# Characterization of Virulence Hallmarks of *Pseudomonas* Strains Isolated from Urinary Tract Infections


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# Characterization of Virulence Hallmarks of *Pseudomonas* Strains Isolated from Urinary Tract Infections

A thesis submitted to the Department of Microbiology, Quaid-i-Azam University, Islamabad in partial fulfillment of the requirements for the degree of

**Master of Philosophy**
**In**
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**“IN THE NAME OF ALLAH, THE MOST BENEFICENT, THE MOST GRACIOUS, THE MOST MERCIFUL”**

# Dedications

My humble efforts are dedicated to my mother and my siblings for their constant support, sacrifices, prayers, and firm belief in me throughout my entire education.

# Declaration

I hereby declare that the research work presented in the following thesis is my own effort, and the content included in this thesis is my original work. I have not previously presented this work elsewhere for any other degree.

**Aiman Mushtaq**

# Certificate

This thesis, submitted by **Aiman Mushtaq** is accepted in its present form by the department of Microbiology, faculty of biological sciences, Quaid-i-azam university, Islamabad, Pakistan, as satisfying the thesis requirements for the degree of Master of Philosophy in Microbiology.

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| | |
|---|---|
| **ADPRT** | **Adenosine diphosphate ribosyl transferase** |
| **CAUTI** | **Catheter-associated urinary tract infection** |
| **CFU** | **Colony forming Unit** |
| **Ebp** | **Endocarditis and Biofilm-associated Pilus** |
| **ECM** | **Extracellular matrix** |
| **EPS** | **Exopolysaccharides** |
| **Esp** | **Enterococcal Surface Proteins** |
| **GAP** | **GTPase activating protein** |
| **ICUs** | **Intensive care units** |
| **LBA** | **Luria-Bertani agar** |
| **LPS** | **Lipopolysaccharides** |
| **MPA** | **Microtiter plate assay** |
| **MRP** | **Mannose-resistant Proteus fimbriae** |
| **MR-VP** | **Methyl red-Vogues Proskauer** |
| **MTP** | **Microtiter plate** |
| **NAF** | **Non-agglutinating fimbriae** |
| **OD** | **Optical density** |
| **ODc** | **Optical density cut-off** |
| **OPS** | **O-polysaccharides** |
| **PCR** | **Polymerase chain reaction** |
| **PLC-H** | **Phospholipase C- hemolytic** |
| **PLC-H** | **Phospholipase C- non hemolytic** |
| **PMF** | ***P. mirabilis*-like fimbriae** |
| **Pz** | **Phospholipase zone** |
| **QS** | **Quorum Sensing** |
| **ROS** | **Reactive Oxygen Species** |
| **RPM** | **Revolution per minute** |
| **SIM** | **Sulphide indole motility** |
| **T3SS** | **Type III secretion system** |

| | |
|---|---|
| **TBE** | **Tris-borate-EDTA** |
| **TSI** | **Triple sugar iron** |
| **UPEC** | **Uropathogenic *Escherichia coli*** |
| **UTIs** | **Urinary tract infections** |
| **VAP** | **Ventilator-associated pneumonia** |

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## Abstract

Urinary tract infections (UTIs) are the most prevalent bacterial infections, affecting 150 million people worldwide every year. *Pseudomonas* is one of the most common uropathogen, posing a risk to both community and hospital settings due to its ability to thrive in the host environment by secreting various virulence factors that help in causing infections. In the present study, the virulence hallmarks of *Pseudomonas* strains associated with urinary tract infections were determined. Total 50 isolated and purified bacteria were revived from glycerol stocks, and their identification was confirmed as *Pseudomonas aeruginosa* through a sequence of biochemical tests including oxidase, catalase, triple sugar iron test, sulphide indole motility test, citrate utilization test, methyl red-Voges-Proskauer test, and nitrate reduction test. Bacterial Phenotypic detection of virulence factors in *Pseudomonas* isolates was identified by performing various assays. The biofilm-forming potential of *P. aeruginosa* was evaluated both by the Congo red assay and the microtiter plate assay. The motility traits were identified as swimming, swarming, and twitching. Additionally, these isolates were inoculated on egg yolk agar, skimmed milk agar, blood agar, Christensen urea agar, and cetrimide agar to assess their ability to produce extracellular virulence factors, i.e., phospholipase C, proteases, hemolysins, urease, and pigments, respectively. In the microtiter plate assay, 28% of isolates were non-biofilm formers, 46% exhibited weak biofilm formation, 16% showed moderate biofilm formation, and 10% demonstrated strong biofilm-forming ability. In contrast, the Congo red assay identified 86% of isolates as non-biofilm producers, while only 14% were biofilm formers. The three types of motility traits were assessed, and the majority of isolates showed swimming motility (95%), followed by twitching (82%) and swarming motility (62%), indicating their pathogenic potential. A large number of these isolates consistently displayed many extracellular virulence factors, 100% expressed phospholipase and urease production, 98% demonstrated hemolysin production, and 96% exhibited protease activity. Moreover, pigment production was determined by cetrimide agar, where pyoverdine pigment was found in all isolates, while pyocyanin pigment was observed in 82% of isolates. Among these isolates, all were highly pathogenic strains of *P. aeruginosa* with a large number of virulence factors. These strains were acquired in hospital settings and associated with urinary tract infections. There is a pressing need to develop target-based therapeutic strategies to combat *Pseudomonas*-associated urinary tract infections.

# Chapter 01

# Introduction

# Introduction

Urinary tract infections (UTI) are one of the most common bacterial infections. Approximately 150 million incidences of UTIs are reported each year, contributing to over 6 billion dollars in direct medical expenses (Flores-Mireles *et al*., 2015). As indicated by a comprehensive global report, UTIs accounted for more than 400 million cases, with 236,790 fatalities in a single year in 2019 (Yang *et al*., 2022). A rising trend is being observed in the prevalence of urinary tract infections from 1990 to the present globally. About half of the adult female population experiences UTIs at some stage in their lives (Sen, 2009).

Research indicates that the rate of UTIs is comparatively higher in developing countries as compared to developed countries (Gebretensaie *et al*., 2023). It is also a serious health concern in Pakistan, imposing a financial burden on the community. Despite the paucity of data on the morbidity and mortality associated with UTIs in Pakistan, every day innumerable outpatients, ambulatory visits, and hospitalizations are reported to occur due to urinary tract infections. The widespread occurrence of UTIs not only adversely affects the quality of an individual's life but also has a negative impact on the management and treatment of these infections in a third-world countries (Fazly Bazzaz *et al*., 2021).

Urinary tract infections are heterogeneous in their origin, symptoms, and progression, ranging from simple cystitis and urethritis to more complex and life-threatening conditions such as pyelonephritis, bacteremia, and septic shock. Urinary tract infection develops when bacteria enter through the urethra and invade the epithelium of the urinary tract, predominantly affecting the urethra, bladder, and sometimes kidneys. Medical procedures involving urogenital manipulations facilitate the transfer of bacteria into the urinary system (Sihra *et al*., 2018).

Clinical presentation of urinary tract infections varies from asymptomatic colonization of bacteria to severe urosepsis. The most frequent clinical symptoms include frequent urination, flank pain, inflammation in the urinary tract, dysuria, pain in the lumbar or suprapubic region, and bloody urine (Flores-Mireles *et al*., 2015). Poor hygiene, urinary retention, pregnancy, sexual activity, old age, female gender, diabetes, renal

transplantation, renal calculi, prior UTIs, and the presence of any indwelling urinary devices (such as catheters or stents) are all predisposing risk factors for urinary tract infections (Sabih & Leslie, 2023).

Depending on certain risk factors, urinary tract infections can be categorized as complicated UTIs or uncomplicated UTIs. Uncomplicated urinary tract infections are confined to healthy, non-pregnant, or pre-menopausal women who do not have any structural or functional abnormalities in their urinary tract, while complicated UTIs extend beyond the premises of the lower urinary tract and progress to involve the kidneys. This occurrence is most commonly associated with compromised immune systems in specific conditions such as pregnancy, renal failure, renal obstruction, and the presence of calculi or indwelling urinary devices (Zeng *et al*., 2022).

In hospital settings, incidences of urinary tract infections are reported to reach as high as 9.4% (Yang *et al*., 2022). Prolonged hospitalization, compromised immune status, and invasive procedures such as catheterization or urinary stent placement are major contributing factors to the onset of nosocomial urinary tract infections. These hospital-acquired UTIs account for about 40% of all nosocomial infections, with approximately 75% being associated with indwelling catheters only (Jacobsen et al., 2008). The mortality rate is comparatively high in immunocompromised patients with indwelling urinary catheters, ranging from in one-third to two-thirds of cases (Ndomba *et al*., 2022).

The microbial etiology of urinary tract infections is diverse, with *Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Enterococcus faecalis, Staphylococcus saprophyticus,* and *Pseudomonas aeruginosa,* being the most predominant pathogens (Flores-Mireles et al., 2015). Most of the uropathogens originate from the patient's own normal flora, such as *E. coli* and *Staphylococcus*, but sometimes the moist environment of the hospital also favors the colonization of opportunistic pathogens like *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Beceiro *et al.,* 2013). These uropathogens associated with complicated urinary tract infections possess high levels of virulence and antibiotic resistance mechanisms that is challenging for health care facilities (Kot *et al*., 2021).

*Pseudomonas aeruginosa* has emerged as a major opportunistic pathogenic microbe, accounting for approximately 10 to 20% of nosocomial infections (Reynolds & Kollef, 2021). Its remarkable ability to adapt to moist environments has gained significant interest in hospital environments, as its risk increases with the contamination of medical equipment. In addition to its dominance in pneumonia, sepsis, wound and soft tissue infections, *P. aeruginosa* is one of the major contributors to UTIs, particularly catheter-associated urinary tract infections (CAUTIs). It accounts for approximately 12% of all nosocomial urinary tract infections, ranking as the third most prevalent uropathogen following *Escherichia coli* and *Enterococci* (Cole *et al.*, 2014). *Pseudomonas aeruginosa* seldom causes uncomplicated UTIs, but distinguishing an uncomplicated UTI from a kidney infection can be difficult (Bono *et al.*, 2023).

Recently, the World Health Organization (WHO) has designated *Pseudomonas aeruginosa* as a multidrug pathogen of the highest concern for urinary tract infections due to its intrinsic antibiotic resistance mechanism as well as its ability to develop new resistance mechanisms against existing sensitive drugs (J. Newman *et al.*, 2022). In complicated UTIs, the majority of the *Pseudomonas* strains are resistant to all or three major classes of antipseudomonal agents, such as aminoglycosides, beta-lactams, and fluoroquinolones. This resistance mechanism poses a major challenge in the treatment of these infections, resulting in high levels of morbidity and mortality in immunocompromised individuals. Studies suggest that the mortality rate associated with *P. aeruginosa* urinary tract infection is 23%, and this rate rises up to 67% in the case of multi-drug-resistant bacteria (Obritsch *et al*., 2005).

All the pathogenic bacteria that are involved in the development of urinary tract infections are more aggressive in nature in terms of their virulence factors. They possess various extracellular virulence factors, such as toxins and enzymes, that are crucial for colonization, proliferation, and invasion of the urinary tract. Different cellular components of bacteria, such as capsules, pili, lipopolysaccharides, and different adhesins, also enhance the virulence of bacteria by facilitating its adherence and by providing it protection against host immune defense mechanisms. To understand the mechanism underlying the pathogenesis of urinary tract infection, these virulence hallmarks must be thoroughly investigated. Uropathogenic *Escherichia coli*, the most

predominant culprit behind UTIs, has undergone extensive research in terms of its virulence factor, but in comparison, not much has been known about *P. aeruginosa*, whose treatment is more challenging due to its resistance mechanism (J. W. Newman *et al*., 2017).

The virulence factors associated with *Pseudomonas aeruginosa* infections can be classified into two groups: cell-associated factors such as adhesins, lipopolysaccharides, porins, flagellum, and type IV pili; and secreted virulence determinants, which include exotoxin A, proteases, exoenzymes, phospholipases C, elastase, alkaline protease, DNase, alginate, and siderophores (J. W. Newman *et al*., 2017). The adaptation and evolution of these virulence factors in the establishment of chronic lung infections has been studied in detail (Winstanley *et al*., 2016). However, there is scarcity of literature monitoring the characterization of these virulence hallmarks in *P. aeruginosa* UTI populations.

The virulence of *Pseudomonas aeruginosa* is a multifactorial phenomenon driven by various cell-associated factors. The lipid A portion of lipopolysaccharide holds endotoxic properties, having the potential to induce an inflammatory response that can lead to host tissue damage. Outer membrane proteins are essential for bacteria to interact with the host environment by exchanging nutrients and sometimes by resisting the penetration of antibiotics. Moreover, six types of secretion systems, as well as exopolysaccharides, collectively enable *P. aeruginosa* to establish infections in diverse tissues and organs, and develop resistance to antibiotics, making it a formidable opportunistic pathogen (Jurado-Martín *et al.*, 2021).

*P. aeruginosa* releases a broad spectrum of toxins, such as ExoS, ExoT, ExoY, and ExoU. These toxins play a crucial role in disrupting cellular processes of host, particularly by impeding phagocytosis and impairing host defense mechanisms. Similarly, Exotoxin A has an impact on host protein synthesis machinery as it modifies host elongation factor II with ADP ribosylation. Pyocyanin, produced by *P. aeruginosa* is also detrimental to the host cells by inducing oxidative stress as it reacts with molecular oxygen in the host environment resulting in the liberation of reactive oxygen species (ROS) and hydrogen peroxide ($H_2H_2$). Moreover, iron sequestration mechanism

and Rhamnolipids biosynthesis can also contribute to the pathogenicity of *Pseudomonas* infection.

Biofilm formation is an important virulence hallmark of *Pseudomonas aeruginosa* infections that can lead to persistent and life-threatening infections. It is assumed that *P. aeruginosa* UTI is most commonly related to the expression of biofilm-specific genes and the formation of bacterial biofilms on the surfaces of catheters and stents (Gheorghita *et al.*, 2023). These biofilm-forming cells of *P. aeruginosa* can easily evade immune cells and are more tolerant to antibiotics. Alginate, Psl and Pel are the most common exopolysaccharides associated with biofilm formation. Investigating the biofilm formation ability of bacteria and detection biofilm specific genes (algD, pelF, pslD, Ppgl, PAPI-1) expression in UTI isolates of *Pseudomonas* indicate that the major contributing factors involved in the formation of biofilm (Rajabi *et al.*, 2022).

The motility of bacteria facilitates the colonization and dissemination of the bacteria in the host body. *P. aeruginosa* exhibits three distinct motility patterns, designated as swimming, swarming, and twitching. Swimming motility, powered by the rotation of flagella in liquid medium, contributes to *Pseudomonas aeruginosa* ability to navigate through the urinary tract to approach a surface. Twitching and swarming motilities are surface-associated motilities crucial for the movement of *P. aeruginosa* over the surfaces of catheters or the epithelial lining (O'May & Tufenkji, 2011). Assessing the motility profile of UTI isolates of *Pseudomonas* help in understanding its role in the establishment of urinary tract infections.

The majority of *Pseudomonas* strains isolated from clinical sources are proteolytic and lipolytic in nature. These proteases and lipases are also believed to contribute to the pathogenicity of *P. aeruginosa* infections. LasA and LasB elastases, alkaline protease, and phospholipase C are included in a diverse group of lytic enzymes having a role in the persistence of urinary tract infections. Moreover, high levels of hemolysin and urease production are also considered major pathogenic factors for *P. aeruginosa* infections. Therefore, evaluating the enzyme profiling of *Pseudomonas* could serve be an important indicator of its potential invasiveness.

Urinary tract infections are more prevalent in Pakistan, and most of the uropathogens are reported to be untreatable. So, there is a critical need to study the role of these uropathogens in UTIs. *Pseudomonas* is recognized as one of the most resistant and virulent pathogens, particularly associated with indwelling urinary devices. Therefore, the characterization of the virulence hallmarks of *Pseudomonas* can provide us with valuable insights for the development of an evidence-based treatment strategy.

# Aims and Objectives

## Aims:

The aim of the study was to characterize the virulence hallmarks of *Pseudomonas* strains isolated from urinary tract infections.

## Objectives:

- Identification of UTI-associated *Pseudomonas* strains by biochemical characterization.
- Investigation of different virulence factors of *Pseudomonas aeruginosa* (biofilm formation, motility traits, extracellular enzymes, and pigments) by phenotypic assays.
- Optimization of primers for the detection of virulence gene *alg*-D gene in *Pseudomonas aeruginosa*.

# Chapter 02

# Literature Review

# Literature Review

*Pseudomonas* genus represents a large group of gram negative rods classified within the Pseudomonadaceae family of the Gammaproteobacteria (Parte *et al*., 2020). Due to a great metabolic diversity, all species of *Pseudomonas* can thrive in a broad spectrum of environmental condition. Majority of *Pseudomonas* species that have potential to affect humans are attributed to opportunistic infections. *P. aeruginosa, P. putida, P. plecoglossicida, P. oryzihabitans, P. fluorescens, P. stutzeri, P. putrefaciens, P. cepacia and P. maltophilia* are among them (Wilson & Pandey, 2023). *P. mallei* and *P. pseudomallei* are the only two species accountable for specific diseases (i.e. glanders and melioidosis) in healthy individuals (Gilad *et al.*, 2007). It is estimated about 80% of *Pseudomonas* isolated from clinical specimen are *Pseudomonas aeruginosa* (Reynolds & Kollef, 2021). Therefore, *P aeruginosa* has attracted a great attention due to its frequent involvement in human diseases.

## 2.1: *Pseudomonas aeruginosa*

*P. aeruginosa*, like other members of its genus such as *P. fluorescens, P. migulae, P. lini*, and *P. graminis*, is frequently found in water, soil, plants, as well as in humans (Diggle & Whiteley, 2020). The organism has ability to infect hosts regardless of their immune status. Both immunocompetent and immunocompromised patients are exposed to a wide range of *Pseudomonas* infection. However, the reservoirs of community-acquired infections differ from those of hospital-acquired infections. Community-acquired pseudomonas infections, although rare, are reported to be associated with swimming pools and contaminated hot tubs (Huhulescu *et al*., 2011). In Hospital setting, water sources, taps, sinks, disinfectants, sanitizers, and medical equipment such as endoscopes and catheters serve as reservoir, contributing to the spread of nosocomial infections (Reynolds & Kollef, 2021).

*Pseudomonas aeruginosa* is predominantly responsible for **sepsis, pneumonia, and urinary tract infection** in patients with compromised immune defense. It is also associated with respiratory tract infections, especially in people with certain respiratory illness such as obstructive pulmonary illness and cystic fibrosis. The ultimate death that has occurred in majority of cystic fibrosis patients is due to localized *Pseudomonas*

*aeruginosa* infections (Davies, 2002). This pathogen is also one of the leading causative agents of ventilator associated pneumonia (VAP) (Kollef *et al*., 2014).

*Pseudomonas* is also accountable for **wound infections** in patients who have suffered from any burn injury, as well as responsible for **malignant otitis externa** in diabetic patients, which can lead to osteomyelitis of the skull (Tsilivigkos *et al.*, 2023). **Bacteremia** is a common manifestation of pseudomonas infection following surgery or localized infection. Rarely, it can also progress to meningitis, endocarditis, and corneal infections following lumbar puncture, heart surgery, and eye surgery, respectively. *P. aeruginosa* association with **diarrhea** has also been demonstrated in the literature (Chuang *et al*., 2017).

Two additional species, *Pseudomonas cepacian* (alternatively known as *Burkholderia cepacia*) and *Pseudomonas maltophilia* (sometimes referred as *Xanthomonas maltophilia* or *Stenotrophomonas maltophilia*), also cause similar infections, although their occurrence is relatively infrequent. One of the few differences is *Burkholderia cepacian* propagates to systemic infection in patients with cystic fibrosis, while infections caused by *Pseudomonas aeruginosa* are predominantly limited to the lungs (Schwab *et al*., 2014).

*Pseudomonas aeruginosa* is a capsulated rod having size ranging from 1 to 5 μm in length and 0.5 to 1.0 μm in width (Diggle & Whiteley, 2020). Most strains of *Pseudomonas* exhibit unipolar motility with a single flagellum at its one end. Some strains also possess two or three flagella. H antigens on the surface of flagellum's filament enable bacteria to move or swim in liquid medium. Some clinical strains of *Pseudomonas* have pili that possess antiphagocytic properties, as they can facilitate the colonization of bacteria by assisting bacterial attachment to host cells. Like all other Gram-negative bacteria, *P. aeruginosa* cell envelope is also composed of three layers: the innermost cytoplasmic layer, peptidoglycan membrane, and the outer membrane constituting lipopolysaccharide which act as endotoxin (Silhavy *et al., 2010)*.

*Pseudomonas aeruginosa* can easily be recognized by its characteristics bluish-green colour due to the production of both pyocyanin and fluorescein pigments. This bacterium shows positive results for oxidase, catalase and the urease enzyme. Its non-lactose fermenting colonies on differentiation agar media such as MacConkey and

Cysteine Lactose Electrolyte Deficient aid in its laboratory identification. The colony morphology of *P. aeruginosa* varies as well (Gilligan, 2013). The large, smooth and flat edged colony can be observed in clinical setting, whereas tiny, rough and convex is found in nature. The third mucoid type, on the other hand, is found in biological environments and has been discovered in the respiratory and urinary tracts.

*P. aeruginosa* resemble gram-negative rods of *Enterobacteriaceae*, but the key difference is that they are strict aerobes who derive their energy only from oxidation of sugars rather than fermentation. Meanwhile, members of the *Enterobacteriaceae* can ferment glucose (Bartlett *et al*., 1991). In anaerobic conditions (low or no oxygen), it can grow in the presence of arginine and can use nitrate in its respiratory process as an alternative terminal electron acceptor. It exhibits optimal growth conditions at 37 °C but can also tolerate temperatures as high as 42°C and as low as 4 °C. *P. aeruginosa's* ability to thrive at 42° C sets it apart from all other species of pseudomonas. This organism can also survive in low-oxygen atmosphere (Diggle & Whiteley, 2020).

*Pseudomonas aeruginosa* is often considered a prototroph and also possesses the genetic capacity to flourish on minimal growth media, utilizing a single source of nitrogen and carbon (Diggle & Whiteley, 2020).This ability to adapt to different nutritional conditions makes it able to colonize diverse environments. The organism is resistant to high salt concentrations, disinfectants, mild antiseptics, dyes, and even all known antibiotics. These characteristics explain its involvement in the onset of hospital acquired infections (Spagnolo *et al*., 2021).

*Pseudomonas aeruginosa* contributes to 10 to 15% of hospital-acquired infections worldwide (Shi *et al*., 2019). In some studies prevalence rate of *Pseudomonas* strains exceeds 20%, with 76% of the strains being classified as *P. aeruginosa* (Gad *et al*., 2007). Due to its ability to survive on moist surfaces, it can colonize medical equipment. Consequently, patients who have been on catheters are at high risk of acquiring infections related to *P. aeruginosa*. A study conducted in Jimma, Ethiopia, reported the presence of *Pseudomonas aeruginosa* in 49% of urine samples collected from catheterized patients (Bekele *et al*., 2015). In another retrospective observational study carried out on a population with long-term ventilation, *Pseudomonas aeruginosa* emerged as a primary pathogen, along with copathogens such as *Serratia*, *Proteus species, Stenotrophomonas*, and *Burkholderia cepacia* (Sobala *et al*., 2022).

*P. aeruginosa* is not very virulent in contrast to other important pathogenic gram-positive bacteria, such as *Staphylococcus aureus* and *Streptococcus pyogenes*, but it is capable of broad colonization and may aggregate into long-lasting biofilms. If such colonization occurs in vital human organs such as the lungs, urinary system, and kidneys, the consequences might be catastrophic. The mortality rate associated with *pseudomonas* bacteremia can range from 18% to 61% (Y. Zhang *et al*., 2020).

In addition to serious life-threatening infection, another concerning aspect of *Pseudomonas aeruginosa* is its intrinsically advanced antibiotic resistance mechanisms. In health care premises, hospital personnels, medical equipment, food, sinks, taps, mops, and all other damp environment serve as reservoirs for antibiotic resistance genes. Rapid mutations and adaption to acquire antibiotic resistance to all effective antibiotics pose a challenge for treatment of infection. *Pseudomonas aeruginosa* falls within the category of "ESKAPE" pathogens, a highly concerning group of multidrug resistant organisms such as *A. baumannii, K. pneumoniae, S. aureus, E. faecium,* and *Enterobacter*, which urgently requires research and development of novel antibiotics for clinical treatment (Qin *et al*., 2022).

## 2.2: Urinary Tract Infections:

Urinary tract infections are the most prevalent bacterial infection, impacting about 150 million individuals globally each year (Stamm & Norrby, 2001). Incidence pattern of urinary tract infections can vary among general population of Pakistan over time and by geography. Research conducted in Pakistan has reported a substantial range, with some studies indicating a high prevalence rate of approximately 65%, while others have reported prevalence rate as low as 11% (Ullah et al., 2018) (Khatoon *et al*., 2023). But a common pattern frequently observed is the high incidence of UTIs among females in comparison to males (Zubair *et al*., 2019).

The role of urinary tract is filtration of bloodstream by eliminating excessive water and waste products through the production of urine. Urine is produced in kidneys and then travels through the ureters down to the bladder, where it is stored until discharged from the body through the urethra. The peritoneum, a protective membrane, covers the bladder, and it has three layers of muscle tissue that facilitate the expulsion of urine. The protective inner lining of the bladder, referred to as uroepithelium or transitional

epithelium, serves as an important role in preventing the diffusion of urine and adherence of pathogens from penetrating into the underlying tissues of the urinary tract. Between the uroepithelium and the muscle tissue, there is a layer of connective tissues called the lamina propria, which contains cells of immune system, such as lymphocytes and macrophages. These immune cells are crucial factors in initiating an immune response to clear the infection (Walsh & Collyns, 2017).

In a healthy individual, urine is considered a sterile fluid or, sometimes, may contain only a few microbes that could lead to an infection. However, in people with weakened body's defense mechanism, intestinal microbes can enter through the urethra, and establish themselves in the bladder using specific adhesions. These bacteria then multiply, and infect urinary system through the production of toxins and enzymes that aid in their survival (Stamm & Norrby, 2001).

Although uropathogens have potential to affect multiple regions of the urinary tract, but the most common occurrence is a bladder infection, also termed as cystitis. Symptoms of cystitis include painful urination, hematuria (cloudy or bloody urine), and discomfort in the suprapubic region. When bacteria enter the kidney, they can cause a kidney infection (pyelonephritis). Flank pain and fever are indicators of pyelonephritis. In some cases, pathogen crosses the kidney epithelial barrier and can lead to bacteremia (Bono *et a*l., 2023).

Urinary tract infections are clinically classified as uncomplicated or complicated infections based on the existence of predisposing conditions for the development of infection. **Uncomplicated UTIs** affect healthy people without involving any structural or functional urinary tract issues. It usually represents community onset cystitis primarily involving the lower urinary tract such as bladder and urethra. These infections can easily be treated short term antibiotic treatment (Jancel & Dudas, 2002). Uncomplicated UTIs are prevalent among females of all age groups, but they also occur in a specific segment of the male population. Beside the female gender, risk factors for cystitis include a past urinary tract infection, vaginal infection, diabetes, obesity, sexual activity, and genetic predisposition (Storme *et al*., 2019).

In contrast, c**omplicated UTIs** are due to structural and functional abnormality of urinary tract. These infections are more challenging to treat, and they become worse

when there is an existing health problem. Indwelling urinary devices, obstruction and retention of urine, renal calculi, pregnancy, immunosuppression and the kidney transplantation are the factors associated with complicated urinary tract infection. About 80% of complicated UTIs are caused by the use of indwelling devices such as catheters, contributing to one million cases of urinary tract infections every year in the United States (Werneburg, 2022).

Bacteriuria in the bladder can either be symptomatic or asymptomatic. There are multiple ways for the urinary tract to be infected and colonized with bacteria, but if the patient doesn't show any symptoms, it doesn't qualify as an infection. A Colony forming unit above 10^5 per milliliter of urine sample is regarded as clinically significant to diagnose UTI (Hay *et al*., 2016). Asymptomatic bacteriuria is a condition that can be challenging to the diagnosis of UTI, as it involves the growth of more than 10^5 CFU/ml of urine without accompanying symptoms of UTI (Givler & Givler, 2023). Only symptomatic bacteriuria signifies the presence of a urinary tract infection. Therefore, antibiotic treatment should only be recommended after examining the clinical condition of patients to avoid misuse of antibiotics. In the case of asymptomatic bacteriuria, antibiotic treatment is only prescribed if the patient is pregnant, immunocompromised, or undergoing surgery (Totadhri *et al*., 2022).

For catheter associated UTI, a CFU of at least 10^3 obtained from catheter is enough to be considered as positive result. Urinary tract infection can be assessed by an elevated level of polymorphonuclear leukocytes in urine (>10 WBC/hpf), a condition clinically defined as pyuria. However, it is also worth noting that without a significant bacterial count, pyuria could also be associated with diseases like malignancy or kidney stones (Hoberman *et al*., 1994).

## 2.2.1: Predisposing Factors for Urinary Tract Infections:

**Menopause**: In premenopausal women, estrogen hormone is responsible for maintaining the microbiota of lower urinary tract, which helps in proscribing bacterial colonization. At the age of 50, when women enter menopause, estrogen levels drop significantly, and this alternation in urinary tract disrupts the balance of protective bacteria, allowing harmful bacteria to proliferate, which can increase the risk of UTIs (Storme *et al*., 2019).

**Pregnancy:** During pregnancy, various factors such as increased progesterone, reduced peristalsis, bladder displacement, uterine growth, and urinary stagnation in the ureters all contribute to bacterial colonization of the urinary tract. Consequently, the prevalence of asymptomatic bacteriuria in pregnant women is notably higher than in non-pregnant women (Abate *et al.*, 2020) It has been observed that about 25% to 40% asymptomatic bacteriuria cases progress to symptomatic urinary tract infections during pregnancy, posing risks to both mother and the unborn child (Matuszkiewicz-Rowińska *et al*., 2015) (Totadhri *et al*., 2022).

**Diabetes:** In diabetic patients, particularly those with type 2 diabetes, increased glucose levels in renal parenchyma provide an ideal environment for microorganism to grow and proliferate. Furthermore, multiple factors, such as poor metabolic control, compromised immune defense mechanism, and bladder control issues due to autonomic neuropathy, increase the susceptibility of diabetic patients to urinary tract infections. A study conducted in UK revealed that the incidence rate of UTIs was significantly higher in diabetic patients, with a rate of 46.9 per 1,000 individuals, whereas it was 29.9 per 1,000 for those without diabetes (Nitzan *et al*., 2015).

**Catheterization:** In the context of hospital acquired infection, more than 75% of urinary tract infections are secondary to the indwelling urinary catheters (Rhee *et al*., 2016). Prolonged catheterization can disrupt the innate immune defense mechanism by affecting the mucous barrier of the urinary tract, which shields the urinary tract from invading microbes. Moreover, a strong immune response triggered by catheterization can also result in the accumulation of fibrinogen on catheters. Uropathogens then bind to these catheters by expressing fibrinogen binding proteins. The fibrinogen accumulation and protective biofilm environment then favor the proliferation of bacteria, leading to catheter-associated urinary tract infections. If not treated properly, these infections can progress to pyelonephritis and bacteremia.

Several additional risk factors of UTIs include urological conditions like genital prolapse, urinary incontinence, genitourinary malformation, prostatic hypertrophy, renal stones, faecal incontinence, and neurogenic bladder dysfunction. Furthermore, individuals who have undergone kidney transplantation, and particularly those of the female gender face heightened risk. Poor nutritional status, illness severity, increasing

age, and an immunocompromised status, also contribute to the pathogenesis of urinary tract infection (Storme *et al*., 2019).

## 2.2.2: Pathogens associated with Urinary tract Infections:

Majority of urinary tract infections, approximately 95%, are bacterial in origin, but some fungi, viruses, and parasites can also invade and infect the urinary tract. (Patel *et al*., 2019) *E. coli*, *Staphylococcus saprophyticus, Streptococcus agalactiae, Enterococcus faecalis, Pseudomonas aeruginosa, Klebsiella pneumonia*e, *Proteus mirabilis,* and *Candida albican* are major uropathogens implicated in urinary tract infection (Flores-Mireles *et al*., 2015). Uropathogenic *Escherichia coli*, also known as UPEC strain is the most common uropathogens, responsible for about 80% of urinary tract infections (Abate *et al*., 2020). However, in the case of hospital-acquired and complicated urinary tract infections, organisms like *Enterococcus faecalis* and *Pseudomonas aeruginosa* tend to be more prevalent (Shigemura *et al*., 2006).

***E. coli*** is a common resident of the gut microbiota and can enter the urinary tract through faecal contamination. Women are more likely to develop an *E. coli* infection because of short urethra. Different pathotypes of *E. coli* strains have been identified, but the Uropathogenic E. coli (UPEC) strain is the most frequent cause of cystitis and pyelonephritis, harboring different adhesins including S fimbriae, P fimbriae, Type 1 and Type 2 fimbriae, as well as F1C fimbriae. It also possesses other virulence factors such as flagella, toxins, capsule, biofilm formation and iron acquisition systems. Although this stain is highly resistant to many antibiotics, but treatment is easy in comparison to that of the *Pseudomonas aeruginosa* infection (Mancuso et al., 2023).

***K. pneumoniae,*** being the most frequently known agent of nosocomial infections, can cause urinary tract infections, pneumonia, sepsis and many soft tissue infections. (CRISTEA *et al*., 2017) Like UPEC, it also possesses two types of adhesins: one is type 1 fimbriae that colonize the bladder via mannose receptors and the other is type 3 fimbriae that contribute to the formation of biofilms on medical devices like catheters (Werneburg, 2022).

***Proteus Mirabilis*** due to its distinctive swarming motility and flagellar activity can have major impact on urinary tract system. It contributes to only 1% to 2% of all UTIs in healthy individuals, but the incidence rate increases to 45% in the case of complicated

UTI following catheterization. (Jamil *et al*., 2023) *Proteus mirabilis* produces various fimbriae having crucial role in adhesion and colonization of urinary tract. Among these are MRP fimbriae (mannose-resistant Proteus fimbriae), non-agglutinating fimbriae (NAF) and P. mirabilis-like fimbriae (PMF). In addition to fimbriae, two autotransporters such as TaaP (Proteus autotransporter trimeric) and AipA (Proteus autotransporter-mediated adhesion and invasion) also facilitate P. mirabilis' adherence and invasion by binding to collagen I and laminin. (Jansen *et al*., 2004)

***Enterococci***, being the member of human gut microbiota, is associated with opportunistic nosocomial infection. It ranked second after *E. coli* as an etiological agent of urinary tract infection according to a recently conducted study in Pakistan (Bullens *et al*., 2022). Unlike other uropathogens, they do not have pili, instead, Esp (Enterococcal Surface Proteins) and Ebp (Endocarditis and Biofilm-associated Pilus) are two surface proteins that helps pathogen to bind to the host cell.

***S. saprophyticus*** is most commonly responsible for community-acquired urinary tract infections. It binds to the epithelium of urinary tract, particularly ureter and bladder through different types of adhesins i.e., Uaf, Aas, and Sdr. Moreover, urease enzyme produced by S. saprophyticus is also accountable for bacterial colonization, as it neutralizes the acidic environment of urinary tract, which is an important defense mechanism against microbial colonization.

### 2.2.3: Role of *Pseudomonas* in Urinary tract Infections:

The gut or vagina of healthy individual is not the natural habitat of *P. aeruginosa*. So, the most predominant cause of development of pseudomonas urinary tract infections is nosocomial setting. These are more likely to occur in immunocompromised patients, or those who have recently undergone urinary tract instrumentation or surgery. People having structural irregularities in their urinary tract (i.e., complicated UTI) are also at higher risk.

In nosocomial setting, *P. aeruginosa* is most commonly associated with biofilm-mediated infections, such as catheter-associated urinary tract infection (CAUTI), ventilator-associated pneumonia (VAP), and those associated with contact lens, stents, heart valves, grafts, and sutures. Research conducted in European ICUs demonstrated *Pseudomonas aeruginosa* as one of the most prevalent bacteria, accounting for CAUTI,

VAP, and catheter associated with blood stream infections (Litwin et al., 2021). In Pakistan, the increased frequency of CAUTI infections due to multi drug resistant *P. aeruginosa* has also been reported in many studies (Khawaja *et al*., 2021). The increasing incidences of multidrug resistant *Pseudomonas aeruginosa* in nosocomial settings is major threat to healthcare facilities worldwide.

Indwelling devices and catheters favor microbial colonization. Consequently, *P. aeruginosa* initiates the infection by invading the periurethral space. It then ascends to the urethra, bladder, ureter, and subsequently the kidneys. The organism also has the ability to breach the tubular epithelial cell barrier, resulting in bacteremia and sepsis in the absence of any medical intervention (Sadikot *et al*., 2005).

## 2.3: Pathogenesis of *P. aeruginosa*:

**Adhesion:** The first step in the pathophysiology of infection is bacteria's ability to bind to mucin layer, a glycoprotein that acts as the first line of defense by protecting mucous membrane from harmful toxins and invading uropathogens. Flagella and Type IV are key adhesins in *P. aeruginosa* that interact with host cells via epithelial gangliosides, asialoGM1 and asialoGM2. After initial attachment through polar flagellum, bacteria produce type IV pili that hold the bacteria to the surface, permitting cells to spread throughout the adjacent areas (Gellatly & Hancock, 2013).

**Proliferation:** *P. aeruginosa* proliferates in the form of microcolonies. These microcolonies develop into a 3D biofilm structure due to the accumulation of extracellular molecules (such as the exopolysaccharide Pel, Psl, and alginate), as well as extracellular DNA. The structural integrity of this structure is then further strengthened by extracellular appendages and adhesins. At the end of their lifecycle, these biofilms actively disassemble and release motile and pathogenic cells into their surroundings (Kostakioti *et al*., 2013).

**Tissue Damage:** Extracellular bacterial proliferation is the major contributor to epithelial injury and tissue damage. After coming into contact with epithelial cells of host, Type III Secretion System is activated, which introduces cytotoxins directly into the host cell by passing phagocytosis and bacterial clearance mechanisms. Multiple virulence factors, such as alkaline protease, elastase, phospholipase C, pyocin, and pyoverdine, produced by *Pseudomonas aeruginosa*, can also have adverse effects on

host cells. Proteases break down mucins and complement factors, and can facilitate the dissemination of bacteria by breaking tight junctions of epithelial cells. Various lipids present in cell membrane of host can be targeted by phospholipases and lipases produced by bacteria. Furthermore, Pyocyanin and pyoverdine pigments have tendency to disrupt electron transport chain and redox cycling of host cells, in addition to their role in iron acquisition mechanisms (Beasley *et al*., 2020).

## 2.4: Virulence Factors of P. *aeruginosa:*

### 2.4.1: Lipopolysaccharides

LPS produced by *P. aeruginosa* has an essential role in mediating both bacterial virulence and host responses, and is responsible for many symptoms of diseases such as fever and shock. It has three main components; lipid A, core polysaccharide, and outer polysaccharide (also called O-antigen). **Lipid A** consists of a diglucosamine bisphosphate backbone, which can be acylated with different numbers of fatty acids, forming penta or hexa acylated isoforms having different potential to stimulate the host's innate defense mechanism by binding to Toll-like receptor 4 (Pier, 2007). A novel hepta-acylated variant, hepta-1855 with potent effects on neutrophil has also been discovered in a subset of *Pseudomona*s strains isolated from patients with cystic fibrosis (SenGupta *et al*., 2016). Studies also revealed that mutants defective for lipid A synthesis lose their ability to adhere to epithelial cells and forming biofilms on both abiotic and biotic surfaces, suggesting the role of LPS in bacterial adhesion and development of biofilms (Jurado-Martín *et al*., 2021). *P. aeruginosa* lipopolysaccharide also increases the pathogenicity of Enterobacteriaceae when present in a mixed infection by stimulating direct effect on serum resistance, type-1 fimbriae, and biofilms formation in *K. pneumoniae* and *E. coli* (Abdel-Rhman, 2019).

Second domain of LPS, known as **core oligosaccharide** can be divided into two parts: the inner core with 3-deoxy-D-manno-octulosonic acid (KdoI and KdoII) and heptose (HepI and HepII) residues, and the outer core with D-glucose, L-rhamnose and D-galactosamine residues. The core oligosaccharide is crucial not only for maintaining integrity and stability of the outer membrane of bacterial cell, but also possesses antigenic attributes (King *et al*., 2009).

The third key part of LPS, **O-polysaccharides** constitute an elongated chain of repeating polysaccharides that can exist in either linear or branched configuration, determining rough (uncapped) and smooth (capped) forms of LPS. As it protrudes from the outer membrane, it can also contribute in host-pathogen interactions and can prevent bacterial killing by providing protection from oxidative stress and membrane attack complex. Studies also suggest that the presence of O-polysaccharides (OPS) is a major stimulus for neutrophils to undergo NETosis (Neutrophil Extracellular Trap Formation), a defensive mechanism to trap and neutralize pathogens (Pieterse *et al*., 2016).

## 2.4.2: Outer membrane Proteins:

**Porins:** *Pseudomonas aeruginosa* possesses 26 different β-barrel channel proteins to control diverse exchange of nutrients across the outer membrane (Jurado-Martín *et al*., 2021). One of the major and most abundant porin is OprF essential for both outer membrane integrity and pathogenicity of *P. aeruginosa*. OprF is responsible not only for ion and saccharide acquisition but also allows the passage of other substances including toluene, siderophores, nitrates, and nitrites. Furthermore, it is involved in bacterial adhesion (via OprF-lectin B complex), biofilm formation, and modulation of the host immune response. OprF protein has also been proposed as a potential target for vaccine development (Dey *et al*., 2022). The second smallest *P. aeruginosa* porin, OprH contributes to respiratory tract infection by binding laminin and surfactant protein A. Studies also revealed its importance in antibiotics resistance, especially to aminoglycoside and polymyxin antibiotics (Chevalier *et al*., 2017). Similarly, Opr D assists the entry of peptides, amino acids, gluconate, as well as carbapenem antibiotics. Its role in the virulence of pseudomonas infection is attributed to its protease activity, as well as its binding affinity for laminin. Loss of the OprD confers *P. aeruginosa* with an inherent resistance to carbapenems, notably imipenem resistance (Li *et al*., 2012). Furthermore, OprO serves as another porin that can adhere to epithelial cells through interaction with human fibronectin.

**Lipoproteins:** Lipoproteins in *Pseudomonas* are involved in multiple functions such as outer membrane biogenesis, transportation and cell integrity. Lipoproteins such as BamBDE or LptE are the integral part of outer membrane assembly machinery. OprL and OprI maintain cell integrity by interacting with peptidoglycan. OprM, OpmE,

OprN, OprJ, OpmG, OpmB are some additional lipoproteins responsible for efflux of harmful molecules, including antibiotic drugs. (Jurado-Martín *et al*., 2021)

### 2.4.3: Flagellum:

The flagellum of *P. aeruginosa* possesses three major components: flagellin filament, curved hook, and the membrane complex. Flagellin filament consists of thousands of helically arranged flagellin proteins (FliC) ending with a filament cap protein (FliD. Other two proteins FlgK and FlgL are located at the junction of filament and hook. The hook (FlgE) functions as a connector between a rigid flagellin filament and a membrane complex that supports flagella on the cell surface (M *et al*., 2021).

*Pseudomonas aeruginosa* possesses a single flagellum at its polar end essential for both chemotaxis and motility of bacteria. The flagellum filament plays an important role in bacteria's swimming motility by propelling the bacteria to move forward in low viscosity medium through a corkscrew like rotation. Some strains of *P. aeruginosa* also exhibit swarming motility, in which *Pseudomonas aeruginosa* moves through semi-liquid surfaces by a multi-factorial mechanism involving pili, rhamnolipids, and some amino acids in addition to flagellum (Yeung *et al*., 2009).

Beyond its role in motility, the FliC protein is also important for host-pathogen interaction. Various cellular components, such as heparan sulfate proteoglycans, membrane glycoproteins asialo-GM1, and surfactant protein-A of alveolar cells, are reported to have binding sites for FliC protein (Jurado-Martín *et al*., 2021). Similarly, FliD protein is responsible for binding to human respiratory mucin. (Haiko & Westerlund-Wikström, 2013). These molecular interactions can activate the TLR5 pathway. Furthermore, flagellar hook proteins such as FlgE confer resistance to surfactant protein A-mediated phagocytosis, indicating importance of flagella in the pathogenesis of infection beyond adhesion and motility (S. Zhang *et al*., 2007).

### 2.4.4: Type IV Pilli:

Pili are long, hair-like structures that not only mediate bacterial attachment to cell surfaces but are also important in development of biofilms and *Pseudomonas* twitching motility. They are mainly polymers of the Pilin A, the primary pilin protein and some minor pilins (FimU, PilE, PilW, PilV, PilX,). These minor pilins are further classified as core and non-core minor pilins, having a role in pili biogenesis as well as pili

interactions with cell surfaces, respectively. Approximately forty mapped genes have been identified that can regulate the activity of pilin A, with PilS and PilR being the most common (Craig *et al*., 2019).

One of the distinctive features of type IV pili, **twitching motility**, is accomplished through sequential actions of type IV fibres extension, tethering, and retraction, which propel the cell to move forward. PilB and PilT are two cytoplasmic ATPases that polymerize and depolymerize PilA subunits, leading to the extension and retraction of the pilus, respectively (Burrows, 2012). PilY1, a minor pilin, plays an important role in attachment induced virulence of *P. aeruginosa* as it recognizes host receptors on epithelial cells, and then bind to the integrins in an RGD-dependent manner (Siryaporn *et al.*, 2014). Furthermore, Pilin A C-terminal also mediates interaction with the host cells glycolipids such as asialo-GM1 and asialo-GM2. Some studies suggest clinical strains of *Pseudomonas* don't use these gangliosides to interact with host cells during attachment process (Schroeder *et al*., 2001).

## 2.4.5: Biofilm Formation:

*P. aeruginosa* possesses the intrinsic property to adhere to the catheter surfaces and form biofilms, increasing the risk of UTIs in patients with prolonged bladder catheterization. Bacterial proliferation begins in the form of microcolonies, which subsequently combine together to form biofilms. Biofilms are structured communities of bacteria embedded in a protective extracellular matrix (ECM). Over half of the extracellular matrix of *P. aeruginosa* consists of three exopolysaccharides: alginate, Pel, and Psl. Additionally, the ECM of bacteria also possesses DNA and proteins (Gheorghita *et al*., 2023).

Biofilm development is a multifactorial process that can occur in almost every environment, involving both biotic and abiotic surfaces. These may consist of either a single species or sometimes involve polymicrobial species. In the case of short-term catheter-associated UTIs, single species is responsible for development of infections, whereas long-term CAUTIs are polymicrobial infection commonly associated with gram-negative rods (Jacobsen *et al*., 2008). Biofilms protect the invading bacteria from the host's immune defense mechanisms as well as antimicrobial agents, hindering the treatment of infections. Therefore, the presence of biofilms significantly contributes to the pathogenicity of *P. aeruginosa*, often resulting in persistent and recurring infections.

The process of biofilm formation begins with an increased concentration of c-di-GMP, which triggers the secretion of adhesion molecules as well as exopolysaccharides (EPS). The resultant EPS then stabilizes the attachment, initiating the development of biofilm architecture. Cup fimbriae and an outer membrane adhesin CdrA, also contribute in strengthening the structural integrity of biofilms. Some QS-controlled extracellular enzymes (esterases, elastases, and lipases) also influences the formation of Pseudomonas biofilms by affecting properties of the extracellular matrix. Eventually, biofilm cells progress towards the final stage of their life cycle with the dispersal of planktonic cells all around (Jurado-Martín *et al*., 2021).

**Alginate**: Alginate is the major exopolysaccharide that primarily consists of an O-acetylated linear polymer of L-guluronic and D-mannuronic acids. These acetyl groups contribute to its property of high viscosity. Enzymes required for the synthesis of alginate are mainly encoded by the algD operon, whose expression is controlled by the algT σ-factor. Strains of *Pseudomonas aeruginosa* that have mucoid characteristics tend to produce more of this exopolysaccharide, which contributes to the maturation, architecture, and stability of biofilms (Orgad *et al*., 2011). In others strains that do not possess mucoid features, either the pel or psl gene is actively expressed. Besides its significant role in biofilm formation, it provides bacteria with a protective shield against immune-based phagocytosis of host cells. Due to its viscosity and mucoid characteristics, alginate can effectively retain water and nutrients, and may also affect the penetration of antibiotics into the biofilms (Mann & Wozniak, 2012).

## 2.4.6: Toxins:

*P. aeruginosa* consists of five secretory systems that are commonly recognized to secrete a broad spectrum of exotoxins to target their host. However, the most significant one is Type III Secretion system (T3SS), which is known to inject four different toxins i.e., ExoU, ExoS, ExoY and ExoT directly into host cells via injectosome during infection process.

**ExoU** is one of the major cytotoxins in T3SS, most commonly associated with epithelial damage in many diseases of the soft tissues, respiratory tract and urinary tract (Foulkes *et al*., 2019). ExoU possesses phospholipase A2 activity that is responsible for rapid cell death by degrading the cell membrane of both phagocytes and epithelial cells of the host. In addition to its cytotoxic effects, it can also induce a proinflammatory

response by increasing eicosanoid production, which is involved in activation of the NF-κB signaling pathway, resulting in the secretion of IL-8 (Jurado-Martín *et al*., 2021).

**ExoY,** being an adenylate cyclase, can increase intracellular concentrations of cAMP, cCMP, cGMP, and cUMP, resulting in activation of protein kinases, which are responsible for disrupting the actin microtubule and endothelial barrier and eventually cell death. ExoY also has one binding site that enables it to bundle actin filaments directly within the host cell. (Mancl *et al*., 2020) Another remarkable virulence feature of ExoY is that it suppresses the secretion of proinflammatory cytokines by blocking the expression of TAK-1 (transforming growth factor β-activated kinase 1) (Jurado-Martín *et al*., 2021).

**ExoT** is the one of the most abundant exotoxin found *P. aeruginosa* nosocomial infection (Javanmardi *et al*., 2019). It is a bifunctional exotoxin possessing two enzymatic activities, such as adenosine diphosphate ribosyl transferase (ADPRT) and GTPase activating protein (GAP). Both these activities synergistically responsible for evasion of phagocytosis and disruption of epithelial barrier. GAP domain is also involved in triggering pro-apoptotic pathways along with ExoT and can impede the healing mechanism (J. W. Newman *et al*., 2017).

**ExoS**, similar to ExoT, also exhibits the same bifunctional activity. During the initial stage of infection, when ExoS enters neutrophils by T3SS, the ADPRT domain of ExoS inhibits ROS production by neutrophils, which is an essential component of the immune system, thereby evading phagocytosis. As the infection progresses into the lungs, ExoS also gains entry into type I pneumocytes, causing disruption of the alveolar-capillary barrier. Additionally, ExoS is also involved in the ribosylation and inactivation of a broad range of virulence-associated proteins (Jurado-Martín *et al*., 2021).

## Exotoxin A:

Unlike other toxins, it is released by Type II secretion system. After binding to the host cells receptors, Exotoxin A is internalized into the host cell via clathrin-coated pits (a receptor mediated endocytosis). Once inside the host cell, it undergoes conformational changes and releases ADP-ribosyl transferase. This enzyme is responsible for inhibiting the protein synthesis mechanism within the host cell by deactivating EF-2 (elongation

factor 2), which is crucial for translocating mRNA within ribosomes. This toxin also activates two caspases associated with apoptosis, inhibiting the secretion of IL-18, IL-10, IL-8, IL-6, and TNF-α (Jurado-Martín *et al*., 2021).

### 2.4.7: Secreted Enzymes:

### 2.4.7.1: Phospholipase C:

Phospholipase C secreted by Type II Secretion System (T2SS) of *Pseudomonas aeruginosa* is a potent virulence hallmark of urinary tract infections. Both types of phospholipases; PLC-H (hemolytic) and Plc-N (non-hemolytic) are capable of hydrolyzing phosphatidylcholine, a crucial component of lipid bilayer of cell membrane. Hemolytic phospholipase can additionally target sphingomyelin. Similarly, non-hemolytic phospholipase can hydrolyze phosphatidylserine in addition to phosphatidylcholine. Although non-hemolytic PLC lacks pathogenic activity, PLC-H emerges as an important virulence factor, having a significant impact on suppressing neutrophil respiratory bursts by selectively hydrolyzing the phosphodiester bonds in the phosphatidylcholine, as it is crucial for the production of ROS during the respiratory burst (Terada *et al*., 1999). The hemolytic activity of Phospholipase C can also help the bacteria in the iron acquisition mechanism through the lysis of red blood cells and liberation of heam (J. W. Newman *et al*., 2017). According to a research experiment, when mice is administered with high doses of purified PLC-H, it results in increased vascular leakage, vital organ dysfunction, and ultimately the death of mice (Berk *et al.*, 1987).

### 2.4.7.2: Alkaline Proteases:

Alkaline protease, a zinc-dependent metalloendopeptidase, is released through Type I secretion system. It possesses proteolytic activity and disrupts two essential components of the endothelium: fibronectin and laminin. Alkaline protease also aids in phagocytotic evasion by disrupting host complement proteins such as C2, C3, and C1q, as well as cytokines such as TNFα and IFN-γ. It is also known to cleave flagellin subunits and to activate sodium channel of epithelial cells. Both of these actions impair bacterial movement, making it more difficult for the mucociliary system to clear bacteria from mucosal surfaces. Alkaline protease also plays a significant role in the establishment of urinary tract infections. Its role in raising pH of urinary tract and

increasing iron availability through the breakdown of transferrin creates a favorable environment for bacterial growth. Moreover, increased amino acid metabolism during protein degradation fulfills the metabolic needs of bacteria (J. W. Newman *et al.*, 2017).

### 2.4.7.3: Elastases:

Elastases is another protease released by T3SS that contribute to the pathogenicity of *Pseudomonas aeruginosa* infection by targeting host elastin proteins. These zinc-dependent metalloproteases, encoded by the lasA and lasB genes, are primarily regulated by quorum sensing systems. LasB elastase (also recognized as pseudolysin) is the most common protease that can compromise the bacterial clearance mechanism by degrading host SP-A and SP-D surfactant proteins, various immunoglobulins and cytokines (IL-2, IL-6, TNF-α, IFN-γ). LasB elastase also have impact on biofilm formation by regulating rhamnolipids, and can also degrades exogenous flagellin, evading Toll-like receptor 5 recognition. In contrast, LasA degrade glycine-glycine bonds within elastin. In this way they can increase the effectiveness of other LasB protease, regardless of its limited elastinolytic activity. According to some studies, LasA expression is found to be correlated with antibiotic resistance to *P. aeruginosa* (Dehbashi *et al.*, 2020).

### 2.4.7.4: Urease:

Several uropathogens are capable of encoding the urease enzyme, which is crucial for the colonization and persistence of bacteria during urinary tract infections. Urease production in *P. aeruginosa* is also regarded as one of the important virulence hallmarks of bacteria. This enzyme acts as a catalyst for the hydrolysis reaction of urea into ammonia and carbamic acid, which are further hydrolyzed to form bicarbonates. As a result, pH of urine increases, resulting in formation of calcium and struvite crystals both in urine and on catheters. Moreover, ammonia overload is also detrimental to uroepithelial cells, as it can directly damage tissues. In *P. aeruginosa*, this enzyme is also crucial for pH regulation in biofilms (Bradbury *et al.*, 2014).

## 2.4.8: Pyocyanin Production:

Pyocyanin produced by *Pseudomonas aeruginosa* also emerged as an important virulence factor. (Hall *et al.*, 2016) It usually imparts a blue-greenish colour to *P.*

*aeruginosa* colonies, making it visually identifiable. It can be detrimental to host cells as it can interrupt the cell cycle and degrade DNA. Pyocyanin-producing pseudomonas strains can provoke oxidative stress by generating ROS, resulting in considerable cell lysis and the consequent release of eDNA. Pyocyanin interacts with extracellular DNA to create biofilms in urinary tract infections through increasing cell-to-cell contacts and influencing cellular surface characteristics. Recent experimental data shows that this is the mechanism through which biofilms develop in UTIs. (Abdelaziz *et al.*, 2023)

### 2.4.9: Iron acquisition mechanisms:

Iron acquisition mechanisms is important for *Pseudomonas aeruginosa* to successfully invade and colonize the urinary tract. Bacteria produce iron chelating compounds like pyoverdine and pyochelin that enable it to consume ferrous ion from the host lactoferrins and transferrin. Similarly, Pyocyanin, can also contribute in iron chelation process through the Feo system. Furthermore, haem proteins can target host haemoglobin, which is then transferred by the Phu and Has iron absorption systems. *P. aeruginosa* may also have capacity to capture siderophores as generated by other microbes, such as *E. coli*'s enterobactin.

### 2.4.10: Rhamnolipids:

Rhamnolipids regulated by the quorum sensing (QS) system serve as a surfactant, and are an important factor related to the pathogenicity of Pseudomonas. In cystic fibrosis patients, they facilitate *Pseudomonas aeruginosa* to infiltrate epithelia barrier of lungs (ZulianThello *et al.*, 2006). Several studies have shown that rhamnolipids, along with other factors, play a crucial role in swarming motility by overcoming the surface tension of the surrounding environment and facilitating the movement of bacteria across semifluid surfaces. In swarming motility, bacteria coordinate their behavior to spread and migrate in a group, showing an intricate pattern known as tendrils. Such tendril production in swarming motility depends on the biosynthesis of rhamnolipid (Caiazza *et al*., 2005).

All these virulence factors found in *Pseudomonas aeruginosa* not only contribute to the bacteria's survival and adaptability within the host but also have a notable impact on the modulation of the host's immune response. A versatile ensemble of virulence factors, along with its highly advanced antibiotic resistance mechanism, make

*Pseudomonas* a formidable pathogen to treat. Therefore, understanding the in-depth study of these virulence hallmarks of *Pseudomonas* can help us assess the role of this bacterium in urinary tract infections and develop new therapeutic approaches.

In Pakistan, as in other developing nations, UTIs are on the rise as an emerging bacterial infection. The growing incidences of infections caused by multi-drug-Resistant (MDR) Pseudomonas aeruginosa pose a rising concern in our region. Therefore, there is a pressing need to develop targeted-based therapeutic approaches to mitigate the issue of antibiotic resistance. In Pakistan, there is a scarcity of available data on the virulence profile of Pseudomonas strains associated with urinary tract infection. So, it is imperative to characterize the virulence mechanism of the pathogen and establish a systematic surveillance system to monitor this resilient pathogen.

# Chapter 3

# Materials and Methods

## 3.1: Study Design:

It is a retrospective-research that was conducted from February 2023 to January 2024 at the Molecular Microbiology Laboratory, Department of Microbiology, Quaid-i-Azam University, Islamabad.

## 3.3: Bacterial Culture from Glycerol Stock:

Pre-isolated bacteria associated with urinary tract infections were revived from glycerol stock. Nutrient broth was prepared by adding powdered media (13 g/1000 mL) into distilled water. After being autoclaved at 121°C for 15 minutes, media was poured into tubes aseptically, and the tubes were incubated at 37°C for 24 hours for sterility testing. The next day, the glycerol stock of pre-isolated bacteria was removed from the freezer and an amount of the glycerol stock was then transferred into nutrient broth tubes using a sterile inoculation loop. All the inoculated tubes were incubated at 37°C for 24 hours.

## 3.4: Culturing of Refreshed Isolates:

Nutrient agar was prepared by adding powder media (28 g/1000 mL) into distilled water, and then it was sterilized by autoclaving at 121°C for 15 minutes. Media was then poured aseptically into petri plates by the pour-plate technique. Inoculating loops were sterilized by passing them through the flame until they turned red. A small amount of the bacterial culture was obtained from nutrient broth and spread across a small section of the agar surface by quadrant streak method to get purified and single colonies. Plates were incubated at 37°C for 24 hours, and the colony morphology of all isolates was observed the next day.

## 3.5: Identification of *Pseudomonas*:

Pure bacterial culture was then subjected to series of biochemical tests. Identification and confirmation of *Pseudomonas* was done according to Berge's Manual of Determinative Microbiology.

### 3.5.1 Gram Staining:

Gram staining is the most commonly used method in microbiology laboratories for the differentiation of two primary types of bacteria (Gram-negative and Gram-positive) due to the difference in their cell wall composition. Gram-positive bacteria, possessing a

thick peptidoglycan layer, have ability to retain the crystal violet stain and exhibit purple or violet appearance under the microscope, while Gram-negative bacteria lose crystal violet stain during the decolourization process due to the thin peptidoglycan layer. Therefore, the pink colour of the counterstain becomes dominant, leading to the characteristic pink or red appearance of Gram-negative bacteria.

First, the smear was prepared by mixing a freshly isolated bacterial colony with a drop of normal saline placed on a glass slide. After being air dried and heat fixation of bacterial cells to the slide, crystal violet was added as the primary strain for one minute. In the second step, the stain was rinsed with tap water, and iodine was added as mordant for another minute. After washing the iodine, decolourizer was applied to the slides for a few seconds, followed by an immediate rinse with water. In the final step of Gram staining, the counterstain safranine was applied for 1 minute. Dried slides were observed under a microscope at 100X using immersion oil.

### 3.5.2 Lactose Fermentation Test:

Gram-negative bacteria were streaked down on MacConkey agar, which inhibits the growth of Gram-positive bacteria due to the selective action of crystal violet and bile salts present in the media. This medium is also used to distinguish lactose-fermenters from non-lactose fermenters as it contains phenol red, a pH indicator that gives the medium a pink colour under acidic conditions.

To prepare the MacConkey Agar medium, the powdered medium (51.5 grams) was mixed with distilled water (1000 mL). The mixture was thoroughly combined and sterilized using an autoclave at 121°C for 15 minutes (15 psi). After sterilization, the medium was cooled down to a temperature of 40–45 °C., The medium was poured aseptically into Petri plates using the pour plate technique, and the plates were incubated for sterility testing. Isolates were inoculated onto the prepared MacConkey Agar plates using a streaking technique, and the plates were placed in an incubator for 24 hours at 37°C. The next day, colonies were observed for lactose fermentation.

### 3.5.3: Oxidase Test:

It is a biochemical test used to detect the presence of the Cytochrome Oxidase C enzyme. This enzyme for the is crucial for the oxidation of cytochrome c by transferring

electrons to molecular oxygen during the electron transport chain. The artificial electron donor used in the test is tetra-methyl-p-phenylenediamine dihydrochloride, commonly known as Kovac's oxidase reagent, which is oxidized to a dark blue-coloured product if the bacteria possess the cytochrome oxidase enzyme.

This test was performed by adding 1 drop of 1% oxidase reagent on a filter paper. A single isolated colony from 24 hours fresh culture plate was picked up using a sterile loop or wooden stick, and was rubbed on the wet area of filter paper and observed for colour change.

### 3.5.4: Catalase Test:

Catalase enzyme present in bacteria is essential for the breakdown of hydrogen peroxide into water and oxygen to remove the toxic effect of hydrogen peroxide. To indicate the presence of this enzyme in bacteria, a dilute solution of hydrogen peroxide is used as a catalase reagent. Oxygen is liberated as a byproduct of hydrogen peroxide decomposition, and bubble production is observed in the case of positive results

Following slide method, a clean glass slide was used onto which a drop of 3% $H_2O_2$ was placed. A pure bacterial culture was mixed with it with the help of sterile wooden stick, and results were observed within 2 minutes.

### 3.5.5: Sulphide Indole Motility Test:

Sulphide Indole Motility (SIM) media is a multi-test agar used to detect three main characteristics of bacteria; indole production, sulphide production as well as motility of bacteria. SIM media is semi-solid due to addition of low concentration of agar, and motile bacteria can diffuse from stab line and make media turbid indicating its motility. Casein peptone in SIM media is rich in tryptophan, and bacteria producing tryptophanase enzyme convert tryptophan into indole which was visually detected by the presence of distinct red layer at the surface of media when Kovac’s reagent (p-dimethylaminobenzealdehyde) is added. This medium also constitutes sodium thiosulfate and ferrous ammonium sulphate. If a microbe hydrolyzes proteins rich in the sulfur containing amino acid cysteine, the sulfur is released as $H_2S$. This hydrogen sulfide gas reacts with ferrous ammonium sulfate, resulting in the formation of ferrous sulfide, which is detected as a distinctive black precipitate within the medium.

SIM media was prepared, autoclaved, and poured into test tubes. A single isolated colony of pure culture was taken with the help of a stabbing needle and inoculated deep into SIM media by stabbing from the center. After 24 hours of incubation, the next day, tubes were carefully examined for motility and H2S production, and then Kovac's reagent (3 drops) was added to detect indole formation.

### 3.5.6: Triple Sugar Iron Test:

The Triple Sugar Iron (TSI) biochemical test is based on the microorganism's ability to ferment different sugars. The media comprises 0.1% glucose, 1% lactose, and 1% sucrose as sole carbon, as well as phenol red as a pH indicator, which changes the colour of the media during fermentation. The presence of red slant and yellow butt is indicative of glucose fermentation only. If both the slant and butt are yellow, it suggests an acidic reaction, signifying the fermentation of all three sugars (lactose, sucrose, and glucose). If both slant and butt are red, this represents an alkaline reaction, which means there is no fermentation of any sugar. Moreover, cracks or bubbles in the agar media is indicative of gas production, and the blackening of the medium refers to hydrogen sulfide production.

TSI media was autoclaved and poured into test tubes (about 3 mL), making good butts with short slants by tilting the tubes at 45 degrees. Pure isolated culture was then inoculated by first stabbing deep into the butt and then streaking on the surface of slants. After providing 37°C incubation for 18 to 24 hours, results were noticed by carefully examining the colour change.

### 3.5.7: Citrate Utilization Test:

The citrate test is based on bacteria's ability to utilize citrate and ammonium salt as the sole sources of carbon and nitrogen. In Simmons citrate agar, when bacteria utilize citrate, alkaline carbonates and bicarbonates are produced. The colour of the media changes from green to blue due to the pH indicator Bromothymol blue.

Simmon citrate agar was autoclaved and poured into test tubes placed in an inclined position for making slants. After media solidification, the slant surface was streaked with fresh bacterial culture. Test tubes were plugged properly, and after 18–24 hours of incubation, results were observed.

## 3.5.8: Methyl Red and Vogues Proskauer Test:

The Methyl Red and Vogues Proskauer **(**MRVP) test is used to distinguish acid-forming bacteria from acetoin-forming bacteria. This test uses a broth medium containing glucose to determine which fermentation pathway was utilized by bacteria to utilize glucose. Two pathways are most commonly used by bacteria to utilize glucose, i.e., mixed fermentation (detected by the MR test) and 2,3 butanediol fermentation pathways (detected by the VP test).

MR-VP broth was prepared, autoclaved, and then poured into test tubes. Isolates were then inoculated in MR-VP broth and incubated at 37°C for 48 hours. Following incubation, half of the inoculated MR-VP broth was transferred to another test tube. Methyl red reagent (5 drops) was added to one tube labeled as MR, and the second tube was subjected to two VP reagents. Firstly, 12 drops of 5% Barritt's reagent A (alpha-naphthol) were added, and then, after a few seconds, 4 drops of 40% Barritt's reagent B (40% KOH) were added. The tubes were left unstirred, and results were recorded.

## 3.5.9: Nitrate Reduction Test:

This test is used to detect an organism's ability to produce the nitrate reductase enzyme, which can facilitate the reduction of nitrate in the medium to nitrite. The latter may undergo further reduction to nitrous oxide, nitric oxide, or nitrogen. The nitrite produced is then allowed to react with sulfanilic acid, forming a nitrite-sulfanilic acid complex. Subsequently, a reaction with α-naphthylamine leads to the formation of a red precipitate. If no colour change was observed, zinc dust was added to confirm whether the test organism had reduced the nitrite to a gaseous end product.

First, nitrate reduction media was prepared by adding potassium nitrate (1 g/L), meat extract (3 g/L) and peptone (5 g/L) into distilled water. After being autoclaved and poured into tubes, the media was inoculated with heavy inoculum bacteria and incubated at 37°C for 24 hours. Reagent A (0.5g α-naphthylamine) and reagent B (0.6g Sulfanilic acid) were prepared by dissolving them in 100mL of 30% acetic acid. Two drops of each reagent were added to each test tube. Reagents were mixed vigorously by shaking the tubes, and colour change was observed. If there is no colour change within

2 minutes, zinc dust was introduced into tube using by dipping a wooden stick, and colour change was observed.

## 3.6: Phenotypic Identification of Virulence Factors of *Pseudomonas aeruginosa*:

### 3.6.1: Biofilm Formation Assays:

### 3.6.1.1: Congo Red Assay:

Congo red assay is a qualitative technique used to identify the biofilm forming ability of bacterial isolates. Extracellular polymeric substances in bacterial biofilms usually bind to Congo red dye resulting in change of colour from red to black.

Congo red agar was prepared by adding sucrose (36g/L), brain heart infusion (37g/L), agar (10.5g/L) and Congo red dye (0.8g/L) in distilled water. All the constituents were autoclaved at 121 °C for 15 min. *P. aeruginosa* isolates were inoculated on Congo red agar using streaking technique, and results were observed after incubation period of 24 hours.

### 3.6.1.2: Microtitre Plate Assay:

The biofilm-forming ability of these isolates was quantified by performing a microtiter plate assay (MPA). In this method, the optical density of a microtitre plate with stained biofilms was measured and compared with cut-off values. The following steps were performed:

I. **Development of biofilms on Microtitre Plate:**

   The inoculum was first prepared by cultivating the bacteria in brain-heart infusion broth (BHI) and then incubated at 37°C for overnight incubation. The next day, the inoculum's turbidity was adjusted to 0.5 McFarland's standard. A 200 µL of bacterial culture was poured into 96-well flat-bottomed microtitre plates and incubated at 37°C. Wells containing sterile BHI broth were taken as negative controls, whereas wells with known biofilm-forming strains were taken as positive controls.

II. **Washing and Staining:**

After incubation, media was removed from wells by gently tapping the plate without disturbing biofilms. The microtitre plate was rinsed twice with phosphate buffered saline (200 µL) to eliminate unattached bacterial components. Two hundred µL of 95% ethanol was then added for the fixation of biofilms and kept on the microtitre plate (MTP) for 30 minutes at room temperature. After applying 200 µL of 0.1% crystal violet dye, biofilms were allowed to be stained for ten minutes. MTP was rinsed twice again with PBS to remove excessive dye. The plate was left to be incubated at room temperature for half an hour to completely dry the wells. 200 µL of 33% acetic acid was then introduced into each well to resolubilize the dye attached to adherent cells, and after 10–12 minutes of incubation, OD was taken.

**III. Quantification of Biofilms:**

Biofilms were quantified by taking the optical density of each well at 590 nm on the automatic microplate reader and comparing it with the cut-off value. By using the following formula, the cut-off value (ODc) was calculated:

ODc = average OD of negative control + (3 x standard deviation of negative control)

## 3.6.2 Motility Assays:

Motility tests were carried out in sterile petri dishes using different concentrations of agar to assess the swimming (0.3%), swarming (0.5%), and twitching (1%) motility of Pseudomonas.

### 3.6.2.1: Swimming Motility:

Isolates were first refreshed by inoculating a single colony into nutrient broth and allowing it to incubate for 24 hours. Swimming motility medium was prepared by adding 0.3% agar to nutrient broth. After being autoclaved, 25 mL of media was poured into each plate. A sterile pointed toothpick was then immersed in the overnight bacterial culture and stabbed into the agar layer of the plate, but ensuring not to reach the base of the petri plates. Following 24 hours of incubation at 37 °C, plates were observed for swimming motility (de Sousa *et al.*, 2023).

### 3.6.2.2: Swarming Motility:

Like swimming motility, isolates were first cultured into nutrient broth through 24 hours incubation period. Swarming motility medium was prepared by adding 0.5% agar and 0.5% D-glucose into nutrient broth. After being autoclaved and poured the media into petri plates, bacteria were introduced into swarming media by using sterile toothpick that was dipped into overnight bacterial suspension, and a spot was placed on the surface of agar plate without being penetrating inside agar layer. Following 24 hours incubation at 37 °C, swarming motility was assessed by examining swarming pattern and surface area of turbid zone (de Sousa *et al*., 2023).

### 3.6.2.3: Twitching Motility:

Twitching motility was assessed on a 1% Luria-Bertani agar (LBA) medium, which was prepared by adding yeast extract (5 g/L), tryptone (10 g/L), gar (10 g/L), and NaCl (5 g/L) in distilled water. After being autoclaved for 15 minutes at 121°C, 10 mL of 1% LBA media was dispensed into each plate. Using a sterile toothpick, a small section from the outer periphery of a freshly streaked bacterial culture was picked and blended in a sterile agar area until a smooth bacterial culture was obtained, which was then stabbed perpendicular to the bottom of the plate, reaching the agar-glass interface. After inoculation, plates were inverted into a humidified chamber and incubated at 37°C for 48 hours. Following the incubation period, twitching motility was assessed by observing the presence of an interstitial colony of bacteria that had twitched across the plate between the agar and glass interface. To enhance the visibility of interstitial colonies, plates were flooded with TM developer solution (10% glacial acetic acid and 50% methanol) for 30 minutes, which made them appear as a distinctive white halo against the background of the agar plate (Turnbull & Whitchurch, 2014).

### 3.6.3: Hemolysin Production:

The hemolysin production assay is based on the lysis of red blood cells (RBCs). Hemolysins are proteins produced by many bacteria to obtain nutrients through the lysis of red blood cells. Three types of hemolysins are present in bacteria, i.e., alpha, beta, and gamma. β-hemolysins cause complete lysis of RBCs, α-hemolysins cause partial lysis of RBCs, and γ-hemolysins result in no lysis of RBCs.

Blood agar base (40 g/1000 mL) was prepared and autoclaved at 121°C for 15 minutes. Once the medium reached a temperature between 45 and 55°C, sterile defibrinated blood (5–10%) was aseptically introduced into the medium, and then the media was poured into plates. To evaluate hemolysin production, freshly cultured *Pseudomonas* isolates were streaked down on a blood agar plate and incubated for 24 hours at 37°C.

## 3.6.4: Phospholipase Production:

Phospholipase production in *Pseudomonas* isolates was studied using egg yolk agar assay. Egg yolk contains phospholipids, especially lecithin, which serve as a substrate for assessing the enzymatic activity of phospholipase. Phospholipase enzyme produced by bacteria enzymatic results in the hydrolysis of phospholipids, releasing fatty acids and glycerol. The released fatty acids can react with calcium ions to form insoluble calcium salts, leading to the formation of a visible zone of precipitation or clearing around the microbial colonies.

Egg yolk agar was prepared by using tryptic soya agar with 10% egg yolk suspension. After being sanitized with ethyl alcohol, eggs were broken aseptically with a sterile spoon, and their yolks were put into a sterile falcon. An equivalent volume of autoclaved distilled water was then added to the egg yolk to make a 1:1 egg yolk suspension. After centrifugation at 500 rpm for 30 min, the supernatant was added to a cooled medium of tryptic soya agar media that was separately autoclaved for 15 min at 121 °C. The media was mixed gently and poured into plates. Egg yolk agar plates were inoculated with 5 µl of bacterial suspension that was prepared with normal saline according to a 0.5 McFarland solution. After a 24 hours incubation period, the precipitation zone (Pz) was then quantified using the following formula (Çelik, 2020) protocol:

$$Pz = \text{Colony diameter} \div (\text{Colony diameter} + \text{Precipitation zone diameter})$$

## 3.6.5 Protease Production:

Skimmed milk agar assay is used to assess the proteolytic activity of *Pseudomonas*, particularly their ability to produce protease enzymes. These protease enzymes are

capable of degrading casein, a major protein in milk, resulting in the formation of clear zones around the colonies.

Skimmed milk agar was prepared by using a modified basal medium (yeast extract 2.5 g/L, peptone 5 g/L, agar 1.5 g/L, and D-glucose 1 g/L) supplemented with 2.5% skimmed milk powder. A 10% skimmed milk powder mixture was prepared separately in distilled water and sterilized by heating on a hot oven plate. This skimmed milk mixture was then poured into basal media that had already been autoclaved for 15 minutes at 121°C. After being dispensed into plates, these skimmed milk agar plates were inoculated with 5 ul of bacterial suspension that was prepared with normal saline according to a 0.5 McFarland solution. Plates were incubated at 37 °C overnight, and the zone of hydrolysis was measured.

### 3.6.6: Urease Production:

Urease production in *Pseudomonas* was determined by using Christensen urea agar media consisting of 2% urea and phenol red as a pH indicator. Urease enzymes produced by bacteria hydrolyze urea to carbon dioxide and ammonia, which increases the pH of the medium, resulting in a colour change from yellow to bright pink.

First of all, urea base was prepared by adding peptone (1 g/L), potassium phosphate monobasic (2g/L), agar (20 g/L), sodium chloride (5 g/L), dextrose (1 g/L), and phenol red (0.012 g/L) in distilled water. Urea solution was separately added to the cooled media after passing through filter paper and then immediately poured into test tubes inclined at 45 degrees to make slants. *Pseudomonas* isolates were streaked on the slant of agar, and colour change was observed after 24 hours of incubation.

### 3.6.7: Pigment Production:

Cetrimide agar is used to identify pigment-producing isolates of *Pseudomonas*. *P. aeruginosa* strains produce pyocyanin during their metabolic process, imparting a blue-green colour to colonies. Pyoverdine production is enhanced under iron-limiting conditions. Cetrimide agar promotes the manifestation of this characteristic by providing an environment with restricted iron availability.

First of all, cetrimide agar was prepared according to the manufacturer's guidelines. After being autoclaved and poured into plates, *Pseudomonas* isolates were allowed to grow on cetrimide agar by using the simple streak method. Plates were then incubated at 37 °C for 24 hours and observed for pigment production. To determine pyoverdine production, colonies of *P. aeruginosa* were exposed to UV light.

## 3.7: Optimization of Primers for the Detection of Virulence Gene *alg-D* in *Pseudomonas* Isolates

### 3.7.1: DNA Extraction using Phenol Chloroform Method:

DNA of *Pseudomonas* isolates was extracted using the phenol chloroform method based on the principle of liquid-liquid extraction. *Pseudomonas* isolates were initially inoculated into nutrient broth. After overnight incubation, the bacterial cultures were transferred into 2 mL Eppendorf tubes. The Eppendorf tubes were then centrifuged at 1000 rpm for 15 minutes to form a pellet at the bottom of the tubes, and the supernatant was discarded. The pellet was then resuspended in 450 µL of Tris-EDTA buffer (TE buffer) to maintain the stability of DNA by providing a suitable pH environment.

Following this, 45 µL of 10% sodium dodecyl sulfate (SDS) and 5 µL of proteinase kinase were then introduced into each Eppendorf to disrupt the cell membrane and denature protein. A 1:1 phenol-chloroform mixture was prepared according to sample requirements, and 500 µL of this mixture was added to each Eppendorf tube containing the lysate. After centrifugation at 400 rpm for 20 minutes, the two distinct layers were separated.

The upper aqueous phase containing DNA was then pipetted into a new Eppendorf. A 50 µL of sodium acetate (3M) and 300 µL of chilled isopropanol were then added to precipitate the DNA. Following centrifugation at 14000 rpm for 5 minutes, the supernatant was discarded, and the pellet was washed with 1 mL of 70% ethanol. Eppendorf tubes were centrifuged again at 3000 rpm for 30 sec. Ethanol was discarded, and the DNA pellet was dried over blotting paper until the ethanol was evaporated completely. The DNA pellet was then resuspended in 100 ul of TE buffer and stored at -20 $^{o}$C for future use.

## 3.7.2: DNA quantification and estimation:

Ethidium bromide fluorescence method was used to estimate the extracted DNA. This protocol is based on the principle of ethidium bromide's fluorescence emission upon intercalation with nucleotides when exposed to UV light. For the visualization of DNA products, a 1% agarose gel was prepared by adding 1 g of agarose to 100 mL of TBE (Tris-Borate-EDTA) buffer. After mixing and heating it well in the microwave for 1-2 minutes, 5 µL of ethidium bromide was added to the agarose gel to stain the DNA once it is cooled down. The mixture was then poured into a gel tray fixed with combs. The solidified gel was then placed in an electrophoresis tank, and TBE buffer was poured into it. A 2 µL of DNA was mixed with 2 µL of loading dye (bromophenol blue solution) and loaded into the wells next to DNA marker (thermos-scientific 1kb ladder). After this, the gel was allowed to run at 120 volts for 30 minutes. The DNA extracted was then visualized on agarose gel by using UV trans-illuminator and Gel documentation system.

## 3.7.3: Polymerase chain reaction

## 3.7.3.1: PCR based detection of *alg D* gene:

**a) Primers:**

After the DNA extraction, PCR amplification of *alg D* gene was accomplished by using following set of primers.

**Table 3.1: List of primers for *alg D* gene of *Pseudomonas.***

| | Sequence | Base pairs | product size |
|---|---|---|---|
| Forward primer | TTTGGTTTGGGCTATGTGG | 19 | 917 |
| Reverse primer | TGGCTGGTGATGAGATCAA | 19 | |

**b) Amplification:**

Amplification was done in autoclaved and properly labeled PCR tubes. A reaction mixture was prepared using PCR water, forward and reverse primers, and the master mix. The volume used in preparing the reaction mixture is given in Table 3.2. The reaction mixture (9 µL) along with of DNA sample (1 µL) was added to each PCR tube, and the tubes were placed carefully in the gradient PCR. Amplification was performed for 35 cycles. Each cycle is comprised of initial denaturation at 95°C for 5 minutes, final denaturation at 94°C for 30 seconds, annealing at 57◦C to 62◦C for 45 seconds, initial extension at 72°C for 45 seconds, and final extension at 72°C for 5 minutes.

**Table 3.2: Volume used for PCR reaction mixture for amplification of alg D gene of *Pseudomonas***

| Reaction components | Volume (µL) |
|---|---|
| Master mix | 5.0 |
| Forward primer | 0.2 |
| Reverse primer | 0.2 |
| PCR water | 3.6 |
| DNA | 1.0 |
| Total volume | 10.0 |

## 3.7.4 Gel Electrophoresis for the visualization of PCR products:

To analyze the amplified products, a 2% agarose gel was prepared by adding 2g of agarose to 100 mL of TBE buffer. The mixture was then boiled in a microwave oven until the gel was completely dissolved. Ethidium bromide was then added to the cooled gel to visualize the products under UV light. After being solidified and placed into the electrophoresis tank, PCR products were introduced into the gel by putting 2 µL of each into separate wells. This assembly was subjected to an electrical field of 90 for 40 minutes. The gel documentation system was then utilized to detect the presence of intended PCR products.

# Chapter 04
# Results

Fifty bacterial isolates were revived from glycerol stock into nutrient broth, and were incubated at 37◦C for 24 hours. After incubation, turbidity was observed in glass tubes, as shown in Figure 4.1.

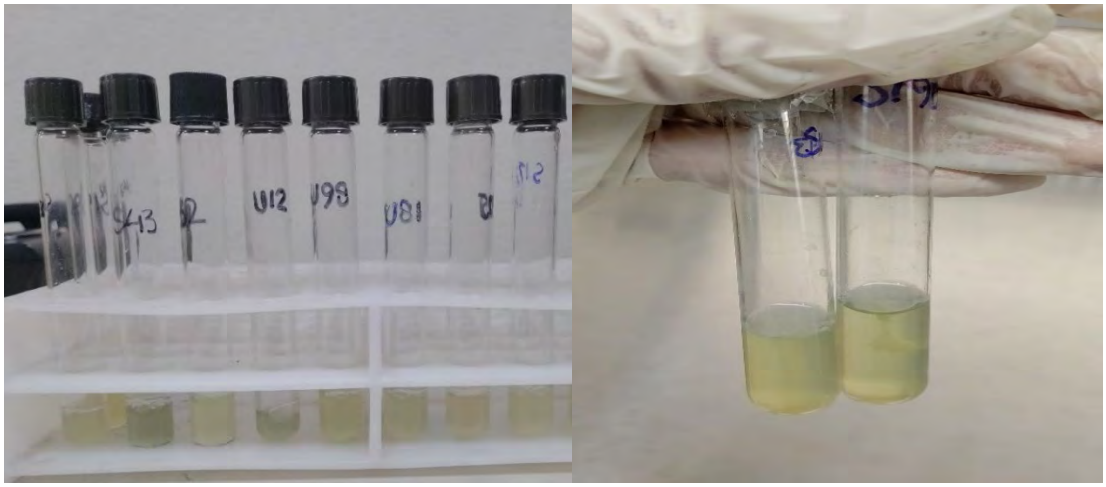


**Figure 4.1:** Nutrient broth tubes containing bacteria culture revived from Glycerol stock following 24 hours incubation period

## 4.1: Colony Morphology on Nutrient agar:

All 50 isolates were streaked on nutrient agar by the simple streak method. Following incubation, the colony morphology of the isolates was observed. A distinctive feature of *P. aeruginosa*, such as flat, translucent, large colonies with irregular spreading edges and a metallic sheen was observed in 26 isolates, while 24 isolates showed round, mucoid, smooth, convex colony morphology, as shown in Figure 4.2.

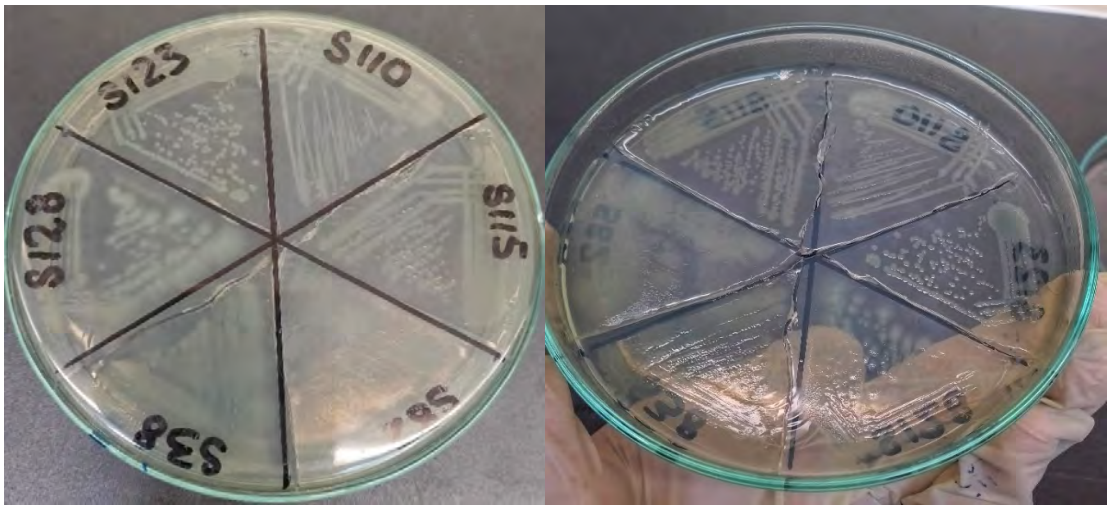



**Figure 4.2:** Colony morphology of isolates S128, S38, and S82 shows large, flat,

spready colonies on nutrient agar, while isolates S123, S110, and S115 show mucoid and convex colony morphology

## 4.2: Identification of *Pseudomonas* Isolates:

### 4.2.1: Gram Staining:

All these freshly isolated colonies from nutrient agar were subjected to Gram staining after making a smear of these isolates. Subsequently, the stained bacterial cells were examined under high-field magnification (100X) using oil immersion. All isolates were Gram-negative short rods (Figure 4.3.).

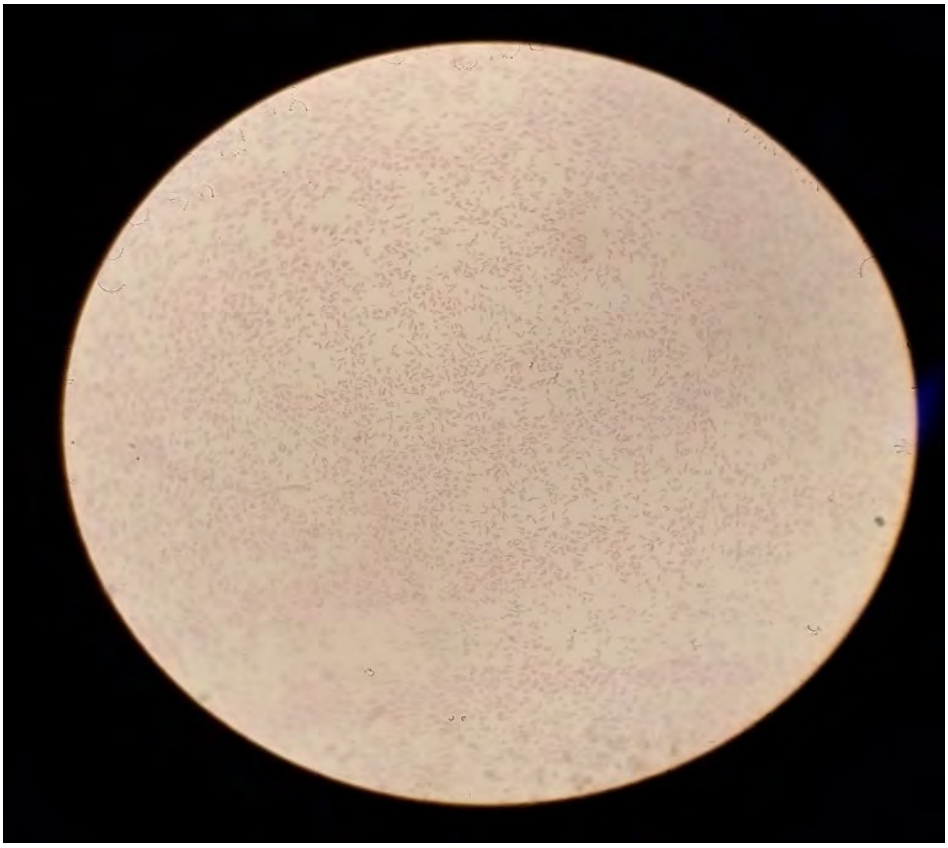


**Figure 4.3:** Gram negative coccobacillus appearance of isolate S190 after Gram Staining

### 4.2.2: Lactose Fermentation Test:

After Gram staining, these 50 isolates were streaked on MacConkey agar media to detect the lactose fermenting ability of bacteria. Lactose-fermenting bacteria produce

acid during the fermentation of lactose, leading to the formation of pink or red colonies on MacConkey agar. In contrast, non-lactose fermenters don't use lactose for fermentation, and therefore produce pale and colour less colonies on MacConkey agar. All the tested 50 isolates appear to be non-lactose fermenters (Figure 4.4), showing different colony morphology. Some isolates impart characteristics greenish colour surrounding media.

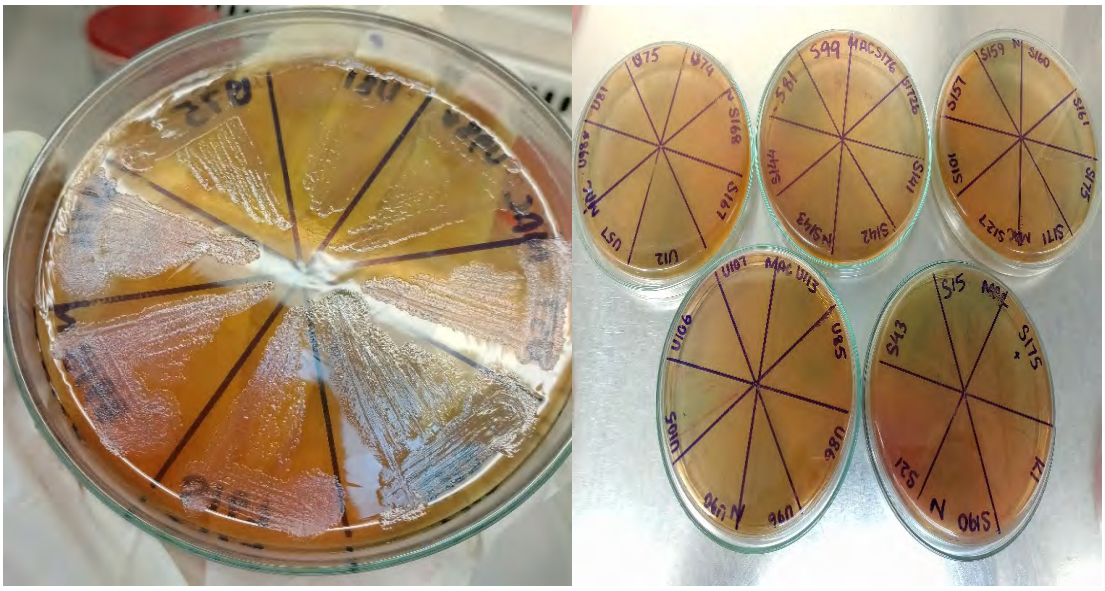


**Figure 4.4:** Isolates showing non-lactose fermenting ability as colourless colonies on MacConkey agar

## 4.2.3: Oxidase Test:

Freshly-cultured 50 isolates were exposed to an oxidase reagent on filter paper, and results were observed within a short period of time (usually 10–30 sec). The majority of bacteria exhibited an immediate, strong reaction with the oxidase reagent, while a very few showed a late oxidase-positive response. All isolates showed oxidase-positive results, as shown in Figure 4.5.

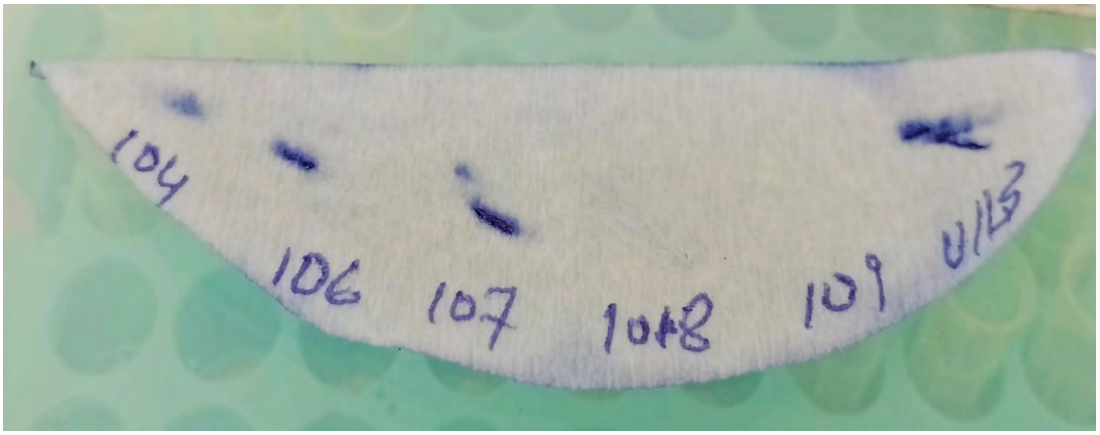



**Figure 4.5**: Isolate U106, U107 and U113 showing immediate oxidase positive reaction, *E.coli* strains (U108 and U109) were taken as negative control for Oxidase Test

## 4.2.4: Catalase Test:

A catalase test was performed on freshly isolated bacteria using a hydrogen peroxide reagent, and results were observed within a few seconds. Bubble production was formed by all of the tested isolates, interpreting their positive results for the catalase enzyme (Figure 4.6.)

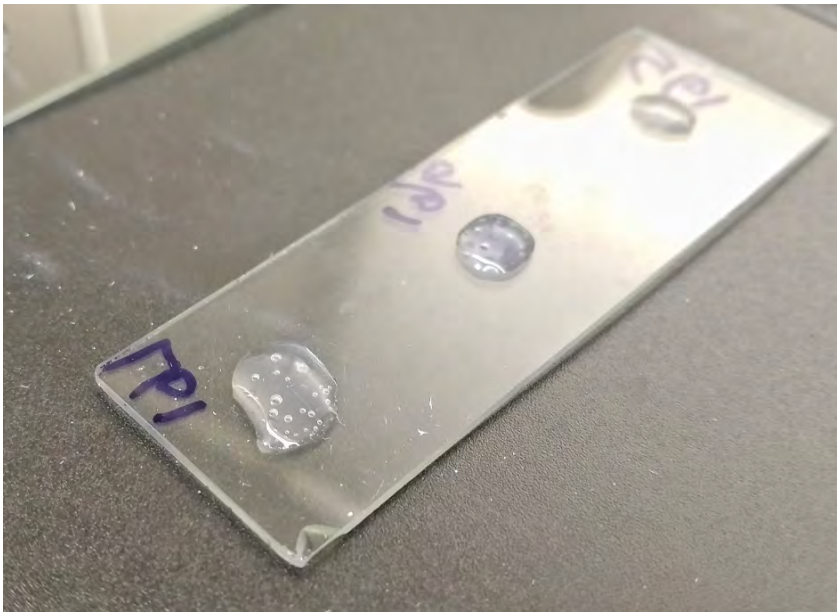


**Figure 4.6:** Isolates showing positive reaction for catalase enzymes by bubble production after reacting with catalase reagent

## 4.2.5: Sulphide Indole Motility Test:

All 50 isolates were inoculated in SIM media to detect the ability of bacteria to produce sulfur dioxide and indole, as well as their motility. All tested isolates showed negative reactions to indole and sulfur dioxide production. None of the isolates showed a black precipitate of ferrous sulfide or a distinct red colour band on addition of Kovac reagent. When carefully examined, all isolates were motile, growing around the stab line, resulting in turbidity in the medium (Figure 4.7).

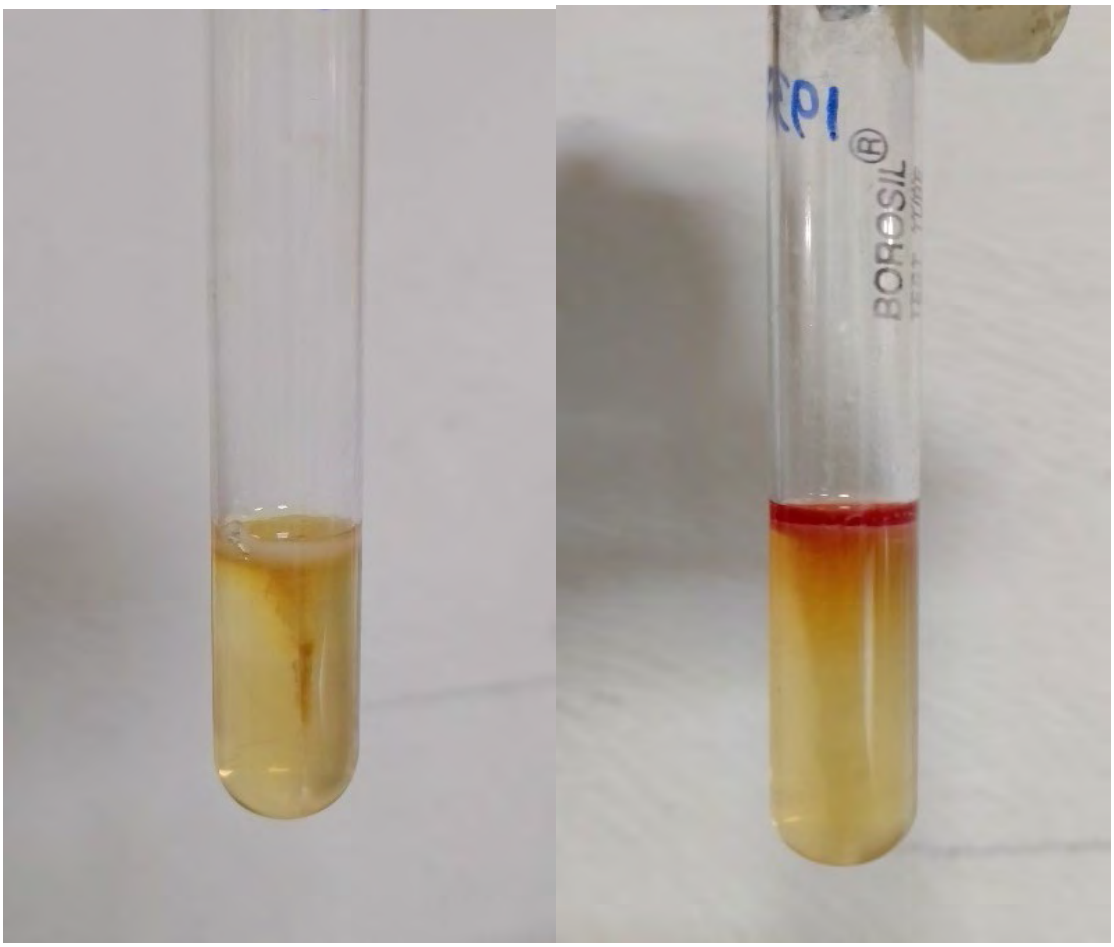



**Figure 4.7:** SIM Test (a) Isolate U62 showing motility around stab line with no reaction with Kovac's reagent (b) *E. coli* strain was taken as positive control for Indole Production

## 4.2.6: Triple Sugar Iron Test:

Isolates were inoculated in each tube of TSI media, assessing their ability to ferment different sugars with gas or $H_2S$ production. All the tested isolates showed alkaline

reactions characterized by red slant and red butt, which indicated that none of the sugars (glucose, lactose, and sucrose) present in the media was fermented by these isolates, as illustrated in Figure 4.8(a). Moreover, no bubbles, cracks, or blackening of the medium were observed in any of these isolates.

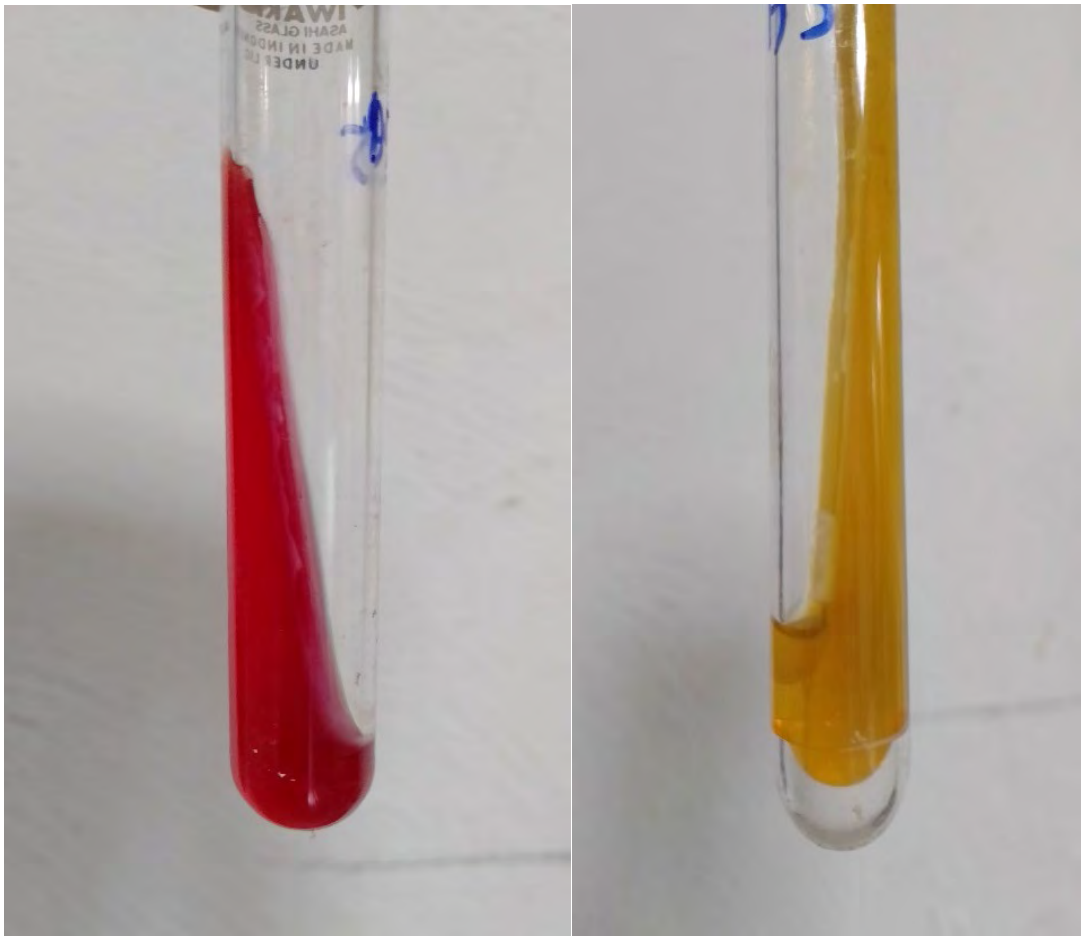


**Figure 4.8:** TSI test (a) Isolate U81 showing alkaline reaction with no sugar fermentation (b) Control strain of *E. coli* showing acidic reaction in which all the three sugars were fermented with production of gas

## 4.2.7: Citrate Utilization Test:

Following SIM and TSI tests, these isolates were cultured on Simmon citrate media to assess their ability to utilize citrate as the sole carbon source for their metabolic processes. In this agar, only those bacteria can grow well that are capable of transferring citrate from the medium into cells. Therefore, the lack of bacterium growth and the

green colour of the media are indicative of negative results. All tested isolates exhibited a positive reaction by turning the media blue, as shown in Figure 4.9(a)

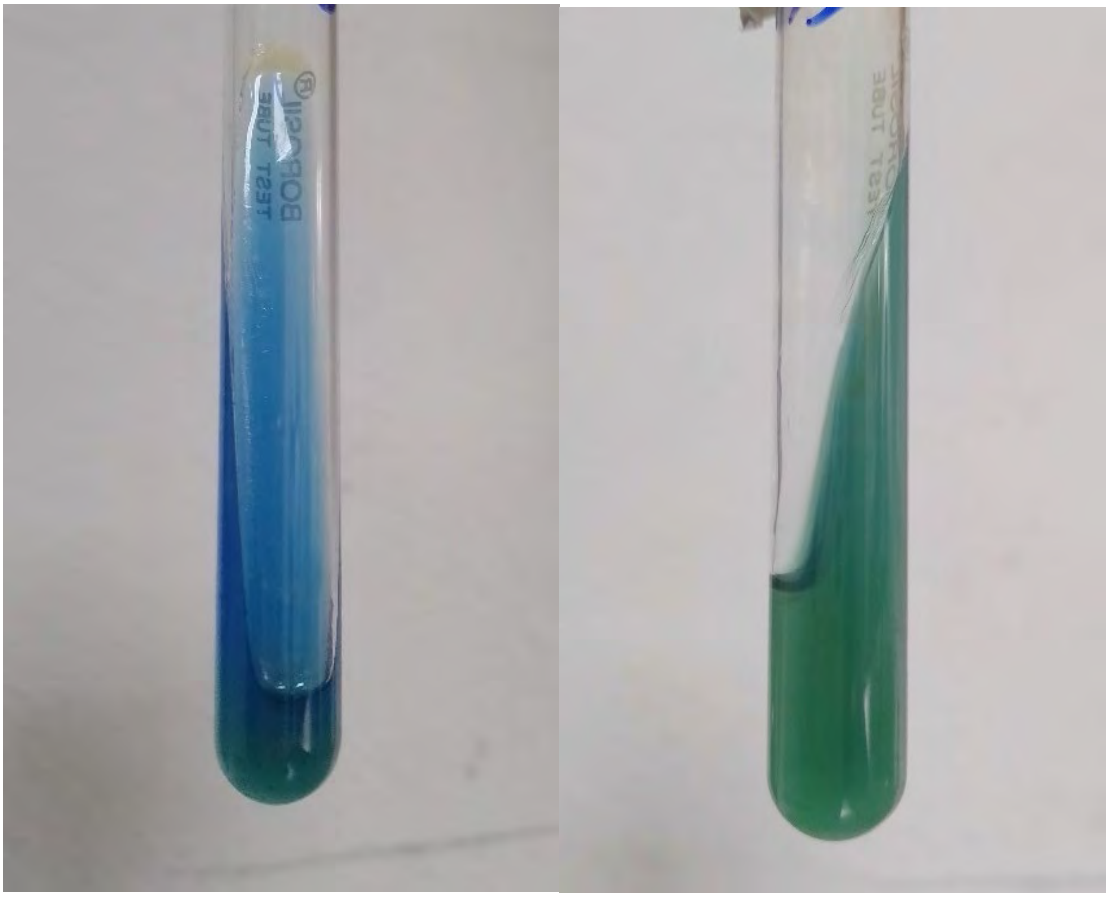


**Figure 4.9:** Citrate Utilization Test (a) S190 isolate showing Citrate Positive Reaction (b) *E. coli* strain as a negative control for citrate utilization test

## 4.2.8: Methyl Red and Vogues Proskauer Test

All the isolates were then subjected to the MR-VP test, another set of two biochemical tests commonly used in the identification of gram-negative bacteria, along with SIM, TSI and citrate tests. In a positive reaction, the respective medium turns red after the addition of reagents for both the MR and VP tests. For the VP reaction, it is important to wait for 30 minutes to 1 hour to observe the colour change. All isolates were negative for both the Methyl red and Voges-Proskauer tests (Figure 4.10 a).

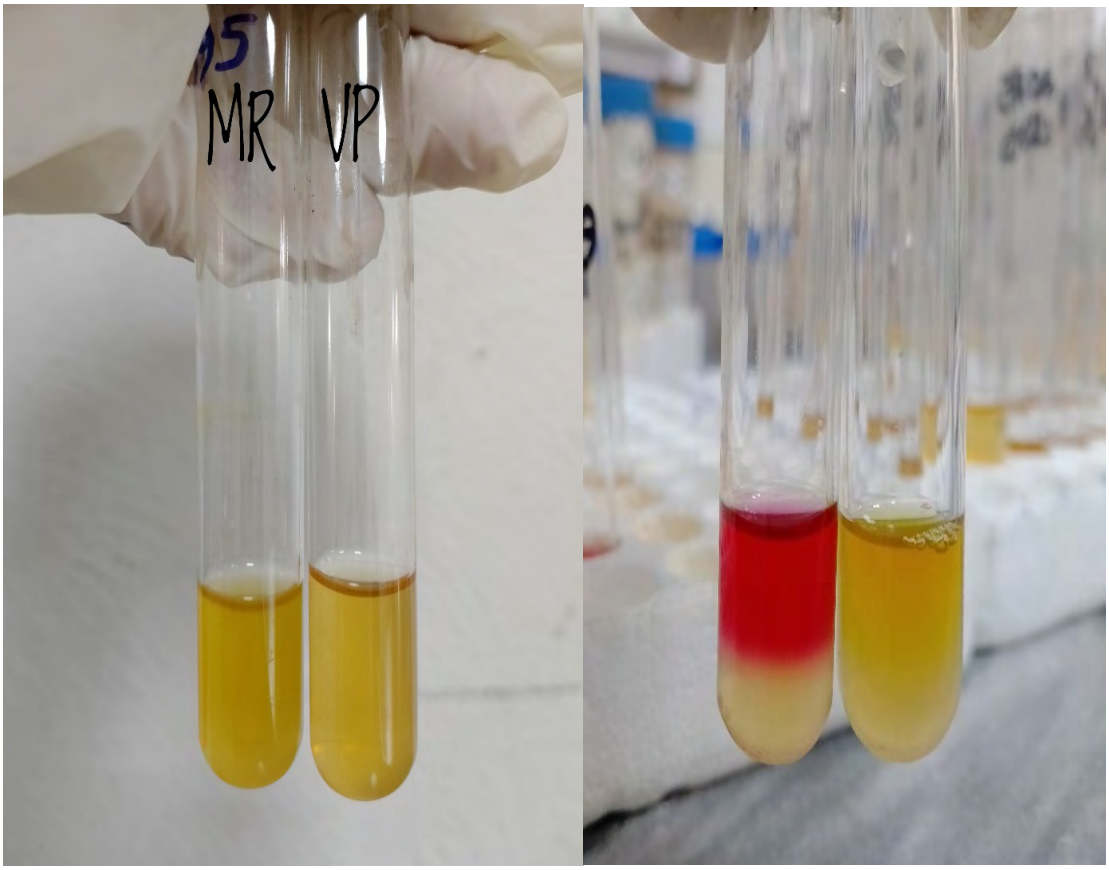



**Figure 4.10:** MR-VP test (a) Isolate S172b showing negative reaction for both MR and VP tests (b) *E. coli* Strain as a positive control for MR test

### 4.2.9: Nitrate Reduction Test:

All the isolates were inoculated into nitrate medium to assess the ability of bacteria to reduce nitrate to nitrite. Following incubation, 21 isolates produced red precipitation, as shown in Figure 4.11(a) on the addition of reagents (sulfanilic acid and α-naphthylamine), while the remaining 29 showed no discernible colour change, as shown in Figure 4.11(b). To verify if nitrate had been further reduced to a gaseous end product, zinc dust was introduced into each tube. None of the tubes changed colour with the addition of zinc, indicating all of the nitrate has been previously consumed to nitrite and subsequently to nitric oxide, nitrous oxide, and nitrogen. The absence of colour change following zinc addition also indicates a positive reaction, as illustrated in Figure 4.11(c). All the tested isolates were positive for the nitrate reduction test.

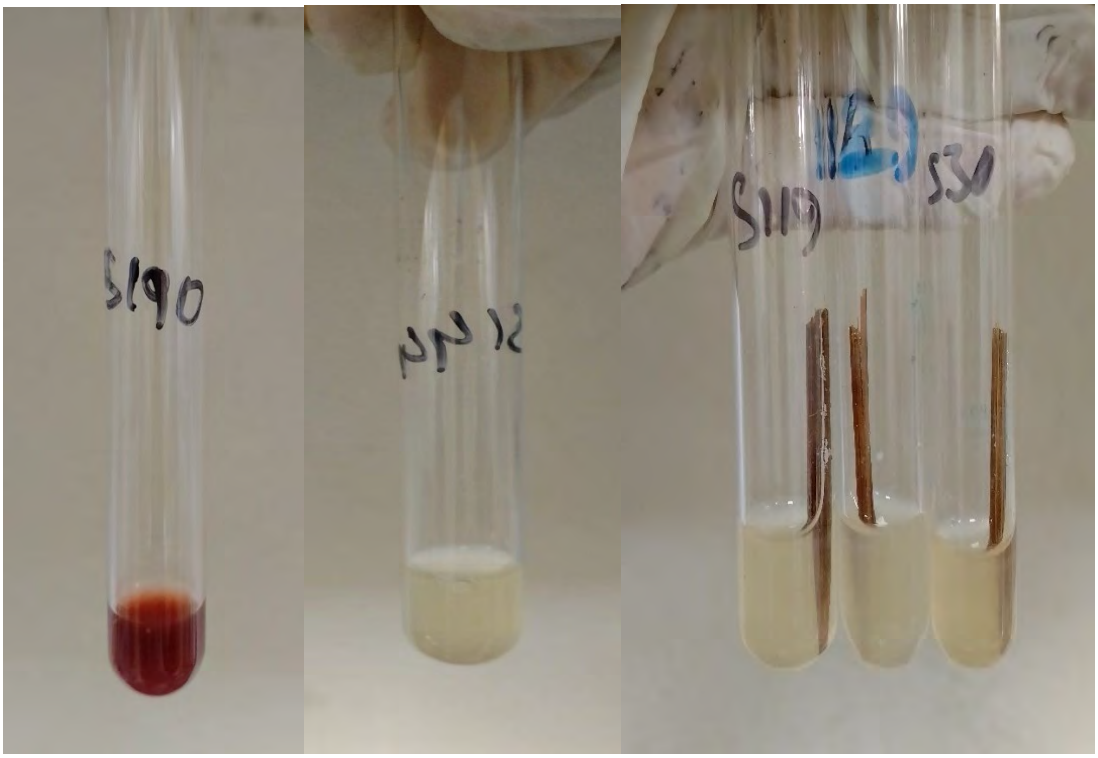



**Figure 4.11: Nitrate Reduction Test: (**a) Isolate S190 showing red precipitation on the addition of reagents (b) Isolates S144 remain colourless on addition of reagent (c) No colour change observed when zinc dust was added to negative reaction

Based on the evaluation of colony morphology, biochemical testing, and the presence of green pigmentation, these 50 bacterial isolates were categorized as *Pseudomonas aeruginosa*.

## 4.3: Phenotypical Identification of Virulence Factors in *Pseudomonas aeruginosa*:

### 4.3.1: Biofilm Formation Assays:

#### a) Congo Red Assay:

All the isolates of *Pseudomonas aeruginosa* were cultured on Congo red agar to determine the biofilm-forming ability of the bacteria. The interpretation of the Congo red assay was based on the distinctive colour of colonies. Pink or orange-coloured colonies were categorized as non-biofilm formers, while those ranging from grey to dark black were differentiated into weak, moderate and strong biofilm formers.

Out of the 50 tested isolates, the vast majority (n=43; 86%), exhibited red colonies, indicating no biofilm formation. While five isolates (10%) showed a slight colour change towards grey, showing a weak biofilm-forming potential, Furthermore, two isolates (4%) were considered moderate biofilm formers, displaying a grayish-black colour. None of the isolates was dark blackish in colour, a characteristic feature of strong biofilm formers, as shown in Figure 4.12.

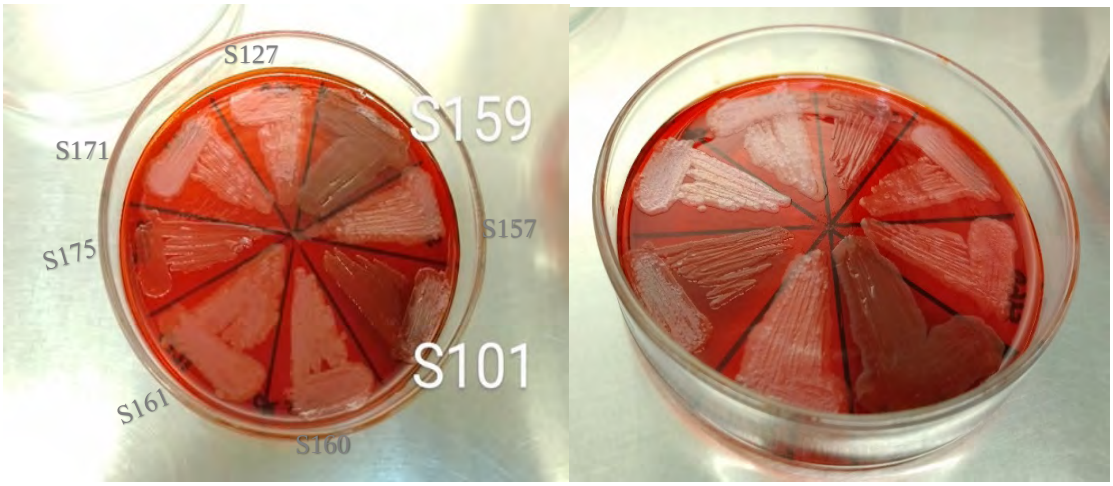



**Figure 4.12**: Isolates S159 and S101 of *P. aeruginosa* showing moderate biofilm-forming potential on Congo red agar, isolates S175 with weak biofilm formation, and isolates S127, S171, S160, S161, and S157 with no biofilm formation

### b) Microtitre Plate Assay:

All the isolates of *Pseudomonas aeruginosa* were assessed for biofilm-forming ability on 96-well microtitre plates using the crystal violet staining method as per the established protocol. A cut-off ODc was established for each plate, against which the optical density (OD) of each isolate was compared to interpret the results as described by (de Sousa et al., 2023).

- No biofilm formers if OD isolate is equal to or less than ODc
- Weak biofilm formers if OD isolate is up to 2 x ODc
- Moderate biofilm formers if OD isolate is up to 4 x ODc
- Strong biofilm formers if OD isolate is greater than 4x ODc

A total of 50 isolates were analyzed for microtiter plate assays. Among these, 14 (28%) were identified as non-biofilm formers, 23 (46%) showed weak biofilm-forming potential, 8 (16%) exhibited a moderate level of biofilm formation, and 5 (10%) were determined to be strong biofilm formers.

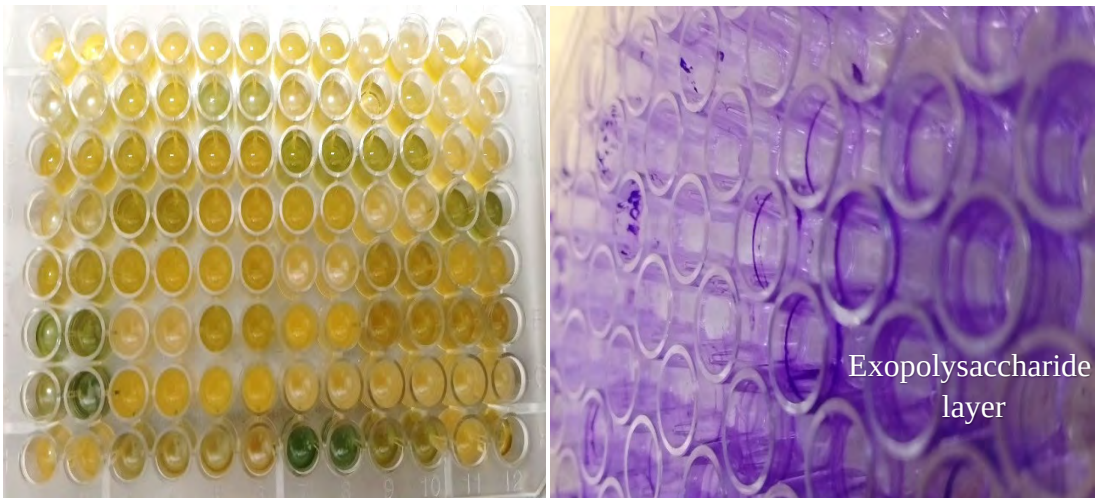
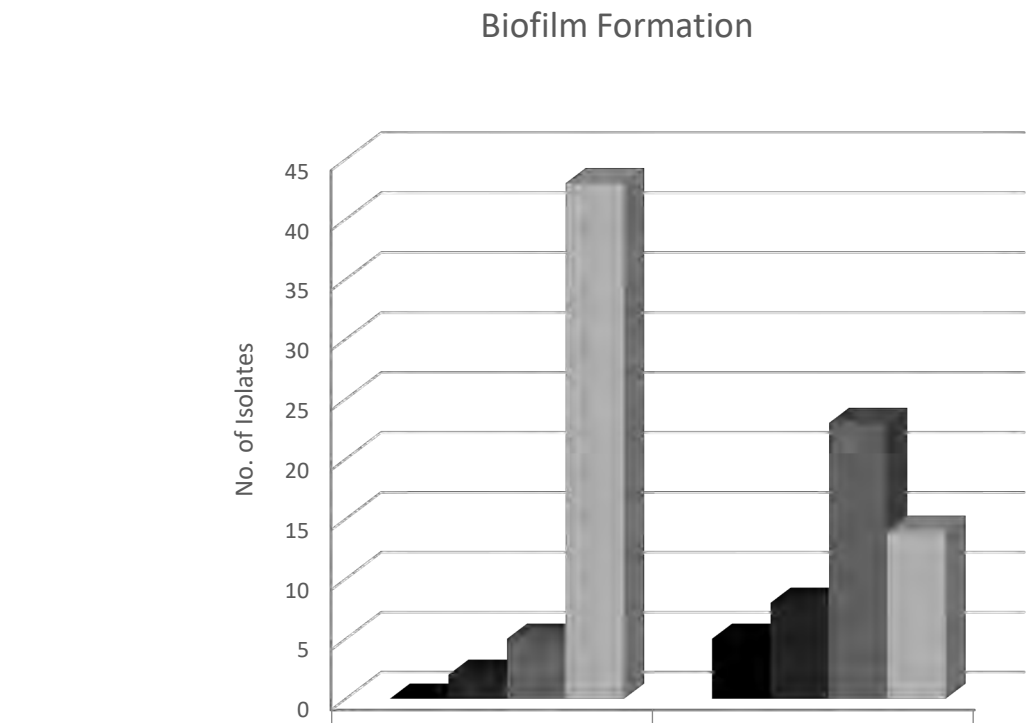



**Figure 4.13:** (a) Growth of *P. aeruginosa* isolates on microtiter plate after the incubation period of 24 hours at 37°C (b) Exopolysaccharides layer on the wall of microtiter plate after being stained with 0.1% crystal violet




| | Congo Red | Microtitre Plate |
|---|---|---|
| Strong Biofilm Formers | 0 | 5 |
| Moderate Biofilm Formers | 2 | 8 |
| Weak Biofilm Formers | 5 | 23 |
| Non Biofilm Formers | 43 | 14 |

**Figure 4.14:** Comparative analysis of biofilm forming potential of different isolates of *P. aeruginosa* using Congo red assay and Microtiter plate assay

## 4.3.2: Motility Assays:

All the confirmed *P. aeruginosa* isolates were assessed for swimming, swarming, and twitching motility by culturing them on different concentrations of agar plates. Following incubation, the diameter of the growth zone was measured in millimeters, and then the surface area of each representative isolate was calculated to determine motility characteristics. A total growth area greater than 20 $mm^2$ was considered motile. If the value was less than 20 $mm^2$, it was interpreted that bacteria were able to grow, but are non-mobile.

### a) Swimming Motility:

Fifty isolates of *P. aeruginosa* were examined for their swimming motility characteristics by inoculating them on media with a 0.3% agar concentration. Majority isolates (n=45; 90%) were positive for swimming motility, while the remaining 5 (10%) isolates did not show this characteristic, as shown in Figure 4.15 (a)(b).

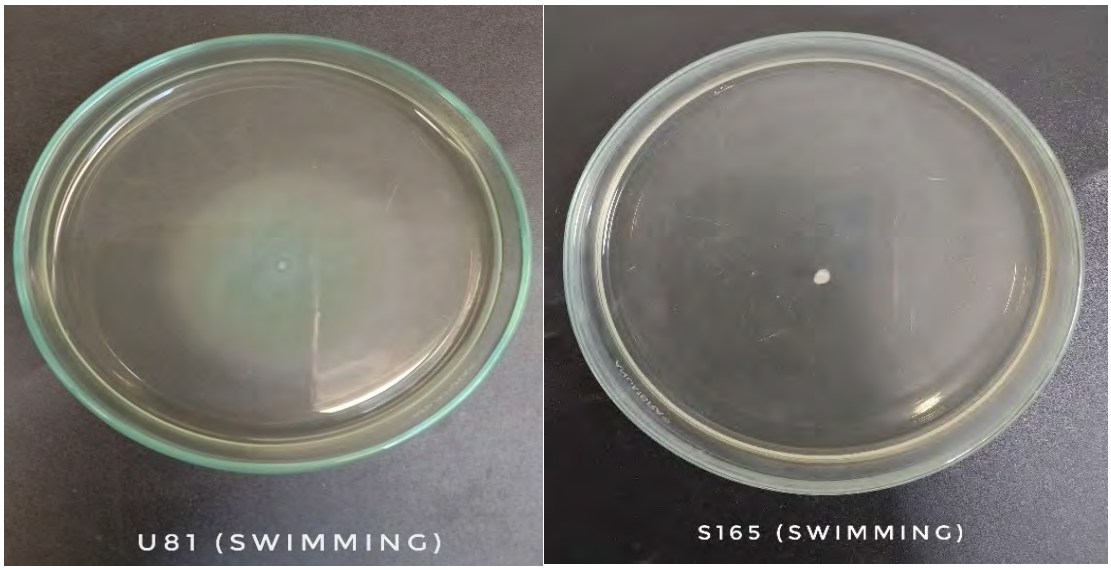



**Figure 4.15**: (a) *Pseudomonas* isolate U81 showing characteristics swimming pattern on media with 0.3% agar concentration (b) *Pseudomonas* isolates S165 showing negative result for swimming motility

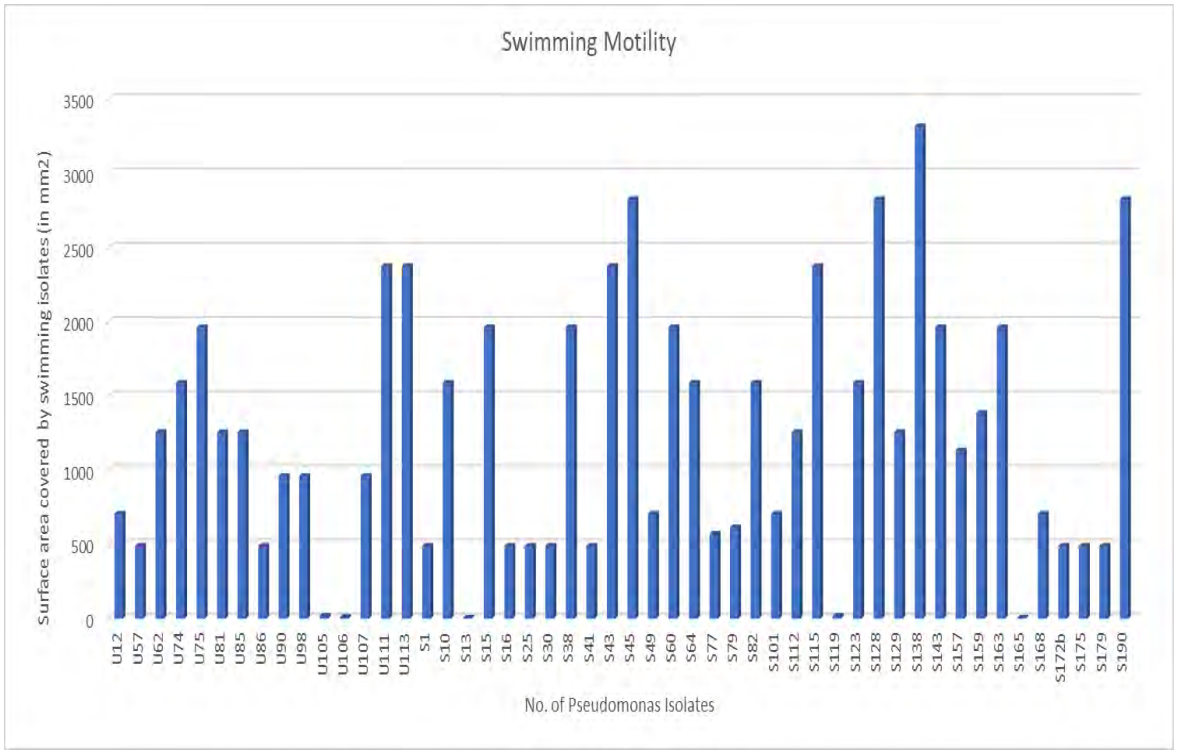


**Figure 4.16**: Surface areas (in mm²) covered by tested isolates of *P. aeruginosa* after 24 hours of incubation on swimming media (0.1% agar concentration), the majority of *P. aeruginosa* isolates represented by blue bars had a surface area greater than 20 mm², showing a swimming motility trait; however, isolates (U105, U106, S13, S119 and S165) were considered negative for swimming motility as their surface areas were less than 20 mm²

## b) Swarming Motility:

Fifty *P. aeruginosa* isolates were investigated for their swarming motility by inoculating them on media with a 0.5% agar concentration. Following 24 hours of incubation, 31 (62%) isolates were demonstrated to be positive for swarming motility as their surface area was greater than 20 mm², while 19 isolates were considered negative for this motility trait. However, the vast majority 23 (74%) of positive isolates showed a dendritic swarming pattern, as shown in Figure 4.17 (a).

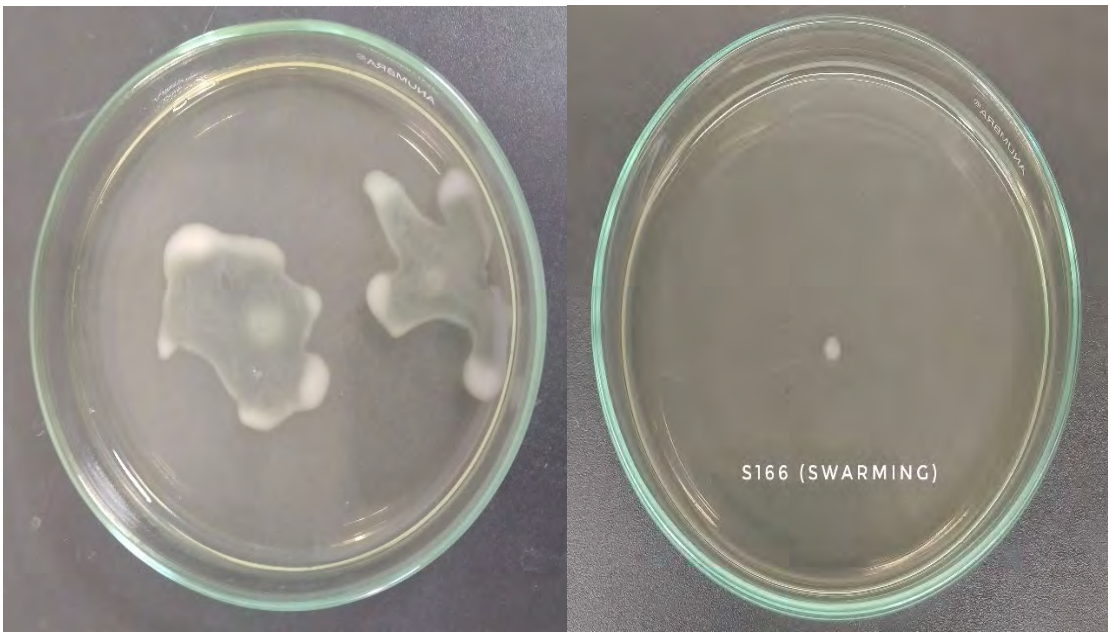



**Figure 4.17**: (a) *P. aeruginosa* isolate S128 showing dendritic swarming pattern on media with 0.5% agar concentration (b) Isolate S166 showing negative result for swarming motility

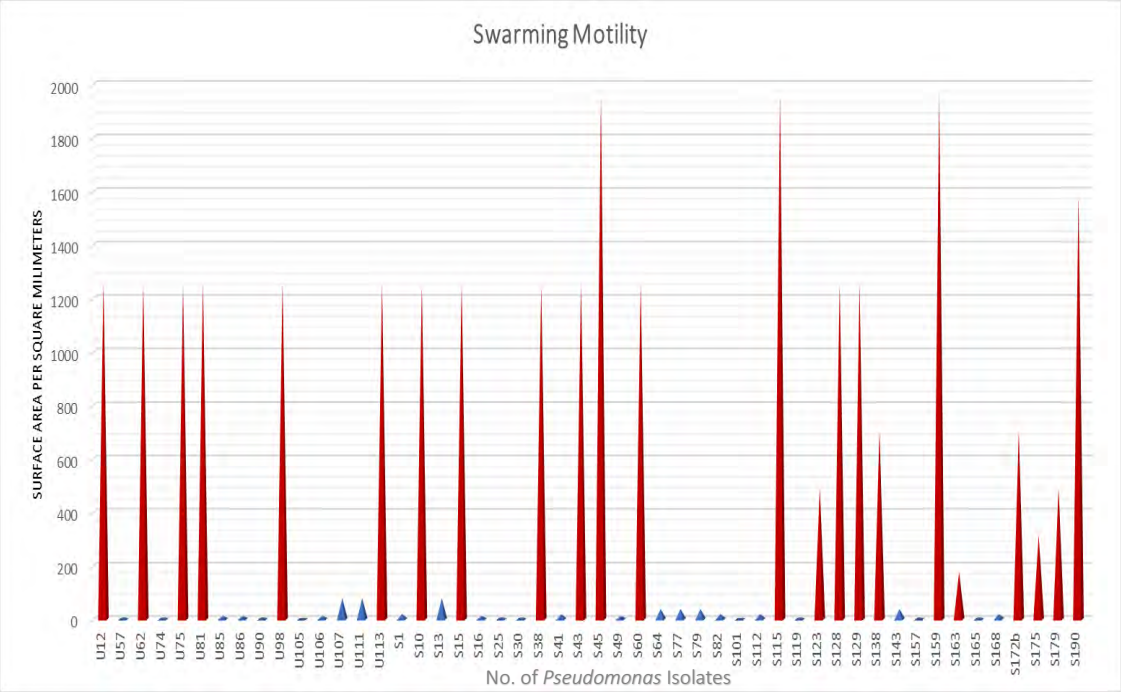



**Figure 4.18:** Surface areas covered by *P. aeruginosa* isolates after 24 hours of incubation on swarming media (0.3% agar concentration), all the isolates represented by red bars exhibited a dendritic swarming pattern, covering a significant surface area. Isolate U107, U111, S13, S64, S77, S79, and S43 showed swarming motility as their surface area was greater than 20 mm², but they didn't exhibit a dendritic swarming pattern

## c) Twitching Motility:

All these *P. aeruginosa* isolates were then assessed for their twitching motility by inoculating them on media with a 1% agar concentration. Following incubation and flooding with TM developer, plates were inspected for the presence of interstitial colonies at the agar-glass interface. Out of 50 isolates, 41 (82%) demonstrated positive twitching, which was characterized by the presence of an interstitial colony, as shown in Figure 4.19(a). The remaining 9 (18%) isolates showed a negative result for twitching motility. (Figure 4.19).

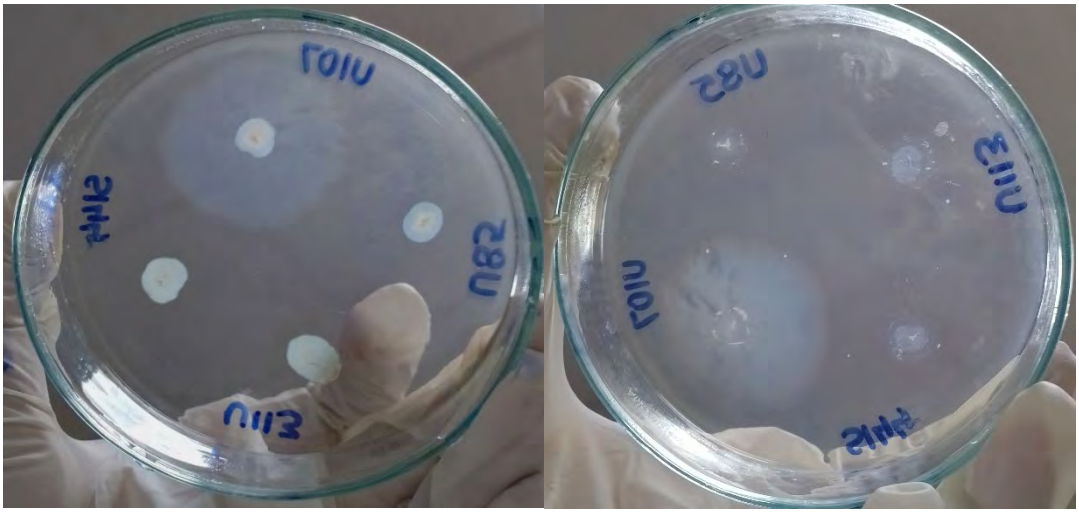


**Figure 4.19:** (a) 1% LBA media plate before flooding with TM developer; isolate U107 showing clear interstitial colony when light was shinned upon growth (b) 1% LBA media plate after being flooded with TM developer solution to enhance visualization of interstitial colonies; isolates U113 and S144 also show small interstitial colony on agar-glass interface, and were considered positive for weak twitching motility. However, no interstitial colony of isolate U85 indicated negative twitching motility

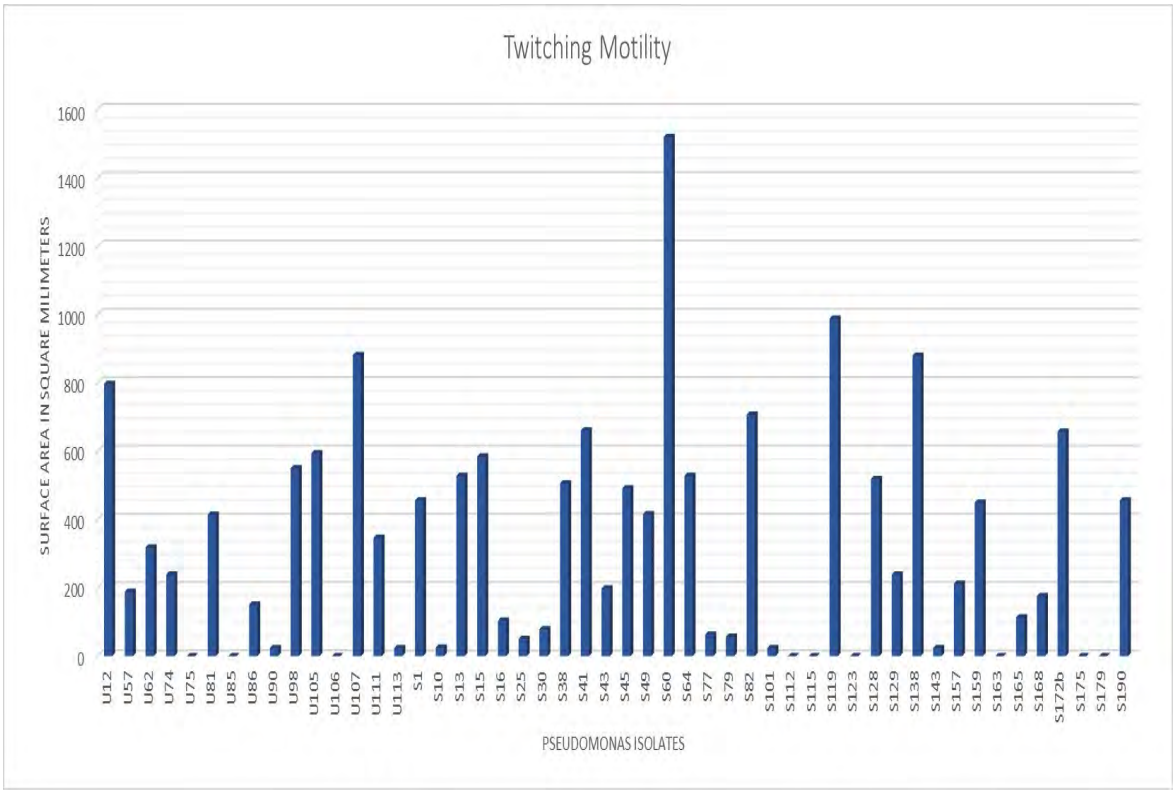


**Figure 4.20:** Surface areas of interstitial colonies (in mm²) on twitching media with 1% agar concentration. *Pseudomonas* isolates U75, U85, U106, S112, S115, S123, S163, S175 and S179 showed negative results for the presence of twitching motility.

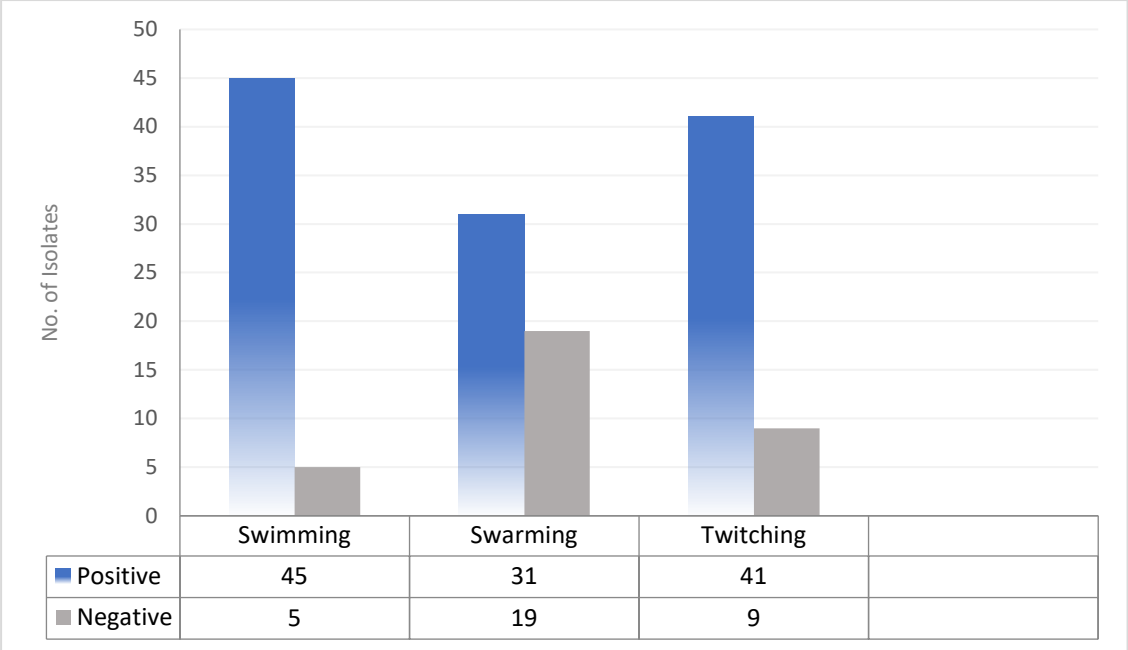



**Figure 4.21**: Comparative analysis of swimming, swarming and twitching motility in isolates of *P. aeruginosa*. The swimming motility was the most predominant motility trait observed in 90% of *Pseudomonas* isolates, followed by twitching motility detected in 82% of isolates, whereas 62% isolates were demonstrated to be positive for swarming motility as their surface area was greater than 20mm²

## 4.3.3: Hemolysin Production:

All 50 isolates were streaked on blood agar media to check the hemolysin production ability of *Pseudomonas aeruginosa.* Complete lysis of RBCs that indicates a clear zone around the colony is considered beta hemolysis. In alpha hemolysin, partially lysed RBCs create a greenish zone around the colonies due to the reduction of hemoglobin to methemoglobin, while gamma hemolysins show no zone around the colony. 49 out of the 50 tested isolates produced beta hemolysis as shown in Figure 4.22(a), indicating the production of active hemolysins. However, there was one isolate that showed gamma hemolysis, indicating no hemolysin production.

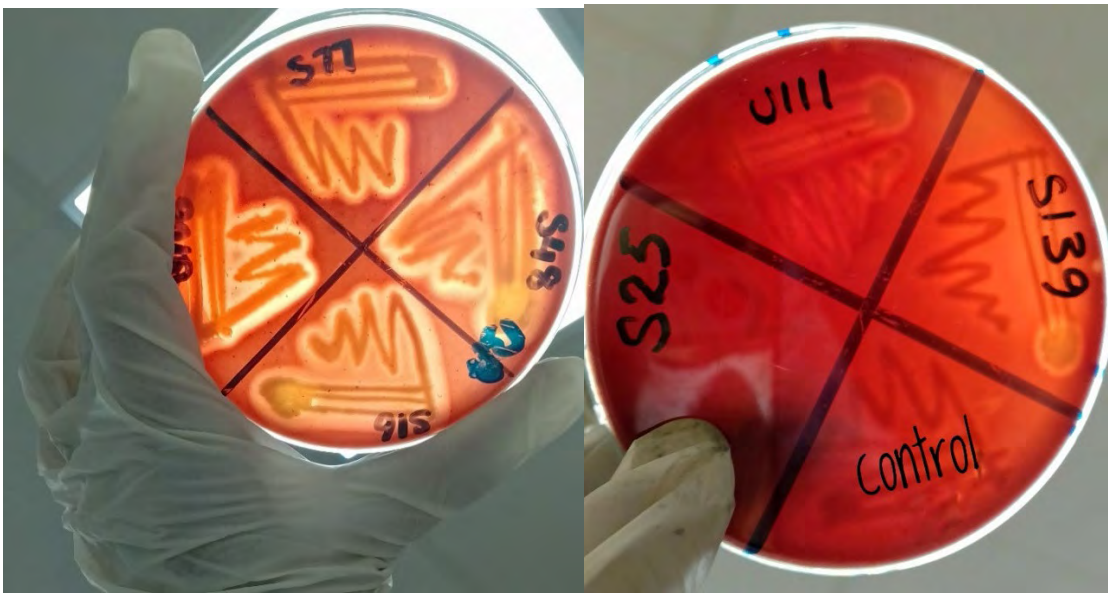



**Figure 4.22:** (a) *P. aeruginosa* isolates S48, S16, S77, S106 showing beta hemolysis on Blood agar media (b) *P. aeruginosa* isolate S25 is negative for hemolysin production

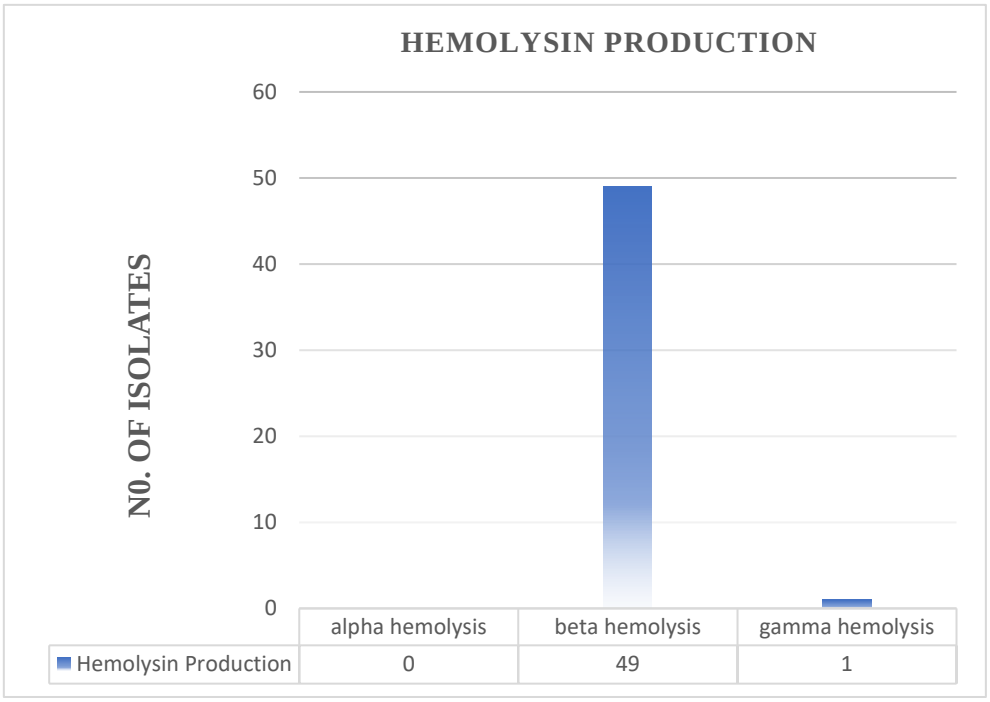



**Figure 4.23:** Distribution of *P. aeruginosa* isolates based on hemolysis potential

## 4.3.4: Phospholipase Production:

All isolates of *P. aeruginosa* were inoculated on egg yolk agar to assess their ability of producing phospholipase C enzyme. Following incubation, petri dishes were reviewed for halo zones, and results were interpreted according to the method described by Çelik, (2020). The ratio of colony diameter to the total diameter including the halo zone, was measured to find the value of the phospholipase zone (Pz value). Bacteria were considered phospholipase producers if their determined value is below 1.00. All the tested isolates of *P. aeruginosa* were positive for phospholipase production, as shown in Figure 4.24.

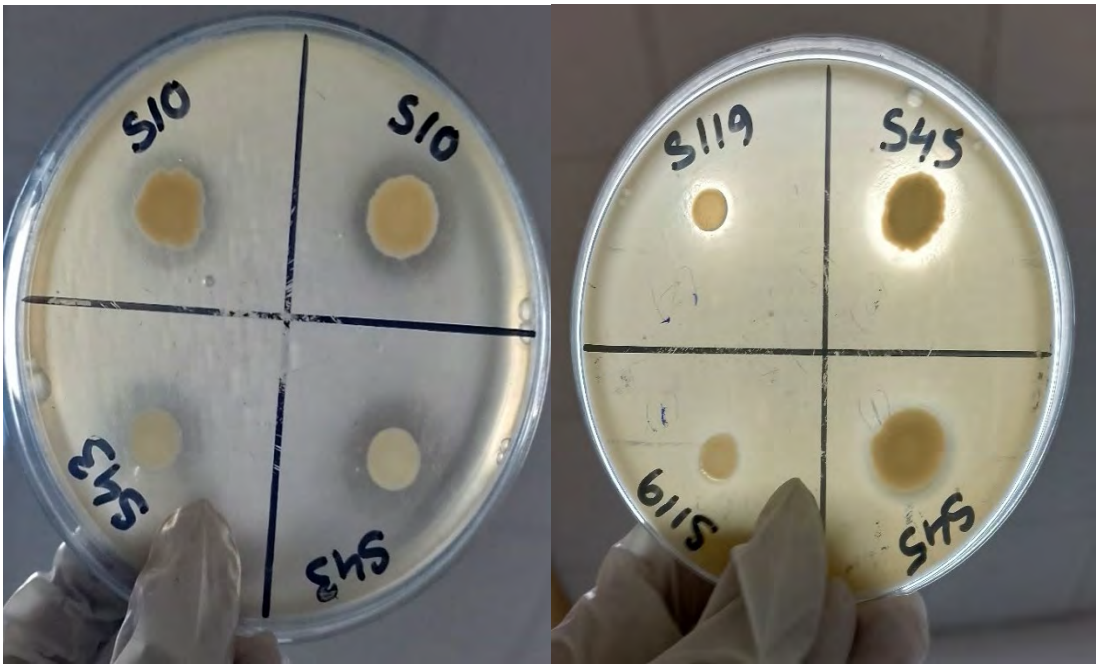



**Figure 4.24:** (a) *P. aeruginosa* isolates S10, S43 showing halo zones around colonies on Egg Yolk agar, indicating phospholipase activity (b) S119 is weak phospholipase producer while S45 is considered as strong

## 4.3.5: Protease Production:

All the *P. aeruginosa* isolates were inoculated on skimmed milk agar to access their protease activity. Following 24-hours incubation period, the presence of a halo zone around the colonies was interpreted as a positive result, and the absence of this zone as a negative result, as interpreted by Silva et al., 2021. Out of the tested 50 isolates, 48 (96%) were found to produce proteases as shown in Figure 4.25.


**Figure 4.25:** (a) *P. aeruginosa* isolates showing halo zones around colonies on skimmed milk agar indicating protease activity (b) Isolate S119 showing negative result for phospholipase production.

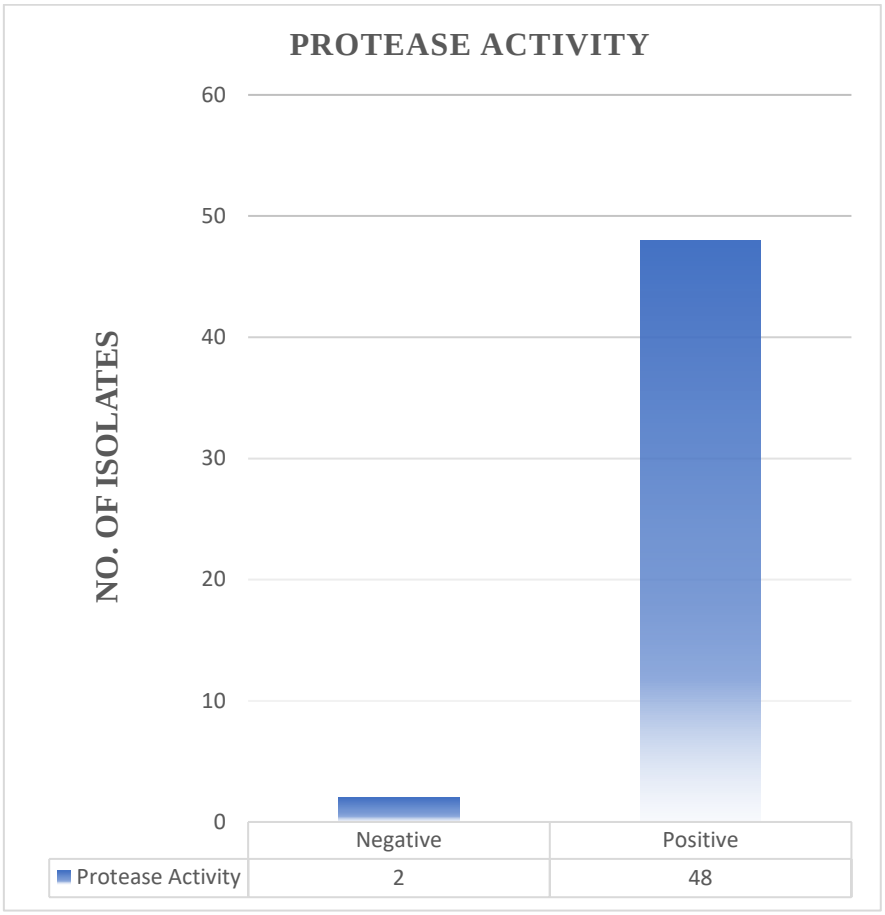



**Figure 4.26:** Distribution of *P. aeruginosa* isolates based on protease activity

## 4.3.5: Urease Production:

All the isolates were then streaked on urea agar tubes to assess the urease production ability of *P. aeruginosa* isolates. Following the 24-hour incubation period, all the tubes were carefully examined for the change in colour of the media from yellow to bright pink. All 50 tested isolates (100%) observed this distinct colour change, indicating urease production, as illustrated in Figure 4.27(a)

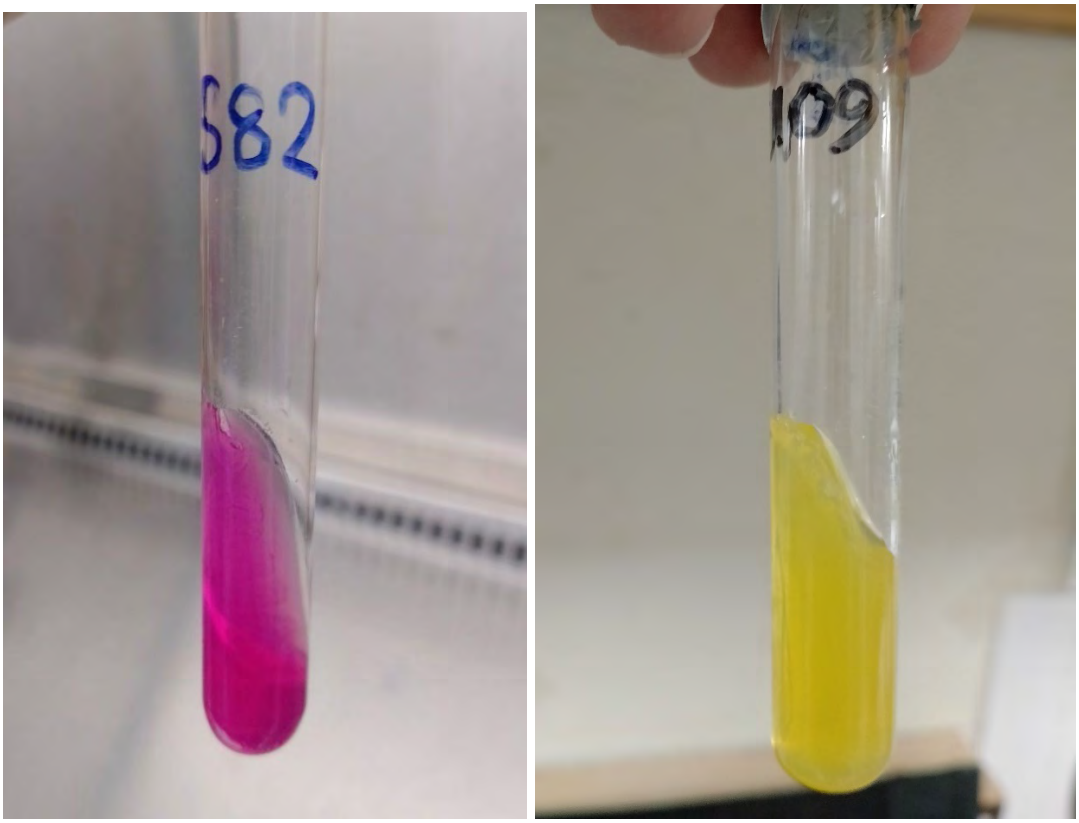



**Figure 4.27:** (a) *P. aeruginosa* isolate S82 showing positive result for urease production (b) *E. coli* U109 taken as negative control for urease production.

## 4.3.6: Pigment Production:

All the isolates of *P. aeruginosa* were streaked on Cetrimide agar to visualize their pigment production and incubated at 37°C for 24 hours. Pigment production was evaluated by observing the colour manifestations of each pigment in the cetrimide agar. Pyocyanin is blue-green in colour; pyoverdine is yellow-green, pyorubin is a reddish pigment; and black to brown pigmentation is indicative of pyomelanin. Moreover, the

production of pyoverdine was confirmed when the cultures emitted fluorescence upon exposure to ultraviolet light.

All the isolates were positive for pyoverdine production (Figure 4.28 a), while 41 (82%) isolates produced pyocyanin pigment, as shown in Figure 4.28(b). Pyorubin and pyomelanin pigments were not observed in any of the observed isolates.

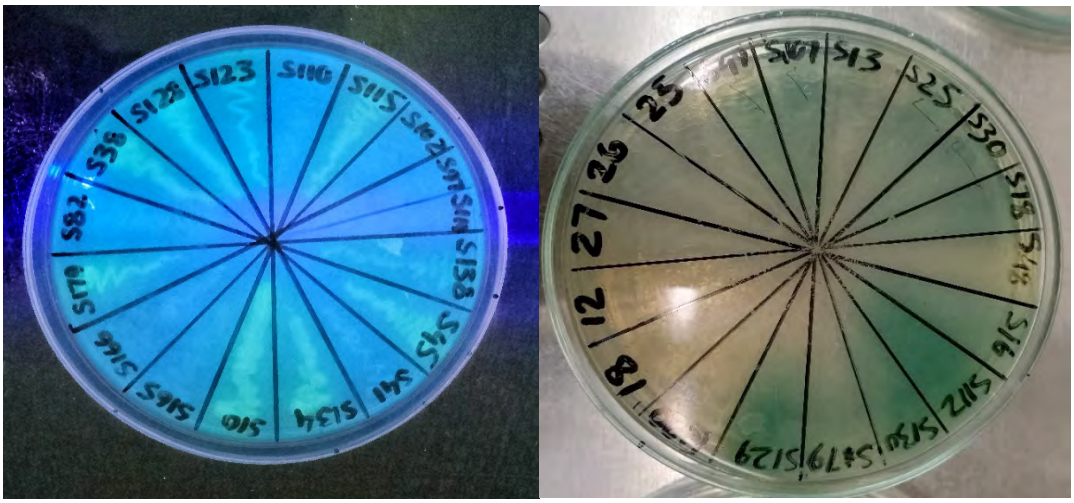


**Figure 4.28:** (a) On Cetrimide agar, isolates of *Pseudomonas aeruginosa* exhibiting fluorescence on UV exposure (b) Green Pigmentation (Pyocyanin production) on Cetrimide agar

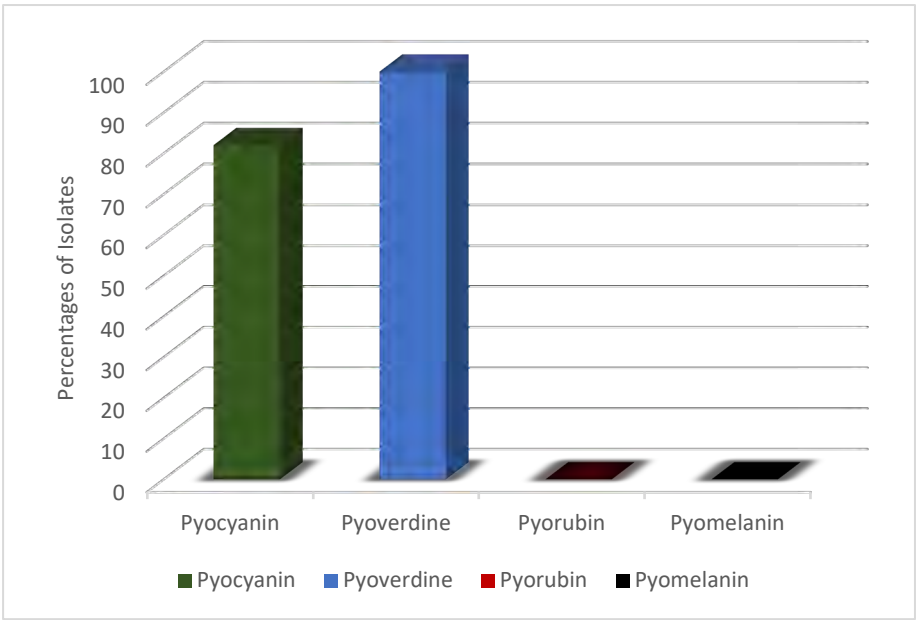



**Figure 4.28:** Percentage distribution of *P. aeruginosa* isolates based on pigments production

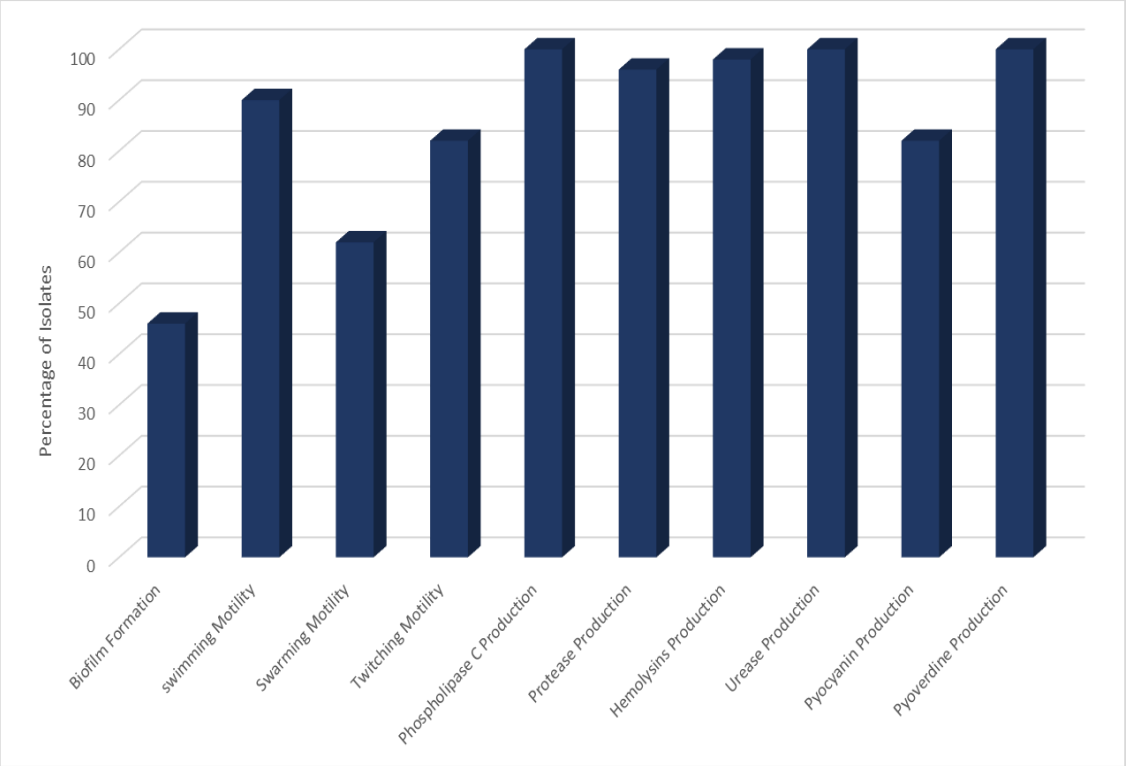



**Figure 4.29**: Percentage Distribution of *Pseudomonas aeruginosa* isolates based on various virulence factors

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## 4.4: Optimization of PCR conditions for the Detection of Virulence gene *alg*-D in *P. aeruginosa:*

After DNA extraction and amplification of the *alg D* gene using a gradient PCR machine with six different temperatures i.e., 57°C, 58.6°C, 59.2°C, 60.4°C, 61.1°C, 62°C, PCR products were visualized on a gel documentation system following gel electrophoresis. Different bands appeared at the expected product size of 917 base pairs, as illustrated in Figure 4.30. The strong band was observed at an annealing temperature of 62°C. Therefore, 62°C is identified as the optimal annealing temperature for amplifying the *alg*-D gene. Further details regarding the optimized PCR conditions are shown in Table 4.1.

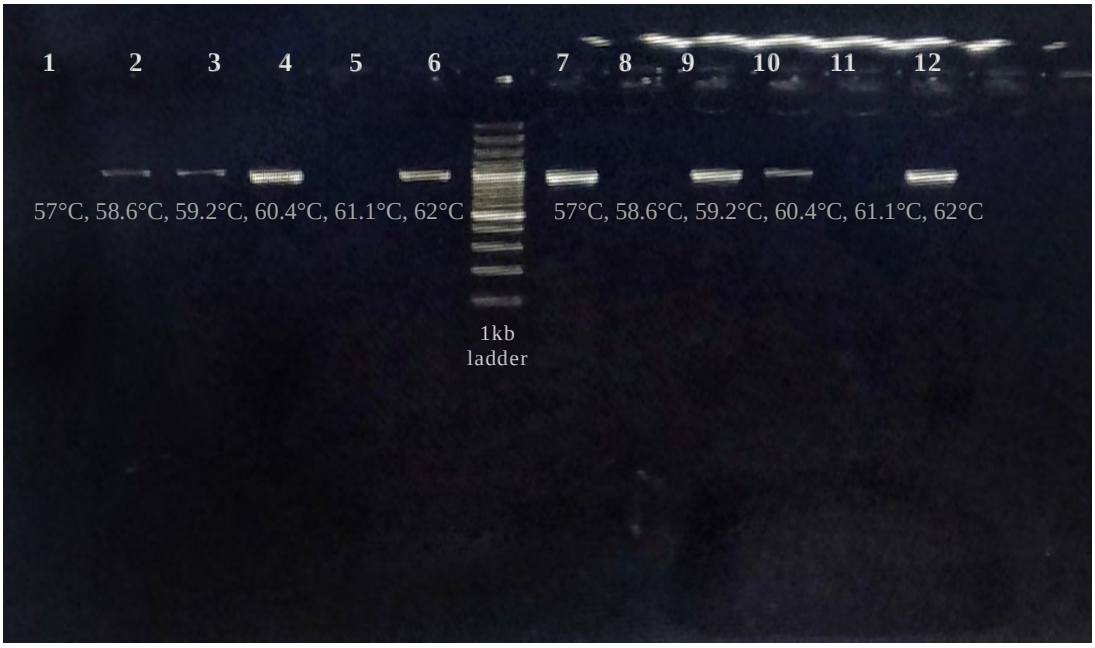


**Figure 4.30:** Amplified PCR product *alg*-D gene from *Pseudomonas* isolates. Lanes 1 to 6 show the PCR product for the isolate S101 at varying annealing temperatures ranging from 57°C to 62°C. Similarly, PCR results for the *alg*-D gene of *Pseudomonas* isolate S159 are shown in lane 7 to 12.

**Table 4.1: Optimized PCR Conditions for the amplification of *alg D* gene**

| | Initial Denaturation | Final Denaturation | Annealing | Initial Extension | Final Extension |
|---|---|---|---|---|---|
| Temperature | 94°C | 94°C | 62°C | 72°C | 72°C |
| Time | 5 min | 30 sec | 45 sec | 45 sec | 5 min |

# Chapter 05

# Discussion

## Discussion:

UTI is a widespread global public health concern, affecting diverse age groups and populations. There were various variants and classes of UTIs, usually based on symptoms, location in the urinary tract, and related risk factors. In Pakistan, like other countries, UTIs is a significant burden with notable prevalence, impacting both community and hospital settings. A recent study conducted in Swabi, Pakistan by Khatoon *et al.*, (2023) reported that 65% of suspected cases of UTIs are positive for bacterial growth, with Gram-negative bacteria being the most prevalent.

*Pseudomonas* emerges as a significant contributor to urinary tract infections, with the reported global prevalence ranging from 7–12% (Sathe *et al*., 2023). In Pakistan, a study conducted by Ahmed *et al.,* (2022) reported *Pseudomonas aeruginosa* as the second most prevalent uropathogen (12.8%) after *E. coli*. This study was conducted to characterize the virulence hallmark of UTI isolates of *Pseudomonas* to understand the role of various factors in development of urinary tract infections.

In current study, total 50 pre-isolated Gram-negative bacteria associated with urinary tract infections were revived from glycerol stock and were subjected to the biochemical testing according to the Berge's Manual of Determinative Microbiology. All the revived isolates were Gram-negative rods positive for the oxidase enzyme, a characteristic feature that differentiates *Pseudomonas* species from other members of the *Enterobacteriaceae* family. Oxidase-positive isolates were then assessed for their ability to ferment sugar in TSI media, they exhibited an alkaline reaction (red/red). Additional biochemical tests, such as citrate positive, catalase positive, indole negative, $H_2S$ negative, and MR-VP negative, supported the identification of these isolates as *Pseudomonas* species. The same methodology was also followed by a study conducted in Iraq by Midhat *et al.,* (2023) and Hussein *et al*., (2023) to differentiate of *Pseudomonas* isolates from other Gram-negative bacteria.

*Pseudomonas aeruginosa* is one of the most common and clinically significant uropathogen among *Pseudomonas* species. In the present study, all *Pseudomonas* isolates were categorized as *P. aeruginosa* on the basis of pigment production, fluorescein production, as well as positive results in the nitrate reduction test. All isolate were confirmed as *Pseudomonas aeruginosa,* no species was detected and confirmed.

A similar study conducted in India by Kl *et al*. (2017) reported *P. aeruginosa* as the primary uropathogen among *Pseudomonas* species, accounting for 84% of documented cases. However, *Pseudomonas putida*, has also been identified in 16% of *Pseudomonas*-associated UTIs in the same study. Various earlier researches conducted are also in accordance with current study indicating *P. aeruginosa* as a major uropathogen (Pobiega *et al*., 2016; Ullah *et al*., 2018; Abate *et al*., 2020).

Biofilm formation, diverse motility traits (swimming, swarming, and twitching), extracellular enzyme production such as urease, protease, and phospholipase, as well as pigments produced by *Pseudomonas aeruginosa*, contribute to the pathogenicity of urinary tract infections. In the present study, the phenotypic identification of these virulence factors was assessed through virulence assays. The biofilm-forming potential of *P. aeruginosa* associated with UTI was evaluated both by qualitative and quantitative methods using the Congo red assay and the microtitre plate assay, respectively. Hemolysin production was examined on blood agar plates by observing the lysis of red blood cells. Protease production was observed on skimmed milk agar through the degradation of casein. The assessment of phospholipase C activity was evaluated on egg yolk agar, while urease production was noted on Christensen urea agar media. Various motility traits of bacteria, including swimming, swarming, and twitching, were assessed by preparing motility media with different concentrations of agar. Moreover, pigment production was visualized on cetrimide agar, which serves as a specific medium for this purpose. Similar virulence factors of *P. aeruginosa* were studied in previously conducted studies in Portugal by de Sousa *et al.*, (2023), in Egypt by Edward *et al*., (2023), in Venezuela by Rodulfo *et al*., (2019), and in the USA by Murray *et al.*, (2010).

The biofilm producing ability of *Pseudomonas* makes them more resistant to antibiotics and less affected by immune mechanism of the host. Biofilm can be formed either on biotic and abiotic surfaces. In this investigation, biofilm ability of *Pseudomonas aeruginosa* was observed through qualitative and quantitative method following described protocol. In the qualitative assessment, the majority of isolates (86%), were identified as non-biofilm producers, while 10% were categorized as weak biofilm producers. A small proportion (4%) exhibited a moderate biofilm-producing phenotype showing dark grey colour. In contrast, in the quantitative assessment using a microtiter

plate, a notable variation in results emerged, revealing 28% of isolates as non-biofilm formers, 46% exhibiting a weak biofilm-forming potential, 16% demonstrating a moderate level of biofilm formation, and 10% displaying a strong biofilm-forming ability. This divergence in results can be attributed to the sensitivity and specificity limitations of the Congo red assay, which relies on visual assessment and may categorize isolates differently, leading to disparities in the classification of biofilm-producing abilities. The observed variability in this study results finds support in a study conducted by Lima *et al*. (2017), where only 15% of *Pseudomonas aeruginosa* isolates were identified as biofilm producers through a qualitative approach. However, the number increased up to 75% when their biofilm-forming ability was assessed by the quantitative microtiter plate method, which measures the actual biomass of exopolysaccharide produced and offers an accurate quantification of biofilm production.

In current study, a high percentage (72%) of *P. aeruginosa* isolates exhibited positive biofilm formation potential. A study conducted in Egypt, 89.4% *Pseudomonas* isolates were biofilm formers with 51.6% classified as weak, 35.5% as moderate, and 12.9% as strong biofilm formers (Edward *et al*., 2023). Interestingly present study also depicted the lower percentage of strong biofilm formers compared to moderate and weak. Another study, particularly focusing on UTI isolates of *P. aeruginosa,* demonstrated that 92% of the isolates were biofilm producer (de Sousa *et al.,* 2023*)* This emphasizes the importance of *P. aeruginosa* biofilms forming potential in the development of urinary tract infections. A notable variation in results has been observed across different studies, suggesting potential influences from geographical and clinical factors. Studies conducted by Rodulfo *et al.,* (2019) and Naik *et al.,* (2021) reported relatively low percentages of biofilm production by *P. aeruginosa,* accounting for 40% and 48% in Venezuela and India respectively.

The type of motility displayed by *Pseudomonas aeruginosa* is considered important virulence hallmarks of bacteria in the development of infections, as it enables bacteria to move and colonize diverse environments. A comprehensive understanding of the motility characteristics of *Pseudomonas* isolates is crucial for assessing their potential to cause infections. The prevalence of these motility traits may contribute to the overall virulence of the isolates. In this work, results of three type of motility assays revealed

that the vast majority of isolates had three types of motility traits, where swimming motility was detected 90% *Pseudomonas* isolates, while 82% were positive for twitching motility. Additionally, 62% of these isolates were positive for swarming motility too. Some swarm colonies differed in morphology among these isolates. In current study, a dendritic swarming pattern was the most frequently observed phenomenon, occurring in 74% of isolates demonstrating positive swarming motility. The findings of present work align with a study conducted in the USA, where swimming was the highest motility trait observed in 83% of *Pseudomonas* UTI isolates, followed by twitching motility (75%), and swarming motility (63%) (Murray et al., 2010). A recent study conducted in Portugal by de Sousa *et al.,* (2023) also suggests that isolate perform better when doing the swim movement compared to swarming or twitching movements. A relative high prevalence of these motility traits has been observed in catheter-associated *Pseudomonas* infections, was described by Olejnickova *et al*., (2014) where over 95% of *Pseudomonas aeruginosa* isolates exhibited both swarming and swimming motility traits suggesting a potential role for these traits in the initial phases of infection development. Rodulfo *et al*. (2019) reported the comparatively low frequency of swimming (37.5%), swarming (42%), and twitching (40.3%) traits in *Pseudomonas aeruginosa,* this differ could be due to specimens being obtained from skin and bronchial secretion.

Hemolysin production has an important role in the pathogenesis of *Pseudomonas aeruginosa*. In current study, the majority of isolates (98%) produced beta hemolysis. Only one isolate was gamma hemolytic. Such a high frequency is in line with another study in Egypt conducted at Benha University, where 92% of isolates of *Pseudomonas* were found to express beta hemolysins (Ismail et al., 2021). Such similar results have been observed in studies conducted by Rodulfo *et al*., (2019) and Edward *et al.,* (2023) where hemolysin production was detected in 85.6% and 80.1% of clinical isolates of *P. aeruginosa*, respectively. The high frequency of beta hemolysis suggests that this activity is common among pathogenic *Pseudomonas* isolates. The consistency of this findings across different studies, including those conducted internationally, strengthens the reliability of this trait as virulent factors in infections.

In this study, *Pseudomonas* isolates from urinary tract infections were assessed for their ability to produce the phospholipase C (PLC) enzyme, and all were determined to be

phospholipase producers. In Brazil, a study conducted by Silva *et al*. (2021) on clinical isolates of *Pseudomonas* reported the similar finding to present work, where 80% of isolates showed positive results for phospholipase production. In a similar research conducted in Turkey with a similar methodology, 60.46% of *Pseudomonas* isolates were found to be positive for PLC activity (Çelik, 2020). But it is notable to mention all these isolates were taken from soil and water in case of study from Turkey, which could be the reason for this variation in phenotypic traits among clinical and environmental isolates of *Pseudomonas aeruginosa* (Grosso-Becerra *et al*., 2014). Current research specifically focuses on *Pseudomonas* isolates from urinary tract infections in which high prevalence of the phospholipase C enzyme positive isolates is seen, hence current study suggests a potential link between phospholipase C activity and the virulence of *Pseudomonas aeruginosa* in the development of UTIs.

Protease production was observed in 96% of *Pseudomonas* isolates associated with urinary tract infections in this investigation. These results are in harmony with those reported in Brazil, where protease was observed in more than 90% of isolates (Silva *et al., 2021)*. A study conducted in Egypt also found that 79.8% of *Pseudomonas* isolates possess alkaline protease activity (Edward *et al.*, 2023). There is a slight difference in the percentage, but the overall trend of high protease production in *Pseudomonas aeruginosa* was same. The high frequency of protease production in UTI-associated *Pseudomonas* isolates is noteworthy. Proteases play a crucial role in bacterial pathogenesis by facilitating tissue invasion, nutrient acquisition, and evasion of the host immune response. The high prevalence of protease production in the studied isolates suggests its significance in the pathogenesis of urinary tract infections.

In current investigation, the analysis of pigment production in Pseudomonas isolates on Cetrimide agar demonstrated a high percentage of pyocyanin (82%) and pyoverdine (100%), while none of the isolates produced pyorubin or pyomelanin. These results align with findings from other studies in different geographical regions, such as one conducted in Brazil, where no pyorubin was detected with limited percentage of pyomelanin. The study on clinical isolates from Brazil reported 40% positive isolates, which was relatively lower than current study. Pyoverdine pigment was the most frequently found bacterial phenotype, consistent with many other studies (Silva *et al*., 2021; Rodulfo *et al*., 2019). In Egypt, Edward *et al*. (2023) reported that 74%, 9.6%,

and 1% of *Pseudomonas* isolates exhibited pyoverdine, pyocyanin, and pyomelanin, respectively. The high percentage of pyocin production in present study suggest an increased capacity for these Pseudomonas isolates to combat competitors and establish a niche in the urinary tract to enhance the pathogenicity in urinary tract infections.

Urease production is considered an important marker of virulence in *Pseudomonas aeruginosa* as demonstrated by (Bradbury et al., 2014). Their study established a strong correlation between the urease-positive phenotype and increased virulence in *Pseudomonas aeruginosa*. In this work, all the tested isolates of Pseudomonas associated with urinary tract infections were positive for urease production. Current study showed a higher prevalence of urease positivity compared to those reported by Naik *et al*. (2021) in India for ocular infections. In their study, the prevalence was 48% and 52% for drug-sensitive and drug-resistant bacteria, respectively. This discrepancy raises interesting questions about the potential role of urease in the virulence and pathogenesis of *Pseudomonas aeruginosa* caused urinary tract infections.

Overall, a significant presence of highly virulent strains of *P. aeruginosa* were detected and confirmed in urinary tract infections. Such wide spread of this isolates in community setting is a grave health issue in Pakistan, which need serious attention. This study provides valuable insights on the virulence profile of *Pseudomonas* strains associated with UTIs, and these findings can be useful in the future for effective diagnostic and therapeutic approaches for managing UTIs caused by *P. aeruginosa.*

# Conclusion

The main findings of this study are:

- Detection of highly pathogenic strains of *P. aeruginosa* with multiple virulence factors from Pakistani population with urinary tract infections.
- Notably, 72% of UTI-associated *Pseudomonas* isolates possessed biofilm-forming potential, suggesting its role in infection persistence and antibiotic resistance.
- The high prevalence of swimming (90%), swarming (62%), and twitching (82%) together in isolates of *P. aeruginosa* isolates indicate these strains have important virulence factor for the initial establishment of UTIs.
- The widespread prevalence of extracellular virulence factors such as hemolysins (98%), phospholipases (100%), urease (100%), and proteases (96%) indicates the involvement of these factors in the pathogenicity of UTIs.
- The strikingly high rates of pigment production, such as pyoverdine (100%), and pyocyanin (82%), further characterize these UTI-associated *Pseudomonas* isolates as highly virulent.

# Future Prospectives

The present study was conducted to evaluate the virulence factors of *Pseudomonas* that have a role in UTIs. This study adds insights to the limited existing literature in Pakistan on the virulence profile of *P. aeruginosa* and opens doors to several promising future prospects in understanding and addressing urinary tract infections caused by *Pseudomonas* strains.

- Further research on the virulence genes found in these highly pathogenic *Pseudomonas* strains must be conducted to understand the molecular mechanism behind the pathogenesis of UTIs.
- Investigating the correlation between these virulence factors and antibiotic resistance can provide us with valuable information about the impact of these factors on the drug resistance profile.
- The identification of a wide range of virulence factors in *Pseudomonas* isolates opens new avenues for the development of targeted-based therapeutic strategies.
- Such a targeted therapeutic approach to the specific virulence hallmark of *Pseudomonas* can improve clinical outcomes and reduce the risk of antibiotic resistance.
- These virulence factors can also serve as potential targets for vaccine development to combat *Pseudomonas* infections.
- Comparative studies with *Pseudomonas* strains from other geographical regions can provide insights into the variations in virulence profile. This comparative approach will help to establish a systematic surveillance system to monitor strategies for managing *Pseudomonas*-related UTIs.

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