Screening of *lasI* and *lasR* Genes in Clinical Isolates of *Pseudomonas aeruginosa*

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CERTIFICATE

This thesis submitted by **Muniba Zainab Naqvi** is accepted in its present form by the Department of Microbiology, Quaid-i-Azam University, Islamabad, as satisfying the thesis requirement for the Degree of Master of Philosophy in Microbiology.

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بسم الله الرحمن الرحيم

DECLARATION

The material and information contained in this thesis is my original work that was carried out at the Department of microbiology, Quaid-i-Azam University, Islamabad. I have not previously presented any part of this work elsewhere for any other degree.

Muniba Zainab Naqvi

Dedication page

I would like to dedicate my work to Almighty ALLAH the most Merciful, and nothing is possible without His help, Our Holy Prophet Hazrat Muhammad (P.B.U.H) and His Progeny (A.S). As every challenging work needs self-efforts as well as guide and support of elders who are near to our heart, so I will dedicate this work to my beloved Parents and respected Teachers.

LIST OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

PQS	Pseudomonas Quinolone Signal
IQS	Integrated Quorum Sensing Inhibitors
PYA	Pyocyanin
QS	Quorum Sensing
CF	Cystic fibrosis
HAIs	Hospital Acquired Infections
CAIs	Community Acquired Infections
VAP	Ventilator Associated Pneumonia
UTI	Urinary Tract Infections
BSI	Blood Stream Infection
CVC	Central Venous Catheter
PSS	Protein Secretion System
EPS	Extracellular Polymeric Substances
eDNA	Extracellular DNA
AMR	Antimicrobial Resistance
WHO	World Health Organization
GIT	Gastrointestinal Tract
OMPs	Outer Membrane Proteins
RGP	Regions of Genomic Plasticity
ICU	Intensive Care Unit
HIV	Human Immunodeficiency Virus

NGAL	Neutrophil Gelatinase-associated Lipocalin
T2SS	Type two Secretory System
MCC	Mucociliary Clearance
RLs	Rhamnolipids
LPS	Lipopolysaccharides
AHL	Acyl-Homoserine Lactone
SAM	S-adenosylmethionine
Acyl-ACP	Acyl-acyl carrier protein
HCN	Hydrogen Cyanide
GNR	Gram-Negative Rods

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ABSTRACT

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacillus which causes variety of clinical illnesses in both humans and animals. It is practically hard to eradicate this pathogen from hospitals due to its remarkable potential for adaption to unfavorable environmental conditions. Quorum sensing system is a density-based communication mechanism that allows bacteria to communicate with one another. *P. aeruginosa* has four types of quorum sensing systems las, rhl, pseudomonas quinolone signal (PQS) and integrated quorum sensing system. Our study focused on the las system among the four systems of quorum sensing. We began our research by detecting phenotypic expression of QS las system by assaying biofilm formation, PYA production, protease production and elastase production in clinical *P. aeruginosa* isolates in order to understand QS and its controlled virulence factor expression. After that, the genotypic assay was done to examine the prevalence of QS las genes i.e *las I* and *las R* genes. So we determined the QS regulated virulence factors and correlated to the presence of Las system. Sensitivity testing for antibiotics were also carried out. In this study we use 175 isolates of *Pseudomonas aeruginosa* collected from the Armed Forces Institute of Pathology (AFIP). For the identification of *P. aeruginosa* strains in the samples each sample has undergone through multiple tests like morpho-cultural, biochemical, and molecular techniques. The biofilm ability of all 175 *P. aeruginosa* isolates was tested. Biofilm formers were 95.43% (8.57 %, 60.57%, 26.29% strong, moderate and weak biofilm formers), whereas non-biofilm formers were 4.57%. Protease producers were 91% , pyocyanin producers were 74% , elastase producers were 60 %. A PCR assay was used to identify the las QS system and *lasI* and *lasR* genes. According to the findings, 90 % have las system genes. *lasR* gene was found in 52 % of the isolates and *lasI* was found in 71% of the isolates. The las QS system genes were found in 77% of pyocyanin producers, 93% of the protease producers and 89% of elastase producers. Two QS las genes and associated virulence factors are found in a large number of *P. aeruginosa* isolates. There was a correlation between them as well. As quorum sensing is essential for the expression of a battery of virulence factors as well as for biofilm formation in *P. aeruginosa* thus the results of my research suggest that the inhibition of QS regulatory process would be effective for removing and reducing the drug resistance.

INTRODUCTION

Pseudomonas aeruginosa is a member of the Kingdom Monera, phylum *Proteobacteria,* order *Pseudomondaceae*, genus *Pseudomonas*, and species *Pseudomonas aeruginosa* (Diggle & Whiteley, 2020). It is ubiquitous Gram-Negative bacteria (Moradali et al., 2017).Mostly all animals and human-affected surroundings have this bacterium. It can be isolated from a variety of environments, particularly water and soil. Carle Gessard, a French pharmacist, described *P. aeruginosa* for the very first time in his research in 1882. Pseudomonas 15 is made up of two Greek words: Pseudo, which means "false," monas, which means "single unit," and aeruginosa, which comes from the Latin word aerugo, which means "rusted copper" (Diggle & Whiteley, 2020). *Pseudomonas aeruginosa* is a rod-shaped, motile, heterotrophic bacterium with a width of around 5µm-lµm and a length of 1µm-5 µm. It is a facultative aerobic that may develop via aerobic respiration, whereas for anaerobic respiration uses nitrate as a terminal electron acceptor. *P. aeruginosa* can also grow anaerobically using arginine, but its fermentation abilities are limited, resulting in very slow or no growth. *P. aeruginosa* can consume over a hundred organic molecules as an energy and carbon source, but as a prototroph, it can grow on a salt growth medium using only one carbon or energy source. The optimal temperature for *P. aeruginosa* development is 37°C, however it may live in a broad range of temperatures, ranging from 4°C to 42°C. These bacteria can degrade aromatic polycyclic hydrocarbons present in soil, but it has also been discovered in water reservoirs that have been contaminated by people and animals, such as sinks and sewages.

The genome of *Pseudomonas aeruginosa* is quite large and complicated. With 5570 predicted genes, it has the biggest genome in the prokaryotic world. PA01, was the first wild type genome discovered in 2000 and it was isolated from wound infection and is still being widely used for research. Its genome is around 6.3Mbp.The genome of *P. aeruginosa* encodes a large number of proteins involved in virulence factors, transport adaptation, and diverse regulatory roles (Kung et al., 2010).

Pseudomonas aeruginosa may infect a variety of organisms, including humans, insects, plants, and nematodes. Its infection is especially dangerous in those with CF infection. Because of the extensive use of antibiotics, CF patients are more likely to get lung infection. As the infection progresses, the lungs become increasingly infected, and

eventually fail to function, resulting in patient death (Kidman, 2020).

P. aeruginosa infection has become a big concern in HAIs, and it is especially dangerous in the case of immunocompromised individuals (Bassetti et al., 2018). Among all Gram-negative rods, *P. aeruginosa* plays a key role in creating various forms of infections (El Zowalaty et al., 2015). Bloodstream infection is a dangerous illness caused by *P. aeruginosa* and its species, which are the primary cause of hospital-acquired bacteremia, accounting for 4% of all cases and being the third most prevalent cause of Gram negative bloodstream infections (Magill et al., 2014). Different studies have found that BSI patients infected with *P. aeruginosa* had a higher risk of mortality than BSI patients infected with another Gram-negative bacterium (Thaden et al., 2017) or BSI patients infected with *Staphylococcus aureus* (Kang et al., 2003). *P. aeruginosa* is the leading cause of bloodstream infection in the respiratory system, accounting for 25%, and the urinary tract, accounting for 19%, followed by CVC, skin, and soft tissues (Thaden et al., 2017).

P. aeruginosa is the most common cause of Ventilator-Associated Pneumonia in Europe and the United States (Fernández-Barat et al., 2017; Koulenti et al., 2017; Lambiase et al., 2009). Prolonged mechanical ventilation, earlier colonization by *P. aeruginosa*, older age, past antibiotics therapy, cancer, shock, or admission to a hospital place for a longer period of time are all risk factors for VAP, and consequently results in the high incidence rate of *P. aeruginosa* infection. According to studies, the isolation of multi-drug resistance pathogenic organisms is strongly linked to ventilator-associated pneumonia (Fernández-Barat et al., 2017; Venier et al., 2011). Pneumonia caused by multidrug-resistant *P. aeruginosa* has a higher death rate in hospitalized patients than non-MDRs (Micek et al., 2015).

One of the most serious consequences of HAIs is urinary tract infection (del Barrio-Tofiño et al., 2017; Weiner-Lastinger et al., 2020). Around 7-10% of instances of UTI patients reported and admitted to hospitals are caused by *P. aeruginosa* (Ferreiro et al., 2021). Pseudomonal UTI patients are at a higher risk of developing chronic conditions such as diabetes, cognitive deficits, and hypersensitivity. With a high mortality rate of about 20% (Lamas Ferreiro et al., 2017).

P. aeruginosa causes a variety of soft tissue and skin infections, ranging from benign infections like post-surgical infections and cellulitis to extremely life-threatening infections. It is the most isolated pathogenic organism from persistent decubitus ulcer

infection, trauma-related infections, and neutropenic cellulitis and infections at many surgical locations (Bassetti et al., 2018).

The most prevalent and primary cause of infection at the site of burn injuries is *P. aeruginosa* (Azzopardi et al., 2014; Weinstein & Mayhall, 2003). According to several earlier research, *P. aeruginosa* caused 57 percent of positive tissue culture or swab infections in burn patients, whereas about 15 percent of Pseudomonal bloodstream infection caused in burn patients.

Virulence factors cause a variety of chronic infections in *P. aeruginosa*. Protease, rhamnolipid elastases, alkaline proteases, pyoverdine, pyocyanin, alginate, flagella, and fimbriae are only a few of the extracellular released and cell-associated virulence factors that cause persistent infections. By stimulating and enhancing the pathogenesis of *P. aeruginosa*, all of these virulence factors play a role in producing various types of infections. Presence of the polar flagella enables chemotaxis, bacterium adhesion to the surface, induction of the anti-inflammatory response, and movement. Type-IV pili assists the bacteria to attach to various surfaces and aid in biofilm development by allowing bacteria to establish micro-colonies. It also aids bacteria in evade the host's defense system. Fimbriae is an important part of a bacteria's outer surface, and its primary job is to keep bacteria attached to host receptors (Zaranza et al., 2013).

The two most significant siderophores found in this bacterium are pyochelin and pyoverdine. In a severe environment when iron is lacking, siderophores are responsible for chelating the iron and allowing *P. aeruginosa* to live even in a harsh environment. Pyochelin is responsible for the inflammatory response during any chronic infection, whereas pyoverdine is linked to the generation of green fluorescence (Z. Pang et al., 2019a). Exo-enzyme synthesis causes tissue injury, actin polymer breakdown, IgG and IgA cleavage, and cytoskeleton disruption, making *P. aeruginosa* infections more chronic. *P.aeruginosa's* PSS (protein secretion system) has role in virulence, adaptability, and adhesion. The most significant are T3SS and T2SS.

P.aeruginosa was acknowledged among the most fatal bacteria. WHO has mentioned it as the priority pathogen for the purpose of Research and Development of new antibiotics (Organization, 2017).It shows antibiotic resistance to many agents through adaptation and by the inherent mechanisms of antibiotic resistance ,so this gives rise to high mortality rate (Z. Pang et al., 2019b). The phenomenon of biofilm formation

impedes the treatment processes and also aids them to persist in the medical environments (Moradali et al.,2017; Thi et al., 2020).

Van Leeuwenhoek was the first one who discovered microbial biofilms with simple microscope on the tooth surfaces. Biofilm is a framework of complex multicellular aggregates embedded in the self-generated extracellular polymeric substances. This architecture enables the bacteria to cling to different surfaces. Bacteria in the form of biofilm is sheltered from the stresses of the surroundings which hampers their phagocytosis hence assisting their community development hence they persist for the longer time periods (Yang et al., 2011) (Friedman, & Kolter, 2005; Hall-Stoodley & Stoodley). Biofilm of *P. aeruginosa* produces matrix which essentially composed of extracellular DNA (eDNA), lipids, proteins, polysaccharides (Rehm et al., 2011; Stremple et al; 2013).The matrix comprises of over 90% of the biomass of the biofilm.

According to the National Institutes of Health, biofilms are responsible for around 80% of microbial illnesses (NIH, 2002). Biofilm-based infections cause persistent infections in a range of organs and tissues, such as osteomyelitis, vaginitis, cystic fibrosis, urethritis, conjunctivitis, endocarditis with native-valve infections, non-healing wounds, and some paediatric infections of the respiratory tract, such as rhinosinusitis and otitis media (Filardo et al., 2019; Sessa et al., 2019; Lebeaux et al., 2014).

Because of the biofilms antibiotic resistance emerges as a transient phenotypic trait based on the physical conditions of the biofilm-forming cell population as well as biofilm specific properties that allow the bacterial population to restrict antibiotic activity and diffusion.

Bacteria frequently live as community rather than as planktonic states. They are able to communicate by sending and receiving a sequence of signals or messages. One of the most common kinds of communication mechanisms is quorum sensing, in which the word quorum means "threshold" and sensing means "feel," which is abbreviated as QS system. The QS system is a cell-to-cell mechanism in which bacteria control the expression of specific genes depending on the cell density. QS System can be considered as multi-cellular activity in the uni-cellular bacterial world, in which bacteria estimate the cell density population and utilize the knowledge to control gene expression (Häussler, 2010; Wenseleers et al., 2010).

P. aeruginosa has four types of quorum sensing systems las, rhl , pseudomonas

quinolone signal (PQS) (Wang et al., 2018) and integrated quorum sensing inhibitors (IQS) (J. Lee et al., 2015). The QS circuits of *P. aeruginosa* have four different pathways and they are arranged in a hierarchical order, in which the Las system is present at the top position (J. Lee et al., 2015).

Las system has three components: LasR , a response regulator (RR), N-3-oxo-dodecanoyl homoserine lactone (AHL; 3-O-C12-HSL), a similar AI molecule, and LasI which is employed for the AI- synthase. The rhl system is made up of rhlR , N-butyryl homoserine lactone (C4-HSL) as an AI molecule, and rhI, which regulates the AI- synthase (Wang et al., 2018). Despite the fact that the las and rhl QS systems are separate systems, still they work in a hierarchal manner in which las system dominates the rhl system.The LasR-3-O-C12-HSL complex controls *lasR, lasI, rhlR, rhlI, and pqsR* genes expression (Wang et al., 2018).

Many virulence factors (Meena et al., 2020; Wang et al., 2018) including flagella and fimbriae, pyocyanin (PYA), pyoverdine, alkaline proteases, protease IV, elastases, and rhamnolipids (Al-Wrafy, Brzozowska, Górska, Gamian, & Doswiadczalnej, 2017) are controlled by *P. aeruginosa* QS systems via a complex of AI and R protein (Van Delden & Iglewski, 1998).The pathogenicity of these bacteria is mostly determined by their ability to produce these virulence factors. For example extracellular proteases play role in tissue destruction, elastase is released by about 75% of clinical *P. aeruginosa* (Kuang et al. 2011), and PYA disrupts the functioning of ciliated airway epithelial cells and results in increasing tissue damage (Britigan et al., 1999).*P. aeruginosa* QS systems are also involved for biofilm formation, swarming, and twitching motility (Wolska et al., 2016). Biofilm is an important virulence factor of *P. aeruginosa* infections because it assists the bacteria in evading immune defenses and protecting them from antibiotics (Al-Wrafy et al., 2017). In order to create a biofilm, free-swimming bacteria use a type IV pilus and flagellum structure to connect to a surface. The bacteria then proceed to create microcolonies via twitching motility. The bacteria then release QS-dependent AI compounds up to a particular concentration.

Finally, microcolonies are intricated in extracellular polymeric substance (EPS) which necessitates the use of the las and subsequently the rhI QS systems for biofilm development (Alasil et al., 2015).The QS system and associated virulence factors of *P. aeruginosa* have been intensively explored in human isolates (Sırıken et al., 2021).

Antibiotic resistance is a major threat to infection control systems and patient care in

any health-care facility across the world. AMR is causing rising healthcare costs, as well as increasing mortality and morbidity. "Combat drug resistance — No action today, no cure tomorrow," proclaimed the World Health Organization in 2011 (Kiran et al., 2011).

In general, *P. aeruginosa* antibiotic resistance mechanisms may be divided into three categories: adaptive, intrinsic, and acquired resistance. Intrinsic resistance is a form of resistance mechanism in which resistance is genetically encoded in an organism's core DNA. An adaptive mechanism is one in which resistance is induced by environmental stimuli, whereas a process in which resistance is induced by getting resistance genes from another organism is known as acquired mechanism (Tenover, 2006).

The presence of Beta-lactamases such as AmpC and OXA-50, as well as the existence of efflux pumps, low outer membrane permeability of *P. aeruginosa* (12-100 times less than E. coli) are some of the factors that contribute to *P. aeruginosa* intrinsic resistance (Girlich et al., 2012).

The emergence of adaptive resistance is influenced by external stressors. For instance, certain antibiotics and stress factors may be present. As adaptive resistance is unstable and transient, it differs from acquired resistance. This sort of resistance, unlike acquired mutational resistance, is not permanent, and it may be deactivated by removing stress factors (Moradali et al., 2017). Adaptive resistance frequently involves regulatory processes, which result in genetic changes, protein synthesis changes, or target site changes. For example, biofilm formation, MexXY and TCSS induction (Coleman et al., 2020; Hocquet et al., 2003). Due to the longer period of selection pressure during prolonged antibiotics therapy of any chronic infection, as well as the immunological response of host effectors acquired resistance is widespread in isolates of Cystic Fibrosis (Dettman et al., 2016).

Antibiotics must pass across the outer membrane of bacteria to work on their intracellular targets (Shaikh et al., 2015). The accumulation of antibiotics in *P. aeruginosa* cells is fundamentally reduced owing to the lower permeability of its outer membrane. The porin inside its outer membrane is another factor that reduces the rate at which antibiotics may permeate the cell's outer membrane (Lambert et al., 2002). Porin is involved in not just the transport of chemicals and nutrients across the cell membrane, but also the stability, signaling, and adherence of the cell membrane (Achouak et al., 2001; Langendonk et al., 2021).

P. aeruginosa has 26 porins, the most prevalent of which is OprF (Chevalier et al., 2017). Antibiotic resistance is aided by efflux pumps. Outer membrane porin channel proteins, cytoplasmic membrane transporters, and periplasmic liker proteins make up these efflux pumps. There are 12 expressed efflux pumps in *P. aeruginosa*, however only four causes antibiotic resistance (Dreier & Ruggerone, 2015).

Lipopolysaccharide is found in Gram-negative bacteria and functions as a leaflet of *P .aeruginosa's* outer membrane. Antimicrobial resistance, virulence factors, and the host-pathogen interaction all benefit from it (Lam et al., 2020). The lipopolysaccharide of *P. aeruginosa* has three distinct domains i.e. the O-antigen, Lipid-A, and core oligosaccharide regions (Gellatly & Hancock, 2013). Any change in lipopolysaccharide can aid in bacterial cell resistance to polymixin antibiotics and CAPS (Denis et al., 2019). Many pathways and phenotypic expression interactions among various regulatory genes are involved in the production of high levels of resistance to polymixin antibiotics (Jochumsen et al., 2016) but the aminoarabinosylation of lipid A and the functional arm operon are needed for colistin and polymixin resistance in *P. aeruginosa* (Lo Sciuto et al., 2018).

The biofilm formation process declines the susceptibility of *P. aeruginosa* to many treatments done through antimicrobials, hence it is necessary to diagnose *P. aeruginosa* diseases at the initial level prior to the formation of biofilm. Although the rate of acute and chronic infections are elevating globally, so in order to circumvent the spread of this disastrous Gram-negative bacterium there is a dire need of adopting new therapeutic approaches to replace conventional antibiotics and strategies (Thi et al., 2020).

Our study focused on the las system among the four systems of quorum sensing. We began our research by detecting phenotypic expression of QS las system by assaying biofilm formation, PYA production, protease production and elastase production in clinical *P. aeruginosa* isolates in order to understand QS and its controlled virulence factor expression. After that, the genotypic assay was done to examine the prevalence of QS las genes i.e *las I* and *las R* genes. So we determined the QS regulated virulence factors and correlated to the presence of Las system. Sensitivity testing for antibiotics were carried out.

AIM AND OBJECTIVES

AIM

The aim of this study is to screen *lasI* and *lasR* genes in clinical isolates of *Pseudomonas aeruginosa.*

OBJECTIVES

1. Antimicrobial susceptibility testing to analyze the antibiotic resistance profile of the isolates.
2. To screen the phenotype of the QS las system by:

a. Determining biofilm formation ability of the isolates.

b. Characterizing clinical isolates of *P. aeruginosa* as weak, moderate and strong biofilm formers.

c. Determining the ability of the isolates to produce PYA or pyocyanin pigment.

d. Determining the ability of the isolates to produce protease enzyme.

e. Determining the ability of the isolates to produce elastase enzyme.

3. Screening of quorum sensing genes *lasI* and *lasR* by PCR.

LITERATURE REVIEW

3.1. Characteristics of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is considered to be the major etiological agent for the critical infections related to wounds and surgeries since so long. Frequently *P. aeruginosa* is contemplated as an opportunistic pathogen or secondary intruder. It is an aerobic, motile, glucose non-fermenting, non-sporing, Gram-negative bacillus with a single flagellum at the pole which has the ability to survive under 5°C- 42° C. It usually thrives in diverse range of living and non-living environments. Usually, it is found in environments with humidity. Its smells fruity, unable to do fermentation of glucose and it produces fluorescence when exposed to violet light. It is one of the ESKAPE pathogens. It is from Pseudomonadaceae family, so it is a member of γ-proteobacteria. Among 12 members it is one of the subtypes (Todar, 2008). *P. aeruginosa* can live in two forms free or planktonic and sessile or biofilms. Almost 10-20% nosocomial infections are caused by this dangerous pathogen (Fazeli et al., 2012).This pathogen has paramount importance in clinical field as well as it is crucial from the epidemiological perspective. Among all Gram-negative non-fermenting rod-shaped bacteria *Pseudomonas aeruginosa* is the major etiological agent for many opportunistic and hospital-acquired infections particularly in immunocompromised hosts (M. H. J. C. C. Kollef , 2013; Rocha et al., 2019). It can dwell in wide range of habitats i.e water, plants and soil. It can infect the normal healthy person, but this happens very rarely and in case if a person with normal immunity extracts the bacteria, he suffers through a mild infection. In case of people with weak or debilitated immunity (i.e cystic fibrosis, AIDS or HIV, wound burn, the people who have soft tissue infections , urinary tract infections, skin , bone and joint infections , meningitis or bacteremia, the people having prosthetic devices i.e catheter or mechanical ventilator or those individuals who have undergone any surgery or chemotherapy. Several other important factors which play pivotal role to acquire pseudomonad infections are unhygienic practices in the course of surgeries, age, use of prosthetic devices, poor health conditions. Among the normal and healthy people only mild type infection happens like ear infections or skin rash that can be extracted by unchlorinated swimming pools (Gillespie & Hawkey, 2006; Pitt et al., 2006).

3.1. Discovery

Luke mentioned about this bacterium for the first time in 1862. He did this discovery when he was examining the bluish-green pus coming out from an infection in human. Before that Sedillot noticed the same colored pus on the surgical bandages (Al-Wrafy et al., 2017). Later on the bluish green color of the pus was found to be due to the presence of a pigment called pyocyanin.

3.2. Habitat

Pseudomonas aeruginosa has an extraordinary ability to adapt to its surrounding environments and its metabolism is highly proficient (Arai, 2011). It can dwell in diverse range of habitats such as hospital areas (Kerr & Snelling, 2009) ,soil, environments contaminated with oil (Haritash & Kaushik, 2009), human body (Rybtke et al., 2015) (Streeter & Katouli, 2016) , water (Mena et al., 2009), drains and sinks. Therefore it is considered to be a highly ubiquitous bacteria of water and soil (Stover et al., 2000) (Shrivastava et al., 2004) (Crone et al., 2020). *Pseudomonas aeruginosa* is not a part of normal flora in human GIT. However, among the hospitalized patients it may colonize the skin and upper respiratory tract. Its nutritional needs are very limited, it can get energy from wide range of carbon sources, and it can also survive the anaerobic environments by using nitrogen as an alternative electron acceptor. It can endure the temperature up to 42°C (Kung et al., 2010).

3.3. Genome

In the year, 2000 the wild type strain PAO1 of this pathogen was sequenced for the first time which was originally isolated from a wound and is being extensively used for research purposes. The genome of *Pseudomonas aeruginosa* is quite large and complicated because of its extraordinary ability of adaptation to the surrounding environment, high proficiency of the metabolism and its exemplary opportunistic pathogenic nature. In the prokaryotic world the genome of *P. aeruginosa* is among the largest genomes which is about 6.3 Mbp with 5570 open reading frames (Kung et al., 2010).Quite high amount of proteins are encoded by its genome which are engaged in virulence functions , regulatory functions, versatility , transport and adaptability of species in versatile ecological niches. The proteins encoded by its genome have very crucial and important roles pivotal for their survival and functioning. Only 0.3% genes of the total genome encode the antibiotic resistance proteins. A huge part of the genome encodes for diverse range of enzymes which performs functions like transportation of

nutrients and metabolism. Large number of genes encode for the outer membrane proteins (OMPs) which perform the functions like motility, metabolic diversity, efflux of virulence factors and antibiotics, adhesion etc.

The contemporary paradigm explains that the genome of pseudomonas bacteria comprises of two major parts:

1. Core Genome - Highly conserved portion of genome in all the strains. It is 90% of the whole genome.

2. Accessory Genome – Not present in all of the strains, they exist in the form of "regions of genomic plasticity" or RGP's which are the cluster of gene segments at a particular locus. Accessory genome has been classified into four main types. Each of it is formed by the combination of different functional modules.

I. Conjugative and integrative elements
II. Transposons and integrin + sequences
III. Replacement Islands
IV. Prophages and phage like-elements
V. Replacement islands.

Its genome is rich in GC bases content which is about 66% (Mathee et al., 2008).

3.3.1. Pathogenicity islands

Pathogenicity islands are specialized type of genomic islands that code for diverse virulence factors. Because 10% of genes in Pseudomonas aeruginosa are arranged into pathogenicity islands, the genome of this species is highly adaptable. Pathogenicity Island is made up of a high number of genes that code for the ability to easily acquire mobile genetic elements, as well as resistance genes. In the less virulent reference strain PAOL of pseudomonas, Pathogenicity Islands PAPI-1 and PAPI-2 are missing. A highly virulent wild type reference strain PA14 has been fully sequenced and carries two well-characterized pathogenicity islands. The first is PAPI-1, and the second is PAPI-2. PAPI-1, a 108-kilobyte pathogenicity island, is integrated into a lysine tRNA gene. A considerably smaller 11kb island is incorporated into a separate tRNA lysine gene (Harrison et al., 2010).Bacterial infections remain a major cause of rising mortality and morbidity despite 60 years of active development of antimicrobial agents. Similarly, P. aeruginosa has established a variety of antibacterial resistance mechanisms, leading to a rise in antibacterial resistance in recent years. Flagellum and pili, exo-enzymes, protein secretion system, lectins, iron chelation, lipopolysaccharide,

toxins, siderophores, secreted enzymes, motility, and quorum sensing are various virulence features found in this opportunistic pathogen (Lovewell et al., 2014).

3.4. Mortality rate

This pathogen causes serious and life-threatening infections with high mortality rates in specific conditions, such as neutropenic patients, who have a mortality rate of 30-50 percent, nosocomial pneumonia, which has a mortality rate of 45-75 percent, and urinary tract infections, which are caused by *Pseudomonas aeruginosa* and are acquired in hospitals, which account for 12 percent of infections. It can also cause ophthalmic infection when infected contact lenses are used. Neonatal Ophthalmia is caused by this infection. However, it is uncommon for it to develop infections in the digestive tract. In immunocompromised patients, Pseudomonas infection can cause brain abscesses, meningitis, and septicemia. A major consequence of Pseudomonas aeruginosa infection of the lungs is chronic bronchopulmonary obstructive disease, often known as cystic fibrosis, a disorder that can cause chronic inflammation of the lungs due to continuous bacterial immunological activation (Mesaros et al., 2007).

3.5. Epidemiology

3.5.1. Nosocomial infection caused by *Pseudomonas aeruginosa*

P. aeruginosa is responsible for 11 percent to 13.8 percent of all nosocomial infections (D.-j. Kim et al., 2012; Lizioli et al., 2003). In intensive care units, the greatest rate of nosocomial infection by *P. aeruginosa* has been documented, ranging from 13.2 percent to 22.6 percent ((Erbay et al., 2003; Lizioli et al., 2003; Sedlak-Weinstein et al., 2005). *P. aeruginosa* is the second most prevalent cause of ventilator-associated pneumonia and healthcare-associated pneumonia (M. H. Kollef & Kollef, 2005). *P. aeruginosa* has been described as one of the most prevalent causes of nosocomial infections in the ICU.

3.5.2. Bloodstream infections caused by *Pseudomonas aeruginosa*

According to research, *P. aeruginosa* causes 46% of bloodstream nosocomial infections, this is less than infections produced due to the gram-negative bacteria in the similar kind of individuals (Lynch 3rd & Zhanel, 2022; Thaden et al., 2017).*P. aeruginosa*-caused bacteremia was more common in patients with bone marrow and solid organ transplants than in the general population of a hospital (McCarthy, 2018). In patients of lung, heart, and bone marrow transplants, *P. aeruginosa* is a common source of nosocomial infection (Hogan et al., 2020).When a patient develops

Bronchiolitis obliterans, *P. aeruginosa* is a common cause of late-onset pneumonia during lungs transplantation (Yoshiyasu & Sato, 2020).

3.5.3. Urinary tract infections caused by *Pseudomonas aeruginosa*

P. aeruginosa is said to be responsible for roughly 16 percent of surgical infections, 16.3 percent of UTIS in ICUs, and 9 percent of all hospital admissions (Bassetti et al., 20018; Driscoll et al., 2007; Litwin et al., 2021). It's also been found that *P. aeruginosa* infection was more common in hospital acquired UTI patients who used urinary catheters than in UTIS patients who did not use it. This occurred 10.5 percent of the time in patients who used urine catheters and 4.1 percent of the time in patients who did not use urinary catheters (Litwin et al., 2021).

3.5.4. Burn wound infections caused by *Pseudomonas aeruginosa*

The majority of research have established that *P. aeruginosa* is among the most important infectious organisms in burn patients, and those studies have revealed that *P. aeruginosa* colonization on burn sites was common during the first week of hospitalization (Sarker et al., 2022; Wardhana et al., 2017) . The prevalence of infection varies depending on the facility, although burn units are the most usually reported (Elmassry et al., 2020).

3.5.5. Skin Infections caused by *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is the most common pathogen in the case of individuals having toxic epidermal necrolysis and disturbed skin microflora and burn patients being the greatest example. It has shown high occurrence in diabetic foot infection patients (Tocco-Tussardi et al., 2017).

3.5.6. HIV caused by *Pseudomonas aeruginosa*

In patients with both acquired and primary immunodeficiency, *P. aeruginosa* is the most commonly encountered pathogenic bacterium. *P. aeruginosa* was shown to be the most prevalent cause of septicemia in patients with primary immunodeficiency in a cohort study. It is also responsible for 14 percent to 21 percent of bacteremia in acute leukemia patients (Samonis et al., 2013). A study was carried out to determine the occurrence of *P. aeruginosa*-caused bacteremia in HIV-positive patients and common population of that same hospital. The researchers discovered that the incidence was ten times more among the HIV-positive individuals (Karmen-Tuohy et al., 2020). The most common pathogen recovered from 111 HIV-positive nosocomial pneumonia patients was *P. aeruginosa* (Garcia Garrido et al., 2020). After autopsies were done on 233 HIV-

1 positive patients, it was shown that P. aeruginosa was the most common cause of bacterial Bronchopneumonia, accounting for 16 of the 98 positive cases(Driscoll et al., 2007).

3.5.7. Cystic Fibrosis caused by *Pseudomonas aeruginosa*

In a patient with recurrent and chronic Sino-pulmonary tract infection, Pseudomonas aeruginosa is the most prevalent cause of cystic fibrosis. It was decided to conduct a longitudinal research, according to which cultures of respiratory samples from three-year-old children were obtained serologically in order to screen *P. aeruginosa*, which was identified in 97.5 percent of Cystic Fibrosis patients (Parkins et al., 2018). Cystic Fibrosis affected individuals who have positive *P. aeruginosa* respiratory cultures are more likely to be hospitalized, have a higher risk of weight loss, and have worse lung function than those who do not have *P. aeruginosa* positive respiratory cultures (Wolter et al., 2013).

3.6. Virulence factors and mechanisms

Bacterial infections continue to be a major cause of rising death rate and morbidity despite 60 years of active formation of antimicrobial medicines. Similarly, *P. aeruginosa* has established a variety of antibacterial resistance pathways, leading to a recent rise in antibacterial resistance. Flagellum and pili, exo-enzymes, protein secretion system, lectins, iron chelation, lipopolysaccharide, toxins, siderophores, secreted enzymes, motility, elastase, pyocyanin, cytotoxin, alkaline phosphate exotoxin, and pyoverdin and quorum sensing are just a few of the virulence features found in this opportunistic pathogen (Lovewell et al., 2014).These virulence variables have the greatest impact on immunocompromised people. The most prevalent cause of nosocomial infections is *P. aeruginosa*, which shows resistant to most medications, so it is a prime target for researchers looking for innovative ways to combat many bacterial infections (El Zowalaty et al., 2015). Targeting bacteria's virulence factors is one of the most important strategies to eradicate infectious agents, since it not only prevents antibiotic resistance but also activates the immune system, allowing infectious organisms to be eliminated from cells (Azam & Khan, 2019). Hemolysin, pyocyanin, alkaline protease, siderophore, pyoverdin, flagellum, lipopolysaccharide, type-IV pili, exotoxin-A, quorum sensing, alginate, type-VISS, proteases, biofilm formation, and motility are among the virulence factors of *P. aeruginosa* (CANTACUZINO, 2015; Viadero Valderrama, 2020).

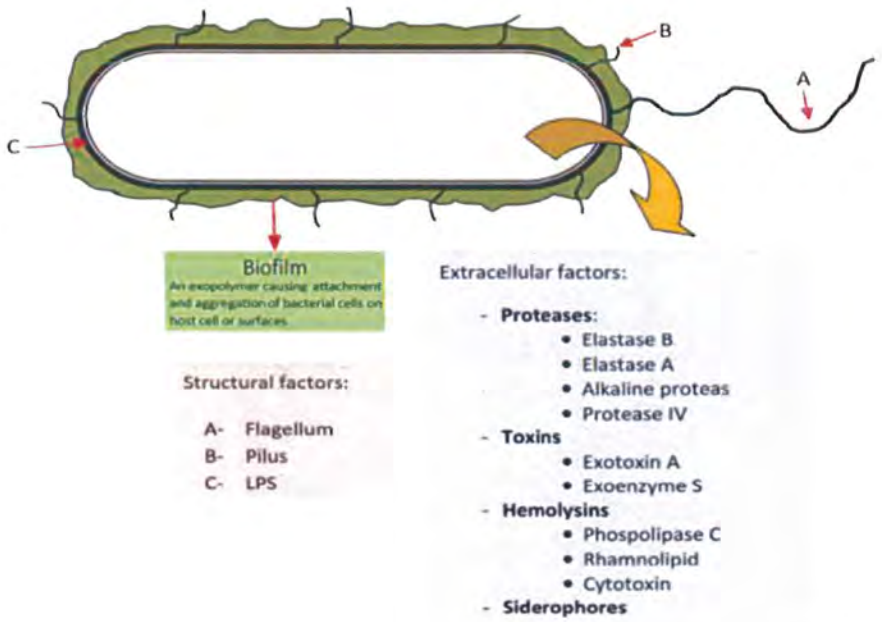


Figure 3.1 Virulence factors in P .aeruginosa (Moghaddam et al., 2014)

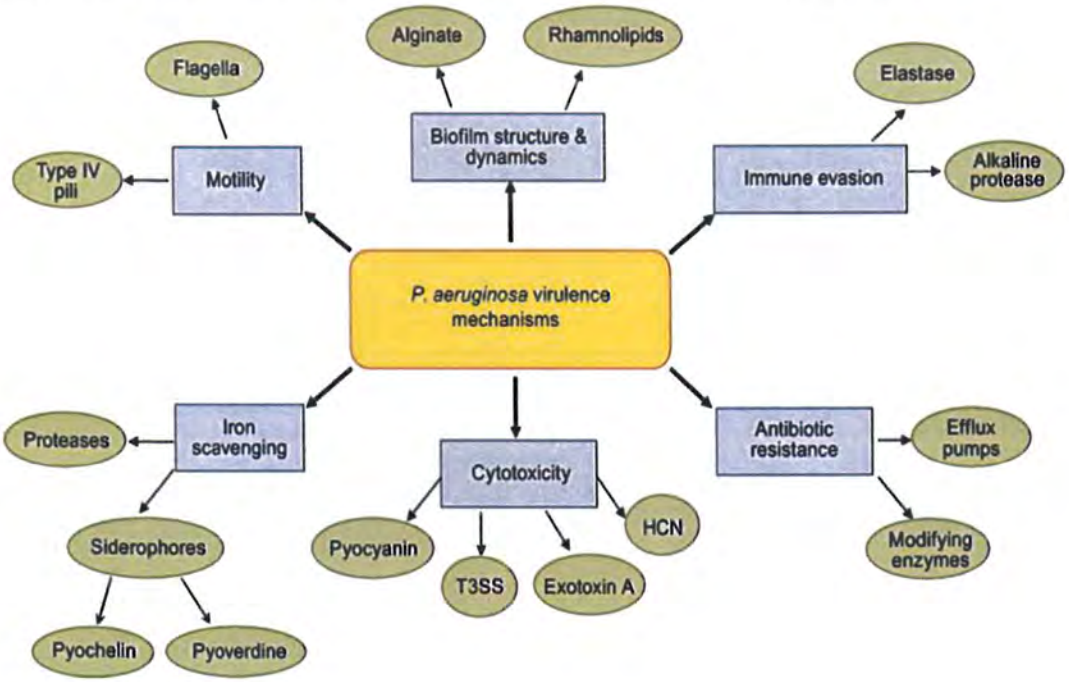


Figure 3.2 Virulence Mechanisms Recruited during P. aeruginosa Infections (J. Lee & Zhang., 2015).

3.6.1. Iron Scavenging

3.6.1.1. Siderophores

They are the iron chelating chemicals released by bacteria in its surroundings where free iron is not accessible because host cells obtain lactoferrin and transferrin from the environment. As a result, siderophores aid in the bacterial sequestration of Fe via iron chelation. Pyochelin and pyoverdine are generated as siderophores. Both serve as major transportation systems and signaling molecules (Cézard, Farvacques, & Sonnet, 2015).

1.6.1.2.Pyoverdine

Pyoverdine is a very fluorescent siderophore in *P. aeruginosa*. NGAL (neutrophil gelatinase-associated lipocalin) is a bactedrial siderophores pyoverdine scavenger generated by host cells that inhibits bacterial growth. Host cells inhibit the development of bacterial infections by scavenging apo siderophores and bacterial ferric NGAL which is a 25kDa protein also known as lipocalin 2 or siderocalin (Golonka, San Yeoh, & Vijay-Kumar, 2019).

3.6.1.3 Pochelin

These siderophores coexist with pyoverdine and have a decreased affinity for causing persistent infection (Damron, Oglesby-Sherrouse, Wilks, & Barbier, 2016).

3.6.2. Motility

3.6.2.1 Flagellum and type IV pili

P. aeruginosa colonizes and exploits new habitats by using a single polar flagellum to move about and chemotact (Rundell, Commodore, Goodman, & Kazmierczak, 2020). Twitching movement, swarming movement, and swimming movement are the three forms of mobility demonstrated by the pathogen. Neutrophophil phagocytosis, that is the first line of defense against any infection, initiates the formation of extracellular neutrophilic traps by swimming flagellari movement (Lovewell, Patankar, & Berwin, 2014). Through retraction and expansion of the type IV pili, twitching motility contributes to bacterial adhesion to mucosal cell surfaces(Saunders et al., 2020). In pili dependent phage infection, type IV pili play a role in virulence and adaptability. Swarming movement necessitates intricate multicellular coordination of bacteria over viscous surfaces. Isolates derived from cystic fibrosis lung infection patients, which indicate the function of motility during chronic infection by downregulating flagellin. The significance of motility in UTI, however, is unknown. According to studies, 90% of isolated swim and 70% twitch.Acute infections are often linked to swarming motility

(Talà, Fineberg, Kukura, & Persat, 2019). Type IV pili is a key surface adhesin that communicates with the Chp chemosensory system via a signal transduction pathway. Type IV pili are the motorized fimbriae that play a key role in biofilm development and pathogenicity by causing surface-specific twitching movements. Sequential extension, attachment, and withdrawal of type IV pili to the corresponding surfaces offer intimate connection to the surfaces. The Chp system is a key regulator of type IV pili, and the lack of Chp proteins causes twitching motility and Type IV pili assembly to be disrupted.

3.6.3. Cytotoxicity

3.6.3.1. Pyocyanin

This is a pigment which is blue green in color. Pyocyanin is another one of *P.aeruginosa's* secreted compounds that induces ciliary dysfunction in the respiratory tract, which produces oxidative stress in the host cells by disrupting mitochondrial electron transport and catalase. Pyocyanin is a cytotoxic blue pigment with a physiological pH. Pyocyanin's main function in UTI is to prevent urothelial cells from healing themselves, causing inflammation and discomfort (Esoda, 2019).

3.6.3.2. Exotoxin A

Exotoxin-A is secreted by a Pilus-like structure that secretes proteins in the extracellular environment, including proteases, lipases, alkaline-phosphatases, and phospholipases. Exotoxin-A is responsible for tissue invasion and injury (Viadero Valderrama, 2020).

3.6.3.3. Type III secretion system

A double membrane integrated nano machine delivers effector proteins to the plasma membrane and cytoplasm of eukaryotic target cells, allowing for colonization and invasion. T3SS is linked to a high risk of death and acute invasive responses in infected patients. Effector proteins with various functions are expressed differently in different Pseudomonas strains and isolates. There four effector proteins are Exo S, Exo Y, Exo U, and Exo T(Azimi et al., 2016). ExoS and ExoT both have an ADP-ribosyltransferase activity and a GTPase-activating function. They can protect *P. aeruginosa* from being phagocytosed by interacting with the actin cytoskeleton (Foulkes et al., 2019). T3SS is linked to bacterial pneumonia because it inhibits wound healing by changing and disrupting the epithelial barrier, resulting in bacterial pneumonia symptoms.

3.6.4. Immune evasion

The invasion of tissues is aided by theses virulence factors generated by *P. aeruginosa*.

3.6.4.1. Elastases

Collagen, IgA, IgG, and complement systems of the host cells are all cleaved by elastases. Epithelial cells tight junctions and MCC (mucociliary clearance) are altered by elastase mediated disruption of the epithelial barrier. SP-A and SP D (surfactant proteins), which are critical in nonspecific immunity, are likewise degraded by *P. aeruginosa* elastases (Galdino, Branquinha, Santos, & Viganor, 2017).

3.6.4.2. Alkaline proteases

The lysis of fibrin is aided by alkaline proteases, which prevents fibrin production. They play role in the inactivation of certain host defense system's critical proteins i.e cytokines, IFN-γ, complement, and antibodies.

3.6.4.3. Hemolysins

Pseudomonas aeruginosa has hemolysins, such as lecithinase and phospholipases, which work together to break down lecithin and lipid. These proteins promote invasion while also causing cytotoxicity in host cells (Brindhadevi et al., 2020).

3.6.4.4. Cytotoxin

Pathogenic bacteria produce cytotoxin (leukocidin), a pore-forming protein that has lethal effects on the cells of their hosts.

3.6.5. Biofilm structure and dynamics:

3.6.5.1. Alginate

In individuals with a persistent respiratory tract infection, pathovars of this species release alginate, which is a kind of exopolysaccharide. These alginates assist bacteria adhere to solid surfaces by protecting them from un-favorable environmental conditions (Garrison, Mahoney, & Wuest, 2021). Alginate lyses enzyme is produced by *P. aeruginosa* which breaks the polysaccharides into the subunits of small size, and it has been suggested that both degradation and biosynthesis mechanisms are critical in the infection process (Skariyachan, Sridhar, Packirisamy, Kumargowda, & Challapilli, 2018). Its pathogenicity is also linked to the structure of its cells and the presence of extracellular virulence factors (Moradali, Donati, Sims, Ghods, & Rehm).

3.6.5.2. Rhamnolipids

Jarvis and Johnson discovered rhamnolipids from *Pseudomonas aeruginosa* and reported them in 1949 (Jarvis & Johnson, 1949). *P. aeruginosa* produces primary rhamnolipids in a quickly agitated liquid medium having limited nitrogen or iron contents during the stationary growth phase(Guerra-Santos, Käppeli, Fiechter, &

Biotechnology, 1986).*Pseudomonas aeruginosa* can grow and produce rhamnolipids from a variety of carbon sources. Antibacterial and antifungal properties are also demonstrated by rhamnolipids (Gunther IV, Nunez, Fett, Solaiman, & microbiology, 2005). Rhamnolipids (RLs) are glycolipid molecules that are diverse in structure. They are biosurfactants in nature generated primarily by *Pseudomonas aeruginosa*, and they are key virulence factors in *P. aeruginosa* pathogenesis (Laabei et al., 2014).

3.6.6. Lipopolysaccharide

Lipopolysaccharide makes up most of the of *P. aeruginosa's* outer membrane. The components of bacterial lipopolysaccharide include O-antigen (distal polysaccharides), endotoxin (also known as lipid-A), and a non-repeating oligosaccharide sequence (Rocha, Barsottini, Rocha, Laurindo, Moraes, & Rocha, 2019). Lipopolysaccharides are involved in the stimulation of the host's adaptive and innate immune responses, which is the primary cause of inflammation and, as a result, mortality, and morbidity (Schechner et al., 2009).

3.7. Biofilm formation

Introduction

Pseudomonas aeruginosa is a common Gram-negative opportunistic pathogen which produces severe acute as well as chronic infections .Its infections are not only nosocomial but also fatal (Gale, Maritato, Chen, Abdulateef, & Ruiz, 2015; Thi, Wibowo, & Rehm, 2020).It usually affects immunosuppressed human hosts i.e cancer patients, patients undergoing post-operative period, dreadfully burnt people or the victims of Human Immunodeficiency Virus (HIV) (Gale et al., 2015; Gomila et al., 2018; W. Wu, Jin, Bai, & Jin, 2014).In the year 2017, *P. aeruginosa* was acknowledged among the most fatal bacteria. WHO has mentioned it as the priority pathogen for the purpose of Research and Development of new antibiotics (Organization, 2017).It shows antibiotic resistance to many agents through adaptation and by the inherent mechanisms of antibiotic resistance ,so this gives rise to high mortality rate(Pang, Raudonis, Glick, Lin, & Cheng, 2019). The phenomenon of biofilm formation impedes the treatment processes and also aids them to persist in the medical environments (Moradali, Ghods, Rehm, & microbiology, 2017; Thi et al., 2020). Biofilm formation is the universal trait of the bacteria. Biofilm is a framework of complex multicellular aggregates embedded in the self-generated extrapolymeric substances. This architecture enables the bacteria to cling to different surfaces. Bacteria in the form of biofilm is sheltered from the

stresses of the surroundings which hampers their phagocytosis hence assisting their community development hence they persist for the longer time periods (Thi, Wibowo et al)(Yang et al., 2011) (Branda, Vik, Friedman, & Kolter, 2005; Hall-Stoodley & Stoodley).Quorum sensing is a process which occurs in biofilm and help them to persist for longer periods of time. This is a communication process in which each cell of the aggregate interacts with each other and work in coordination this team work enables bacteria to endure harsh environmental conditions hence they regulate they sense their surroundings , so they maintain the cell density and behave accordingly (Römling & Balsalobre, 2012)(Bassler & Losick, 2006; Liang Wu & Luo, 2021).Consequently complex structures are established which are often found in chronic infection patients i.e chronic lung infection, chronic wound infection and rhinosinusitis(Römling & Balsalobre, 2012).Approximately more than 90% of the chronic infections of wounds are poorly healed as they have been brought out due to biofilm development. It has been estimated that round about 6.5million people got diagnosed with chronic infection of wounds solely in US, this badly affected their economy (25 billion USD) and gave rise to many complications in health care settings (Sen et al., 2009).As biofilm formation declines the susceptibility of *P. aeruginosa* to many treatments done through antimicrobials hence it is necessary to diagnose *P. aeruginosa* diseases at the initial level prior to the formation of biofilm. Although the rate of acute and chronic infections are elevating globally , so in order to circumvent the spread of this disastrous Gram-negative bacterium there is a dire need of adopting new therapeutic approaches to replace conventional antibiotics and strategies (Thi et al., 2020).

3.7.1. Historical basis

Biofilm are the aggregates of microorganisms embedded in a sticky matrix of extracellular polymeric substances in this way these cells could adhere to many surfaces in the form of colonies. Following scientists have gave their contributions in the discovery of biofilms. Since then, the studies on biofilms in different settings like industrial areas, ecological areas and public health concerned areas have synchronized each other.

Table 3.1 Different Scientists and their work in Biofilm discovery

Scientists	Contribution for Biofilm Discovery	References
Van Leeuwenhoek	First one who discovered microbial biofilms with simple microscope on the tooth surfaces	
Heukelekein and Heller	Described the "bottle effect" that describes how marine microorganisms' proliferation and bacterial activity increased when they attached to the surface. "	(Donlan, 2002; Heukelekian & Heller, 1940).
Zobell	Concluded that bacterial population on the surfaces was substantially greater as compared to the outside medium surrounding it (seawater here)	(Zobell, 1943)
Jones et al.	Observed that biofilms comprises of mixed microbiota (based on the morphology) which were grown on the tricking filters in a waste water treatment plant. Transmission and Scanning Electron Microscopes were used for this purpose. Polysaccharide nature of the extracellular polymeric substances was also found.	(Jones, Roth, & Sanders III, 1969)
Characklis	In 1973, examined microbial slimes in the water systems of industrial area and remarked that they were very firm and tremendously resistant to the disinfectants like chlorine.	(Characklis, 1973)

Costerton et al.	In 1978, proposed a theory on biofilms based on the dental plaque and sessile colonies establishment in streams flowing in the mountains. Attachment of microorganisms to the non-living and living objects and the advantages stem through this ecological niche were mentioned in this theory.	(Costerton, Geesey, & Cheng, 1978)

3.7.2. Biofilm characteristics

Biofilms are much more resistant as compared to the planktonic cells as they have the ability to adapt in their environment easily. Moreover, the metabolism and the phenotypes of biofilm formers is quite different from free-floating cells relating to growth rate, surface molecule expression, virulence factors, and nutrition consumption. Horizontal gene transfer allows bacteria in biofilms to exchange resistance genes, allowing antibiotic resistance to spread quickly across species boundaries in biofilm populations. Antibiotic resistance genes are exchanged between the cells in the biofilms through bacterial horizontal gene transfer mechanism hence spreading antibiotic resistance in the biofilm communities (Donlan & Costerton, 2002).

3.7.3. Biofilm composition

Biofilm of *P. aeruginosa* produces matrix which essentially composed of extracellular DNA (eDNA), lipids, proteins, polysaccharides (Ghafoor, Hay, Rehm, & microbiology, 2011; Strempel et al., 2013).The matrix comprises of over 90% of the biomass of the biofilm. It provides a framework for the attachment to living and non-living surfaces and shields the embedded bacterial cells from stringent conditions of the surrounding environment i.e immune responses of the host and antibiotics. It further dispenses wide range of other compounds encompassing cystolic proteins, essential nutrients and enzymes for the biofilm. Coordination and interaction between the cells is also assisted by the matrix. (Jackson, Starkey, Kremer, Parsek, & Wozniak, 2004). It comprises three types of exopolysaccharides Psl , Pel, Alginate that help the biofilm framework form, adhere to surfaces, and stay in place (Billings et al., 2013; Ghafoor et al., 2011).

3.7.4. Phases of biofilm development

A mature biofilm develops in five phases:

First phase

It involves planktonic bacterial cells attachment to any surface with the help of type-IV pili or flagella. This attachment is reversible. The proteomic study of PA01 wild-type has determined that the formation of biofilm and bacterial responses are materials specific. *P. aeruginosa* can change the presence and quantity of its proteins according to the nature it senses and then responds accordingly (Guilbaud et al., 2017).

Second phase

During stage two the bacterial cells that have been attached to the surface, undergo change by switching from reversible attachment to irreversible. During this stage, cells are firmly attached to the surface so that became resistant to any physical attempts I (Armbruster & Parsek, 2018), detach them.

Third phase

During this stage, the attached bacteria start multiplication and the production of biofilm matrix components and convert themselves into a more organized structure called Micro colonies.

Fourth phase

During this stage micro-colonies further, develop into an extensive and three-dimensional structure that appears like a Mushroom shape, and then biofilm starts maturation. In this stage resistance toward antibiotics increase.

Fifth phase

The matrix cavity in the mid of the micro-colony gets rapture by autolysis of cells for the discharge of dispersed cells.

Sixth phase

This stage allows the cycle of biofilm to repeat in the same above manner (Ma et al., 2009;Rasamiravaka et al., 2015)

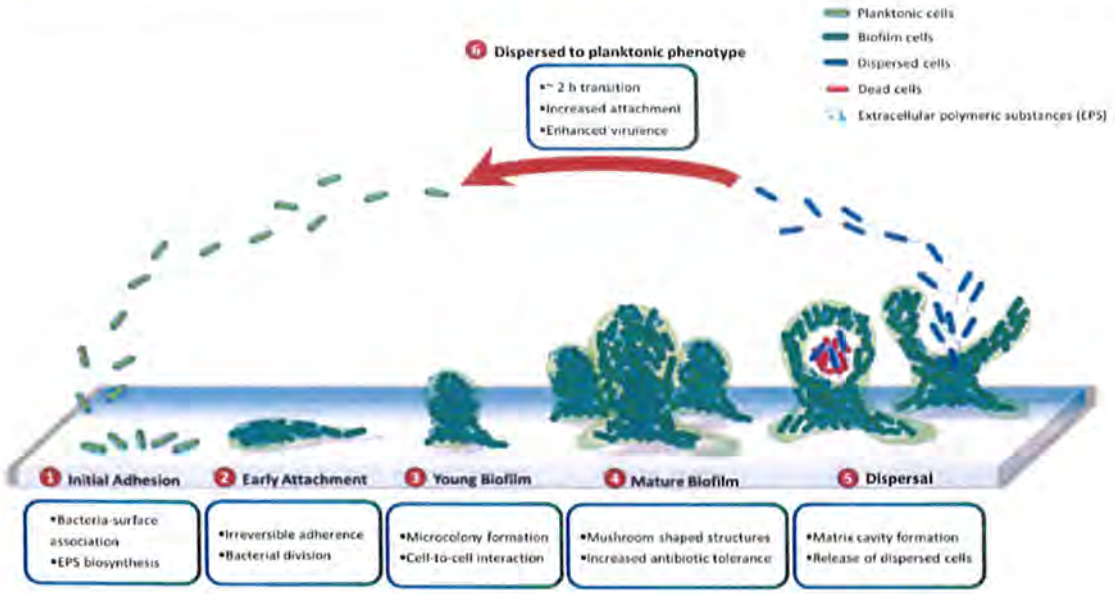


Figure 3.3 Demonstration of Biofilm formation cycle in *P. aeruginosa (Thi et al., 2020)*

3.7.5. Biofilm, a cause of infectious diseases

According to the National Institutes of Health, biofilms are responsible for around 80% of microbial illnesses (NIH, 2002). Biofilm-based infections are those that are inherent to the host tissues and are exploited to generate an opportunistic and persistent infection in a range of organs and tissues, such as osteomyelitis, vaginitis, cystic fibrosis, urethritis, conjunctivitis, endocarditis with native-valve infections, non-healing wounds, and some paediatric infections of the respiratory tract, such as rhinosinusitis and otitis media. Periodontitis, caries, gingivitis, and halitosis are only a few of the numerous oral illnesses (Filardo, Di Pietro, Tranquilli, & Sessa, 2019; Hamilos, 2019; Lebeaux et al., 2014). Infections caused by biofilms on medical devices such as UTI and intravascular catheters, heart valves, pacemakers, breast implants, orthopaedic implants, endotracheal tubes, and contact lenses fall into another class of biofilm associated infections (Gominet, Compain, Beloin, & Lebeaux, 2017; Lebeaux et al., 2014; Zimmerli & Sendi, 2017).

3.7.6. Antibiotic resistance in biofilms: causes and consequences

Antibiotic tolerance is a non-heritable and transient phenotypic trait based on the physical condition of the biofilm-forming cell population as well as biofilm-specific properties that allow the bacterial population to restrict antibiotic activity and diffusion. Antimicrobial agents must overcome some of the mechanisms that cause biofilm

formation, such as the efflux pump, high number of resistant mutants, molecular exchange, persistent cells, high cell density, and substance delivery (Hathroubi, Mekni, Domenico, Nguyen, & Jacques, 2017). Infectious diseases are the consequence of biofilm formation.

3.7.7. Penetration of antibiotics

Antibiotics have a tendency to enter the biofilm matrix and harm the cells that are coated. The EPS has an impact on the number of molecules that make it to the inner layer of the biofilm and interaction with antibiotic molecules occurs. By connecting with charged antibiotic molecules, the extracellular polymeric matrix acts as a physical barrier, providing a safe haven for microbial colonies. Pel exopolysaccharides have the capacity to distribute cationic antibiotics, such as aminoglycosides, in the case of P. aeruginosa (Colvin et al., 2011; Nadell, Ricaurte, Yan, Drescher, & Bassler, 2017).

3.7.8. The buildup of an antibiotic-degrading enzyme in a matrix

As a defense strategy, biofilm-forming microbes may store a large number of Beta lactamases in the matrix. In the case of P. aeruginosa, accumulating Beta lactamases in the biofilm matrix cause Ceftazidime and Imipenem to be hydrolyzed. As a result, removing Beta lactamases from biofilm can aid antibiotics such as Ampicillin in reaching deeper layers (Anderl, Franklin, & Stewart, 2000; Bagge et al., 2004).

3.7.9. DNA molecules in biofilm matrix

Extracellular DNA is the most prevalent and important component of bacterial biofilm matrix. Biofilm's capacity to exhibit resistance to various antibiotics can be boosted by the presence of eDNA. The capacity of eDNA to modify the outer membrane of bacteria, as well as the ability to chelate cations like Mg, is a way by which it promotes biofilm resilience. It can reduce the magnesium ion concentration in the membrane. Antibiotic resistance is also provided by eDNA through horizontal gene transfer across biofilm-forming bacteria (Hall & Mah, 2017; Wilton, Charron-Mazenod, Moore, & Lewenza, 2016).

3.8. Cell-to-cell communication and immune evasion mechanisms

Generally bacteria use intracellular communications to govern their environmental systems and cell densities, allowing them to operate and respond optimally in response to demographic and environmental factors (Decho, Norman, & Visscher, 2010; Horswill, Stoodley, Stewart, Parsek, & chemistry, 2007). Bacterial pathogens take benefit of this strategy and produce many infections by coping the host defense system.

They evaluate cell density via intercellular communications and signal transmitter factor concentrations, so when the density reaches a point where the immune system can no longer cope, bacteria release virulence factors, preventing the host immune system from responding quickly. The process is regulated by a bacterial mechanism known as "Quorum Sensing" (Y. H. Li, Tian, expression, & bacteria, 2016; Moghaddam et al., 2014; Waters & Bassler, 2005)

3.8.1. The system of Quorum Sensing in bacteria

Bacteria is among the most simple and ancient biological entities which are unicellular organisms with very basic communication, reproduction and feeding mechanisms but recent researches shows that these simple entities also have some complex and intriguing mechanisms just like the multicellular organisms. The mechanism of quorum sensing regulates the communication both among and between species that is concerned with nutrient deprivation, biofilm development and other environmental stressors like disinfectants, antibiotics, bacterial colonization, the identification of vexing species, the maintenance of normal intestinal flora and presence of normal flora of intestine etc (Rutherford & Bassler, 2012) (Miller & Bassler, 2001).

3.8.2. What is quorum sensing?

Bacteria frequently live as community rather than as planktonic states. They are able to communicate by sending and receiving a sequence of signals or messages. One of the most common kinds of communication mechanisms is quorum sensing, in which the word quorum means "threshold" and sensing means "feel," which is abbreviated as QS system. The QS system is a cell-to-cell mechanism in which bacteria control the expression of specific genes depending on the cell density. QS System can be considered as multi-cellular activity in the uni-cellular bacterial world, in which bacteria estimate the cell density population and utilize the knowledge to control gene expression (Häussler, 2010; Wenseleers et al., 2010).

3.8.3. How is it mediated?

In the phenomenon of QS, bacteria synthesizes low molecular weight chemical molecules that spread easily and cluster in the environment; these messenger molecules are known as "Auto-inducer" or "Self-inducer." Many bacteria, according to recent research, employ a collection of messenger chemicals to coordinate their actions. The signaling pathways are triggered when the concentration of chemicals outside the bacterium surpasses the threshold level and the bacteria respond to the messages by

modifying the expression of genes and influences physiological functions collectively (Egland & Greenberg, 1999; C. Fuqua, Parsek, & Greenberg, 2001).

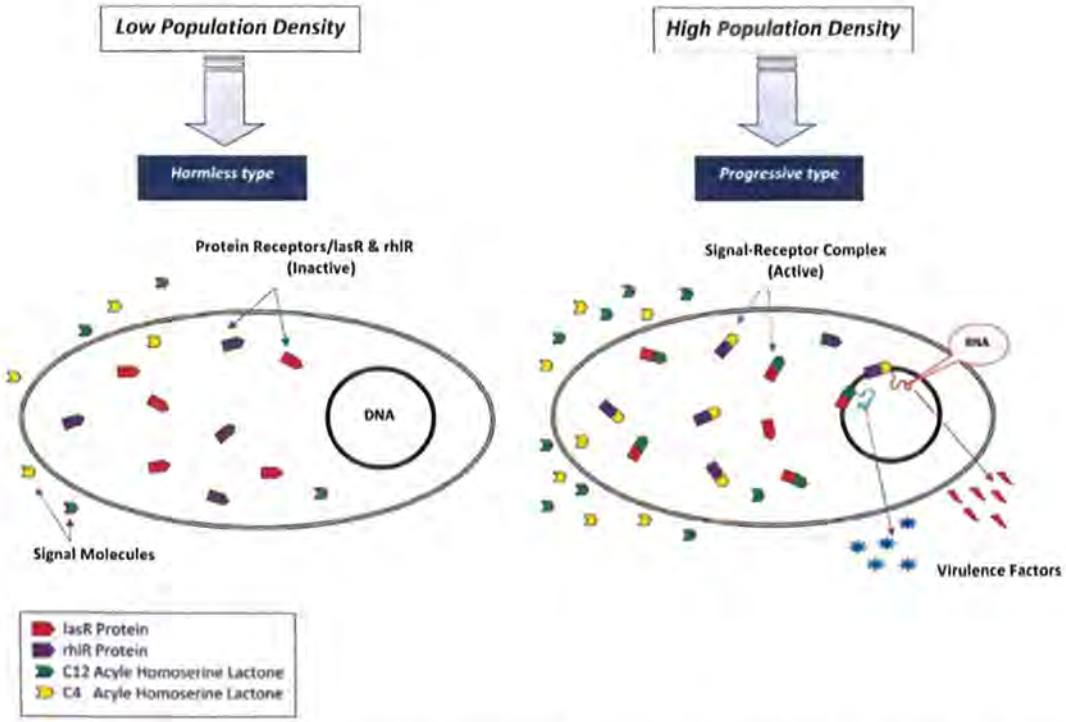


Figure 3.4 The Quorum Sensing in the bacterial Population, based on QS in *P. aeruginosa* (Moghaddam et al., 2014)

3.8.4. Quorum Sensing in gram-negative bacteria

Quorum sensing complexes have been discovered in over 25 Gram-negative bacteria species in the last decade. Quorum sensing regulates a wide range of key bacterial functions, including bioluminescence, swarming, swimming, twitching, antibiotic biosynthesis, biofilm formation and dissemination, conjugation, sporulation, and the creation of virulence markers (Atkinson, Chang, Sockett, Cámara, & Williams, 2006; Von Bodman, Bauer, & Coplin, 2003).QS System of Vibrio fischeri, Pseudomonas aeruginosa and Agrobacterium tumefaciens are highly considered. On the other hand, studies illustrate that changes in this system has occurred with time as well as with each microorganism's specific needs. The bioluminescence feature of Vibrio fisheri is the subject of the majority of quorum sensing research.

3.8.5. History of Quorum Sensing

***luxI-luxR* System**

The research on the prototype luxI-luxR system of Vibrio fischeri led to the

development of idea of quorum sensing in *P. aeruginosa*. This system is made up of two regulatory proteins:

LuxI: Autoinducer synthase enzyme. It is responsible for the biosynthesis of acyl homoserine lactones (AHL/acyl-HSL) or autoinducer, a kind of messenger molecule.

LuxR: Promoter binding protein

The concentration of autoinducer rises as the cell population grows. At a threshold concentration, the autoinducer gets inside the bacterial cell and attaches to a protein called R protein. This complex stimulates the target gene transcription, allowing bacteria to coordinate gene expression more efficiently with cell population changes (Figure 3.4) (Stevens & Greenberg, 1997; Visick, Foster, Doino, McFall-Ngai, & Ruby, 2000; Von Bodman et al., 2003).In recent research, various unusual systems other than LuxI/R have been discovered; for instance, in *Pseudomonas aeruginosa*, the *lasI* and *lasR* control the synthesis of virulence proteins. Many bacteria have homologous *luxI and luxR* genes with a comparable mechanism and performance (Visick et al., 2000).

3.8.6. Autoinducers

The quorum sensing system produces autoinducers, which are tiny diffusible signaling molecules generated by bacteria which are classified into two types:

- ✓ **Autoinducers type-I** - Unique to each specie because of the specific carbon chain in each Gram-negative bacteria attached to acyl homoserine ring (AHL) and helps in communication within the species.
- ✓ **Autoinducers type-II** – Not unique for each specie, composed of furanosyl borate diester and helps in communication between the species such as bacteria with fungi and protozoa (C. Fuqua et al., 2001; Parsek & Greenberg, 2000) (Figures 3.5 A and 3.5 B).

Synthesis of Autoinducer in Pseudomonas aeruginosa

las I and *las R* expresses synthase enzymes which synthesizes the autoinducers by the help of S-adenosylmethionine (SAM) and Acyl-acyl carrier protein (Acyl-ACP) as the substrates.

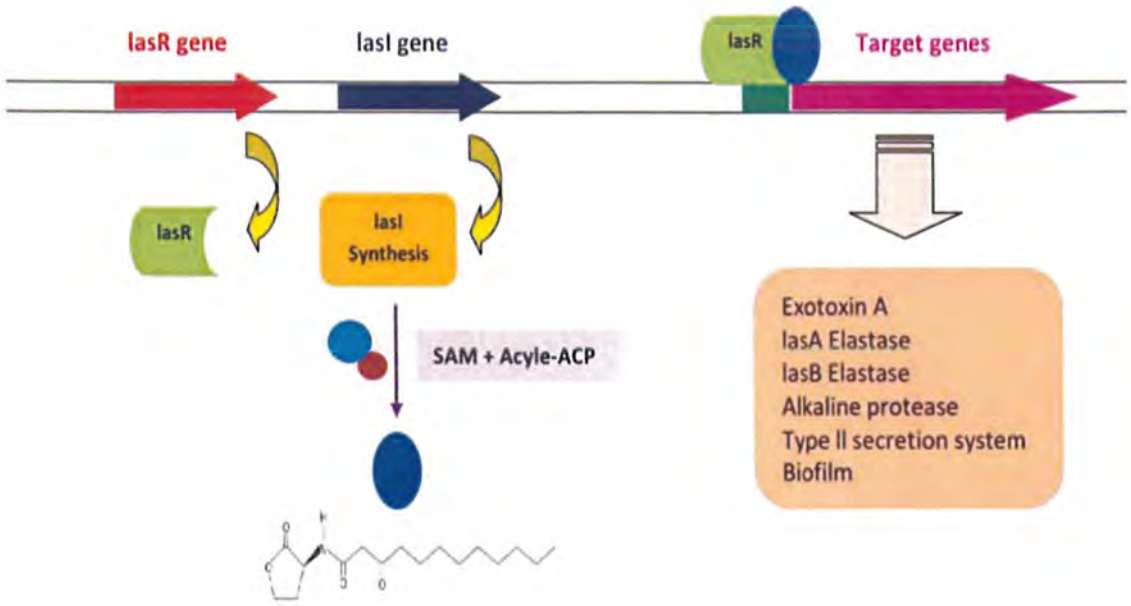


Figure 3.5 (A) The function of *las* genes in the *Pseudomonas aeruginosa* QS system and the pathogenic genes under their control (Moghaddam et al., 2014).

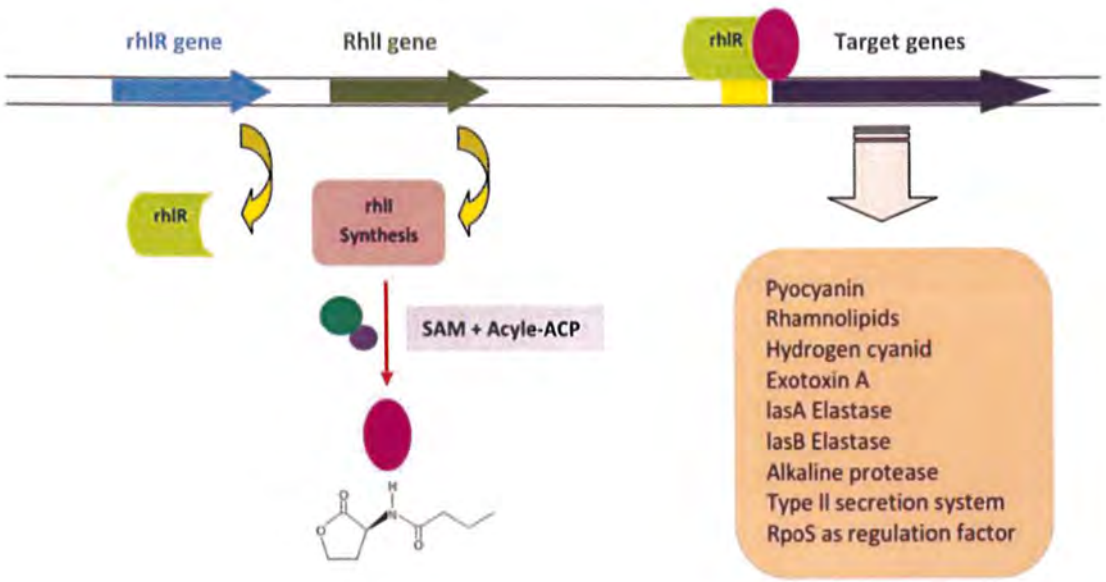


Figure 3.5 (B) The function of *rhl* genes in the *Pseudomonas aeruginosa* QS system and the pathogenic genes under their control (Moghaddam et al., 2014)

3.8.7. Quorum Sensing system of *Pseudomonas aeruginosa*

3.8.7.1. Types of Quorum Sensing in *P. aeruginosa*

The QS circuits of *P. aeruginosa* have four different routes which generate their respective auto-inducer molecules:

1. **Las** –Its AI is N-3-oxo-dodecanoyl-L-homoserine lactone (3O-C12-HSL)
2. **Rhl** – Its AI is N-butyryl-L-homoserine lactone (C4-HSL)
3. **PQS** –Its AI is 2-heptyl-3-hydroxy-4-quinolone (Pseudomonas Quinolone signal, PQS)
4. **IQS** –Its AI is 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde (Integrated Quorum Sensing Signal , IQS)

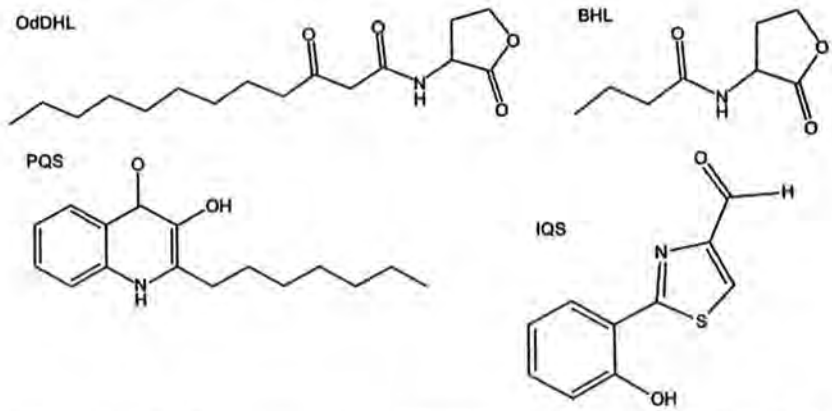


Figure 3.6 Structure of *P. aeruginosa* Quorum Sensing (QS) Signals (J. Lee et al., 2015)

3.8.7.2. The main genes controlled by QS in *P. aeruginosa*

The key constitutive genes in the quorum sensing system of *P. aeruginosa* are the two pairs of genes.

Las genes: *lasI* and *lasR* genes encode C12-HSL auto-inducer synthase and R protein. QScR is a regulatory gene which produces the inhibitor protein of *las R*.

Rhl genes: *rhlI* and *rhlR* genes encode synthase and R protein respectively (Figure3.5B). This pair of QS systems comprises a synthase enzyme that makes C4-HSL auto-inducer but only a little quantity of N-hexanoyl-L-homoserine-lactone (HHL) (Girard & Bloemberg, 2008).

The QS genes of *Pseudomonas aeruginosa* are dependent and linked to each other for their functioning, Las genes regulate the expression of the rhl genes by the help *lasR*s R protein.

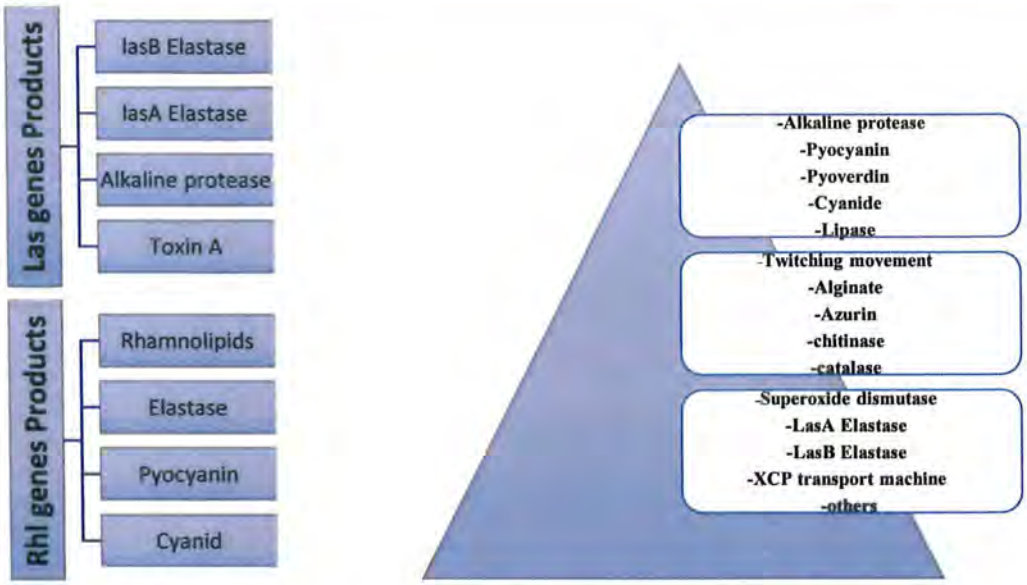


Figure 3.7 Virulence genes products Regulated by QS system of *P. aeruginosa*

rhl genes products may also be coupled with the *las* gene (Figure 3.9).

Name of *las* gene

The role of the *las* gene was initially assessed through the enzyme activity of lasB elastase, which is why it was given the name *las* gene in the *P. aeruginosa* QS system.

Name of *rhl* gene

The *rhl* gene was given the name rhl because of its important involvement in rhamnolipid formation. Rhl regulates the *RhlAB* operon, which encodes the rhamnosyl transferase enzyme, which is required for rhamnolipid synthesis.

3.8.7.3. The Las and Rhl Quorum Sensing systems

Components of Las system:

The three components of the las system

- LasR, a response regulator (RR)
- N-3-oxo-dodecanoyl homoserine lactone (AHL; 3-O-C12-HSL) which is a similar AI molecule.
- *lasI*, which is employed for AI- synthesis.

Components of Rhl system

Similarly, the Rhl system is made up of:

- rhlR
- N-butyryl homoserine lactone (C4-HSL) as an AI molecule.
- *rhlI*, which controls AI synthase (Wang et al., 2018)

- ❖ Despite their differences, the las and rhl QS systems have a hierarchy: the former is dominant over the latter(Van Delden & Iglewski, 1998)(Srken, z, and coworkers, 2021).

The las and rhl Quorum Sensing systems

The discovery of the QS systems in *P. aeruginosa* has encouraged more study into their functioning, regulons, and the molecular processes by which the *las* and *rhl* circuits stimulate the expression of QS-responsive genes.

- ✓ The receptor proteins LasR and RhlR are activated and form complexes when they bind with their corresponding autoinducers OdDHL and BHL.
- ✓ The LasR-OdDHL and RhlR-BHL complexes bind to the conserved *las-rhl* boxes present in the target gene promoters, inducing their transcriptional expression (Schuster & Greenberg, 2007; Whiteley & Greenberg, 2001; Whiteley, Lee, & Greenberg, 1999).
- ✓ The regulons are present in a continuity, with few genes responding strongly to OdDHL (e.g. *lasA*), some to BHL (e.g. *rhlAB*), and others to both signals equally well (Schuster & Greenberg, 2006; Schuster, Lostroh, Ogi, & Greenberg, 2003). These genes account for almost 10% of the genome of *P. aeruginosa*, and hence constitute for the bulk of physiological processes and virulence phenotypes (Schuster & Greenberg, 2006).Some of the most important virulence genes are given here.

Table 3.2: Quorum sensing (QS) regulated virulence factors and their effects to the human host (J. Lee & Zhang, 2015)

QS regulated genes	Proteins or virulence factors	Effects to host during infections	Benefits of *P. aeruginosa*
lasB	Elastase	Degradation of elastin, collagen, and other matrix proteins	Extracellular iron acquisition from host proteins
lasA	Protease	Disruption of epithelial barrier	Staphylolytic activity, host immune evasion

			and enhanced colonization
toxA	Exotoxin A	Cell death	Establishment of infection; enhanced colonization
aprA	Alkaline protease	Degradation of host complement system and cytokines	Immune evasion and persistent colonization
rhlAB	Rhamnosyl-transferases (rhamnolipids	Necrosis of host macrophage and polymorphonuclear lymphocytes	Immune evasion; biofilm development
lecA	Lectin (galactophilic lectin)	Paralysis of airway cilia	Establishment of infection; enhanced colonization
hcnABC	Hydrogen cyanide	Cellular respiration arrest; Poorer lung function	Enhanced colonization
phzABCDEFG, phzM	Pyocyanin	Oxidative effects dampen host cellular respiration and causes oxidative stress; Paralysis of airway cilia; Delayed inflammatory response to *P. aeruginosa* infections through neutrophil damage	Establishment of infection; enhanced colonization; immune evasion

✓ *lasR* also triggers RsaL expression, which is a transcriptional repressor of *lasI*. When RsaL binds to the bidirectional *rsaL-lasI* promoter it suppresses both of the genes to be expressed, resulting in a negative feedback loop that balances OdDHL levels by counteracting the positive signal feedback loop (Rampioni et al., 2007).Despite the fact that LasR/OdDHL and RsaL have no competition for the common binding site on the *lasI* promoter, repression done via RsaL is greater than activation through LasR (Rampioni et al., 2007).RsaL also suppresses the expression of several QS target genes, such as pyocyanin and cyanide biosynthesis genes (Rampioni et al., 2007).

- After that, several positive and negative regulatory proteins were discovered, each controlling the *las* and *rhl* systems in a different way.

➢ LuxR homologues QscR and VqsR perform important regulatory roles.

▪ Heterodimers of QscR and LasR/OdDHL are created and RhlR/BHL and hence inhibits them from binding to the promoter region of responsive genes present in downstream position in this way the effects of *las* and *rhl* QS signaling are reduced (Ledgham et al., 2003).QscR also binds to OdDHL, which it uses to activate its own regulon (S. A. Chugani et al., 2001; C. Fuqua, 2006; Schuster & Greenberg, 2006).

▪ The LasR/OdDHL complex regulates VqsR, which is a positive regulator of the Las QS system (L.-L. Li, Malone, & Iglewski, 2007).

➢ QslA which is an anti-activator has been discovered. It binds with LasR by protein-protein interactions and prevents the binding of LasR with promoter sequence of *las* responsive genes. Regardless of OdDHL concentrations, QslA has an inhibitory impact on LasR. QslA regulates the overall QS activation threshold by interfering with LasR's capability to induce the downstream genes to be expressed and initiate a QS response (Seet & Zhang, 2011).

➢ Furthermore, the AHL-acylases PvdQ and QuiP, quorum quenching enzymes that degrade AHL signals, are important in balancing the amount of AHL signals in *P. aeruginosa* (Huang, Du, McClellan, Barrett, & Hazlett, 2006; Sio et al., 2006).There are a number of additional AHL-based QS super-regulators, which are included in the table below.

Table 3.3 : Super-regulators of QS in *P. aeruginosa (J. Lee & Zhang, 2015)*

Regulator	Mechanism of action
AlgR2	Negative transcriptional regulator of *lasR* and *rhlR*
DksA	Negative transcriptional regulator of *rhlI*
GacA/GacS	Positive transcriptional regulator of *lasR* and *rhlR*
MvaT	Negative transcriptional regulator (global regulation)
QscR	Negative regulator (anti-activator) of LasR protein
QslA	Negative regulator (anti-activator) of LasR and PqsR proteins
QteE	Negative post-translational regulator of LasR and RhlR
RpoN	Negative transcriptional regulator of *lasRI* and *rhlRI*
RpoS	Negative transcriptional regulator of *rhlI*
RsaL	Negative transcriptional regulator of *lasI*
RsmA	Negative transcriptional regulator of *lasI*
Vfr	Positive transcriptional regulator of *lasR* and *rhlR*
VpsR	Positive transcriptional regulator of *lasI*

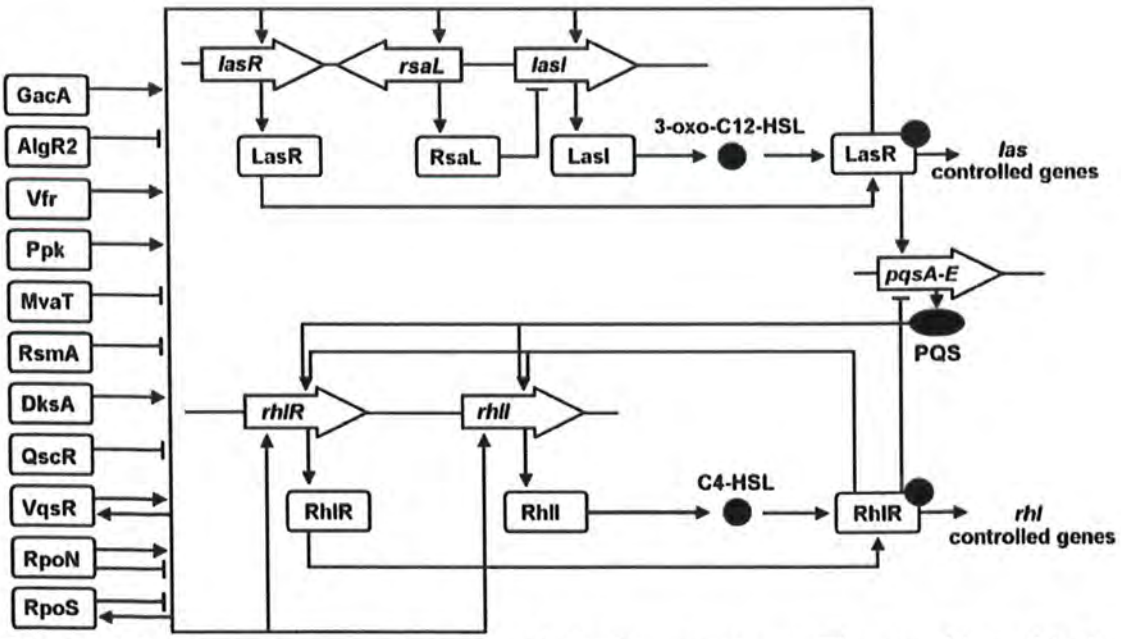


Figure 3.8 The quorum sensing network in *P. aeruginosa*. The two hierarchically arranged las and rhl quorum sensing circuits are subject to modulation by a number of additional regulators, which finetune the quorum sensing response of the organism (activation ⟶, inhibition ⊣)(Juhas, Eberl, & Tümmler, 2005)

3.8.7.4. Role of Quorum Sensing in biofilm formation *Pseudomonas aeruginosa*

Each cell within bacterial communities must work together to form *P. aeruginosa* biofilms (Yan & Wu, 2019).Quorum sensing (QS) allows *P. aeruginosa* to communicate between individual cells and eventually organize collective behavior, which is critical for the adaptability and survival of large communities. In response to variations in cell density as well as environmental signals or stressors, *P. aeruginosa* enters the QS mode (Mukherjee & Bassler, 2019). QS includes the creation, secretion, and accumulation of signaling molecules termed autoinducers (AI), whose specificity and concentration are recognized by transcriptional regulators (W. C. Fuqua, Winans, & Greenberg, 1994), resulting in population-wide expression of certain sets of genes. In addition to the formation of biofilms. QS has been connected to the control of virulence-factor synthesis, stress tolerance, metabolic adjustment, and host-microbe interactions, in addition to biofilm development (Römling & Balsalobre, 2012) Understanding and manipulating these chemical communication channels may lead to novel targets for antimicrobial and antibiotic alternatives or complements.

The hierarchy of quorum sensing circuit in *Pseudomonas aeruginosa*

The QS circuits of *P. aeruginosa* have four different pathways: Las, Rhl, PQS, and IQS.

- These QS circuits are arranged in a hierarchical order, in which the Las system is present at the top position (J. Lee et al., 2015).
- During the initial exponential growth phase, both the Las and Rhl systems are activated by a rise in cell density(Choi et al., 2011), although the PQS and IQS systems are active during the late exponential growth phase, especially under iron limitation (Oglesby et al., 2008)and phosphate famine conditions(Rampioni et al., 2016).
- The generated AIs are transported across the cell membranes, first to the outside and subsequently to the inside, possibly by free diffusion, efflux pumps, or outer membrane vesicles (Mashburn & Whiteley, 2005)
- The delivered 3O-C12-HSL binds to the regulator protein LasR, and the resulting complex activates the *lasI* synthase gene, triggering an autoinduction feed-forward loop (Seed, Passador, & Iglewski, 1995).
- The LasR–3O–C12–HSL also stimulates the expression of the *rhlR* and *rhlI* genes and *pqsR* and *pqsABCDH* genes, which encode for Rhl (J. Lee et al., 2015) and PQS (Déziel et al., 2004) systems.

- The RhlR–C4–HSL complex, like the Las system, triggers the second autoinduction feed-forward loop by inducing *rhlI* gene expression (Winson et al., 1995).
- The PqsR–PQS complex activates the *pqsABCDH* genes and feeds back to stimulate *rhlRI* gene expression in the PQS system (McKnight, Iglewski, & Pesci, 2000)
- RhlR inhibits the expression of both the *pqsR* and *pqsABCDH* genes, which has been proposed as a means to control the appropriate ratio between 3-oxo-C12-HSL and C4-HSL, and hence the activation of the PQS pathway (Cao et al., 2001)
- The IQS system was discovered relatively recently compared to the other QS systems. The identity of the transcriptional regulator in the IQS system is still unclear, despite the fact that its binding to the IQS has been identified to activate the *pqsR* gene (J. Lee et al., 2013; Meng, Ahator, & Zhang, 2020).

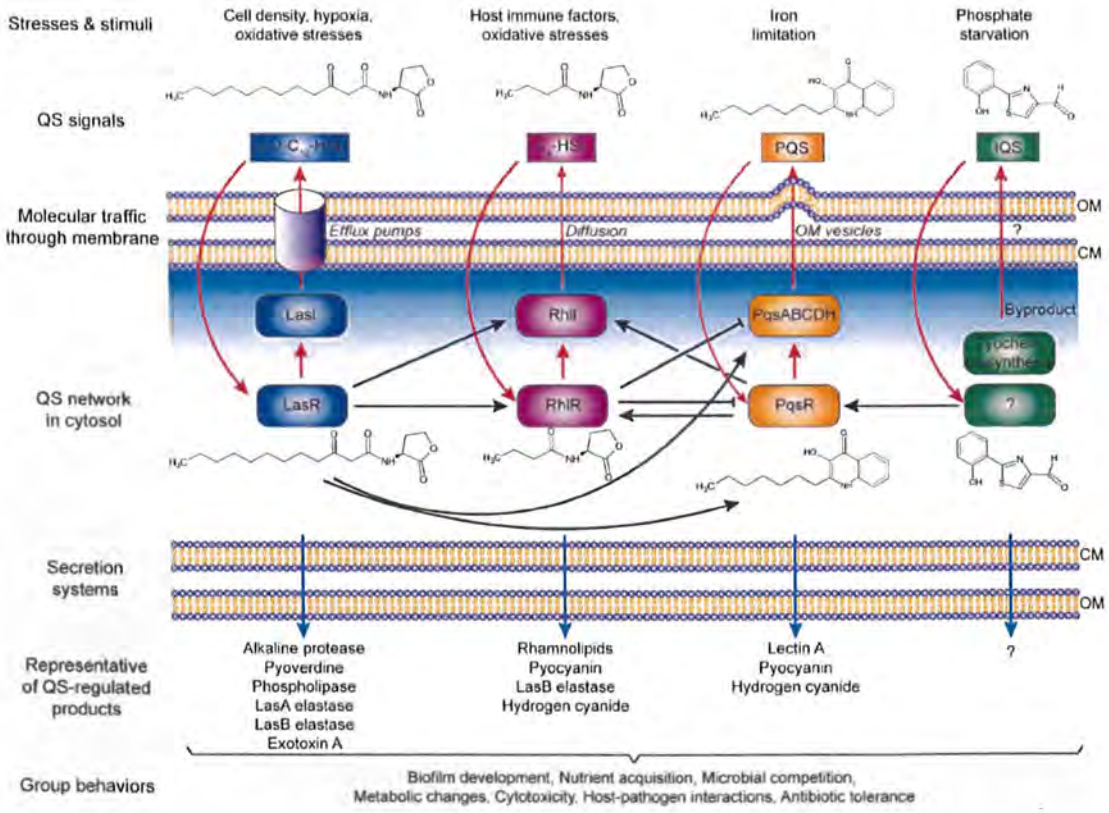


Figure 3.9 Hierarchical Quorum Sensing(QS) network in *Pseudomonas aeruginosa* (Thi et al., 2020)

Initially the role of quorum sensing in the regulation of biofilm development was discovered by (Davies et al., 1998).This study concluded that the las system is essential for the formation of a basic biofilm architecture. Later (Sauer, Camper, Ehrlich, Costerton, & Davies, 2002) supported this idea, demonstrating that the las system is critical in the final phases of biofilm growth. Another recent research suggests that both of *P. aeruginosa's* quorum sensing systems are involved in biofilm growth regulation (Hentzer, Givskov, Eberl, & O'Toole, 2004)(Juhas, Eberl et al. 2005). All the three systems Las, Rhl, and PQS systems of P. aeruginosa's QS network play critical roles in the synthesis of functional components that influence biofilm growth (Figure)(Thi et al., 2020). Rhamnolipids (Pamp & Tolker-Nielsen, 2007), pyoverdine (Banin, Vasil, & Greenberg, 2005), pyocyanin (Das et al., 2015), Pel polysaccharides (Sakuragi & Kolter, 2007), and lectins (Diggle et al., 2006) are only a few examples.

- Rhamnolipid (i.e., biosurfactant) that keeps the pores and channels between microcolonies open, allowing fluids and nutrients to flow through.
- Pyoverdine may sequester iron from the environment and transport it to the cell, which is a key component in biofilm formation. When iron is scarce, twitching motility is preferred over sessile growth, preventing the development of biofilms (Visca, Imperi, & Lamont, 2007).
- Pyocyanin is a cytotoxic secondary metabolite that causes cell lysis and the release of the cells' DNA into the extracellular space (eDNA–one of the biofilm components). Pyocyanin binds to eDNA and increases solution viscosity, enhancing physicochemical interactions between biofilm matrix and the surrounding environment and encouraging cellular aggregation.
- Pel polysaccharides may interact with eDNA inside the biofilm matrix via cationic-anionic interactions, enhancing the biofilm structure.
- Lectins are soluble proteins found in the outer membrane that are divided into two types: LecA (which binds to galactose and its derivatives) and LecB (which binds to glucose and its derivatives) (that binds to fucose, mannose and mannose-containing oligosaccharides). These sticky capabilities of lectins aid in the retention of cells and exopolysaccharides in a developing biofilm, as well as attachment to biological surfaces including epithelium and mucosa.

In conjunction with other polymeric chemicals, such molecular and cellular interactions lead to the formation of a strong and mature biofilm (Davies et al., 1998) (Thi et al.,

2020).

3.8.7.5. Environmental triggers and the QS responses in *Pseudomonas aeruginosa*

Environmental stress appear to have a significant impact on the QS systems of *P. aeruginosa*. In the absence of *lasR*, starvation, phosphate depletion, and iron depletion have all been shown to increase RhlR expression and activity (Jensen et al., 2006; Van Delden, Pesci, Pearson, Iglewski, & immunity, 1998).Phosphate depletion has recently been discovered to cause IQS generation even in the absence of a functioning *las* system (J. Lee et al., 2013).This finding has therapeutic implications since a large number of *P. aeruginosa* chronic infection isolates have a loss of function *las* system (Dénervaud et al., 2004; Smith et al., 2006). The functions and molecular processes by which distinct environmental signals and host immunological factors modify the QS systems of *P. aeruginosa* will be explored individually (J. Lee et al., 2015)

Phosphate-depletion stress

Phosphate is vital for all live cells because it plays a critical part in signal transduction events like phospho-relay and as a component of ATP, nucleotides, phospholipids, and other important biomolecules. During the course of pathogen-host contact process, bacterial pathogens are likely to face fierce competition for free phosphates from the host cells. *P. aeruginosa* survival and infection establishment are therefore dependent on its capacity to survive phosphate deficiency and its response mechanisms for exploiting phosphate from external sources. Consequently, phosphate-depletion stress has been demonstrated to affect QS signaling patterns, gene expression, physiology, and pathogenicity of bacterial pathogens in a variety of ways (S. Chugani & Greenberg, 2007; Frisk et al., 2004; Jensen et al., 2006; J. Lee et al., 2013; Zaborin et al., 2009). PhoBR sensor-response regulator system is critical for detecting and transmitting phosphate stress cues (Hsieh & Wanner, 2010), since *phoB* deletion entirely eliminates *P. aeruginosa* pathogenicity and substantially reduces its swarming motility and cytotoxicity(Bains, Fernández, Hancock, & microbiology, 2012).PhoB (and the *pho* regulon) has also been implicated in the prevention of biofilm formation, the degradation of c-di-GMP signals, and the suppression of type III secretion systems (Haddad, Jensen, Becker, & Häussler, 2009), all of which might have a substantial impact on the clinical outcome of *P. aeruginosa* infections.

Iron depletion stress

Iron deficiency also affects the QS networks of *P. aeruginosa* but less than phosphate

depletion stress. A lack of iron causes significant increases in the expression of QS controlled virulence genes such as iron acquisition (ferric uptake siderophores, pyochelin and pyoverdine; ferrous iron transporters like haem and *feo*), exoenzymes that can cleave iron-bound host proteins (alkaline protease, *lasB* elastase), as well as other redox enzymes and toxins (exotoxin A) (Ochsner, Wilderman, Vasil, & Vasil, 2002).*P. aeruginosa* was also observed to be protected from oxidative damage when exposed to iron depletion stress due to lack of oxygen so the production of the virulence component LasB elastase also rises dramatically (E.-J. Kim, Sabra, & Zeng, 2003).Despite of the fact that *P. aeruginosa's* QS systems regulate the few upregulated virulence factors like alkaline protease and elastase still the direct connection between iron starvation and upregulation of main genes of QS like *lasI, lasR , rhlI or rhlR* not found.

ANR and oxygen deprivation stress

P. aeruginosa's cyanide production (cyanogenesis) is influenced by low oxygen tension (Castric, 1994; Castric, 1983). Hydrogen cyanide (HCN), the end product, is a powerful extracellular virulence factor that contributes to high death rates in host species during the infection (Ryall, Davies, Wilson, Shoemark, & Williams, 2008). Increased cell density in *P. aeruginosa* was also demonstrated to significantly boost up the expression of the HCN synthase genes *hcnABC*, which peaks during the bacteria's transition from exponential to stationary growth phase (Castric, 1983). This might point to a cooperative relationship between oxygen deprivation and QS in the cyanogenesis regulatory mechanism, as evidenced by the discovery of ANR, a transcriptional regulator linked to bacterial anaerobic growth.

Starvation stress

Stringent response is that when *P. aeruginosa* quickly adjusts and evokes a speedy reaction to change their metabolic profiles for survival when exposed to unfavorable conditions and nutrient deprivation. This stringent response causes a variety of effects, including inhibition of growth processes, cell division arrest (Greenway & England, 1999; J. Lee & Zhang, 2015) and, most critically, a cell density-independent early activation of the *P. aeruginosa* QS systems (Van Delden, Comte, Bally, & Marc, 2001). There is increased synthesis of downstream virulence factors such as elastase and rhamnolipids is expected to coincide with the rise in BHL QS signal (Schafhauser et al., 2014).Stringent response regulates the QS dependent response in amino acid

deprived environment .RelA is triggered by uncharged tRNA which further induces the production starvation signal ppGpp (Van Delden, Comte, Bally, & bacteriology, 2001).Overexpression of RelA leads to premature transcription of not only *lasR* and *rhlR* but also OdDHL and BHL QS signals (Van Delden, Comte, Bally, & bacteriology, 2001) therefore there is the overproduction of the QS related virulence factors. The starving signal ppGpp enhances the expression of LasR and RhlR, as well as the downstream variables that follow (Baysse et al., 2005; Van Delden, Comte, Bally, & Marc, 2001). Similarly, many other responses of QS system have been observed as a result of starvation stress.

Response to host factors

Generally it was a common perception that opportunistic pathogens such as *Pseudomonas aeruginosa* can only evade the immunocompromised host or can cross the weakened epithelial barrier passively but this concept was changed by Wu and his colleagues later on that *Pseudomonas aeruginosa's* major outer-membrane protein OprF can recognize and bind to human T cell-based cytokine interferon gamma (IFN-γ), which activates the rhl QS system, which increases the expression of lecA and the synthesis of galactophilic lectin virulence protein. In the presence of IFN-γ, another QS-regulated virulence component, pyocyanin, was also discovered to be up-regulated (Licheng Wu et al., 2005). There are other examples in which QS systems responds to many host factors through its virulence factors expression.

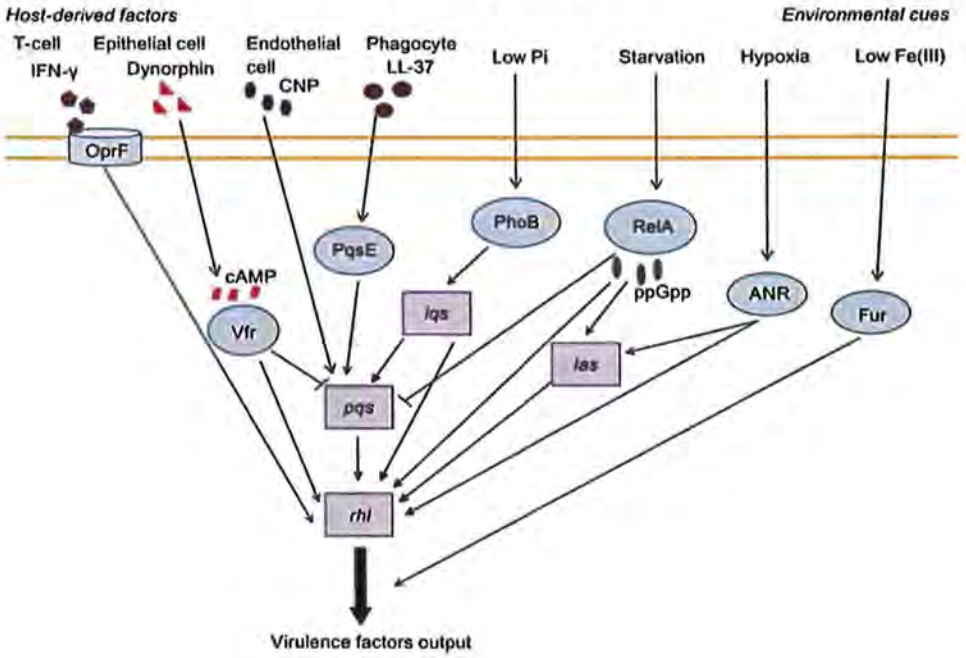


Figure 3.10 *An illustration of various environmental and host variables impacting the*

P. aeruginosa *QS signaling hierarchy. The QS systems, notably* las, iqs, pqs, and rhl *are represented as a single unit for ease of understanding (J. Lee et al., 2015)*

3.9. Antibiotic resistance in *P. aeruginosa*

In recent years, antimicrobial resistance in *P. aeruginosa* has become a major concern. One of the most common causes of resistance development is mutation in its genes. It can also acquire resistance by the acquisition of resistance genes or by the newer genes which are Beta Lactamases such as MBLs, AmpCs, and ESBLS (Kaushik & Basak). Antibiotic resistance is a major threat to infection control systems and patient care in any health-care facility across the world. AMR is causing rising healthcare costs, as well as increasing mortality and morbidity. "Combat drug resistance — No action today, no cure tomorrow," proclaimed the World Health Organization in 2011 (Kiran, Sharma, Harjai, & Capalash, 2011). Antibiotic resistance in *P. aeruginosa* is currently at an all-time high. *P. aeruginosa* infections have been recorded in burn units, cancer units, ICUs, postoperative wards, and other settings. Various antimicrobial drugs with anti-pseudomonal capabilities have been found as a result of substantial advances and developments in scientific study in order to avoid various outbreaks of microbial illnesses caused by *P. aeruginosa*, however, due to the emergence of antibiotic-resistant strains, significant morbidity, increasing mortality, and even life-threatening illnesses continue to occur across the world (Ergin & Mutlu, 1999). Antibiotic resistance in *P. aeruginosa* is caused by a variety of factors (Lambert, 2002). For example, this bacterium is innately resistant to most antibiotics due to its lower cell wall permeability, or it can evolve resistance by obtaining resistant genes from other bacteria via transposons or another source such as plasmid etc. The evolution of resistant bacteria can also be attributed to mutation. New Beta Lactamases are being produced. ESBLs, Metallo beta Lactamases, and AmpC's beta-lactamase are all key causes of resistance. Carbapenems are the antibiotics of last resort in most P. aeruginosa isolates that produce AmpC beta-lactamases and extended beta-lactamases. Carbapenem resistance was largely produced by the production of Carbapenemases and an enhanced efflux system in *P. aeruginosa*. MBLs are the most frequent kind of Carbapenems being produced (Watanabe, Iyobe, Inoue, & Mitsuhashi, 1991).

3.9. Antibiotic resistance mechanism of *Pseudomonas aeruginosa*

The acquired and inherent mechanisms of antibiotic resistance in *P. aeruginosa* are the

two basic categories of mechanisms of antibiotic resistance.

3.9.1. Intrinsic resistance of *P. aeruginosa*

Pseudomonas aeruginosa has innate antibiotic resistance through a variety of mechanisms, including the efflux system, which is responsible for pumping antimicrobial drugs out of the cell; antibiotic-inactivating enzymes are produced by this bacterium, and the limited permeability of its outer membrane contributes to antibiotic resistance (Moore & Flaws, 2011).

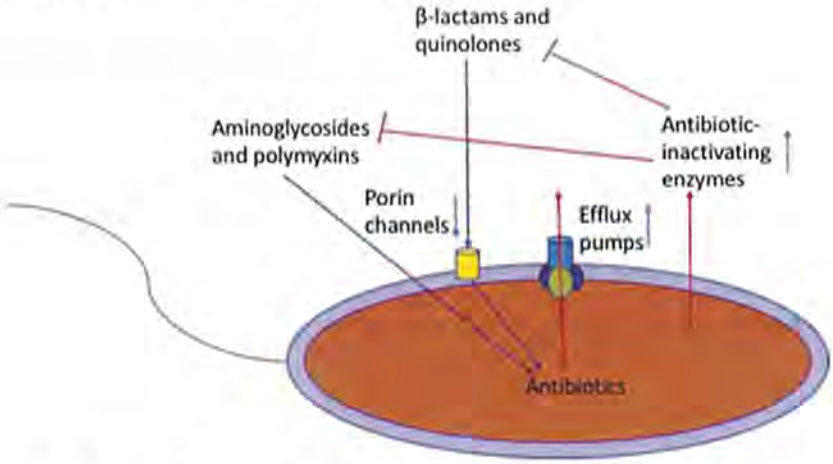


Figure 3.11 Schematic representation of the mechanisms of intrinsic antibiotic resistance in *P. aeruginosa (Pang et al., 2019)*

Outer membrane permeability

P. aeruginosa's outer membrane functions as a barrier, blocking large hydrophilic molecules from passing through. Colistin and aminoglycosides interact with lipopolysaccharides to alter the permeability of the outer membrane, allowing them to pass through, but quinolones and B-lactams require the presence of porin channels to diffuse.

Bacteria generates two main categories of porins:

- The first is generic one that allows practically all kinds of hydrophilic molecules to pass through.
- The second is more specific, with binding sites for certain compounds and the ability to pass these molecules in the most energy-efficient manner following correct orientation.

The majority of bacteria have generic porins, on the other hand P. aeruginosa has a huge number of specific porins (Hancock & Brinkman, 2002; Tamber, Maier, Benz, & Hancock, 2007).

Efflux pump

Multiple efflux pumps are found in *Pseudomonas aeruginosa*, and they are responsible for the efflux of antibiotics and several other compounds through the erial cells. These pumps constitute 3 types of proteins

a) Connective proteins of the periplasm.

b) Cytoplasmic membrane protein transporters that use energy in the form of a proton motive force

c) Outer membrane porin.

With the exception of polymixins, these efflux pumps eliminate the majority of antibiotics. Multidrug efflux (MexA, MexB) refers to a cytoplasmic membrane protein transporter and periplasmic connective proteins, whereas Opr (OprM) refers to an outer membrane porin (Lister, Wolter, & Hanson, 2009; Strateva & Yordanov, 2009).

Antibiotic inactivating enzymes

P. aeruginosa is a member of the SPICE group of bacteria, which produces antibiotic inactivating enzymes. This group of organisms share a number of traits, one of which is the capacity to manufacture chromosomally encoded inducible AmpC-B lactamases. These are Cephalosporinases enzymes that hydrolyze most ß-lactams rather than being blocked by ß-lactamases inhibitors. PoxB, a Class D oxacillinase generated by *P. aeruginosa*, is another endogenous ß-lactam. This enzyme is only found in laboratory mutatants and has no clinical significance (Girlich, Naas, & Nordmann, 2004; Kong et al., 2005).

3.9.2. Acquired resistance of *P. aeruginosa*

P. aeruginosa gained resistance to numerous antimicrobial drugs due to a variety of factors. During the treatment processes like chemotherapy mutational changes or the development of resistance mechanisms are acquired by horizontal gene transfer is an example of these factors. Overproduction of efflux pumps or endogenous ß-lactamases, as well as reduced synthesis of specific porins and altered target locations, can all result from these mutations (Poole, 2011).

3.10 Resistance to β-lactams

Resistance to Beta-lactam antibiotics is caused by a number of factors, among these factors the most important factor is the inactivation of the enzyme β-lactamases. Antimicrobial drugs are inactivated by these enzymes, which cleave down the amide

bonds of the β-lactam ring. They are classed based on their structure and function (Bush, Calmon, & Johnson, 1995). ß-lactams that are most efficient against *P. aeruginosa* are carbapenems. These drugs are resistant to hydrolytic actions majority of β-lactamases, including ESBLS (Falagas & Karageorgopoulos, 2009).

3.10.1. Overexpression of efflux pumps

Pseudomonas aeruginosa possesses several efflux systems that are involved in the development of ß-lactam resistance, although MexCD-OprJ, MexAB-OprM and MexXY-OprM are the most significant. MexAB-OprM is the most often found efflux system of *P. aeruginosa* among clinical isolates, and it can export Meropenem more efficiently as compared to the other two. When the efflux system is overexpressed in certain isolates, it, together with other mechanisms, gives rise to the MDR development. (Poole, 2011; Tomás et al., 2010).

3.10.2. Diminished permeability

Pseudomonas aeruginosa has a specific porin called OprD in its outer membrane that allows carbapenems, particularly Imipenem, to enter the periplasmic spaces. It becomes the basis of Carbapenem resistance If this porin's expression is diminished, or if it undergoes mutational loss (Farra, Islam, Strålfors, Sörberg, & Wretlind, 2008; Poole, 2011), as well as the overexpression of AmpC and efflux pumps (Farra et al., 2008; Poole, 2011; Xavier, Picão, Girardello, Fehlberg, & Gales, 2010).

3.11. Resistance to Fluoroquinolones

Resistance to high-level Fluoroquinolones is caused by target site alteration. Pumps that regulate efflux are also crucial (Drlica et al., 2009; Hooper & Jacoby, 2015).

3.11.1. DNA gyrase and topoisomerase IV mutations

There are two subunits in topoisomerase and gyrase. DNA gyrase (gyrA & gyrB) is the primary target of Fluoroquinolones in *P. aeruginosa*. The mutation occurs more frequently in DNA gyrase than in topoisomerase IV (*parC & parE*). In highly resistant isolates, several mutations in *parC* and *gyrA* have been found, whereas alterations in other subunits have been reported less often (J. K. Lee, Lee, Park, & Kim, 2005; Rejiba, Aubry, Petitfrere, Jarlier, & Cambau, 2008).

3.11.2. Role of efflux pumps

When there is a mutation in the repressor genes of the efflux pumps MexCD-Oprl, MexAB-OprM, MexXY-OprM, and MexEF OprN fluoroquinolone resistance occurs

(Poole, 2001, 2011).

3.12. Resistance of Aminoglycosides

Endogenous efflux pumps depression, transportable Amino-glycoside modifying enzymes and RNA methylases are all factors that contribute to acquired resistance to Amino-glycosides (Poole, 2005, 2011; Ramirez & Tolmasky, 2010).

3.12.2 Aminoglycoside–modifying enzymes

Aminoglycosides are modified and inactivated by Aminoglycoside-modifying enzymes through three main mechanisms: 1) Acetylation, that is done through Amino-glycoside Acetyl-transferases 2) Adenylation, that is done through nucleotidyly-transferases 3) Phosphorylation, that is done through Phosphoryl-transferases (Ramirez & Tolmasky, 2010). Integrons contain genes that code for amino-glycoside modifying enzymes, as well as genes involved in transferrable resistance to other antibiotic classes. So aminoglycoside modifying enzymes become the most important element in the development of MDR in *P. aeruginosa* and other bacteria (Poole, 2011; Ramirez & Tolmasky, 2010).

3.12.3 Efflux systems

Patients with cystic fibrosis have resistance to aminoglycosides in case of *P. aeruginosa*. Without regard to AMEs, different studies have found that overexpression of the efflux pump, MexXY-OprM, is the cause of this form of resistance (Henrichfreise, Wiegand, Pfister, & Wiedemann, 2007; Islam et al., 2009).

MATERIAL AND METHODS

4 Sampling

The Armed Forces Institute (AFIP) of Pathology provided 175 isolates of *P. aeruginosa* from various patients for this study. Standard microbiological methods were employed for the identification of P. aeruginosa strains in the samples.

4.1 Study duration

The study was conducted at the Quaid-i-Azam University Islamabad's Laboratory of Genomics and Molecular Epidemiology, Department of Microbiology. The total duration of the study was almost six months.

4.2 Sources of samples

The researchers took samples from a variety of pathological specimens such as pus, ear swab, blood, urine, CVP tips, sputum, body fluids, Endo-tracheal secretions, catheter tip, chest tube tip , tissue swabs and body fluids.

4.3 Culturing

Isolates were sub-cultured on tryptic Soy Agar plates (TSA) (OXOID, UK), after being refreshed in tryptic Soy broth.

4.4 Morphological identification

Colony appearance and odor were detected during morphological detection of *P. aeruginosa* pigmentation. It's a motile rod-shaped bacterium with polar flagella. Its odor is like tortilla or grape. The size of the colonies was big and they looked opaque. They had mucoid texture and the margins were irregular. Some of the colonies fluoresced blue, green/yellow, and green due to the presence of fluorescent pigments like pyocyanin and pyoverdin,

4.4.1 Gram staining

Principle

Gram staining is based on the capacity of the bacterial cell to maintain the crystal violet dye after being exposed to solvent. Gram-negative bacteria have a lot of lipid in their cell walls, whereas Gram-positive bacteria have a lot of peptidoglycan. All bacteria take up the crystal violet dye at first, but ethanol dissolves the lipid layer of gram-negative bacteria, causing them to lose their primary stain, while ethanol causes the dehydration of the cell wall of gram-positive bacteria, closing pores and blocking CV-iodine complex diffusion, resulting in stained bacteria (Tripathi et al., 2020).

Procedure

4.4.2 Smear preparation

The smear was prepared on newly streaked plates after a 24-hour incubation period. A tiny drop of distilled water was put on the glass slide, then an isolated colony was picked by the help of the sterile inoculation loop and spread uniformly across the slide to form a thin layer. The smear was then heat-fixed using a Bunsen burner carefully because overheating can destroy cell shape.

4.4.3 Staining

The heat-fixed smear was carefully saturated with the crystal violet which is a primary dye and soaked like this for 1 minute. The slide was then cleansed by carefully washing it under running water and turning it slightly. The smear was then treated with Gram's iodine (Mordant stain) and left like this for 30 seconds. Then slide was tilted a bit and washed under running water. Decolonization was accomplished by pouring 95 percent ethanol (demoralizer) in such a way that it dripped down the slide. The slide was washed with tap water after approximately 3-4 seconds. Safranin that is a counter-stain was applied to the smear and left for around 1 minute. The slide was rinsed with running water then air-dried at the end. Now it is ready for the examination.

4.4.4 Microscopy

After that, the slide was examined using a compound microscope at a magnification of 100x with immersion oil to improve the visibility.

4.5 Biochemical identification

The identification of *P. aeruginosa* strains was done by biochemical assays. Oxidase, catalase, citrate, and indole were among the assays performed.

4.5.1 Oxidase test

Principle

This test detects the cytochrome c (indophenol oxidase) producing bacteria. In the presence of this enzyme, the reagent tetramethyl-p-phenylenediamine dihydrochloride (TMPD) is oxidized to indophenol, a purple-colored compound.

Procedure

Prior to the test fresh oxidase reagent was produced. In 10 mL distilled water, 0.1 gram reagent was added. A piece of clean filter paper was put into a sterile Petri dish. The

filter paper was applied with a drop of 1 percent oxidase reagent. A colony was picked using a sterilized inoculating loop and spread on the filter paper. A color shift was seen following 10 to 30 seconds. The color turned to the rich purple-blue shade, indicating the positive oxidase test. No color shift showed negative oxidase test.

4.5.2 Catalase test

Principle

An enzyme produced by those bacteria which thrives in the presence of oxygen in order to inactivate dangerous oxygen metabolites like H_2O_2 is known as catalase enzyme. It converts hydrogen peroxide into its constituent elements i.e oxygen and water, making it non-toxic.

Procedure

Catalase test was done on a sterilized glass slide. 3 percent H_2O_2 was dropped on the slide. With the help of a sterile toot pick, pure colonies were collected from a freshly prepared sample plate and blended with the drop of H_2O_2 on the slide. Positive catalase test was indicated by the presence of bubbles whereas negative reaction was shown by the absence of bubbles.

4.5.3 Citrate utilization test

Principle

This test identify microorganisms which consume citrate as their source of carbon and energy. These bacteria feed on citrate and produce ammonium salts from ammonia, which results in strong alkaline pH of the medium. The bromothymol blue (indicator) changes from green to blue color (pH above 7.6) as a result of the pH change.

Procedure

Initially slants of Simmons citrate agar medium (OXOID, UK) were made by pouring the media in the tubes. After slant preparation, sterile inoculated loop was used to pick a colony and streaked gently on the slants and incubated for 24 hours at 37°C.If the slant's color turn blue from green then the result is positive however if the forest green color retained and no shift in the color is observed then this is the indication of negative result.

4.5.4 Indole Test

Principle

This test is performed for the identification tryptophanase, an enzyme that converts

tryptophanase into ammonia, Indole, and pyruvic acid in bacteria. Kovac's reagent is used for the verification of Indole formation.

Procedure

Peptone water (OXOID, UK) was made as per the specified composition, poured into the test tubes, and then autoclaved. The tubes were inoculated with the test organisms and then incubated at 37°C for 24 hours. Only the control tube was not inoculated. Following the incubation period, 1 ml of the Kovac's reagent was put into each tube, including the control. Tubes were then mixed thoroughly and set aside for about 1-2 minutes. A positive test result was shown by the creation of a red ring. When there was no ring development, the test was considered negative.

4.5.5 Urease test

Principle:

This test identifies the bacteria which has the ability to produce urease enzyme which hydrolyzes urea.

Procedure

Initially slants of Urea agar (HIMEDIA INDIA) were made by pouring the media in the tubes. After slant preparation, sterile inoculated loop was used to pick freshly prepared colonies from the plates and streaked gently on the slants and incubated for 24 hours at 37°C.If the slant's color turns pink from yellow color due then the result is positive and ammonia is produced however if the yellow color retained and no shift in the color is observed then this is the indication of negative result.

4.5.6 Triple Sugar Iron test

Principle

Russell's double sugar agar was improved by Kohn and Krunweide in 1917 by adding sucrose as a third sugar. With the addition of sucrose, coliform bacteria that can ferment glucose more quickly than lactose might be identified. As a differential media, TSI medium is used to detect bacteria's capability to ferment carbohydrates and generate hydrogen sulphide gas. Carbohydrate. Gas is produced as a result of metabolism. The TSI medium is made up of three distinct carbohydrates: sucrose (1%), glucose (0.1%), and lactose (1%), and it distinguishes bacteria based on their capacity to ferment glucose, sucrose, and lactose, as well as produce hydrogen sulphide gas. TSI is the most often used medium for identifying gram-negative bacteria, particularly

Enterobacteriaceae. This medium contains phenol red, as a pH indicator. Slants of TSI agar are aerobic, while butt is anaerobic. Due to fermentation of carbohydrate the pH declines as a result the medium turns into yellow from reddish orange shade. While the medium turns a rich red hue when peptone is alkalized. In addition, the presence of black insoluble precipitates occurs which shows that hydrogen sulphide gas is being produced.

Procedure

Initially autoclaved TSI agar medium was used to make the slants in the test tubes. Fresh colonies were picked from the streaked agar plates with the help of the sterile inoculating needle and stabbed in the butt up to 3-5mm from the bottom and the needle withdrawn. The entire surface of the slant was also streaked later on. Now enclose the tubes with autoclaved cotton plugs and put into the incubator for about 18-24 hours at 37°C.This was done for all the isolates of *P. aeruginosa*. After the incubation period of 24hours the test result results were observed and following interpretations were made:

Result interpretations

Fermentation of Sugar

Table 4.1 Elucidation of TSI test

Observations	Slant	Butt
Glucose Fermentation	Alkaline(red)	Acid (yellow)
Lactose and Sucrose Fermentation	Alkaline(Yellow)	Acid (yellow)
None of the Sugars Fermented	Alkaline (red)	Alkaline (red)

4.6 Glycerol stock preservation

After all the P. aeruginosa isolates were identified and verified, a glycerol stock of all isolates was made and stored at -20°C.

Procedure

To preserve the stock add 500µl of Tryptic Soy Broth (TSB) in all of the sterilized Eppendorf tubes. Then inoculated each tube with the full of loop of colonies and kept in a shaker incubator overnight at 37°C. Each labelled tube was filled with 500l of 40%

glycerol the next day and kept at -20°C.

4.7 Disk diffusion assay / Kirby-Bauer test (Initial screening test)

Principle

This test identifies the sensitivity or resistance of pathogenic aerobic and facultative anaerobic bacteria to a variety of antimicrobial drugs, which aids clinicians in selecting on the most appropriate treatment options for the patients. Antimicrobial filter paper discs are used to culture the pathogens on MHA. The presence or lack of growth surrounding the discs is used to test a compound's capacity to inhibit an organism (Hudzicki, 2009).

Procedure

4.7.1 Preparation of inoculum and plate Swabbing

Each sample was evaluated by removing a single colony from each plate and mixing it in 1ml of Muller-Hinton Broth (MHB). The 0.5 McFarland standard (as a reference) was used to make a bacterial culture suspension with precise turbidity. A sterile cotton swab was dipped in broth culture and then swabbed across the surface of MHA plates (3-way streaking).

4.7.2 Antibiotic discs placement

Antibiotic discs were put on specific positions on each plate with the help of sterile forceps and then the plates were kept in the incubator for 24 hours at 37°C.

Note

The pattern of antibiotic sensitivity of *P. aeruginosa* was previously determined in the Armed Force Institute of Pathology.

4.8 Detection of Quorum Sensing phenomenon

4.8.1 Biofilm formation assay

4.8.1.1 Principle

The biofilm is the aggregation of sessile microorganisms encased inside the autogenous EPS matrix. Biofilm development assay was conducted according to the Microtitre plate method. Optical density of the stained biofilm cells within the Microtitre plate's wells are measured through this quantitative assay.

4.8.1.2 Biofilm formation protocol

In 1ml of MHB, a full loop of overnight grown test organism was added. The McFarland standard was used to adjust the turbidity. A sterile, flat-bottomed, 96-well polystyrene Microtitre plates was used for this assay in which 200µl of the bacterial suspension was poured. This assay was performed thrice for all of the isolates. Only three wells were left un-inoculated, which were used as media controls. Now the plates were put into the incubator for 24hours at 37°C.

4.8.1.3 Biofilm washing

Following the incubation process, all the constituents were eliminated by the help of the pipette. 100µl of the autoclaved phosphate saline buffer solution (PBS) was used to wash the wells thrice. Washing of the wells helps to eliminate detached bacterial cells as well as media constituents, and pure biofilm is left.

4.8.1.4 Biofilm fixation

Following the washing process, few bacteria attached in the wells was removed by the addition of 200µl of 70 percent methanol, which was discarded after only 15 minutes. Later the plate was left to air-dry.

4.8.1.5 Biofilm staining

After fixation, 200µl of 2% crystal violet dye (CV) was added to each well for staining of biofilm cells. CV control was also added in triplicate. After 5 minutes CV was discarded from each well. The wells were then washed 3 times with 100µl of autoclaved PBS solution for the removal of the excess stain. The plate was then air-dried.

4.8.1.6 Biofilm re-solubilization

200µL of absolute ethanol was added in all wells to resolubilized the biofilm cells.

4.8.1.7 Biofilm quantification

Biofilm was quantified using an ELISA auto-reader to evaluate its optical density (OD) at 540nm.

4.8.1.8 Result interpretation

On the basis of the optical density (OD) generated by the bacterial cells, isolates were classified in these groups: Non-biofilm formers, weak biofilm formers, moderate biofilm formers and strong biofilm formers. The cutoff optical density (ODc) was three standard deviations (SDs) over the crystal violets mean optical density.

Table 4.2 Characterization of Biofilm Producers.

Biofilm Pattern	Optical Density (OD)
Non-Biofilm Formers	**OD ≤ OD_c**
Weak Biofilm Formers	**OD </ = 2×OD_c**
Moderate Biofilm Formers	**2×OD_c ≤ 4 × OD_c**
Strong Biofilm Formers	**OD ≥ 4 × OD_c**

4.8.2 Pyocyanin production detection

- First 5 ml of autoclaved LB media was put into the tubes.
- Then 20-25µl of colonies from the preserved glycerol stocks was added into each tube.
- After refreshing the isolates they were placed into the shaker incubator for 24 hours.
- The refreshed culture was then centrifuged at 6000 ×g for around 10 minutes at room temperature
- Then the supernatant was separated and filtered through the 0.2µm filter paper.
- Now pour 3ml of the chloroform solution into the filtered supernatant.
- Then these solutions were centrifuged at 1200 ×g for about 3-5min at room temperature.
- Two phases will be produced in the tubes, under chloroform phase was separated.
- Now 2.5ml of under chloroform phase was mixed with 1ml 0.2M HCL.
- 2000µl samples were transferred to the microtitre plate and absorbance was measured at 530nm in the spectrophotometre (Carlsson, Shukla, Petersson, Segelmark, & Hellmark, 2011).

4.8.3. Protease activity test

- First 1.5 ml of TSB was added into the Eppendorf tubes.
- Then 20-25µl of colonies from the preserved glycerol stocks was added into each tube.
- After refreshing the isolates they were placed into the shaker incubator for 24 hours.

- Prepare skim milk agar plate by adding 2% autoclaved skim milk in autoclaved LB agar and pour into the plates and left to cool down and settle.
- Now pick the colonies from refreshed culture and the agar plates were inoculated with it.
- Then the plates were incubated for about 16-18hours at 37°C.
- After incubation observe the plates for results. A clear zone was formed around the colonies was the indication of positive proteolytic activity (Dong, Zhang, Soo, Greenberg, & Zhang, 2005).

4.9 Molecular level detection of quorum sensing genes

- **DNA extraction**

The DNA template for PCR was prepared using three different ways.

4.9.1 Boiling method

Apparatus

Eppendorf tubes, toothpicks, a pipette, tips, a centrifuge, and a water bath are all needed.

Procedure

- Loop-full of colonies were collected with the help of the sterilized toothpicks from freshly prepared *P. aeruginosa* cultured plate.
- After autoclaving the Eppendorf tubes, they were filled with 1 mL of PCR water and the colonies were properly mixed in the PCR water.
- These tubes were put in a 95°C dry bath, for approximately 10-15 minutes.
- Then for about 15 minutes, centrifugation was carried out at 10,000rpm.
- Each tube's supernatant was transferred to already labelled Eppendorf tubes and kept at -20°C.

4.9.2 Plasmid extraction by alkaline lysis method

- First 1.5 ml of TSB was added into the Eppendorf tubes.
- Then 20-25µl of colonies from the preserved glycerol stocks was added into each tube.
- After refreshing the isolates they were placed into the shaker incubator for 24 hours.

- The refreshed culture was then centrifuged at 14000rpm for around 1 minute on the next day and bacterial pellet was produced which was visible at the bottom of the tube.
- Now three solutions were added step wise.
- After discarding the supernatant very carefully, 200µl of Solution I (10mM EDTA, 50mM Tris-HCl pH 8.0, 100ug/ml RNase A) was added to re-suspend the pellet.
- Following the addition of Solution 1, about 200µl of Solution II (1% SDS, 20mM NaOH) was poured into each of the tube and inverted for the proper mixing of the pellet and the solutions.
- Upon addition of Solution III (3M potassium acetate, pH 3.5) to each of the Eppendorf, the tubes turned cloudy or milky due the white colored precipitate formation.
- After addition of all three solutions centrifugation of the tubes was carried out for 10 minutes at 14000 rpm.
- New Eppendorf tubes were labelled in which supernatant was collected and pellet containing tubes were discarded.
- The supernatant was added up with 900µl of 100 percent ethanol and both of them were properly mixed together, followed by 20 minutes of centrifugation at 14000rpm.
- After this step supernatant was removed and DNA pellet was saved for next step.
- Now 100µl of 70 percent ice-cold ethanol was poured to the DNA pellet and put into the centrifuge for about 30 seconds.
- Again supernatant was removed and left to air-dry for almost 10-30 minutes.
- At the end 50µl of TE buffer (Tris-EDTA buffer) was put on the pellets to re-suspend it and kept at -20°C.
- Plasmids of the *P. aeruginosa* was extracted and stored for several molecular analysis (Sasagawa, 2018).

4.9.3 Phenol-chloroform method

- Refresh the culture in 1.5ml of TSB.
- Now mix them well through vortexing.
- This is followed by centrifugation at 12000rpm for 5min.
- Supernatant was removed and pellet was re-suspended in 450µl of TE buffer, 45µl of 10% SDS and 5µl of Proteinase K (20mg/ml) and gently mixed through vortexing.
- The samples were incubated at 37°C for 1 hour.
- After incubation, 500µl of phenol-chloroform (1:1) was added and mixed properly.
- Then the centrifugation was carried out at 10,000 rpm for 20 minutes.
- Label new Eppendorf tubes and transfer the upper aqueous phase into them.
- 500µl phenol-chloroform was poured into them again and centrifuged at 10,000 rpm for 5minutes.
- Upper aqueous phase was transferred into new Eppendorf tubes through the micropipette tube.
- Now 50µl of 3M sodium acetate and 300µl of Isopropanol / 600µl of ethanol was added and mixed well to the point that DNA precipitation starts and the tubes were incubated for about 15-30min at -20°C.
- Then Centrifugation at 10,000 rpm was carried out for 5 minutes. Now the supernatant was removed.
- The tube were then washed with 1ml of 70% ethanol.
- Then the tubes were centrifuged for 1-2 minutes at 10,000 rpm
- Ethanol was then removed via micropipette.
- The tubes were then left to air dried and made sure no moisture was left in the tubes.
- 100-200 µl of TE buffer was poured into the tubes to suspend the DNA.
- 1-5µl of RNAse enzyme was added to remove all kinds of impurities and pure DNA was extracted and stored at -20°C.

4.9.4 CTAB (Hexadecyl trimethyl-ammonium bromide) method

- First 1ml of TSB was added into the Eppendorf tubes.
- Then 20-25µl of colonies from the preserved glycerol stocks was added into each tube.
- After refreshing the isolates they were placed into the shaker incubator for 24 hours.
- The refreshed culture was then centrifuged at 3000rpm on the next day and the bacterial pellet was produced which was visible at the bottom of the tube.
- Supernatant was discarded and the pellet was used for the next step.
- After this, 20µl NaCl (5M) and 80µl of TE buffer were put on the pellet, which was then vortexed to resuspend the pellet.
- Following this, 10µL of CTAB was poured into each tube and put into the incubator for 20 minutes at the temperature of 60°C.
- After 1 hour, the tubes were taken out of the incubator and 100µl of chloroform was poured and vortexing was done in order to mix all the constituents thoroughly.
- Afterwards the tubes were put at low temperature (on ice) for at least 20 minutes and centrifuged for 10 minutes at 1000rmp in the microfuge.
- Now new Eppendorf tubes were labelled and supernatant was poured into them and pellet containing tubes were discarded.
- In the next step 100µl of phenol-chloroform mixture was poured into the supernatant and then this mixture was thoroughly mixed through vortexing which produced milky solution, following this 5minutes of centrifugation was done at high speed.
- New tubes were labelled and supernatant was added to them and it was then mixed with 100µl of chloroform and set to vortexing, followed by 5 minutes of centrifugation.
- Now label the new tubes for the last time and supernatant was poured into them, in which 200µl of the ice-cold ethanol and 10µl of Sodium acetate were poured for the precipitation of the DNA, ethanol was drained away, but the pellet was saved.

- Now 100µl of 70 percent ethanol was added into the pellet to re-suspend it, followed by centrifugation in order to get the pellet containing DNA.
- The lids of the tubes were kept open to air-dry the ethanol. At the end 20µl of TE buffer (Tris-EDTA buffer) was put on the pellets to re-suspend it and kept at -20°C. (William, Feil, & Copeland, 2012).

4.10.Evaluation and quantification of DNA

Nucleic acid can be quantified by many different ways. Spectrophotometric method of quantification is employed for the pure solution and ultra-violet (UV)-induced emission of fluorescence from intercalated ethidium bromide should be applied when DNA is in small quantity and it might get contaminated. We employed both of these two methods:

4.10 Ethidium bromide fluorescence quantification

Principle

This is based on the intercalation of ethidium bromide between the nucleotides which produces fluorescence when exposed to UV radiation.

Procedure

For this purpose 1% agarose (Life technologies) gel was prepared in 1X TBE buffer. To it 2-3 ul ethidium bromide solution was added. After gel was solidified, 2-3µl of extracted DNA was mixed with 2ul of 6X loading dye (Thermo scientific) and was loaded into wells, next to a DNA marker (Thermo scientific I kb ladder). The gel was then run for 35-40 minutes at voltage of 100 Volts in IX TBE buffer (BIO RAD gel electrophoresis tank). After the adjusted time period, gel was observed under UV trans-illuminator to visualize DNA bands. Bands were photographed by BIO RAD Gel Documentation system.

4.11 Spectrophotometric quantification

Principle:

It is based on the Lambert Beer equation and on the concept of nucleic acid adsorption at 260nm.

Procedure

Materials and methods

To quantify the DNA first of all the several DNA dilutions were prepared by suspending DNA in 1X TE buffer or distilled water.

To calculate the concentration of DNA following formula was applied:

ds DNA concentration µg/ml = abs260 × 50µg/ml

When the diluted samples were used following formula was applied:

ds DNA concentration µg/ml = abs 260 ×50µg/ml × Dilution factor

4.13 Polymerase chain reaction (PCR)

The PCR method was employed to amplify target genes.

4.13.1 Primer stock dilution

A working solution was generated from the lyophilized main stock solution. Initially the Eppendorf tubes were autoclaved to avoid any kind of contaminations and then labelled with the name of the gene. A working solution with 10pmmol/µl total volume was prepared by adding 40µl of PCR water and 10µl of the original stock of the primers into the Eppendorf tubes.

Table 4.3 Primers used in PCR

Target Genes	Primers	Oligosaccharide Sequence	Annealing Temperature (°C)	Product Size (bp)
las I	Forward	5′ CGTGCTCAAGTGTTCAAGG 3′	52	295
	Reverse	5′ TACAGTCGGAAAAGCCCAG 3′		
las R	Forward	5′ AAGTGGAAAATTGGAGTGGAG 3′	55	130
	Reverse	5′ GTAGTTGCCGACGACGATGAAG 3′		

4.13.2 Reaction mixture for PCR

The contents of the reaction mixture are mentioned in the given table:

Table 4.4 Reaction Mixture for 10 Samples of PCR

Reaction Mixture	Volume (10µl)
PCR Water	63
Taq Buffer	10
Magnesium Chloride	6
dNTPs	2
Forward Primer	4
Reverse Primer	4
Taq Polymerase	1
DNA	1.5

4.13.3 Screening of Quorum Sensing genes through PCR

- **Principle:**

PCR is accomplished in different phases on the basis of temperature fluctuations of heating and cooling thermo-cycling reactions: Denaturation, annealing, and extension. The temperature is raised during the first phase of denaturation, which transforms double-stranded DNA to single-stranded DNA by breaking the hydrogen bonds between the two strands. In the second phase of annealing, the primers are annealed to the corresponding sequence of DNA. The final step of extension is the beginning of DNA synthesis by addition of nucleotides to the elongating strand of DNA, these steps kept on repeating for further 25-40 cycles.

- **Protocol:**

PCR (Multigene Optimax) was utilized for the amplification of the quorum sensing genes Las I and Las R, then the reaction mixture of total volume of 10µl per reaction was made with the help of the pipette. Genes were amplified in each cycle.

Table 4.5 Amplification of Genes by Various Cycles of PCR

Sr. No.	Steps		Temperature (°C)	Time	No of Cycles
1	Initial Denaturation		94	5min	1
2	Denaturation		94	30min	35
3	Annealing	*las I*	55	30min	35
		las R	58		
4	Initial Extension		72	30sec	35
5	Final Extension		72	5min	1

4.13.4 Agarose gel electrophoresis

- **Principle**

A standard laboratory procedure for the segregation of the DNA bands on the basis of their size. It aids in the visualization of discrete bands of DNA and helps in the purification of specific genes.

- **Agarose gel preparation**

To make a 1 percent Agarose gel, 0.4 g of Agarose (Invitrogen USA) was mixed with 4ml of 10x TBE buffer and the volume was increased to 40ml by adding distilled water, then put on a hot plate for heating until the solution became clear. When the temperature has reached a certain level, 4ul of ethidium bromide was put into the gel and thoroughly mixed in the solution .For 2% of Agarose gel, 0.6g agarose was used.

- **Gel casting**

Now combs were set into the tray and the mixture was poured into the tray before it gets solidifies. Now it was set like this and wait for about 20-25 minutes for the gel to get solidified. When the gel is properly hardened, combs were carefully removed and put in a gel tank (Clever UK) with 1X TBE buffer.

- **Sample loading**

Now the samples were loaded on the gel. For this a 6X loading dye (Thermo scientific) was carefully mixed the PCR products and picked with the pipette and poured into the wells carefully. The last well was filled with 50bp ladder as standard to detect the exact size of your gene and the electrophoresis apparatus was switched on. Now adjust the current, voltage and time accordingly. Current was set 400mA, Voltage at 70 V and the timer at 40 minutes. After that the gel was carefully taken out to visualize the bands.

- **Gel visualization**

UV Tran illuminator (BIOTOP) was used to illuminate the bands in the gel. Precautions must be taken while dealing with UV light such as gloves and eye protection glasses.

RESULTS

In this study we use 175 isolates of *Pseudomonas aeruginosa*.Each sample has undergone through multiple tests like morpho-cultural, biochemical and molecular techniques.

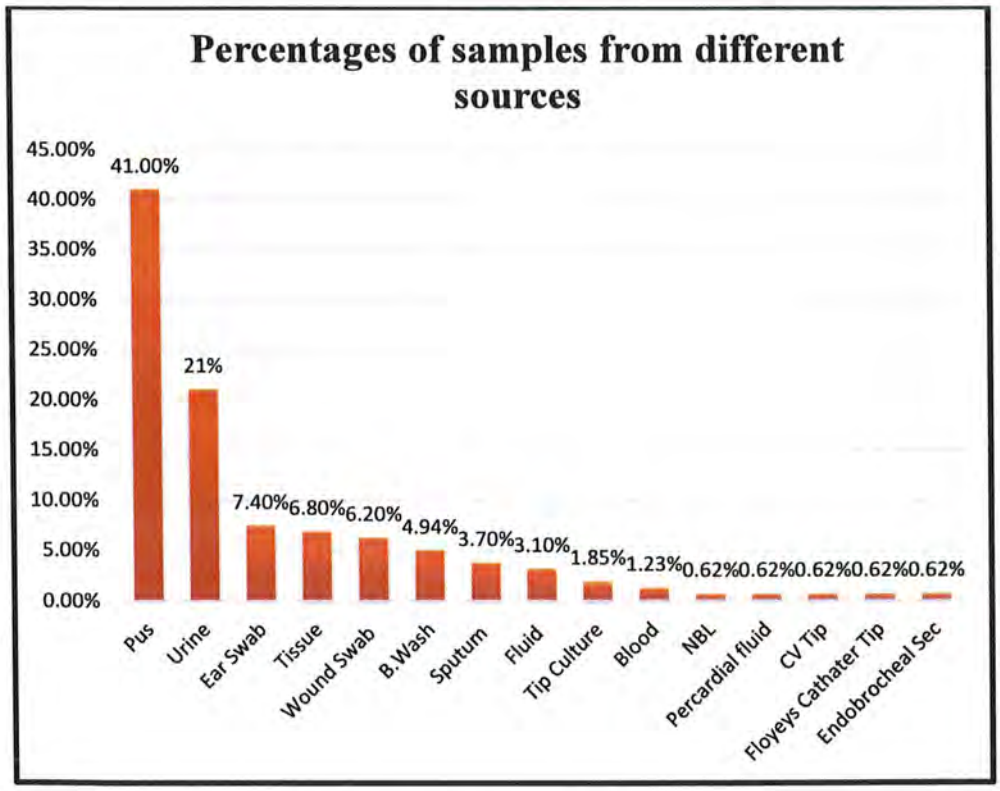


Figure 5.1 Bar graph representing the distribution of samples sources of *P.aeruginosa*

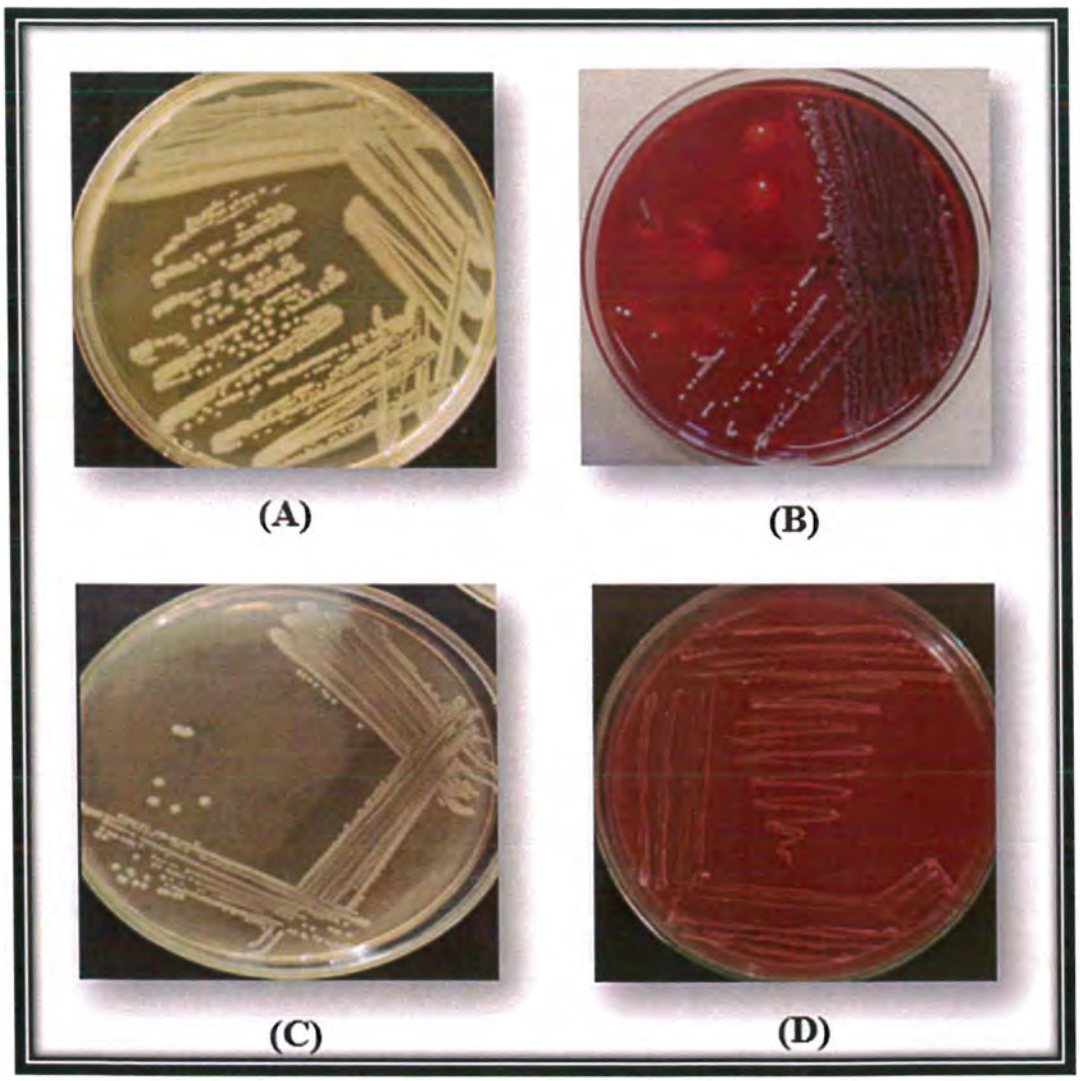
5.1 Morpho-cultural identification

The morphological identification was done on the basis of following two techniques:

1. Colony morphology
2. Gram-staining.

5.1.1 Colony morphology

Each isolate was streaked on several types of media like Nutrient Agar, Trypic Soy Agar and MaCconkey agar and put into the incubator for about 24hours at 37°C.*Pseudomonas aeruginosa* produced slimy,pigmented,and rough and irregular colonies.



igure 5.2 Growth Patterns of *Pseudomonas aeruginosa* (A) demonstrates growth on the Nutrient Agar, (B) demonstrates growth on the Blood agar, (C) demonstrates growth on the Trypic Soy Agar (TSA), (D) demonstrates growth on the MaCconkey agar

5.1.3 Gram staining

Gram staining was done on all of the *P.aeruginosa* isolates. Under the microscope, each isolate showed as pink rods, demonstrating that they are Gram-Negative rods (GNR).

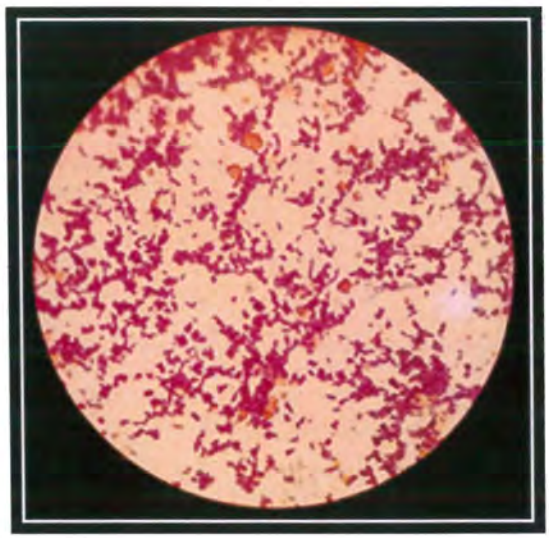


Figure 5.3 Demonstration of Gram-Negative Pink Rods of *P.aeruginosa*

5.2 Biochemical identification

5.2.1 Oxidase test

Each isolate of *P.aeruginosa* was tested for oxidase and all of them demostrated oxidase positive result.

Figure 5.4 Demonstration Oxidase test - Purple color demostrates the positive outcome

5.2.2 Catalase test

Each isolate of P.aeruginosa was tested for catalase enzyme and all of them demostrated catalase positive result.

Figure 5.5 Demonstration of Catalase test - Bubble production demonstrates the positive outcome

5.2.3 Citrate test

Each isolate of *P.aeruginosa* was tested for citrate and all of them demostrated citrate positive result.

Figure 5.6 Demonstration of Citrate Test- Prussian blue color shows the positive outcome

5.2.4 Indole test

Each isolate of *P.aeruginosa* was tested for indole and all of them demostrated indole negative result.

Figure 5.7 Indole test – Ring formation demostrates positive outcome

5.2.5 Urease test

Each isolate of *P.aeruginosa* was tested for urease enzyme and all of them demostrated urease positive result.

Figure 5.8 Urease test - Pink color demostrates positive outcome

5.2.6 TSI test

Each isolate of *P.aeruginosa* was tested for glucose fermentation,lactose fermentatio and sucrose fermentation and all of them demostrated positive result.

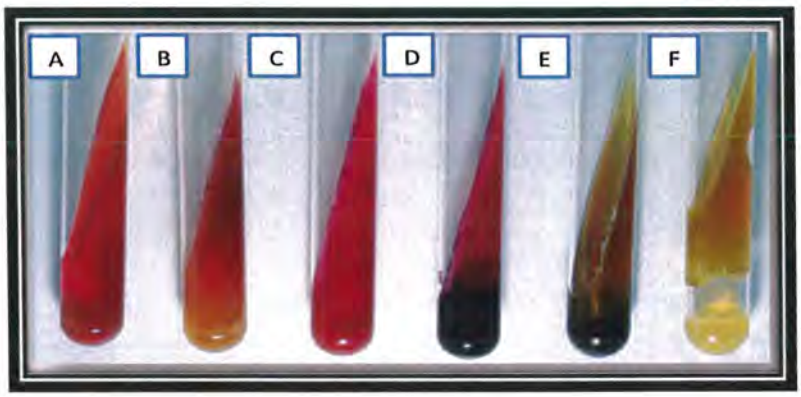


Figure 5.9 Demonstration of TSI test -Red color of the slant and yellow color of the butt represents glucose fermentation. Yellow slant and yellow butt represents lactose and sucrose fermentation. Red slant and Red butt remains red this shows negative results (no fermentation).(A)Demonstrates media control (B) Demonstrates no fermentation in the slant and lactose and sucrose fermentation in the butt (C)Demonstrates no fermentation in the slant and no fermentation in the butt (D)Demonstrates no fermentation in the slant and presence of ferrous sulphide due H_2S production in the butt (E)Demonstrates lactose and sucrose fermentation in the slant and presence of ferrous sulphide due H_2S production in the butt (F)Demonstrates lactose and sucrose fermentation in the slant and in the butt both.

Table 5.1 Biochemical Identification of *Pseudomonas aeruginosa*

Biochemical Test	Test Outcome
Oxidase Test	+
Catalase Test	+
Citrate Test	+
Urease Test	+
Indole Test	-
Triple Sugar Iron (TSI) Test	+

5.3 Antimicrobial susceptibility testing:

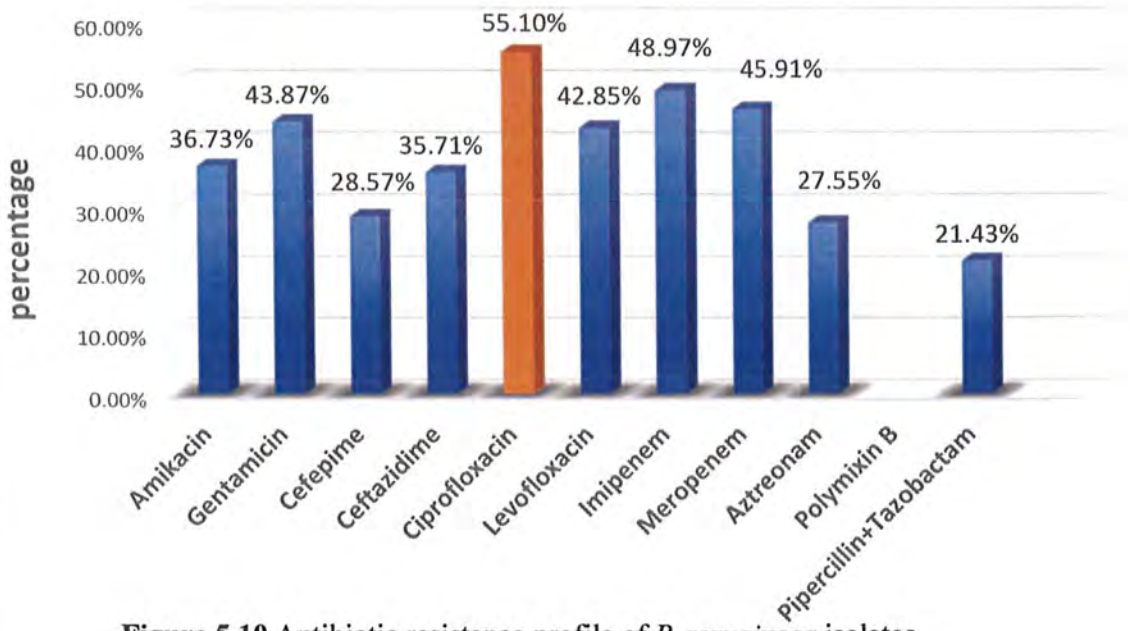


Figure 5.10 Antibiotic resistance profile of *P.aeruginosa* isolates

5.4 Phenotypic identification of quorum sensing genes

5.4.1 Biofilm assay

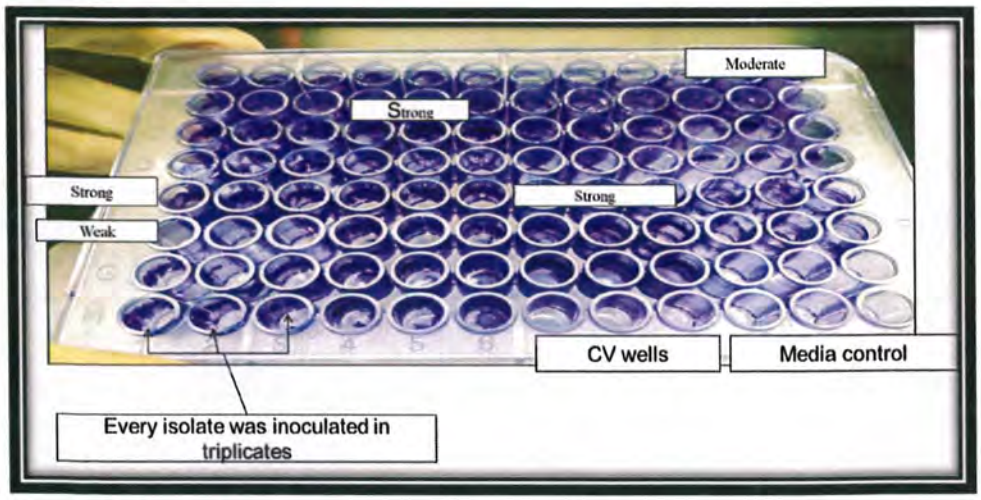


Figure 5.11 Quantification of stained biofilm cells to determine strong, moderate and weak biofim formers.

Characterization of *P.aeruginosa* isolates based on biofilm forming ability

The biofilm ability of all 175 *P.aeruginosa* isolates was tested. Biofim formers numbered 167 whereas non-biofim formers numbered 8.

Table 5.2 Categorization of *P.aeruginosa* on the basis of biofim forming ability

Biofilm Characterization	Percentage
Biofilm formers	95.43%
Strong biofilm	8.57%
Moderate biofilm formers	60.57%
Weak biofilm formers	26.29%
Non-biofim formers	4.57%

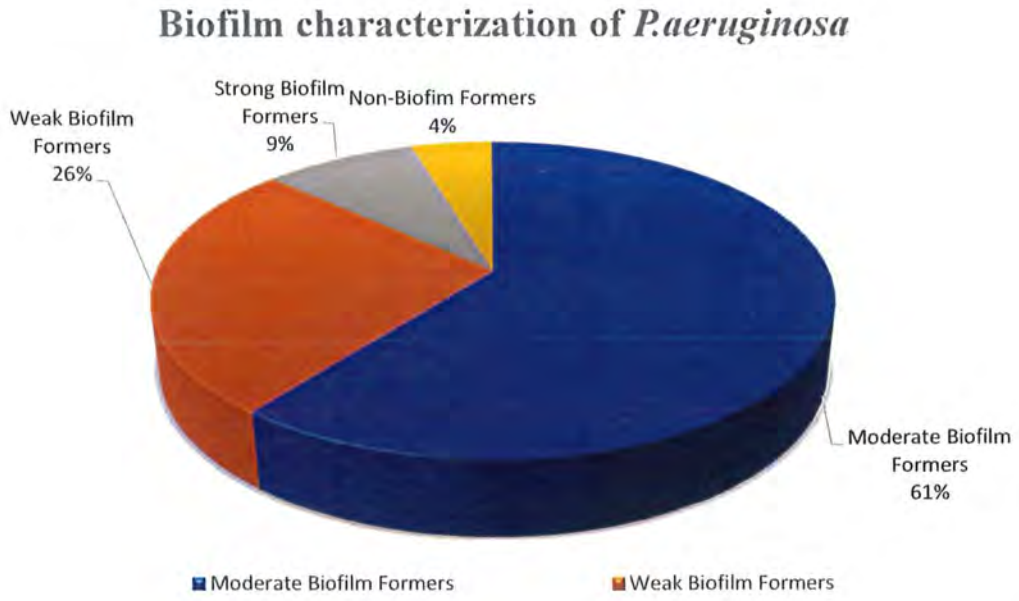


Figure 5.12 Pie-chart demonstrating the data of Strong, Moderate ,Weak and non-biofilm formers.

5.4.2 Pyocyanin assay:

Figure 5.13 Isolates of *P.aeruginosa* shows green fluorescence which is due to the production of green pigment called pyocyanin.

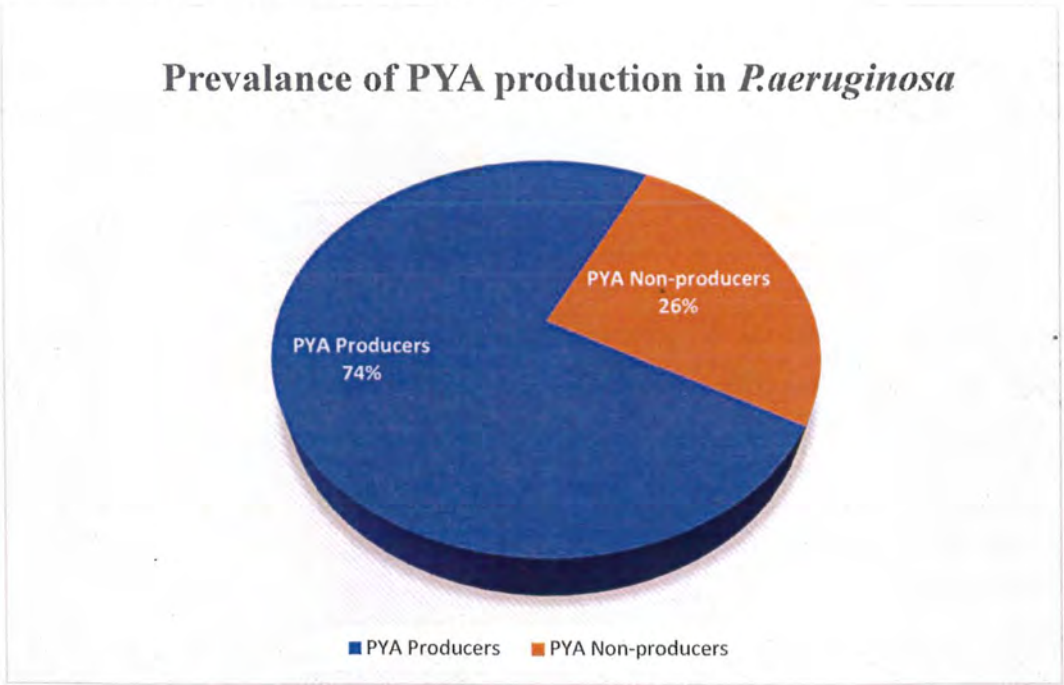


Figure 5.14 Pie-chart demonstrating the data of PYA producers and PYA non producers

5.4.3 Protease detection assay:

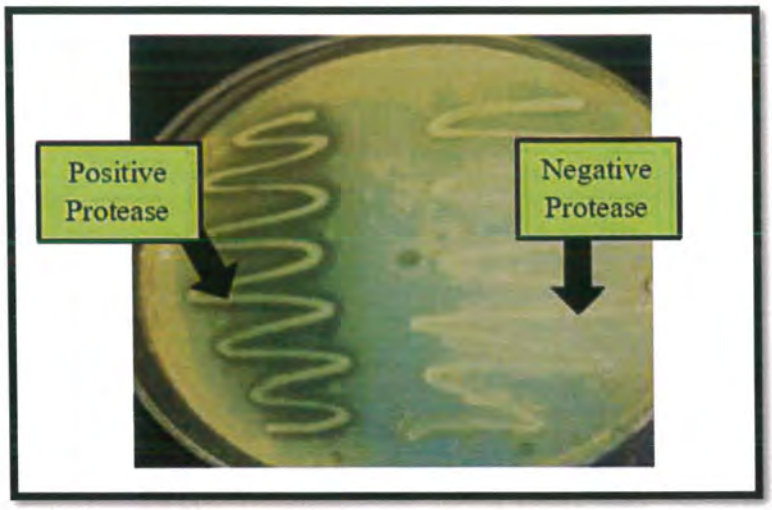


Figure 5.15 A clear zone formed around the colonies is the indication of positive proteolytic activity

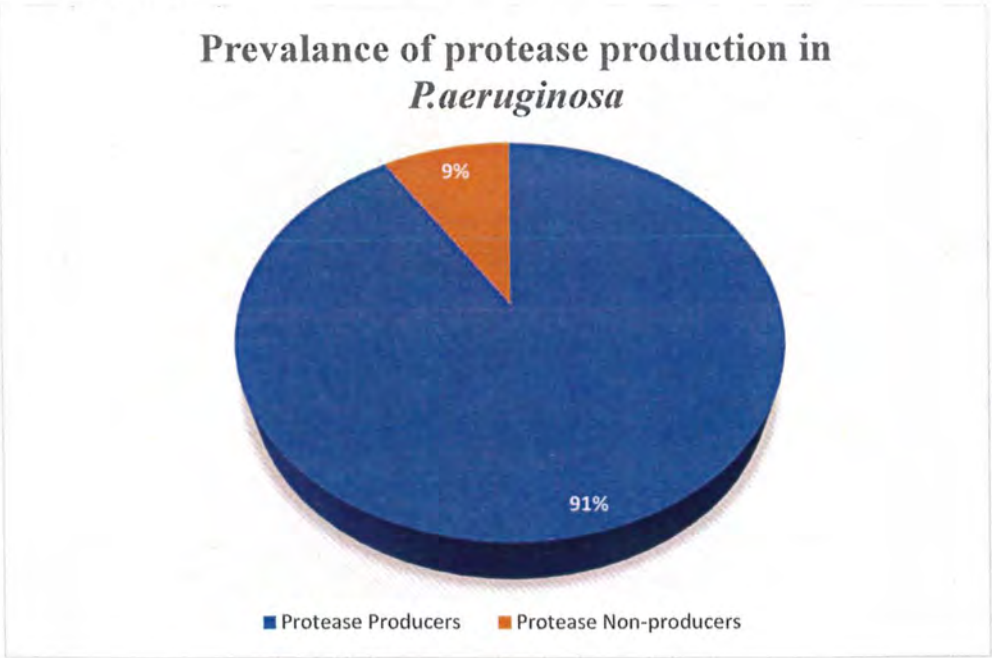


Figure 5.16 Pie-chart demonstrating the data of Protease producers and Protease non-producers

5.4.4 Elastase detection assay:

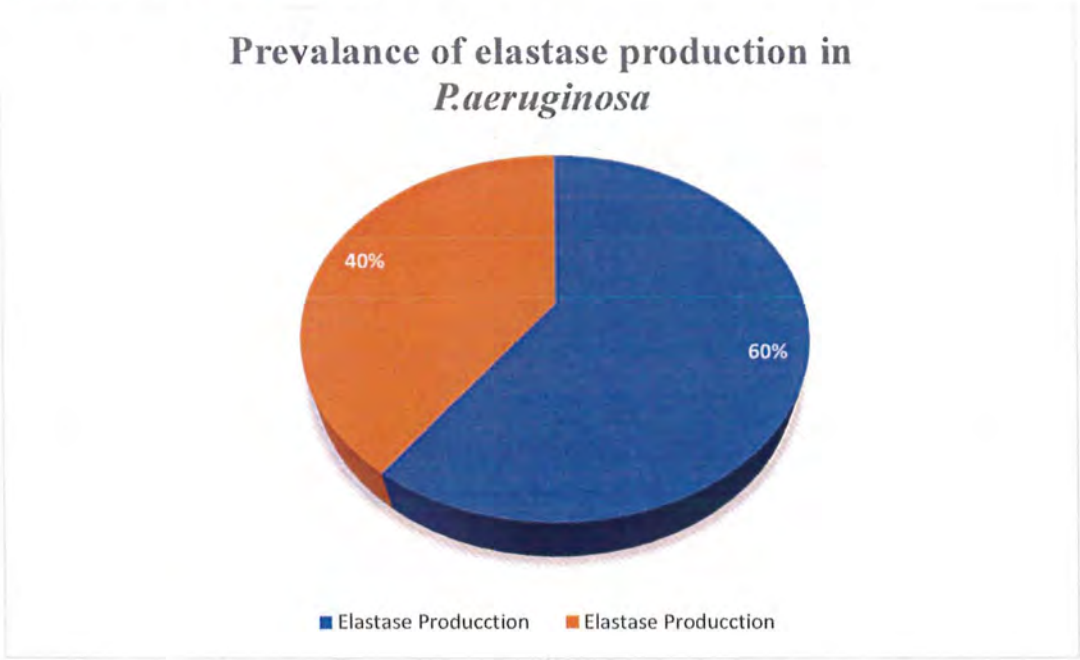


Figure 5.17 Pie-chart demonstrating the data of Elastase producers and Elastase non-producers

5.5 Molecular identification of quorum sensing genes:

5.5.1 DNA extraction :

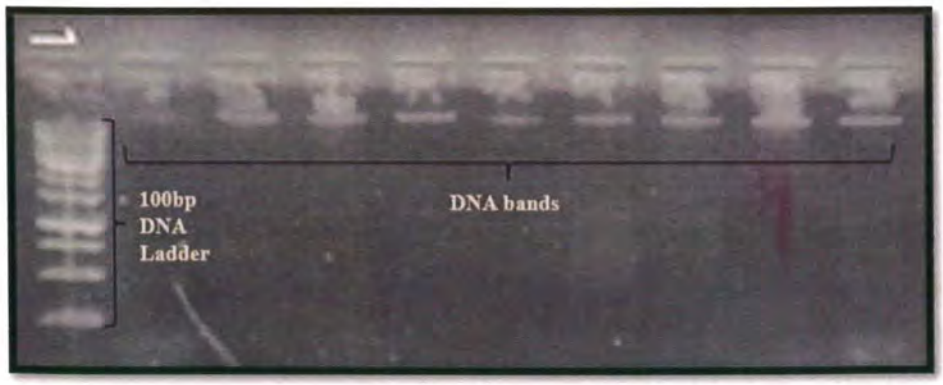


Figure 5.18 Extracted DNA bands run on the 2% Agarose gel with 100bp ladder.

5.5.2 Polymerase chain reaction:

5.5.3 Agarose gel electrophoresis:

Screening of *las R* gene

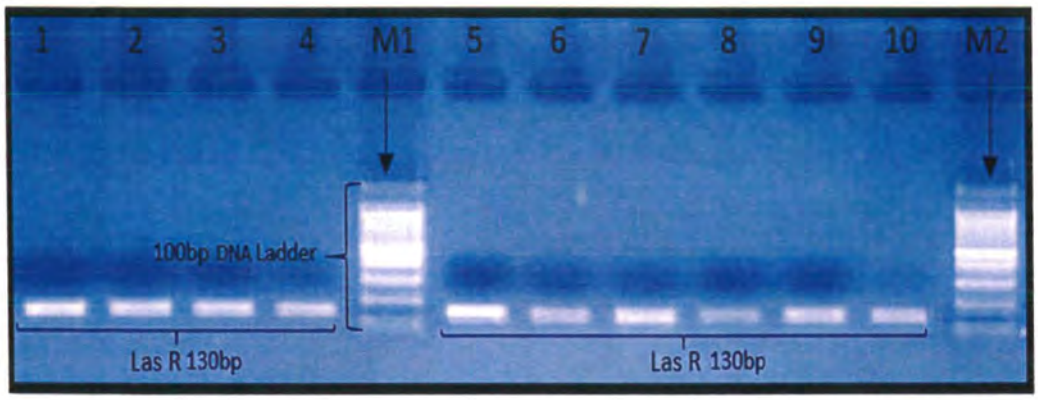


Figure 5.19 *las R* gene on 2% Agarose gel run along 100bp ladder. Amplicon Size is 130bp.

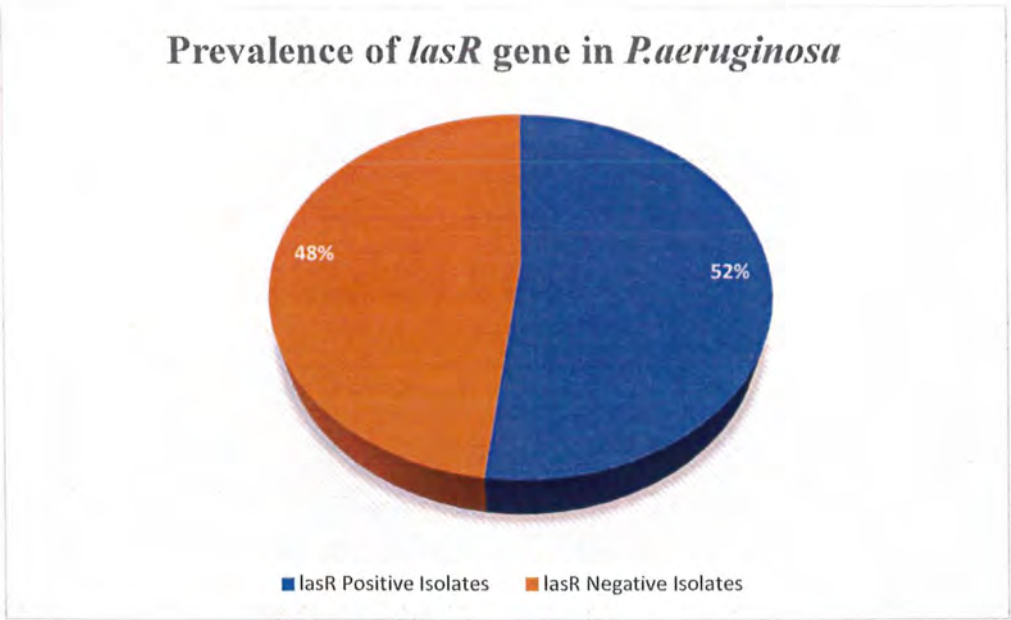


Figure 5.20 Pie-chart representing the prevalence of *lasR* gene

Screening of *las I* gene

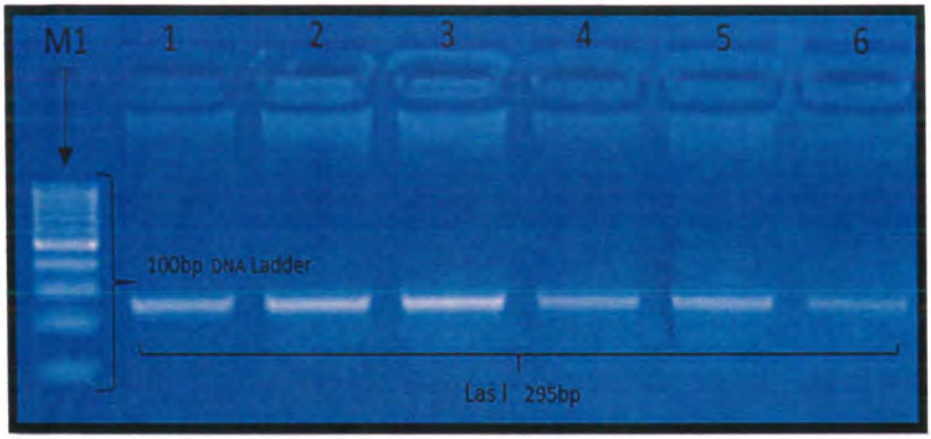


Figure 5.21 *las I* gene on 2% Agarose gel run along 100 bp ladder. Amplicon Size is 295bp.

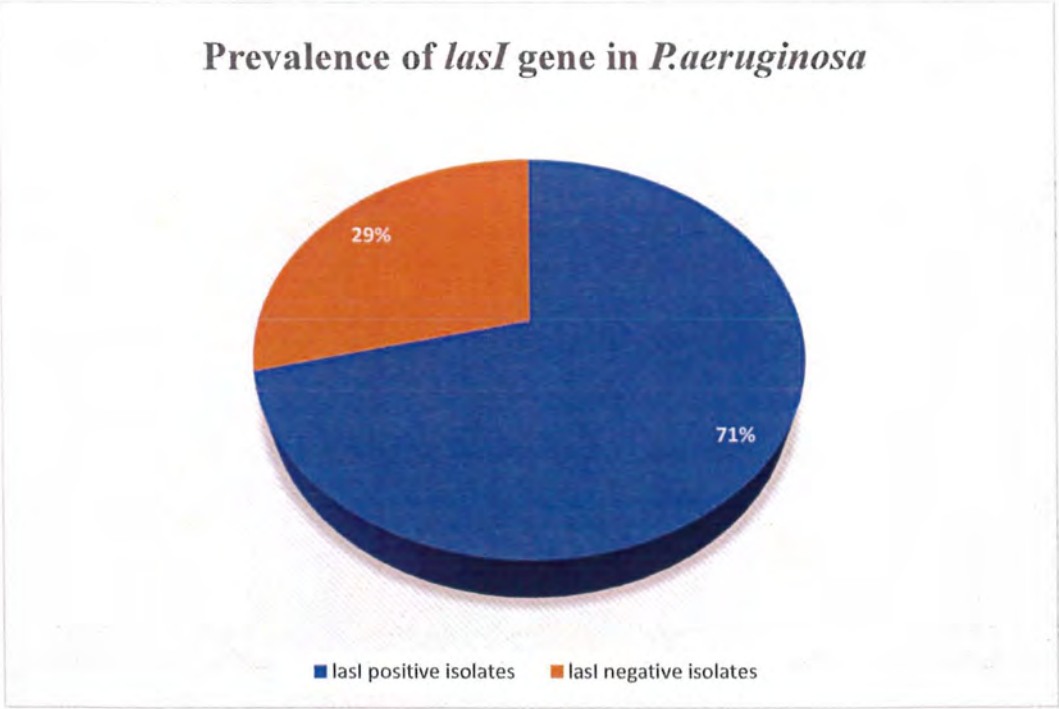


Figure 5.22 Pie-chart representing the prevalence of *lasI* gene

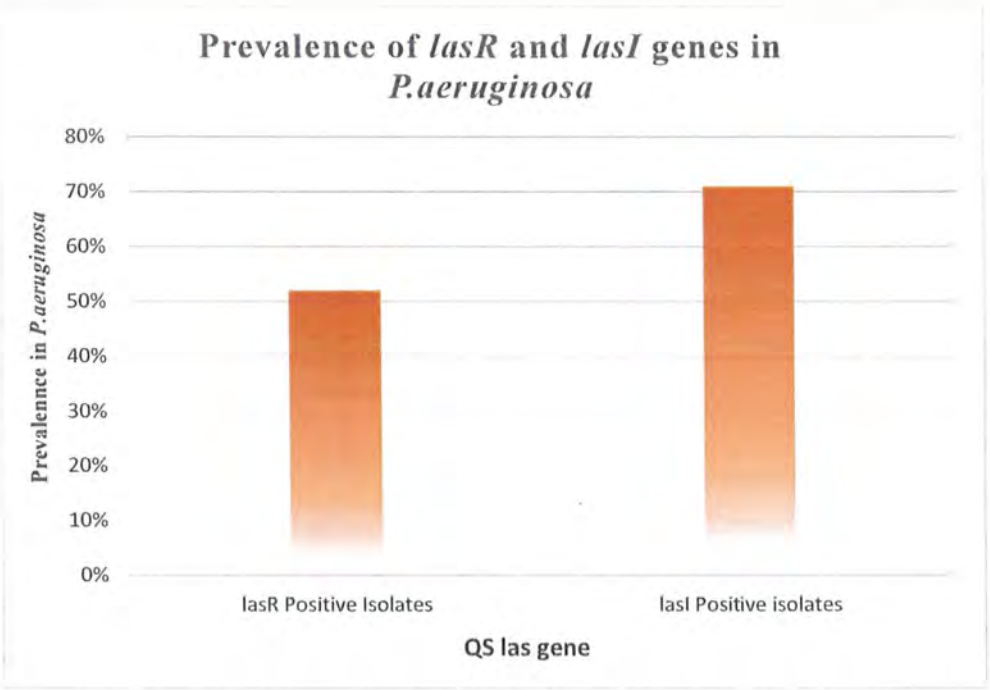


Figure 5.23 Bar graph representing the prevalence of *lasR* and *lasI* genes.

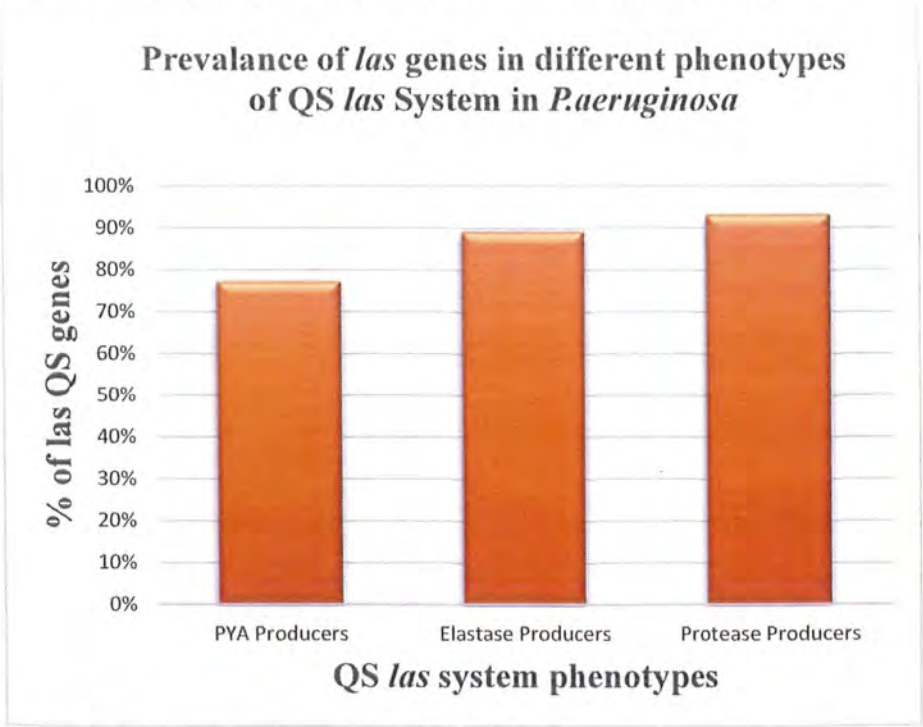


Figure 5.24 Bar graph representing the coorelation of las genes and its different phenotypes

DISCUSSION

Pseudomonas aeruginosa is a Gram-negative, ubiquitous bacillus that causes a variety of clinical illnesses in both humans and animals. It is practically hard to eradicate this pathogen from hospitals due to its remarkable potential for adaption to poor conditions of different environments. So it is a significant nosocomial pathogen (Abdullahi et al. 2013).A quorum sensing system is a density-based communication mechanism that allows bacteria to communicate with one another. *P. aeruginosa* has four types of quorum sensing systems las, rhl , pseudomonas quinolone signal (PQS) (Wang et al., 2018) and integrated quorum sensing inhibitors (IQS) (Lee, Zhang, & cell, 2015). Las system has three components: LasR, a response regulator (RR), N-3-oxo-dodecanoyl homoserine lactone (AHL; 3-O-C12-HSL), a similar AI molecule, and lasI which is employed for the AI- synthase. The rhl system is made up of rhlR, N-butyryl homoserine lactone (C4-HSL) as an AI molecule, and rhI, which regulates the AI-synthase (Wang et al., 2018). Despite the fact that the las and rhl QS systems are separate systems, still they work in a hierarchal manner in which las system dominates the rhl system (Van Delden & Iglewski, 1998).The LasR-3-O-C12-HSL complex controls *lasR, lasI, rhlR, rhlI,* and *pqsR* genes expression (Wang et al., 2018). Many virulence factors (Meena et al., 2020; Wang et al., 2018) including flagella and fimbriae, pyocyanin (PYA), pyoverdine, alkaline proteases, protease IV, elastases, and rhamnolipids (Al-Wrafy, Brzozowska, Górska, Gamian, & Doswiadczalnej, 2017) are controlled by *P. aeruginosa* QS systems via a complex of AI and R protein (Van Delden & Iglewski, 1998).The pathogenicity of these bacteria is mostly determined by their ability to produce these virulence factors. For example extracellular proteases play role in tissue destruction (Rust, Pesci, & Iglewski, 1996), elastase is released by about 75% of clinical isolates of *P. aeruginosa* (Kuang et al. 2011), and PYA disrupts the functioning of ciliated airway epithelial cells and results in increasing tissue damage (Britigan, Railsback, Cox, & immunity, 1999).*P. aeruginosa* QS systems are also involved for biofilm formation, swarming, and twitching motility (Wolska, Grudniak, Rudnicka, & Markowska, 2016).Biofilm is an important virulence factor of *P. aeruginosa* infections because it assists the bacteria in evading immune defenses and protecting them from antibiotics (Al-Wrafy et al., 2017). In order to create a biofilm, free-swimming bacteria use a type IV pilus and flagellum structure to connect to a

surface. The bacteria then proceed to create microcolonies via twitching motility. The bacteria then release QS-dependent AI compounds up to a particular concentration. Finally, microcolonies are intricated in extracellular polymeric substance (EPS) (Pearson, Pesci, & Iglewski, 1997) which necessitates the use of the las and subsequently the rhI QS systems for biofilm development (Alasil, Omar, Ismail, & Yusof, 2015).The QS system and associated virulence factors of *P. aeruginosa* has been intensively explored in human isolates (Sırıken, Öz, & Erol, 2021).Our study focused on the las system among the four systems of quorum sensing. We began our research by detecting phenotypic expression of QS las system by assaying biofilm formation, PYA production, protease production and elastase production in clinical P. aeruginosa isolates in order to understand QS and its controlled virulence factor expression. After that, the genotypic assay was done to examine the prevalence of QS las genes i.e *las I* and *las R* genes. So we determined the QS regulated virulence factors and correlated to the presence of Las system. Sensitivity testing for antibiotics were carried out.

In this study we use 175 isolates of *Pseudomonas aeruginosa* collected from the Armed Forces Institute of Pathology (AFIP). For the identification of *P. aeruginosa* strains in the samples each sample has undergone through multiple tests like morpho-cultural, biochemical and molecular techniques. The majority of the clinical isolates were obtained from pus (41%), urine (21%), ear swab (7.4%), and tissue (6.8%), accordingly. In this study the clinical isolates of *P. aeruginosa* demonstrated the biofilm-forming ability. The biofilm ability of all 175 *P. aeruginosa* isolates was tested. Biofilm formers were 95.43% (8.57 %, 60.57%, 26.29% strong, moderate and weak biofilm formers), whereas non-biofilm formers were 4.57%.Our results were similar to another research conducted by Elnegery *et al.* and Mowafy *et al.* (Elnegery, Mowafy, Zahra, & Abou El-Khier, 2021). In this study they presented biofilm development ability of clinical isolates of *P. aeruginosa* in which biofilm formers were 96% (14%, 38%, 44% strong, moderate and weak biofilm formers) whereas non-biofilm formers were 4%. Similarly results reported by Jabalameli *et al.* (Jabalameli et al., 2012) were also consistent; biofilm formers were 96% (47%, 26%, 22.9% strong, moderate and weak biofilm formers) whereas non-biofilm formers were 4%.On the contrary, Heydari and Eftekhar presented very less percentage of biofilm formers which were 43.5% (66.7%, 33.3% strong and moderate biofilm formers) whereas non-biofilm formers were 56.5% (Heydari & Eftekhar, 2015).The difference in our findings and their findings can be due

to difference in climatic conditions or the protocols.

Protease enzymes play an important role in the pathogenesis of *P. aeruginosa*; they destroy host tissues and promote bacterial proliferation and invasiveness in burn patients (Ahmed & Salih, 2019). Proteases play an important role in an agent's capacity to destroy tissue and invade it (Malloy et al., 2005) and most *P. aeruginosa* isolates, both environmental and clinical, have proteolytic and, in particular, elastase activity. Protease (Klare et al., 2016) a crucial pathogenic component that can help the bacteria adapt to varied environmental circumstances, is encoded by 3% of the whole genome of *P. aeruginosa*(Stover et al., 2000). In this study, 175 *P. aeruginosa* isolates were evaluated for total protease production which showed that 91% of the isolates were positive, whereas 9% were negative. Our results were consistent with (Sırıken et al., 2021) who detected the protease production in 90% isolates. Our findings were matched to those of Khalil *et al.* (Khalil, Sonbol, BADR, & ALI, 2015) who found that *P. aeruginosa* isolates from burn wounds produced a high proportion of protease that is 95%. This supports the theory that protease activity is higher in acute *P. aeruginosa* infections and decreases as the illness progresses (Macin, Akarca, Sener, & Akyon, 2017).Similarly Elnegery *et al.* and Mowafy *et al.* (Elnegery et al., 2021) which showed 86% were positive protease isolates , whereas 14% were negative. The little differences could occur due to environmental factors, human error and instrument errors.

Pyocyanin has the capacity to stop the electron transport chain in a variety of microorganisms and has antibacterial and cell-damaging properties (Laxmi & Bhat, 2016). PYA also aids in the release of extracellular DNA and the creation of biofilms (Klare et al., 2016).PYA is a redox-active compound regulated by the QS system, and *P. aeruginosa* that produces it has an advantage over other bacteria populations when they compete. When PYA synthesis fails even in a single individual isolate is lacking in QS, it will have a poorer tolerance to oxidative stress (Diggle, Griffin, Campbell, & West, 2007).Furthermore, PYA, together with the other virulence factors investigated in this study, allows isolates to persist in harsh conditions, giving them pathogenic qualities. We tested *P. aeruginosa* isolates for their potential to make pyocyanin and found a high rate of pyocyanin synthesis, with 74% positive pyocyanin producers and 26% pyocyanin non producers. This finding is consistent with the study reported by (Sırıken et al., 2021) with 73.3% pyocyanin producers. Similarly Elnegery *et al.* and Mowafy *et al.* (Elnegery et al., 2021) reported 66% positive pyocyanin producers.

Elastase activity was also discovered in 60 % of the isolates according to our findings of the research. Similarly In clinical isolates of *P. aeruginosa*, the elastase ratio is around 75% (Kuang et al., 2011).

The Las system genes were found in 90% of the isolates. Las system plays its role in the early phases of biofilm development. O'Toole and Kolter (O'Toole & Kolter, 1998) discovered that mutations in the las synthase gene produced faulty, consistent, flat, and undifferentiated biofilms. *lasR*-lacking mutants were shown to be less virulent (Zhu et al., 2004). In our study the *lasR* gene was found in 52 % of *P. aeruginosa* isolates. Our results are consistent with the results shown by (Sırıken et al., 2021) who screened the las genes in clinical isolates of *Pseudomonas aeruginosa* and *lasR* genes screened out to be 50%.El-Khashaab *et al.* (El-Khashaab, Erfan, Kamal, El-Moussely, & Ismail, 2016) showed that 94.3 % of P. aeruginosa isolates were *lasR* positive which is in contradiction to our findings. This difference can be due to the difference in climatic conditions specifically temperature because PCR is a highly temperature sensitive process. Minor changes in temperature or the concentrations of the reagents can affect the test results.

In our study the *lasI* gene was found in 71% of the isolates. These results are consistent with the results shown by (Sırıken et al., 2021) who screened the *lasI* genes in clinical isolates of *Pseudomonas aeruginosa* and they were 73%.

The number of *lasI* genes were higher than the number of *lasR* genes. Because the *lasI* gene controls the production of 3-O-C12-HSL signal molecules. These signal molecules then attach to the appropriate receptor of LasR. The LasR-3-O-C12-HSL complex then promotes the expression of a number of target genes (Smith & Iglewski, 2003). A threshold concentration level of 3-O-C12-HSL is necessary for *lasR* activation. For *lasR* activation in *P. aeruginosa*, *lasI*-mediated 3-O-C12-HSL synthesis in the cell must reach a threshold concentration level. As a result, the presence of *lasI* does not always imply the existence of *lasR* and it can be present in las R negative cells (Hill et al., 2005). The transcription levels of the *lasI* gene is very crucial. In *P. aeruginosa*, basic level *lasI* gene transcription have been observed (Hill et al., 2005).The *lasI* gene was also found in few of the *lasR*-negative isolates in our study. This can be due to the fact that the 3-O-C12-HSL regulated by *lasI* is not produced or that *lasI* is not activated as needed for *lasR* expression. Actually other genes, such as *gacA, vfr,* and *relA*, also have a role in the expression of *lasR* (Hentzer et al., 2003; Van

Delden & Iglewski, 1998). Similarly, a study showed once the *vfr* gene was deleted, all *lasR* gene expression in *P. aeruginosa* isolates was removed, and the virulence gene's output dropped (Hentzer et al. 2003).

The biofilm formation of *Pseudomonas aeruginosa* is initiated and controlled by several genes and environmental factors which also includes QS system controlled genes. In *P. aeruginosa*, these systems control around 10% of the genes (Fazli et al., 2014). LasR and RhlR proteins triggers biofilm formation in *P. aeruginosa* QS systems, and the *las* and *rhl* QS systems genes regulate bacterial colonization (Xie et al., 2006) .O'Toole and Kolter also share this viewpoint (O'Toole & Kolter, 1998).According to (Li, Qu, Liu, & Wan, 2014) , *lasI/R* gene expression and biofilm development have a positive correlation ($p < 0.01$).They also found that the las system is the most important for biofilm formation among the QS systems in virulent *P. aeruginosa* strains. The *lasI* mutation in *P. aeruginosa* has been linked to the absence of biofilms (O'Toole & Kolter, 1998).

In our study biofilm formation was detected in 95% of the total 175 isolates. Las system found out in biofilm formers and non-biofilm formers of the isolates. So we can say that the biofilm forming isolates which lacked the *las* system must have the *rhl* system which initiated the biofilm formation process in them. According to Kirisitis *et al.*(Kirisits & Parsek, 2006) the *rhlI* gene aids in biofilm formation, and when a C4-HSL-deficient *rhlI* mutant was found in *P. aeruginosa* isolates, biofilm production capability was reduced by roughly 70%.

C12-HSL/LasR controls the development of virulence factors such as elastase (*lasB*), toxA, and the lasA protease (Bleves, Soscia, Nogueira-Orlandi, Lazdunski, & Filloux, 2005). However, it is unclear whether this trait is controlled by protease II or the elastase activity-regulating genes *lasA* and *lasB*.Our findings demonstrated a very statistically significant difference in protease production between *lasR*-positive and *lasR*-negative isolates (P value=<0.001).

The *rhl* system regulate pyocyanin secretion. PQS also regulates the synthesis of virulence factors that are dependent on rhl, such as pyocyanin (Lee et al., 2015).In the case of *lasR*, Abou shleib and his colleagues observed no statistically significant relationship between pyocyanin production and the *lasR* gene (Aboushleib, Omar, Abozahra, Elsheredy, & Baraka, 2015).In our research, however, we discovered a statistically significant difference in pyocyanin synthesis between *las R*-positive and

las R-negative isolates (P value=0.035) which was consistent with the work done by (Elnegery et al., 2021).

Even in the absence of all studied virulence factors, Schaber et al. (Schaber et al., 2004) found one QS-deficient clinical isolate that produced the infection. According to Karatuna and Yagci (Karatuna, Yagci, & Infection, 2010), QS-dependent phenotypes can be positive for all four QS genes. Despite having all QS genes, Schaber and coworkers identified a *P. aeruginosa* strain that had neither elastase or pyocyanin activity (Schaber et al., 2004).

According to our findings, 90 % have *las* system genes. *lasR* gene was found in 52 % of the isolates and *lasI* was found in 71% of the isolates. The *las* QS system genes were found in 77% of pyocyanin producers, 93% of the protease producers and 89% of elastase producers. Two QS *las* genes and associated virulence factors are found in a large number of *P. aeruginosa* isolates. They showed a correlation between them.

Our study has several drawbacks. Firstly the analysis was based on just few phenotypic virulence variables and if we see there are many phenotypes and virulence factors expressed by the QS system and they are also interrelated and dependent. Only *las* system of genes was screened and discussed which is insufficient to completely understand the quorum sensing expression, as all of the four systems specifically *las* and *rhl* systems of quorum sensing work in a heirachical manner and depend on each other for the production of virulence factors and other functions.

CONCLUSION

The *las* QS system was discovered in the *P. aeruginosa* isolates evaluated in this work, and majority of the isolates showed QS-related biofilm forming capabilities. Furthermore, several isolates exhibited QS-related virulence characteristics, such as PYA synthesis and protease and elastase activity, indicating pathogenicity. These findings are significant in terms of public health. Approximately more than 90% of the chronic infections of wounds are poorly healed and gave rise to many complications in health care settings as they have been produced due to biofilm development. As biofilm formation declines the susceptibility of *P. aeruginosa* to many treatments done through antimicrobials, hence it is necessary to diagnose *P. aeruginosa* diseases at the initial level prior to the formation of biofilm. The rate of acute and chronic infections are elevating globally, so in order to circumvent the spread of this disastrous Gram-negative bacterium there is a dire need of adopting new therapeutic approaches to replace conventional antibiotics and strategies and I think the pathway of quorum sensing can serve as novel target for many antibiotics and natural compounds to eliminate the dreadful consequences of *P. aeruginosa*.

FUTURE PERSPECTIVES

Quorum sensing as drug target:

Due to the spread of antibiotic resistance that today has become one of the main challenges in the field of infectious diseases, therapies avoiding the indiscriminate use of antibiotics are paid special attention. On the other hand, given to the importance of the quorum sensing phenomenon in controlling pathogenicity of bactria such as *P. aeruginosa* , a new generation of antibiotics can be imagined which are designed based on the inhibition of QS system. It is not far from the notion that these inhibitors can be used as a synergism with other medications to reduce their dose. Biofilm that causes resistance against many commonly used antibiotics is regarded as one of the main challenges in treatment. Quorum sensing system plays a fundamental role in the regulation, control and formation of biofilm and many virulence factors. Therefore, it is possible that the inhibition of QS regulatory process for removing and reducing the drug resistance in infectious bacteria to be effective. Therefore, it was suggested that the bacterial QS system can be considered as a suitable target in order to research about the control of bacterial infection. This goal can be achieved using several methods including:

I) The blockage of R proteins activation by HLA antagonists or HLA antibodies and HLA degradation by chemical or enzymatic destruction

II) The metabolism inhibition of AHL by compounds that can compete with substrates which are used in the synthesis of autoinducers.

III) Inhibition of regulatory factors that have a positive effect on QS genes.

Quorum sensing signals as biosensor markers

Based on studies, QS signals can be used as markers for the presence of pathogenic bacteria in clinical and environmental samples by bacterial whole-cell QS biosensors. QS signals should not be employed as the only inputs for microbial biosensors but can be used for detection of pathogenic bacteria in contaminated environments and products such as groundwater, dairy, and meat products.

Quorum sensing signals and anticancer therapy

The 3-oxo-C12-HSL QS signal of *P. aeruginosa* inhibits proliferation and induces apoptosis in human breast cancer cell, therefore, it can be considered as an anticancer drug. However, this feature may be prevented due to some side effects, for example studies showed that 3-oxo-C12-HSL can lead to macrophage apoptosis. Nevertheless, this QS signal is a good starting point for developing synthetic AHL homologs with anticancer toxicity and reducing side effects.

Quorum quenching and biological control

The application of Quorum Quenching (QQ) strategy may be an alternative approach to control bacterial pathogens which employ the AHL based QS mechanism to regulate pathogenicity. The strategy includes several methods to achieve an artificially increased level of AHLs such as the introduction of a gene coding AHL synthase directly to the plant cells, the employment of AHL-degrading bacteria to protect plants and heterologous expression of genes encoding AHL-degrading enzymes in pathogen cells or in plant tissue. Therefore, bacteria misinterpret the population size and the misinterpretation leads to the production of virulence determinants long before the pathogen population is large enough to sustain infection. The AHL degrading enzymes together with QS inhibitors may successfully be applied to disrupt bacterial cell to cell communication and to control bacterial infections.

Many bacteria use quorum sensing as a multicellular system to coordinate gene expression according to the density of their local population. By this mechanism bacteria can regulate metabolic, host interactions and environmental processes. So, this system can be used as a useful target in medicine and other applications such as the production of biochemical, microbial biosensors and mixed-species fermentations. Work of the past few years showed that quorum sensing is essential for the expression of a battery of virulence factors as well as for biofilm formation in *P. aeruginosa* and thus represents an attractive target for the design of novel drugs for the treatment of *P. aeruginosa* infections.

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APPENDICES

A.1 Mueller-Hinton Broth Composition	
Ingredients	**Gram/liter**
Beef dehydrated infusion	2.0
Casein hydrolysate	17.5
Starch	1.5

A.2 Tryptic Soy Agar Composition	
Ingredients	**Gram/liter**
Pancreatic digest of casein	15
Sodium chloride	5
Agar	15
Peptic digest of soybean meal	5

A.3 MacConkey Agar Composition	
Ingredients	**Gram/liter**
Peptone (pancreatic digest of gelatin)	17
Proteus peptone (meat and casein)	3.0
Lactose monohydrate	10
Bile salts	1.5
Sodium chloride	5.0
Neutral red	0.03
Crystal Violet	0.001
Agar	13.5
Distilled water	Add to make one liter

A.4 Simmons Citrate Agar Composition	
Ingredients	**Gram/liter**
Agar	15
Ammonium dihydrogen phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Dipotassium phosphate	1.0
Magnesium sulphate (heptahydrate)	0.2
Bromothymol blue	0.08

A.5 Urea Agar Base Composition	
Ingredients	**Gram/liter**
Agar	15
Monopotassium phosphate	0.8
Sodium chloride	5.0
Peptone	1.0
Disodium phosphate	1.2
Dextrose	1.0
Phenol red	0.012

A.6 Nutrient Agar Composition	
Ingredients	**Gram/liter**
Lab lemco powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
Agar	15

A.7 Oxidase Reagent Composition	
Ingredients	**Amount**
Distilled water	10ml
Tetramethyl-p-phenylenediamine	0.1g

A.8 Catalase Reagent Composition	
Ingredients	**Percentage**
Hydrogen peroxide	3% (10 volume solution)

A.9 Peptone Water Composition	
Ingredients	**Gram/liter**
Peptone	5.0
Sodium chloride	2.0

A.10 10X TE Buffer Composition	
Ingredients	**Gram/liter**
Tris-HCl	15
EDTA	2.92

A.11 10X TBE Buffer Composition	
Ingredients	**Gram/500ml**
Boric Acid	29
Tris Base	54
EDTA	4.65

A.12 Normal Saline Composition	
Distilled water	100ml
NaCl	0.9g

A.13 Phosphate Saline Buffer Composition	
Ingredients	**Amount**
KCl	0.1g
NaCl	4g
Na_2HPO_4	0.7g
KH_2PO_4	0.12g
Distilled water	500ml

A.14 Crystal Violet Solution Composition	
Ingredients	**Amount**
CV Powder	2g
95% Ethyl Alcohol	20ml
Distilled water	80ml

A.15 Loading Dye Composition	
Ingredients	**Amount**
Bromophenol Blue	0.25g
Glycerol	3ml
Distilled Water	7ml

Turnitin Originality Report

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