACCCGGCATTCACTACGCGG	572 bp
exoS-R	TCACTGCAGGTTCGTGACGTCTTTCT	
exoU-F	GGGAATACTTTCCGGGAAGTT	428 bp
exoU-R	CGATCTCGCTGCTAATGTGTT	

Table 2: Conditions of PCR reactions for genes amplification and multiplex PCR

Genes	Steps	Temperature	Time	Cycles
exoT	Initial denaturation	95°c	10 min	1
	Denaturation	94°c	1 min	30
	Annealing	64°c	1 min	
	Extension	72°c	1 min	
	Final extension	72°c	7 min	
	Hold	4°c	-	1
exoS	Initial denaturation	95°c	10 min	1
	Denaturation	94°c	30 sec	30
	Annealing	56°c	1 min	
	Extension	72°c	1 min	
	Final extension	72°c	7 min	
	Hold	4°c	-	1
exoU	Initial denaturation	95°c	10 min	1
	Denaturation	94°c	30 sec	30
	Annealing	56°c	1 min	
	Extension	72°c	1 min	
	Final extension	72°c	7 min	
	Hold	4°c	-	1

3.6 Agarose gel electrophoresis

The amplified products (11µl) were mixed with 3.5 µl of loading dye 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 ladder were utilized. The isolated DNA fragments were visualized and imaged under UV light on a transilluminator (DNA MiniBIS Pro Gel Imaging System.)

CHAPTER IV

RESULTS

4.1 Sample characteristics

A total of 67 confirmed *Pseudomonas aeruginosa* samples were investigated in this study, which were collected from patients admitted to Near East University Hospital. There were thirty-five (52.2%) male patients and thirty-two (47.8%) female patients among the patient groups. The patient's age at admission ranged from 1 to 99 years, with a median of 71 years. Figure 9 shows the distribution of patients by gender and age group. The majority of the patients with *P. aeruginosa* were over 50 years of age.

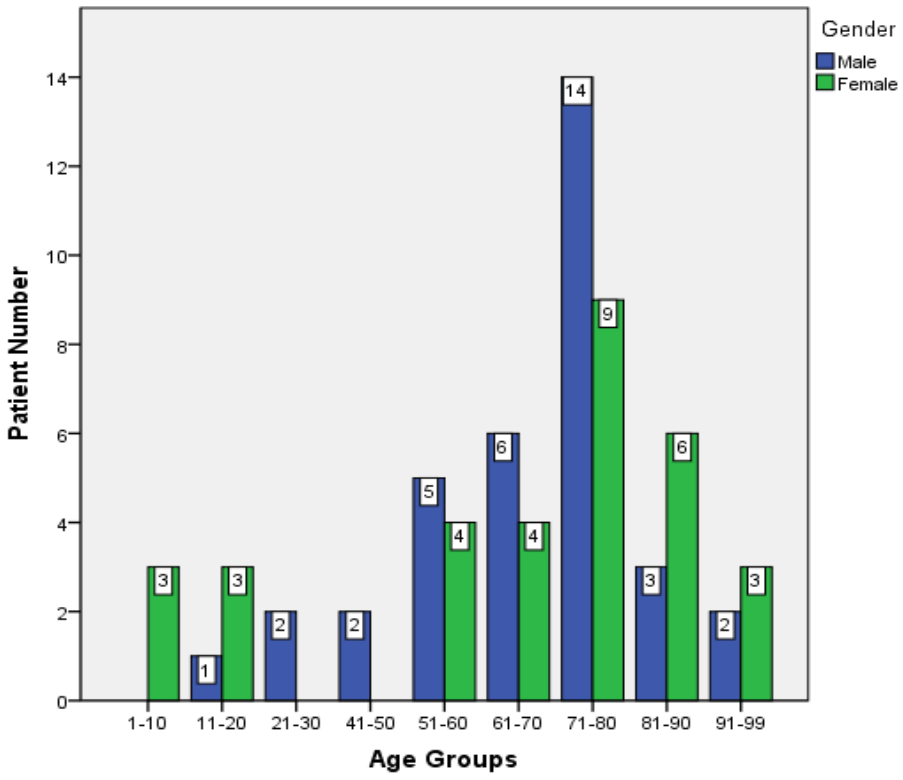


Figure 9. Distribution of patients by gender and age group

Overall, *P. aeruginosa* infections were found in 79.1 % (n=53) of inpatients, with outpatient *P. aeruginosa* infections accounting for the remaining 20.9 % (n=14). Figure 10 shows the distribution of *P. aeruginosa* cases by admission type.

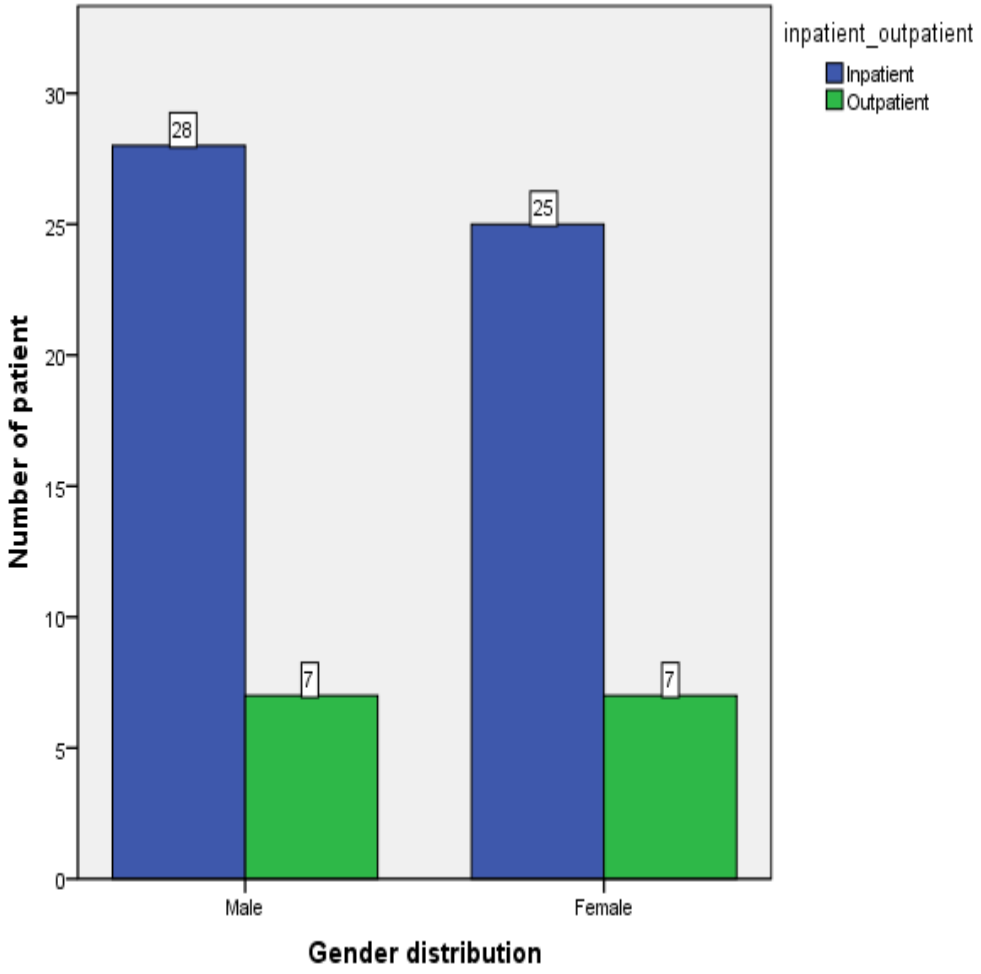


Figure 10. Distribution of *P. aeruginosa* infections in inpatient and outpatient groups by gender

Clinical samples were collected from patients admitted to the following departments: Urology (n=7; 10.45 %), Anesthesiology (n=18; 26.87 %), Respiratory Diseases and Allergy (n=8; 11.94 %), both Pediatrics and Cardiology (n=5; 7.46 %), both Geriatrics and Infectious disease (n=4; 5.97 %), both Internal medicine and Dermatology (n=3; 4.48 %), all Otolaryngology and Neurology (n=2; 2.99%), and the remaining samples were received from Nephrology, Oncology, Orthopedics and Traumatology, General Surgery, Pediatric Immunology and Allergy, and Cardiovascular Surgery departments (n=1; 1.49 %) (Figure 11).

The results indicate that the majority of *P. aeruginosa* infections were observed in the Anesthesiology, Respiratory Diseases and Allergy and Urology departments.

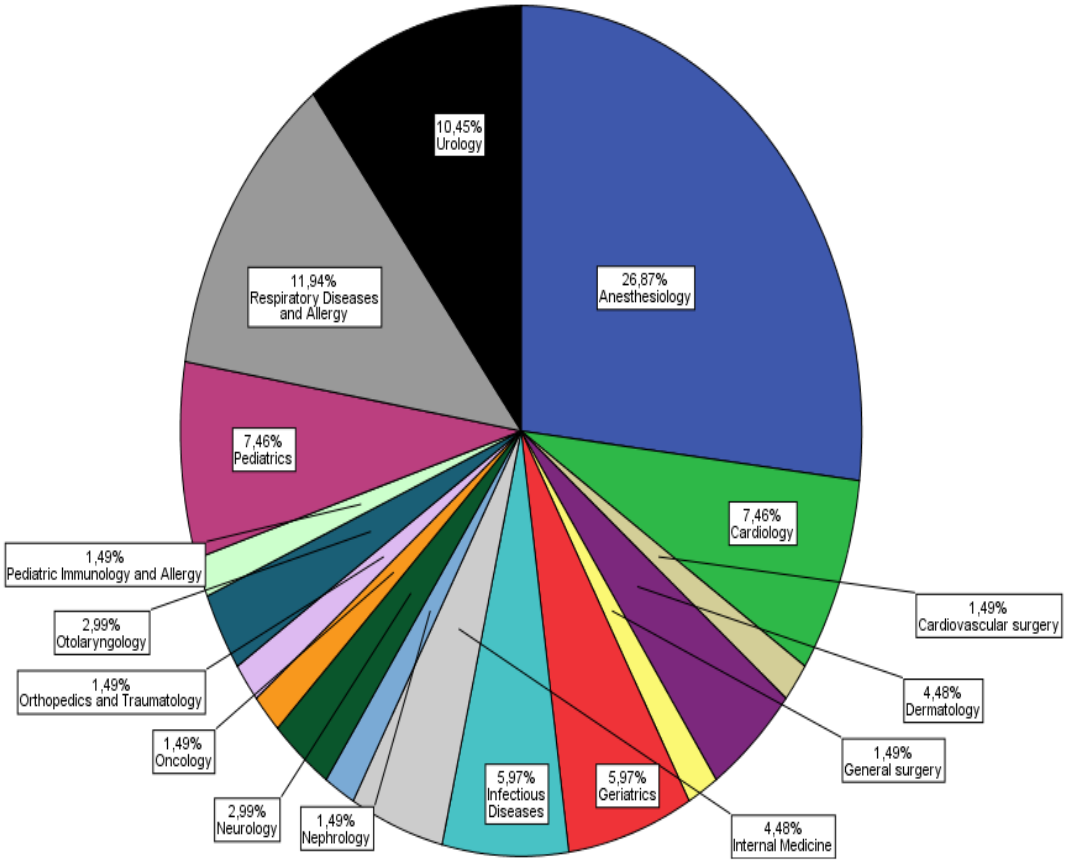


Figure 11: Distribution of samples by hospital departments

When the isolates were distributed based on the source of clinical samples, they were observed to be isolated mostly from urine (n=23; 34.3%), followed by tracheal aspirate (n=18; 26.7%), wound & pus (n=8; 11.9%), catheter tip (n=3; 4.48%), sputum (n=12; 17.9 %), and the remaining rectal, ear discharge and CSF samples (n=1; 1.49%) (Figure 12).

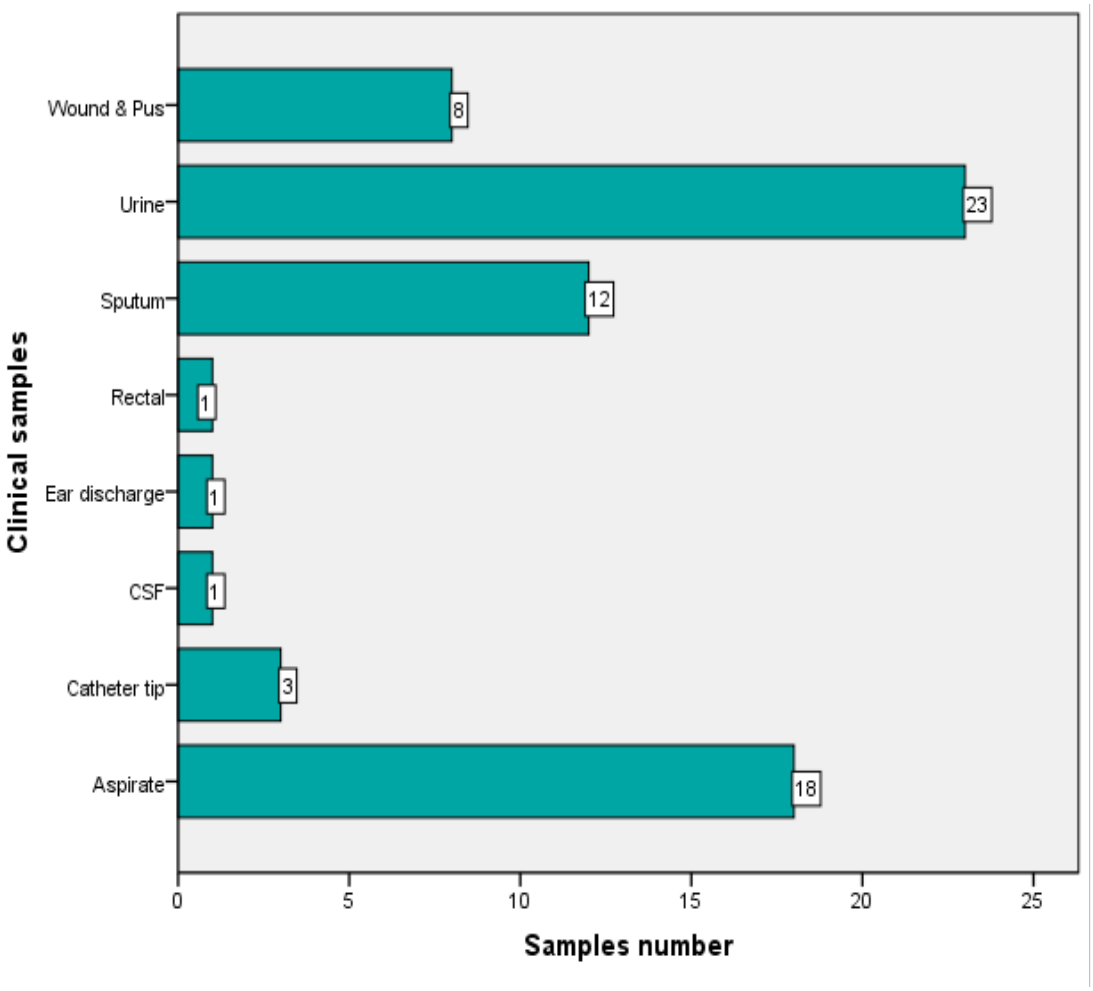


Figure 12: Distribution of isolates according to the source of clinical samples

4.2 Antimicrobial susceptibility patterns of *P. aeruginosa* isolates

Isolates were most resistant to aztreonam, imipenem, netilmicin, and piperacillin/tazobactam (26.9%), followed by cefepime, ceftazidime, levofloxacin, and piperacillin (25.4%) as well as gentamicin (14.9%). The isolates were most susceptible to

colistin (92.5%) and amikacin (85.1%). The antibiotic susceptibility patterns of the *P. aeruginosa* isolates are shown in Table 3.

Table 3. Antibiotic susceptibility patterns of *P. aeruginosa* isolated from patients at Near East University Hospital

Antibiotics	Sensitive	Intermediate	Resistant
Amikacin	57 (85.1)	3 (4.5)	5 (7.5)
Aztreonam	14 (20.9)	29 (43.3)	18 (26.9)
Cefepime	48 (71.6)	_	17 (25.4)
Ceftazidime	49 (73.1)	_	17 (25.4)
Ciproflaxin	53 (79.1)	_	13 (19.4)
Colistin	62 (92.5)	_	3 (4.5)
Gentamicin	56 (83.6)	_	10 (14.9)
Imipenem	43 (64.2)	3 (4.5)	18 (26.9)
Levoflaxacin	32 (47.8)	_	17 (25.4)
Meropenem	46 (68.7)	6 (9)	14 (20.9)
Netilmicin	38 (56.7)	_	18 (26.9)
Piperacillin	38 (56.7)	1 (1.5)	17 (25.4)
Piperacillin/Tazobactam	47 (70.1)	1 (1.5)	18 (26.9)
Tobramycin	46 (68.7)	_	3 (4.5)
Norfloxacin	5 (7.5)	_	_
Trimethoprim/Sulfar	_	_	2 (3)
Tetracycline	_	_	1 (1.5)
Tigecyclin	1 (1.5)	_	1 (1.5)
Ertapenem	1 (1.5)	_	1 (1.5)

[a]The values are presented as No. (%).

4.3 Biofilm formation capacity of *P. aeruginosa* isolates

A quantitative biofilm formation assay performed on 67 *P. aeruginosa* isolates reported a total of 41 (61.2%) isolates to form biofilm and 26 (38.8%) non or weak biofilm producers. Strong biofilm producers were found to be 25 (37.3%), moderate biofilm producers to be 16 (23.9%), weak biofilm producers to be 12 (17.9%), and non biofilm producers to be 14 (20.9%) under tested conditions (Figure 13).

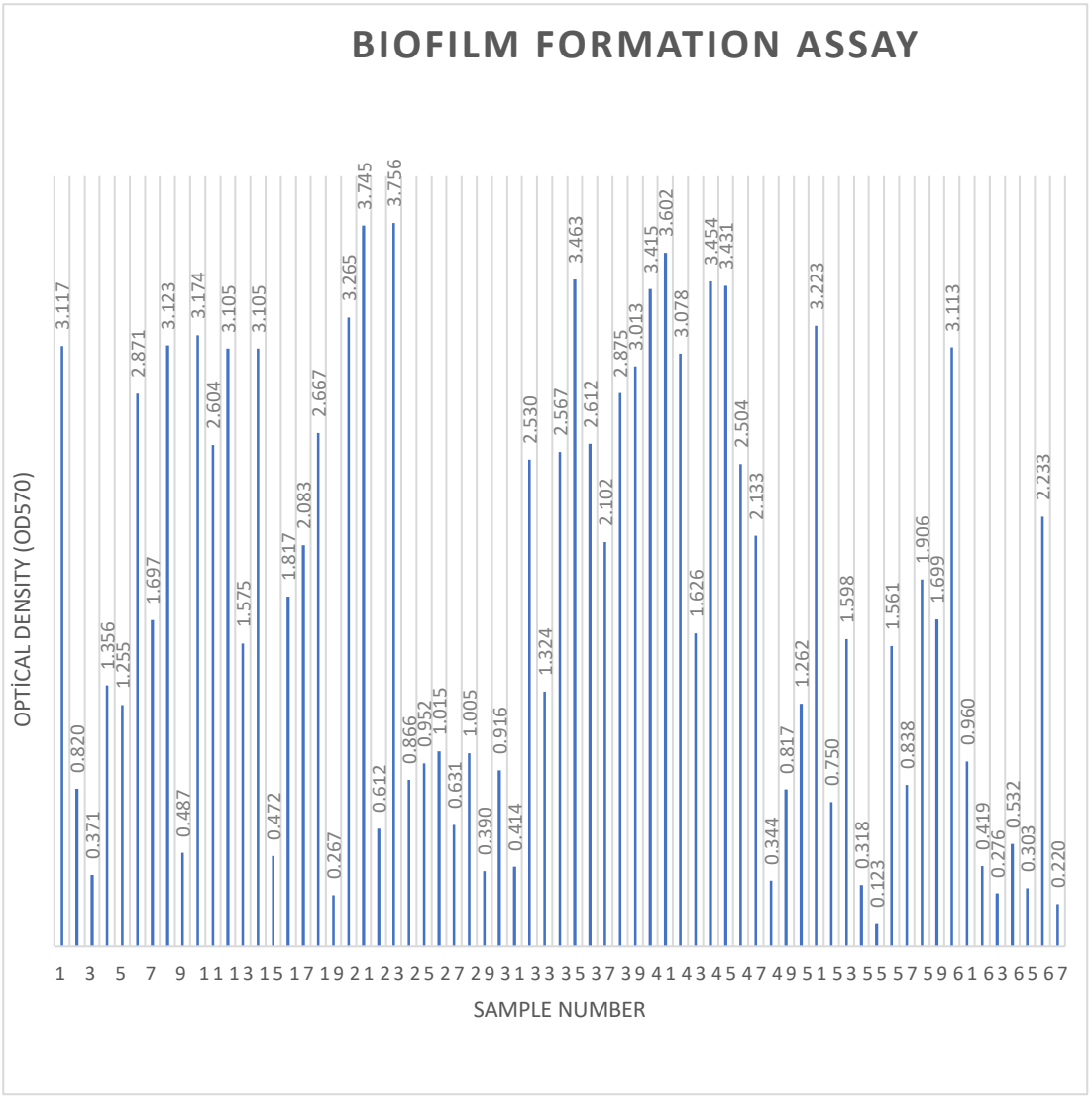


Figure 13. The biofilm-formation ability of 67 *P. aeruginosa* isolates.

When all levels of biofilm-forming isolates (strong, moderate, and weak) were combined, this group of isolates were found to be more prevalent in the inpatients group compared to the outpatients group.

Interestingly, the inpatient group had (26.9%, n=18) and the outpatient group had (10.4 %, n=7) of the strong biofilm forming isolates. Figure 14 shows the distribution of biofilm formation ability by admission type.

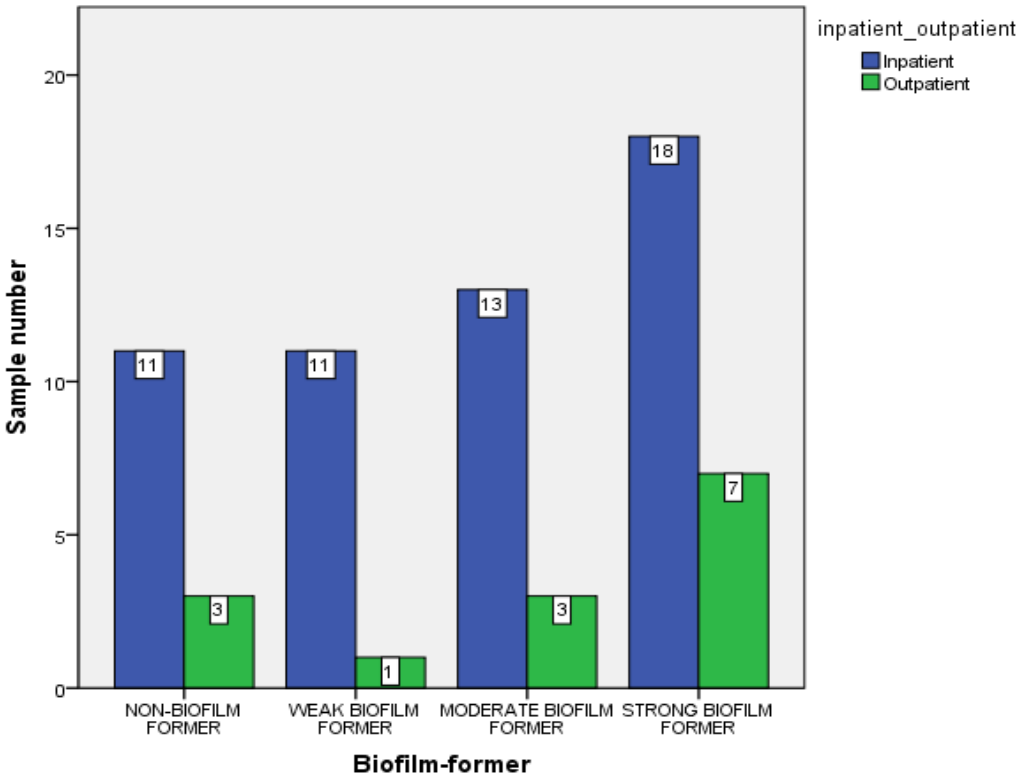


Figure 14. Distribution of cases in inpatient and outpatient groups by biofilm former level

Among the strong biofilm forming isolates, the elderly patient group aged 71-80 years had the highest rate of 10 (40%), followed by the 61-70 and 51-60 year old group. Figure 15 demonstrates the age distribution of patients in terms of biofilm forming capacity.

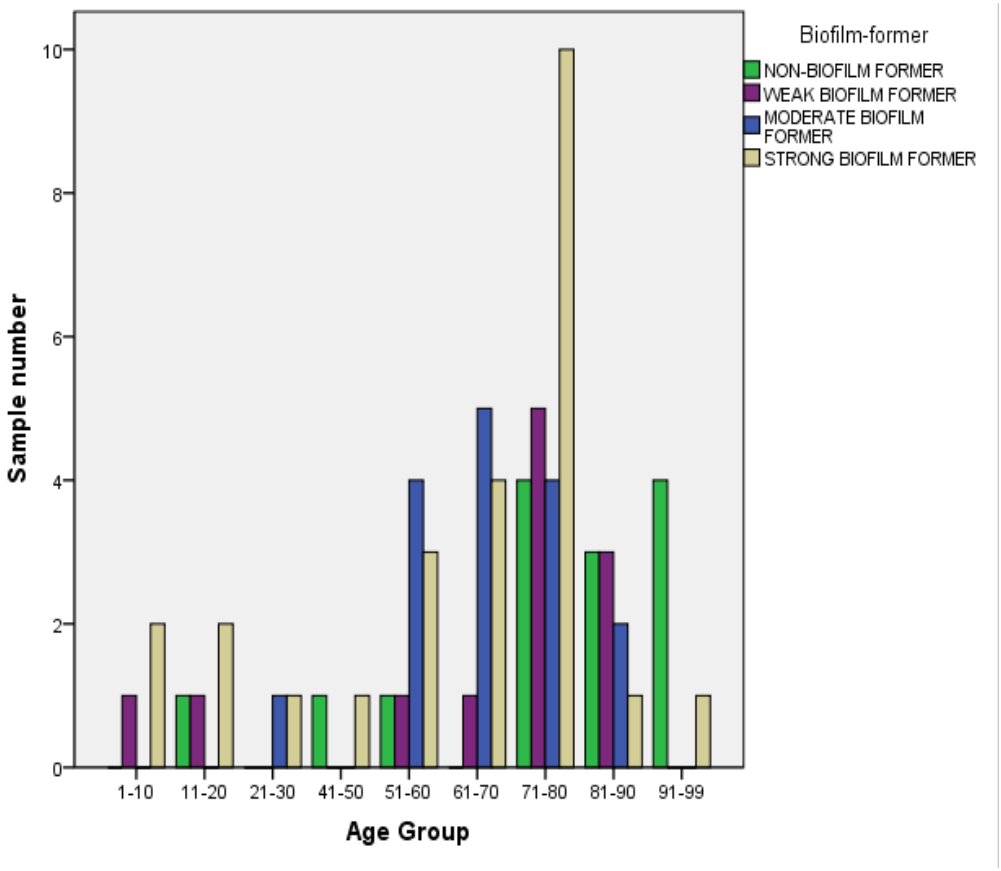


Figure 15. Distribution of patients by biofilm forming capacity and age group

4.4 Amplification of T3SS toxin-encoding genes: *exoT, exoS* and *exoU*

The PCR amplification for the presence of the *exoT* is shown in Figure 16. The amplification for the presence of *exoS* and *exoU* multiplex PCR product is demonstrated in Figure 17. The prevalence of the screened virulence genes in *P. aeruginosa* was 86.6 %, 59.7, and 32.8 % for *exoT, exoS* and *exoU*, respectively.

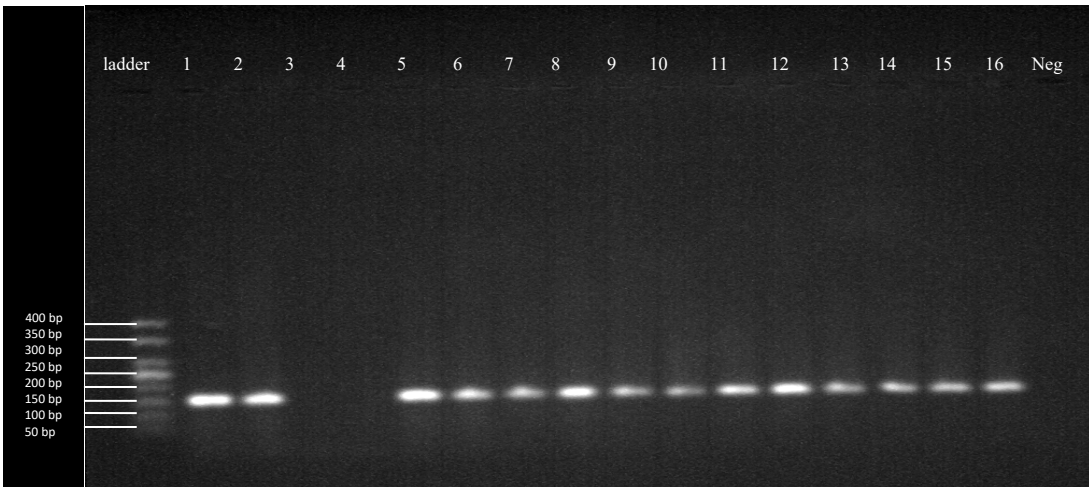
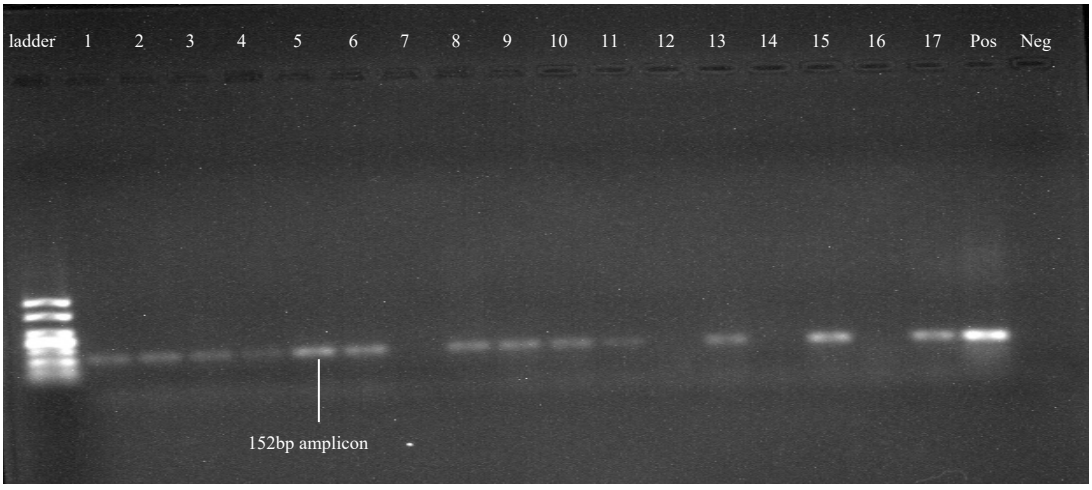


Figure 16 A. Gel electrophoresis image showing positive amplification of 152 bp fragments specific for *exoT* of *P. aeruginosa*. Lines: 'ladder', DNA molecular weight marker 50bp; 3 and 4, negative isolate; 1, 2, 5 to 16, positive isolates; 'neg', negative control.

The following image show amplification of *exoT* (152 bp amplicon) in the remaining clinical samples with 50 bp DNA molecular weight marker.



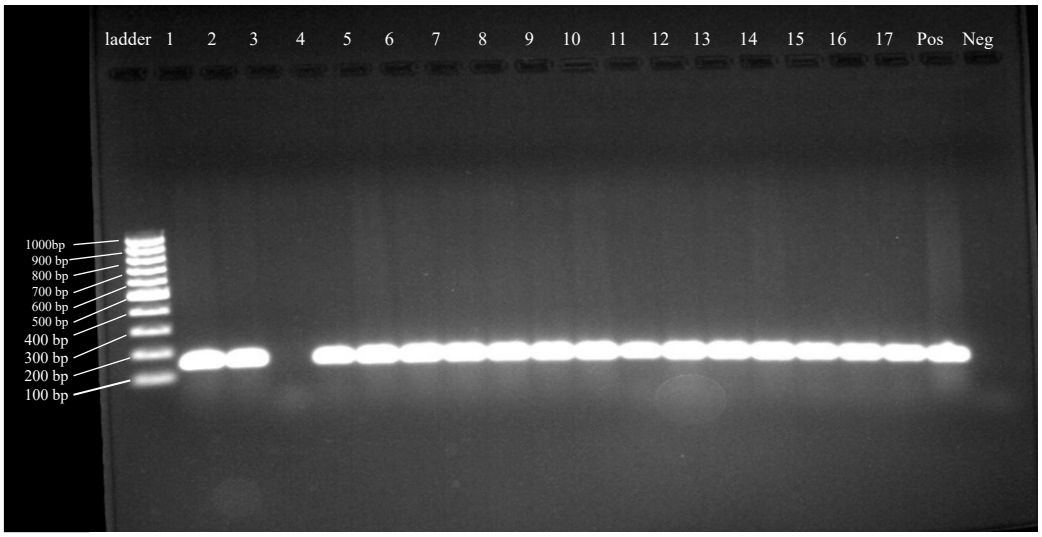
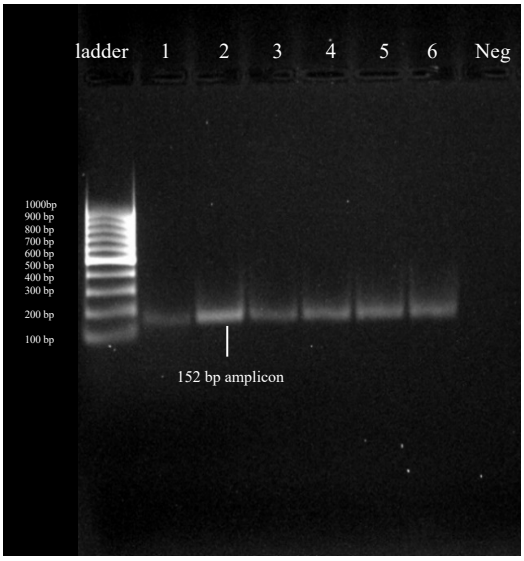
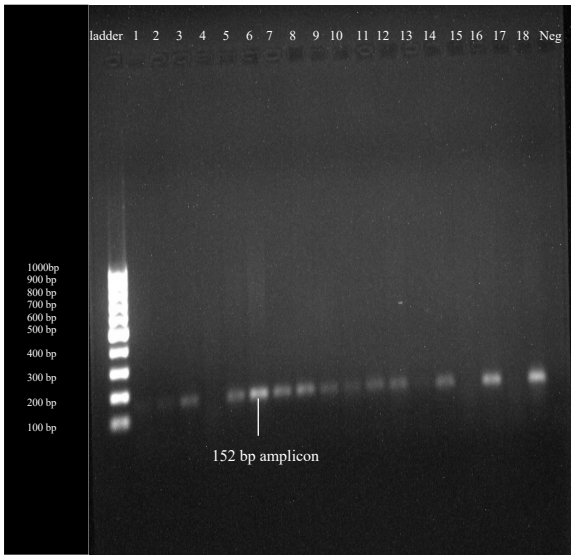


Figure 16. B Gel electrophoresis image showing positive amplification of 152 bp fragments specific for *exoT* of *P. aeruginosa.* Lines: 'ladder', DNA molecular weight marker 100bp; 1, 2 and 4 to 18, positive isolates; 3, negative isolate; 'pos', positive control; 'neg', negative control.





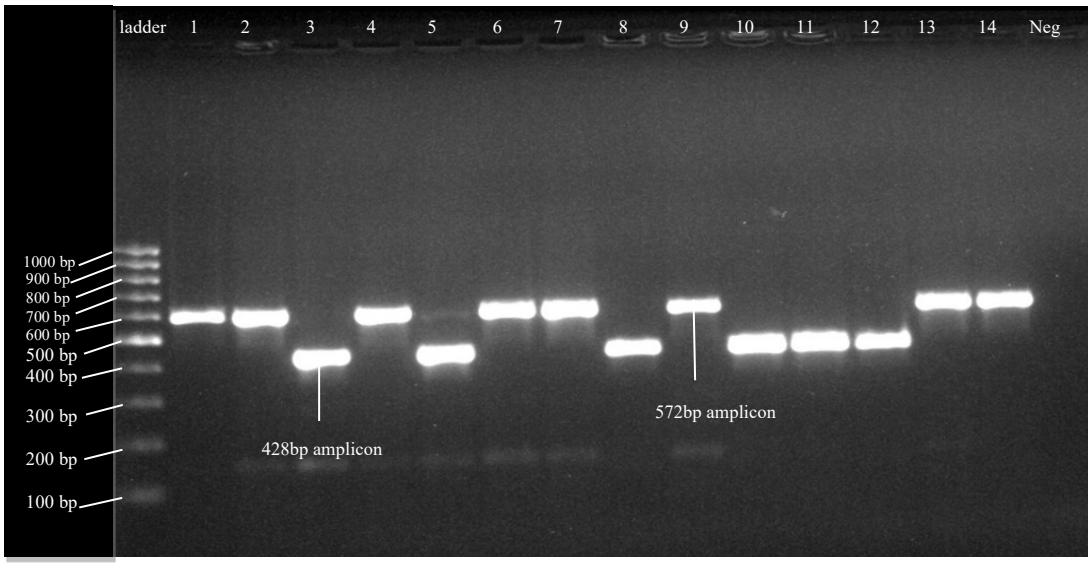
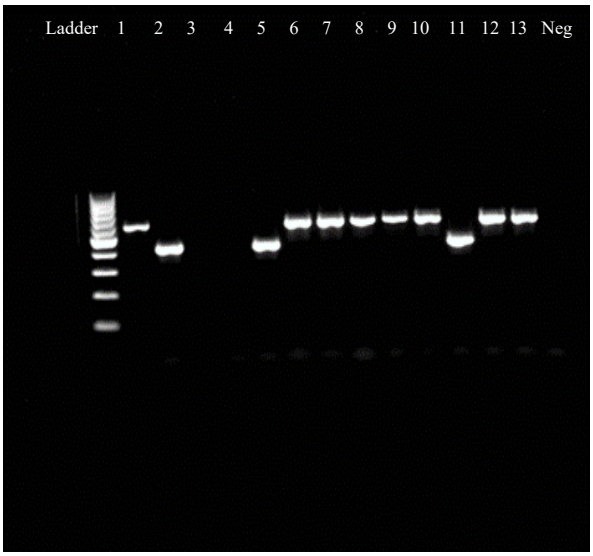
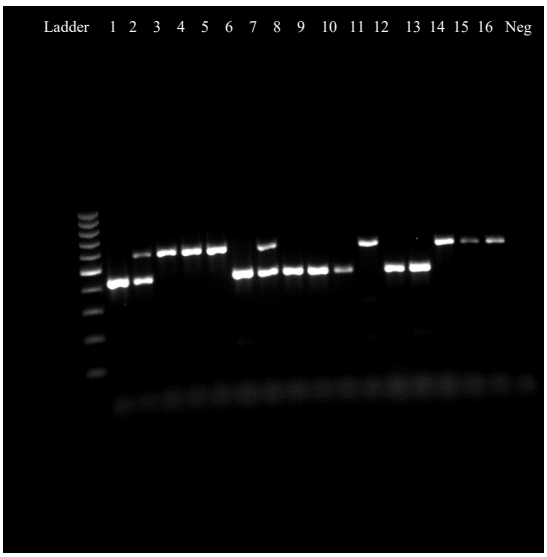


Figure 17. Gel electrophoresis image showing positive amplification of 572 bp fragments specific for *exoS* and 428 bp fragments specific for *exoU* of *P. aeruginosa.* Positive isolates for *exoS* were 1,2,4,6,7,9,13, and 14, while negative isolates were 3,5,8,10,11, and 12. Positive isolates for *exoU* were 3,5,8,10,11, and 12, while negative isolates were 1,2,4,6,7,9,13, and 14. Lines: 'ladder,' DNA molecular weight marker 100bp; 'neg,' a negative control.

The images below show a multiplex PCR product for amplification of the *exoS* gene (572 bp amplicon) and the *exoU* gene (428 bp amplicon) in remaining clinical samples using a 100 bp DNA molecular weight marker.


Ladder 1 2 3 4 5 6 7 8 9 10 11 12 13 Neg


Ladder 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 Neg

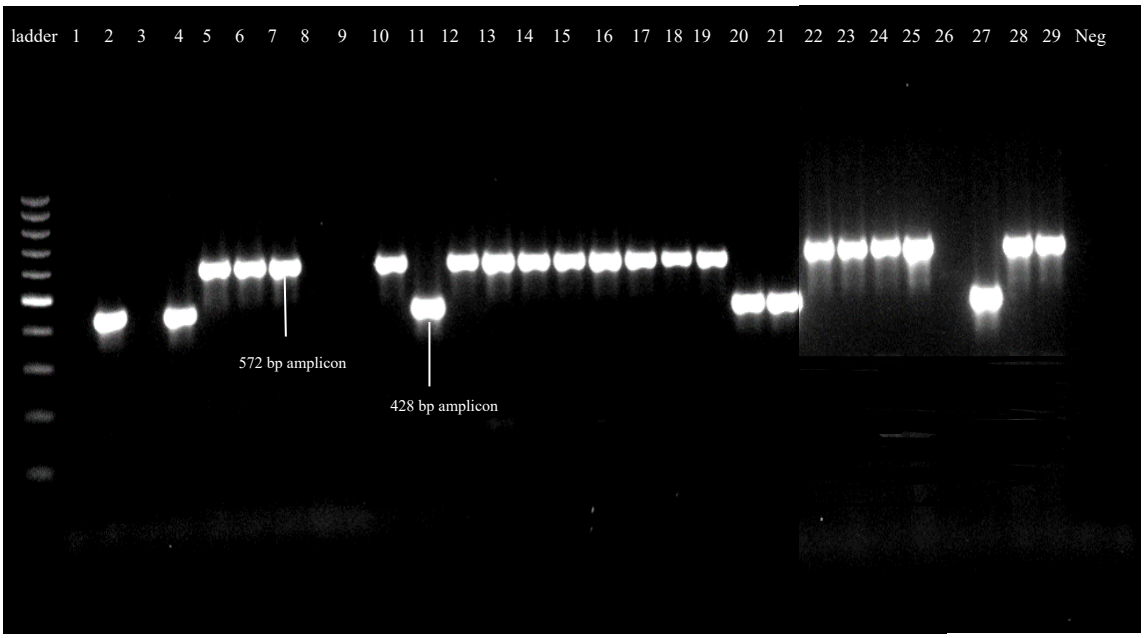

ladder 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 Neg
572 bp amplicon
428 bp amplicon

Table 4. Prevalence of type III secretion genes in *P. aeruginosa* isolates according to sample type

Source	No. of isolates examined	*exoT*	*exoS*	*exoU*
Wound and pus	8	5 (7.5)	4 (5.9)	2 (2.9)
Urine	23	18 (26.9)	14 (20.9)	5 (7.5)
Sputum	12	12 (17.9)	8 (11.9)	4 (5.9)
Rectal	1	1 (1.5)	-	1 (1.5)
Ear discharge	1	1 (1.5)	-	1 (1.5)
CSF	1	1 (1.5)	1 (1.5)	-
Catheter tip	3	3 (4.5)	3 (4.5)	-
Aspirate	18	17 (25.4)	10 (14.9)	9 (13.4)
Total	67	58	40	22

[a]The values are presented as no. of isolates (%).

When the distribution of the exotoxins was analyzed in terms of the sample source, all catheter tip, CSF, ear discharge, rectal, sputum and almost all (94.4%, n=17/18) aspirate samples harbored the *exoT* gene. Prevalence of *exoT* was detected as 78.3% (n=18/23) of the urine isolates, and 62.5% (n=5/8) of the wound-pus isolates. The prevalence of *exoS* was found to be lower (60.8%, n=14/23) in urine isolates, aspirate isolates (55.6%, n=10/18), and in wound-pus isolates (50%, n=4/8). The lowest distribution was observed for *exoU* gene for all sample types. The prevalence of type III secretion genes in *P. aeruginosa* isolates according to sample type is given in Table 4.

CHAPTER V

Discussion

P. aeruginosa is an opportunistic human pathogen that can cause a range of nosocomial infections, particularly in patients with weakened immune systems (Moradali et al., 2017). Infections caused by *P. aeruginosa* strains can be life-threatening, and they have become a global public health problem due to a variety of mechanisms for adaptation, survival, and resistance to a range of antimicrobial drugs (Gill et al., 2016). Moreover, the bacterium has a plethora of virulence factors which have been shown to have a significant impact on pathogenesis and mortality in human infections (Gellatly & Hancock, 2013).

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. Male patients were the most affected subgroup, accounting for 52.23 % (n=35) of the infections, and the age group 71 to 80 years had the highest number of male patients (n=14; 20.89 %) with a median age of 71 years. Infections caused by *P. aeruginosa* were shown to be less common in individuals aged 70 and below. In terms of the distribution of *P. aeruginosa* infections among patients, the data analysis revealed a threefold increase among inpatients (regardless of gender) versus outpatients; indeed, 79.10 % (n=53) of *P. aeruginosa* infections occurred in inpatients, compared to only 20.89 % (n=14) in the outpatient group. Patients who stay in the hospital for a long 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 patient illnesses and deaths (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=18; 26.87%), Respiratory Diseases and Allergy (n=8; 11.94%), and Urology (n=7; 10.4%) 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 (Bhagirath et al., 2016). As a result, it is critical to identify the groups of people 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 TET and ETP. The most effective antibiotics against *P. aeruginosa* clinical isolates were CST, GEN, and AK. These findings are in line with those of Ghanbarzadeh., who found a significant rate of antibiotic resistance to CIP, ATM, CAZ, PIP, AK, and IPM (Ghanbarzadeh Corehtash et al., 2015). Similar results were obtained according to a study conducted by Kamali and colleagues in Iran, in which the most efficient antibiotics against *P. aeruginosa* isolates were reported as LEV (23.75%), CIP (20%), IPM (22.5%), and GEN (18.75%), whereas *P. aeruginosa* isolates showed the highest resistance rate to CAZ (17.5%) (Kamali et al., 2020). Another study that contradicted our findings indicated the highest levels of antibiotic resistance to ceftazidime and the lowest resistance to amikacin in *P. aeruginosa* isolates from clinical and hospital environments (Fazeli et al., 2012). Several of the mechanisms of intrinsic antibiotic resistance that *P. aeruginosa* possesses include restricted outer-membrane permeability, efflux systems that pump drugs out of cells, and the generation of antibiotic-inactivating enzymes. Quinolone antibiotics including ciprofloxacin and levofloxacin block DNA gyrase and topoisomerase IV, which prevent DNA replication. A β-lactam ring is involved in the molecular structures of β-lactam antibiotics such as penicillin, cephalosporin, carbapenem, and monobactam (Pang et al., 2019).

The formation of biofilm has been identified as a significant factor of pathogenicity in *P. aeruginosa* infections (Ghanbarzadeh Corehtash et al., 2015). According to our results, 61.2 % of *P. aeruginosa* isolates produced biofilm. In a research published by Jabalameli et al. in Iran, 96.9 % of the isolates produced biofilm (Jabalameli et al., 2012). Our findings vary from those of Kunwar et al., who found that only about 25% of *P. aeruginosa* isolates produced biofilms. Surprisingly, there was a relatively high rate of outpatients among the strong biofilm forming isolates, which might be attributed to the COVID-19 outbreak. Patients with *P. aeruginosa* could not be hospitalized when isolates were collected during the pandemic period.

P. aeruginosa virulence is determined by a number of factors. The most important factor in *P. aeruginosa* pathogenicity is the production of a group of protein exotoxins. *ExoU, exoS, exoT,* and *exoY* are the four effector proteins identified so far in *P. aeruginosa*. *ExoT* and *exoY* have a moderate impact on virulence, whereas *exoS* and *exoU* play a major role in pathogenesis and raise a significant concern. The current investigation identified three exotoxins in *P. aeruginosa*: *exoT, exoS,* and *exoU*, which were detected in 86.6 %, 59.7%, and 32.8 % of the isolates, respectively. All three genes of the type III secretion system were detected in two isolates. According to Bogiel et al., the *exoT* gene has the highest prevalence rate among *P. aeruginosa* exotoxins, reaching up to 100 %, and *exoS* has a prevalence rate of 58.9 % which is consistent with the findings in the present study (Bogiel et al., 2021). Another study in Poland obtained similar results showing *exoT* (100%), *exoY* (100%), and *exoS* (59%) genes were the most prevalent, while *exoU* (41%) genes were the least prevalent of the tested isolates. In our study, the distribution of the exotoxins was also investigated in terms of the sample type. All catheter tip, CSF, ear discharge, rectal, sputum and almost all (94.4%, n=17/18) aspirate samples were found to harbour the *exoT* gene. Prevalence of *exoT* was detected as 78.3% (n=18/23) of the urine isolates, and 62.5% (n=5/8) of the wound-pus isolates. The prevalence of *exoS* was found to be lower (60.8%, n=14/23) in urine isolates, aspirate isolates (55.6%, n=10/18), and in wound-pus isolates (50%, n=4/8). The lowest distribution was observed for *exoU* gene for all sample types. *P. aeruginosa* also secretes ETA, which is one of the most toxic factors. The *exoA* gene encodes ETA. During this study, the *exoA* gene could not be investigated due to time constraints.

CHAPTER VI

Conclusion

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 Northern Cyprus. The isolates were also found to habour the *exoT, exoS,* and *exoU* 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 simultaneous determination of virulence factors and antimicrobial resistance is the current approach to investigating the microbiological aspects of *P. aeruginosa* infections. This study also highlighted several well-known molecular processes that enable the pathogen to survive in a variety of hostile environments, such as pathogenesis and antibiotic therapy. Further studies are required to explore the mechanisms of host-pathogen interactions and molecular mechanisms of antibacterial resistance in the bacterial strains.

Limitations

Regulation of virulence genes was not investigated in this study. Future studies are needed to elucidate how virulence is regulated during pathogenesis of the bacterium and hence improve the diagnosis and treatment of the disease. To enhance current knowledge, future studies should determine the relationships between biofilm formation and toxins in multi-drug resistant strains. Another limitation can also be stated as the use of a single assay to measure biofilm formation. Fluorescent microscopy techniques can further add value to the existing dataset. This method can quantitatively assess biofilms without the need for resuspension, allowing natural structures to be preserved. Furthermore, the application of dyes and fluorescence can provide more information about cellular viability in terms of space and time.

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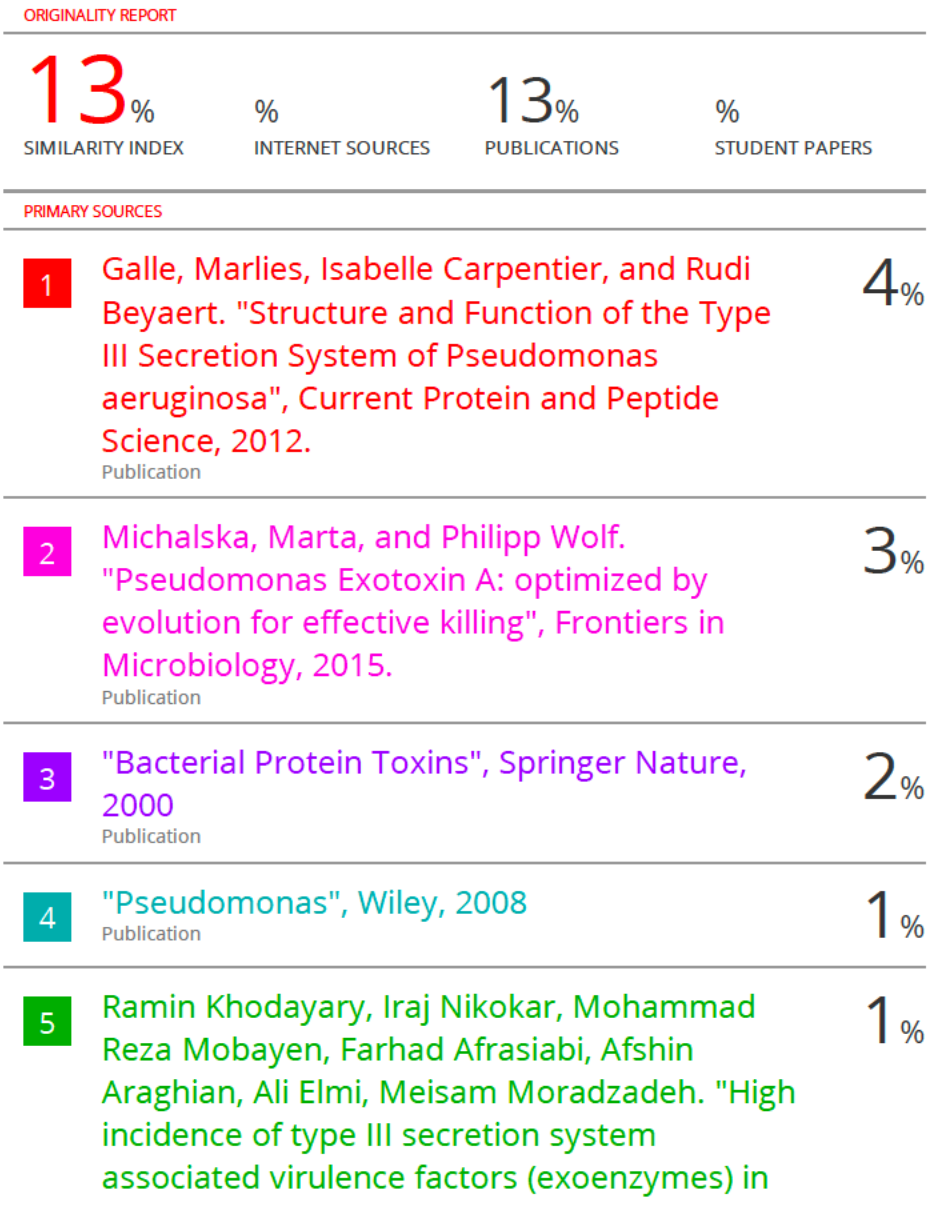
APPENDICES

Appendix A

Turnitin Similarity Report



ORIGINALITY REPORT

13%	%	13%	%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	Galle, Marlies, Isabelle Carpentier, and Rudi Beyaert. "Structure and Function of the Type III Secretion System of Pseudomonas aeruginosa", Current Protein and Peptide Science, 2012. Publication	4%
2	Michalska, Marta, and Philipp Wolf. "Pseudomonas Exotoxin A: optimized by evolution for effective killing", Frontiers in Microbiology, 2015. Publication	3%
3	"Bacterial Protein Toxins", Springer Nature, 2000 Publication	2%
4	"Pseudomonas", Wiley, 2008 Publication	1%
5	Ramin Khodayary, Iraj Nikokar, Mohammad Reza Mobayen, Farhad Afrasiabi, Afshin Araghian, Ali Elmi, Meisam Moradzadeh. "High incidence of type III secretion system associated virulence factors (exoenzymes) in	1%

Appendix B

CURRICULUM VITAE

1. PERSONAL INFORMATION

NAME, SURNAME: DATE of BIRTH and PLACE:	Afnan Ali 13/07/1994 – Riyadh/Saudi Arabia
CURRENT OCCUPATION: MSc Student ADDRESS of CORRESPONDENCE: Gonyeli / Nicosia – North Cyprus TELEPHONE: +905391014054 E-MAIL: afnanelhag@gmail.com	

2. EDUCATION

YEAR	GRADE	UNIVERSITY	FIELD
Sept, 2013 to June, 2017	2.92/4.0	Eastern Mediterranean University	BSc Molecular Biology and Genetic
Nov, 2020 to present	3.93/4.0	Near East University	MSc Medical Microbiology and Clinical Microbiology

3. ACADEMIC EXPERIENCE

PERIOD	TITLE	DEPARTMENT	UNIVERSITY
April 2021	Covid-19 PCR Academy	Medical Microbiology and Clinical Microbiology Medical Genetics	Near East University
September 2021	Microbiology Lab	Medical Microbiology and Clinical Microbiology	Near East University

4. FIELD OF INTERESTS

FIELDS OF INTERESTS	KEY WORDS
Molecular Microbiology	bacteriology, biochemistry, cell biology, eukaryotic organisms, fungi, genetics, microbiology, molecular biology

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	✓	✓