PHENOTYPIC AND MOLECULAR CHARACTERIZATIONS OF *PSEUDOMONAS AERUGINOSA* ISOLATED AMONG PATIENTS ADMITTED AT TIKUR ANBESSA SPECIALIZED HOSPITAL AND YEKATIT 12 HOSPITAL MEDICAL COLLEGE, ADDIS ABABA, ETHIOPIA

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Phenotypic and Molecular Characterizations of *Pseudomonas Aeruginosa* Isolated Among Patients Admitted at Tikur Anbessa Specialized Hospital and Yekatit 12 Hospital Medical College, Addis Ababa, Ethiopia

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ABBREVIATIONS

AMR	Antimicrobial Resistance
ANI	Average Nucleotide Identity
ARG	Antibiotic Resistance Genes
BSI	Blood Stream Infections
CAMHB	Cation-Adjusted Mueller-Hinton Broth
CDC	Centers for Disease Control and Prevention
CLSI	Clinical Laboratory Standards Institute
CoRPA	Colistin-Resistant *P. aeruginosa*
CRPA	Carbapenem-Resistant *P. aeruginosa*
ESBLs	Extended-Spectrum B-Lactamases
ESKAPE	*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter spp.*
EUCAST	European Committee on Antimicrobial Susceptibility Testing
HAI	Health Care Associated Infections
HGT	Horizontal Gene Transfer
HRC	High-Risk Clone
MDR	Multi Drug Resistance
MLST	Multilocus Sequence Typing
PubMLST	Public Multilocus Sequence Typing Database
SNP	Single Nucleotide Polymorphism
ST	Sequencing Type
T1SS	Type I Secretion System
T2SS	Type II Secretion System
T3SS	Type III Secretion System
T5SS	Type V Secretion System
T6SS	Type VI Secretion System
WGS	Whole Genome Sequencing
WHO	World Health Organization
XDR	Extensive Drug Resistance

PUBLISHED PAPERS

Paper I

Olana, M. D., Asrat, D., & Swedberg, G. (2024). Antimicrobial resistance profile, biofilm forming capacity and associated factors of multidrug resistance in *Pseudomonas aeruginosa* among patients admitted at Tikur Anbessa Specialized Hospital and Yekatit 12 Hospital Medical College in Addis Ababa, Ethiopia. *BMC Infectious Diseases*, **24 (1**), 1472. https://doi.org/10.1186/s12879-024-10359-3

Paper II

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ABSTRACT

Background: *Pseudomonas aeruginosa* is a leading cause of hospital-acquired infections and is increasingly resistant to multiple antibiotics, which complicates treatment and infection control. Its ability to form biofilms, express multiple virulence factors, and harbor diverse molecular mechanisms of antimicrobial resistance contributes to its persistence and pathogenicity in healthcare settings. Investigating biofilm formation, virulence traits, and molecular resistance mechanisms of *P. aeruginosa* is essential for understanding its pathogenic potential, informing effective infection prevention strategies, and guiding clinical management.

Objectives: This study aimed to determine the phenotypic and molecular characterizations of *P aeruginosa* among patients admitted at Tikur Anbessa Specialized Hospital and Yekatit 12 Hospital Medical College, Addis Ababa, Ethiopia.

Methods: A cross-sectional study was conducted from August 2022 to September 2025. A total of 422 clinical specimens including blood, urine, burn swabs, and surgical site wound swabs were collected from hospitalized patients at Tikur Anbessa Specialized Hospital (TASH) and Yekatit 12 Hospital Medical College (Y12HMC). Eighty-four isolates identified as *Pseudomonas* species were stored in STGG medium at −80°C and later shipped to Uppsala University, Sweden, for molecular characterization.

Isolation and identification of *P. aeruginosa* were performed using standard microbiological techniques. Antimicrobial susceptibility testing was conducted against 11 antibiotics using the Kirby-Bauer disk diffusion method according to CLSI guidelines. Carbapenem susceptibility was determined by broth microdilution, and colistin susceptibility was tested using the colistin broth disk elution method. Biofilm formation was assessed using the microtiter plate assay.

Whole-genome sequencing (Illumina HiSeq 2500) was performed on the 84 isolates, and downstream genomic analysis was conducted on 64 isolates to characterize virulence factors, serotypes, antibiotic resistance determinants, and phylogenetic relationships.

Bivariate and multivariable logistic regression analyses were used to identify factors associated with multidrug resistance (MDR). The Spearman correlation coefficient was applied to evaluate the relationship between biofilm formation and antimicrobial resistance.

Results: The overall prevalence rate of Pseudomonas aeruginosa among the 422 clinical specimens was 19.6% (83/422). The distribution of isolates across hospitals was 43.4% (n=36) from Y12HMC and 56.6% (n=47) from TASH. Among the positive isolates, the distribution by specimen type was as follows: urine 32.5% (27/84), blood 22.9% (19/84), surgical site wounds 27.7% (23/84), and burn wounds 16.9% (14/84).

High resistance rates were observed for ciprofloxacin (51.8%), ceftazidime (50.6%), and cefepime (48.2%), while low resistance was noted for ceftazidime-avibactam (4.8%) and imipenem (16.9%). The overall proportion of multidrug-resistant (MDR) isolates was 56.6%.

Biofilm production was detected in 95.2% of isolates, with 27.7% classified as strong and 39.8% as moderate biofilm producers. A significant positive correlation was observed between biofilm formation and multidrug resistance.

Previous exposure to ciprofloxacin was identified as an independent factor associated with MDR P. aeruginosa.

Among the 64 isolates subjected to whole-genome sequencing, eight serotypes were identified. The most prevalent serotypes were O6 (50%), O11 (14.1%), O3 (10.9%), O5 (9.4%), O1 (7.8%), O2 (3.1%), O4 (3.1%), and O9 (1.6%). Serotype O6 was most frequent across all infection types, while O1, O3, and O11 predominated in specific specimens.

Serotype-specific MDR rates varied: O11 had the highest MDR rate (88.9%), followed by O5 (66.7%); O6 and O2 had equal distributions of MDR and non-MDR isolates (50% each), whereas O4 and O9 were entirely non-MDR (100%).

Amongst the 241 virulence genes identified, 83.4% were present in nearly every isolate. The virulence genes identified were flagella-related genes, biofilm related genes, secretion system components, secreted factors, and toxin related genes. The most common toxins identified were *exoY* (96.9%), *exoT* (96.8%), *exoS* (95.3%), *toxA* (93.8%) and *exoU+* (6.3%). There were four (6.3%) *exoU+* strains and one (1.6%) *exoU+exoS+* multidrug-resistant strain, all of which were O11 serotypes.

The most frequently detected ESBL gene among MDR isolates was $bla_{\text{CTX-M-15}}$ 10 (15.6%). The most prevalent OXA genes identified was $bla_{\text{OXA-396}}$ 24 (37.5%). At least one-aminoglycoside gene was identified in all MDR isolates. The most frequently identified aminoglycoside gene was *aph (3')-Iib* (57.6%). Two distinct carbapenemase genes identified were $bla_{\text{VIM-2}}$ and $bla_{\text{NDM-1}}$.

Significant *mexR* and *ampD* mutations were found among MDR isolates with 25 (75.8%) and 29 (87.9%) respectively. Most of carbapenem-non-susceptible isolates were exhibited mutations in *mexR* and *nalC* genes. Colistin resistant isolates were contained at least one mutation in *pmrB* gene.

Multi-locus sequencing type analysis revealed two global high-risk clone types (ST235 and ST277) and two novel STs (ST5135 and ST5136).

Conclusion: This study showed the presence of diverse virulence and resistance determinants in P. aeruginosa. The finding of genetically related isolates shows potential clonal spread within hospital. This showed the need for strengthen infection prevention strategies in healthcare settings to monitor spread of resistant strains

Keywords: *Pseudomonas aeruginosa*, biofilm, multidrug-resistance, serotypes, virulence genes, Ethiopia

CHAPTER ONE: INTRODUCTION

1.1. General Introduction

Pseudomonas aeruginosa is a Gram-negative, opportunistic pathogen commonly found in soil, water, and hospital environments (Silby *et al*., 2011) It can cause a wide range of infections, including urinary tract infections, bloodstream infections, burn wound infections, and surgical site infections, particularly in immunocompromised individuals (Folic *et al*., 2021; Lila *et al*., 2018).

Globally, *P. aeruginosa* is among the leading causes of healthcare-associated infections and is frequently isolated from intensive care units, burn wards, and surgical sites (Folic *et al*., 2021). Its prevalence varies by region and healthcare setup, with higher rates reported in low- and middle-income countries due to limited infection prevention and control practices (Sartorius *et al*., 2024). In Ethiopia, *P. aeruginosa* remains one of the most commonly encountered nosocomial pathogens, contributing substantially to treatment failure (Addis *et al*., 2021).

P. aeruginosa is known by its a broad arrays of virulence factors that contribute to its ability to cause variety of infections (Shugang *et al*., 2022). These includes structural components like flagella and pili for motility and adhesion, secreted toxin such as exotoxin A, secretion system that deliver effector proteins to host cells and its capacity to form biofilm (Liao *et al*., 2022). *P. aeruginosa* has been divided into 20 serotypes (O1 to O20) according to the structure of its O-polysaccharide, with O6 and O11 being the most often reported in various studies (Del Barrio-Tofiño *et al*., 2019; Hu *et al*., 2024; Lu *et al*., 2014; Nasrin *et al*., 2022). *P. aeruginosa* is developed resistant to many of the currently available antibiotics and resistant strains become increasingly associated with different infections (Park *et al*., 2012). The main resistance mechanisms that *P. aeruginosa* uses against antibiotic can be broadly categorized as adaptive, acquired, and intrinsic resistance (Pang *et al*., 2019). It is intrinsically resistant to several antibiotics. Intrinsic resistance mechanisms include lower outer membrane permeability, expression of antibiotic inactivating enzymes and efflux

pump (Pang *et al*., 2019), whereas *P. aeruginosa* can acquire resistance through mutation or horizontal transfer of resistance genes (Verdial *et al*., 2023). Adaptive antibiotic resistance in *Pseudomonas aeruginosa* often involves phenotypic changes that enhance survival under antibiotic pressure (Breidenstein *et al*., 2011). One key adaptive strategy is biofilm formation, a structured community of bacterial cells encased in a self-produced extracellular matrix(Spagnolo *et al*., 2021).

Several β-lactamases in *P. aeruginosa* have been shown to hydrolyze carbapenem, cephalosporins, cephems, and/or monobactams (Hosu *et al*., 2021a). The mechanisms of resistance to carbapenems are multifactorial, including permeability defects, efflux pump expression, production of carbapenemases and mutation in *oprD* gene (Mirsalehian *et al*., 2017). Resistance to colistin may be resulted from the horizontal gene transfer (HGT) of resistance by means of plasmid carrying colistin-resistant gene or chromosomally encoded mutation (Cannatelli *et al*., 2013). Plasmids harboring diverse ARG including $bla_{\text{PER-1}}$, $bla_{\text{OXA-198}}$, *mcr-1*and $bla_{\text{VIM-2}}$, that attributed to HGT have been identified in *P. aeruginosa* (Garch *et al*., 2011; Hameed *et al*., 2019; Mei *et al*., 2024; Vilacoba *et al*., 2014).

Multidrug-resistant (MDR) *P. aeruginosa* becomes a significant public health problem and associated with increasing the mortality rate (Abd El-Baky *et al*., 2020). With the increase in CRPA, colistin has been considered as a last-resort treatment choice, while colistin resistance has emerged sporadically worldwide (Wi *et al*., 2017).

These rising threat of antibiotic resistance in *P. aeruginosa*, emphasizes the need for comprehensive genomic-based investigation. Therefore, the current study was aimed to determine the phenotypic and molecular characterizations of *P. aeruginosa* at Tikur Anbessa Specialized Hospital (TASH) and Yekatit 12 Hospital Medical College (Y12HMC) in Addis Ababa, Ethiopia.

1.2. Statement of the Problem

P. aeruginosa is the main cause of HAIs that exhibits rising trends in both incidence and fatality rates (Raoofi *et al*., 2023). In 2019, bacterial AMR was directly attributable to 250 000 deaths in WHO's African region (Sartorius *et al*., 2024). *P. aeruginosa* is one of the leading pathogens for death associated with AMR and HAIs in Africa (Abubakar *et al*., 2022; Wagenlehner and Dittmar, 2022). *P. aeruginosa* AMR was directly responsible for 14,000 deaths in the WHO's African region in 2019 (Sartorius *et al*., 2024).

P. aeruginosa is a member of the ESKAPE pathogens that include also *Klebsiella pneumoniae, Staphylococcus aureus, Acinetobacter baumannii, Enterococcus faecium, and Enterobacter spp,* which are the leading cause of HAI and posing global threat to human health (Ayobami *et al*., 2022). Increasing resistance of *P. aeruginosa* to several classes of antimicrobial agents has been reported from different countries (Hirsch and Tam, 2010; Ssekatawa *et al*., 2018). WHO states that carbapenem resistant *P. aeruginosa* (CRPA) is a priority pathogen that cause a threat to world health and new treatments are desperately needed (Jesudason, 2024). Emergence of colistin resistance, the antibiotics that has been considered as last resort treatment is another concern (Wi *et al*., 2017). In Ethiopia, studies have been shown rising antibiotic resistance in *P. aeruginosa* (Addis *et al*., 2021). According to systematic review study conducted in Ethiopia, pooled MDR in *P. aeruginosa* was 80.5% (Asmare *et al*., 2024). *P. aeruginosa* is a ubiquitous bacterial pathogen that thrives in hospital settings, where its ability to form biofilms on medical devices and tissues enhances resistance to antibiotics and making it a common source of HAIs (Rasamiravaka *et al*., 2015).

Although previous research has demonstrated raising concern of MDR *P. aeruginosa* in Ethiopia, whole genome sequencing of strains have not been fully documented using molecular techniques. Additionally, despite its importance, there is a limited data on molecular epidemiology and genetic relatedness of resistant *P. aeruginosa* strains from Ethiopia. Hence, molecular epidemiology study is important to track the spread of the

isolates, identify resistance genes, improve surveillance, facilitate localized control measures, and to implement public health interventions. Therefore, this study aimed to investigate the genomic-based characterizations of *P. aeruginosa* among admitted patients suspected for UTI, blood stream infection and wound infections at TASH and Y12HMC, Addis Ababa, Ethiopia.

1.3. Literature Review

1.3.1. Historical Background of *P. aeruginosa*

According to 1923 edition of Bergey's Manual, several phenotypic characteristics including Gram-stain, flagella type, and metabolism with relation to oxygen were used in an attempt to differentiate the species of the genus *Pseudomonas* (Bergey, 1923). Stanier *et al*. (1966) established the taxonomical foundation for identification of *Pseudomonas* species using biochemical and physiological traits.

The Pseudomonadaceae family had more than 102 distinct species by 1984, the majority of which were soil saprophytes or plant pathogens. They are oxidase positive and able to grow in wide temperature from 4 to 42°C (Woese *et al*., 1984). Infection associated with *P. aeruginosa* was initially described in 1850 by a French military surgeon, after he observed blue pus in the wound dressings of injured soldiers (Wood *et al*., 2019). The first evidence of MDR in *P. aeruginosa* was reported from the United States of America in 2004 (Obritsch *et al*., 2004). For the first time, strain PAO1 was sequenced in 2000 GC, that sheds light on the mechanisms underlying *P. aeruginosa's* drug resistance and adaptability (Stover *et al.*, 2000). The sequence of multiple strains of *Pseudomonas* genus have since been added including *Pseudomonas syringae* in 2003, *Pseudomonas putida* in 2002, *Pseudomonas fluorescens* in 2005, *Pseudomonas endomophila* in 2006, and *Pseudomonas stutzeri* in 2008 (Silby *et al.*, 2011).

1.3.2. Taxonomy and classification of *Pseudomonas* species

Pseudomonas is a dynamic pathogen that inhabits a several environments, including water, plant, soil, and mammals (Silby *et al*., 2011). To date, over 330 species with correct name have been characterized and validly published (Parte, 2014). There are three main lineages of Pseudomonas genus based on 16S rRNA gene sequences. These are represented by the *P. aeruginosa, Pseudomonas pertucinogen* and *Pseudomonas fluorescens* (Peix *et al*., 2018). The *P. fluorescens* lineage comprises five phylogenetic groups (*P. putida, P. asplenii, P. lutea, P. fluorescens and P. syringae*); the *P. aeruginosa* lineage comprises eight phylogenetic groups (*P. aeruginosa, P. anguilliseptica, P. oryzihabitans, P. stutzeri, P. oleovorans, P. straminea, P. resinovorans and P. linyingens*is) and the genus Azotobacter (Lalucat *et al*., 2020).

Various species of *Pseudomonas* spp. are associated with human infections. Both *P. aeruginosa* and *P. maltophilia* are account around 80% of *Pseudomonas* species from clinical specimens (Gomila *et al*., 2015). Other species including *P. fluorescens, P putida, P. guariconensis, P putrefaciens*, and *P stutzeri* clinical isolate has been reported in different countries (Gomila *et al*., 2015; Yoon and Lee, 2024).

1.3.3. Microbiological Features of *P. aeruginosa*

P. aeruginosa is gram-negative rod bacteria. It drives its energy from oxidation and grows well over a wide range of temperature including at 42° C. It has the capacity to thrive a wide range of molecules including on medium that only provide ammonium sulfate and acetate (Diggle and Whiteley, 2020; Koutsogiannou *et al*., 2013). In addition, it has capacity to develop resistance to many antibiotics, resist antiseptics, and high concentration of salt and different dyes. These ubiquitous nature of *P. aeruginosa* contribute to it's a main cause of nosocomial infections (Koutsogiannou *et al*., 2013). In addition, *P. aeruginosa* has minimal nutritional needs and non-spore-forming isolates (Remold *et al*., 2011).

It is positive for oxidase, Simmon's citrate and l-arginine dehydrolase tests and negative for Voges Proskauer tests, indole and methyl red tests. It produces pyoverdin and pyocyanin that is responsible for yellow and blue green pigment respectively (Neves *et al*., 2014). It

exhibits swimming, swarming and twitching motility in liquid, across semisolid and solid surfaces respectively (Murray and Kazmierczak, 2008).

1.3.4. Virulence Factors and Pathogenesis of *P. aeruginosa*

There are several virulence factors of *P. aeruginosa* that are important in host attachment ,bacterial survival and the invasion of tissues (Alonso *et al*., 2020). The main categories of *P. aeruginosa* virulence factors are surface structures, secreted factors as well as bacterial cell-to-cell interaction (Liao *et al*., 2022).

a) Surface appendages and outer membrane components

These includes flagella, type IV pili and lipopolysaccharide (Liao *et al*., 2022). *P. aeruginosa's* type IV pili are made up of several copies of the 15-kDa protein known as pilin. It is involved in the formation of biofilms, regulation of virulence factors, twitching and swarming motility and adhesion to a variety of surfaces (Floyd *et al*., 2016; Persat *et al*., 2015). Cytoplasmic ATPases drive the repetitive extension and retraction of type IV pili, which promotes bacterial motility and adhesion (Craig *et al*., 2019). The flagella of *P. aeruginosa* are important in adhesion, chemotaxis, bacterial movement, and immune response activation (Ozer *et al*., 2021). Because of its good immunogenicity, vaccination with flagella induced good protective immune-response in animal model (Hassan *et al*., 2018). Lipopolysaccharide (LPS) is a crucial surface structural element that protects the exterior layer of the bacterial membrane and poisons host cells. The structure of LPS is composed of Lipid A, which is covalently attached to a core oligosaccharide and capped by the O antigen (Whitfield and Stephen Trent, 2014). *P. aeruginosa* exhibit heterogeneous compositions of the O antigen, which are extremely varied and play important roles in host–pathogen interactions (Maldonado *et al*., 2016).

b) Secretion system of *P. aeruginosa*

P. aeruginosa consist of five virulence associated secretion systems (T1SS, T2SS, T3SS, T5SS, and T6SS).

Type one secretion system (T1SS) is one of the secretion systems that play an active virulent role during the inflammatory phase in *P. aeruginosa* infection process (Liao *et al*., 2022). It is involved in in the secretion of heme acquisition protein and iron utilization (Bleves *et al*., 2010).

T2SS secretes Elastase A and B (LasA and LasB), Lipase A and C (Lip A and Lip C), alkaline phosphatases and exotoxin A (Bleves et al., 2010). The exotoxin A is responsible for tissue necrosis, while Phospholipase C is for thermos-labile hemolysin (Alonso *et al*., 2020).

T3SS is used by *P. aeruginosa* as molecular syringe to inject host cells and composed of five components (Liao *et al*., 2022). These includes the needle, which connect host cell with bacterial cell, the translocation apparatus, which is responsible for injection of effectors to host cells and pore formation, regulatory proteins that is responsible for controlling of T3SS, chaperones which is responsible for avoiding premature interaction of proteins in cytoplasm, and effectors which are injected and damage host cells (Horna and Ruiz, 2021). The four important effectors of T3SS of *P. aeruginosa* are *ExoS, ExoU, ExoT,* and *ExoY* (Alonso *et al*., 2020). *ExoT* and *exoY* are the most frequently reported toxins while *exoU* and *exoS* are clinically most relevant toxins (Horna and Ruiz, 2021). *Exo S* is functions as an ADP-ribosylating enzyme, which a major cause of cell apoptosis with its C terminal region and GTPase activating protein, which leads disruption of cell-to-cell adhesion with its N-terminal region (Horna and Ruiz, 2021). It is also important for the destruction of immunoglobulin G and A, disruption of normal cytoskeletal organization, and contributes to the resistance to macrophages (Bleves *et al*., 2010).

ExoU is located within downstream of *spcU* gene (Horna and Ruiz, 2021). It has phospholipase A2 activity that induces cytotoxicity to a variety of cells and release of arachidonic acid from host cell (Diaz *et al*., 2008). It is associated with increases risk of tissue injury, bacteremia and diseases severity in *P. aeruginosa* infection (Berre *et al*., 2011). It activates IL-8 through NF-kB activation (Lima *et al*., 2012). It is the only T3SS

effectors, encoded in genomic island environment and transferred through horizontal gene transfer (Song *et al*., 2023). The two most common toxins, *ExoT* and *ExoS* have bi-functional with both GTPase and ADP-ribosylating enzyme activity that involved in apoptosis and cytoskeleton reorganization (Horna and Ruiz, 2021). *ExoY* has a nucleotidyl cyclase activity that is important in mediating permeability and endothelial phosphorylation permeability (Horna and Ruiz, 2021). It needs cofactors like filamentous actin for its activation in host cells. It plays important role in immune evasion by suppressing Transforming Growth Factor β-Activated Kinase 1(He *et al*., 2017).

Different studies have shown the exclusive properties of *exoU* and *exoS* toxins, due to targeted deletion event (Kulasekara *et al*., 2006), although some studies recently reported the presence of both toxins in the single strain (Horna and Ruiz, 2021; Song *et al*., 2023).

T5SS of *P. aeruginosa* is secretes a variety of proteins that participate in bacterial adhesion immune escape and nutrient acquisition. These include *EstA, TpsA* and *TpsB* (Liao *et al*., 2022).

T6SS is a flexible secretion system that able to inject effectors with antibacterial activity, associated with bacterial virulence and biofilm formation (Horna and Ruiz, 2021).

c) Secreted factors

Toxins, proteases, sederoverdin, and exopolysaccharides are among secreted factors that play an important role in the pathogenesis of *P. aeruginosa* infection (Liao *et al*., 2022). Siderophores are iron chelating compounds that aid bacterial survival, especially in iron-limited environments. Pyoverdine (pvd) is a group of green fluorescent compounds that represents the primary iron chelating system in *P. aeruginosa*, while pyochelin (pch) is non-fluorescent compound with iron chelating and sustained inflammatory responses identified in chronic infections (Cornelis and Dingemans, 2013).

There are many protease produced by *P. aeruginosa* including alkaline protease (AprA), elastase (LasA and LasB) and protease IV(Horna and Ruiz, 2021). Alkaline protease is

important in degrading complement components, elastase are important in degrading elastin, and protease IV play role in degrading immunoglobulins, complement components and enhancing bacterial infection through degradation of lactoferrin, transferrin, fibrinogen and elastin (Bleves *et al*., 2010).

Exopolysaccharides secreted by *P. aeruginosa* are important to improve its survival against desiccation, oxidizing chemical and host defense (Liao *et al.,* 2022).

d) Biofilm formation and Quorum sensing

The biofilm is a complex of bacteria aggregated in self-produced extracellular polymeric substances to survive at the time of nutritional shortage, fluctuation of temperature and effect of antibiotics (Harrison *et al*., 2010). Biofilm matrix of *P. aeruginosa* contains extracellular DNA, pyocyanin, rhamnolipids and functional proteins that contribute to the formation of biofilms. The three exopolysaccharides components that involved in biofilm formation are *Psl, Pel* and alginate (Bleves *et al*., 2010). While *pel* and alginate are important in non-mucoid and mucoid strain respectively, *Psl* is important for both types of strains. Alginate is mainly important for biofilm maturation, while *psl* is important in signaling molecule in forming thick biofilm and *pel* for attachment initiation (Thi *et al*., 2020). Bacteria that are isolated from the environment mostly produce *Psl* and *Pel*, whereas strains recovered from cystic fibrosis patients typically secrete alginate (Newman *et al*., 2017).

Bacteria go through various stages to form biofilm (Rasamiravaka *et al*., 2015). First bacteria attach with surface and produce matric components, and then replication and irreversible attachment of surfaces followed by formation of micro colonies and development to mushroom shaped of these microcolonies (Ozer *et al*., 2021). Lastly, matrix cavity is formed at the center of mushroom shaped complex and disruption of this matrix leads release of planktonic bacteria cell (Thi *et al*., 2020).

The development and maturation of biofilm in *P. aeruginosa* requires coordination of each bacterial cell in bacterial communities through cell to cell communication pathways (Khalifa

et al., 2011). Quorum sensing (QS) is a bacterial cell-to-cell communication system that allows the bacteria to identify signal molecules that they secrete and coordinate the expression of several genes that are involved in virulence and maturation of biofilm (Rezzoagli *et al*., 2020). In *P. aeruginosa las, rhl, Iqs,* and *pqs* are the four QS pathway that utilize different auto inducers (Hemmati *et al*., 2024). The two-component transcriptional regulatory system and the quorum sensing system are crucial in controlling the expression of the majority of *P. aeruginosa* virulence factors (Sultan *et al*., 2021).

1.3.5. Serotypes, Virulence Genes and their association with MDR isolates

a) Serotypes and MDR

Serotype specificity is conferred by O-polysaccharide, the most variable portion of LPS, which play important role for virulence (Maldonado *et al*., 2016). Various researchers have developed a number of serotyping schemes, but the International Antigenic Typing Scheme is the most widely used. There are 20 different serotypes of *P. aeruginosa* based on its O-polysaccharide structure (O1 to O20) (Lam *et al*., 2011). *Pseudomonas aeruginosa* produces two distinct LPS types: A-band (common polysaccharide antigen, CPA), a conserved D-rhamnose homopolymer, and B-band (O-specific antigen, OSA), a heteropolymer that varies among ~20 O-serotypes (Lam *et al*., 2011). These LPS types are encoded by separate chromosomal loci and play distinct roles in virulence: B-band mediates serotype specificity and immune evasion, while A-band is antigenically conserved and prevalent in chronic infections (Maldonado *et al*., 2016).

The most prevalent serotypes were O11 (22%), O1 (14%) and O6 (13%) which accounted for around 50% according to research conducted on isolate collected from multicounty (Nasrin *et al*., 2022). Serotypes O11, O12, O6 and O1 were the most frequently reported in European countries, with O11 and O12 commonly high among MDR isolates (Pirnay *et al*., 2009). Other studies showed serotype O6 and O11 were most common in pneumonia (Lu *et al*., 2014), while serotype O5, O6, and O11 were frequently reported in burn wound infections (Estahbanati *et al*., 2002). The most frequently reported serotypes in *P. aeruginosa* infections were O6, O11, and O1 and O12 (Pirnay *et al*., 2009).

Studies have shown that serotype O4, O11 and O12 were the most common serotypes in clinical settings that associated with MDR in *P. aeruginosa* (Del Barrio-Tofiño *et al*., 2019). Additionally, previous research demonstrated that certain serotypes are more virulent than others; for example, isolates of serotype O11 were found to produce *ExoU*, more frequently than other serotypes and linked to greater tissues damage (Berre *et al*., 2011).

b) *ExoU* toxins

Systematic review study revealed that 32% of clinical *P. aeruginosa* isolates were *exoU* toxins producers (Javanmardi *et al*., 2019), whereas a study carried out in Spain reported that 31.1% of clinical strains were *exoU* toxins producers (Alonso *et al*., 2020). Furthermore, *exoU* toxin was reported in *P. aeruginosa* strains from food products (2.5%) in Côte d'Ivoire (Benie *et al*., 2017) and drinking water (7.6%) in China (Wei *et al*., 2020).

c) Other Toxins

The prevalence of *toxA* and *exoY* has been reported 97% and 93% in Iran, respectively (Haghi *et al*., 2018), whereas prevalence rate for *exoT* was 83% (Javanmardi *et al*., 2019), and prevalence of exoS was 73% (Kiyaga *et al*., 2022). Similarly, prevalence of *aprn*, *phzM* and *phzS* were 98.8%, 96.5% and 91.9%, respectively (Heidari *et al*., 2024). Frequency of *pvd* and *wzz* were 26.7% and 18%, respectively (Kiyaga *et al*., 2022).

1.3.6. Clinical Outcomes of *P. aeruginosa* Infections

P. aeruginosa is an opportunistic pathogen that causes both acute and chronic infections particularly affecting critically ill and immune deficient patients (Juan *et al*., 2017). It founds on skin, pharynx, and as intestinal flora (Rezzoagli *et al*., 2020). *P. aeruginosa* is associated with a wide spectrum of infection from skin infections to systemic infection (Bassetti *et al*., 2018; Elfadadny *et al*., 2024) as shown in Figure 1.1.

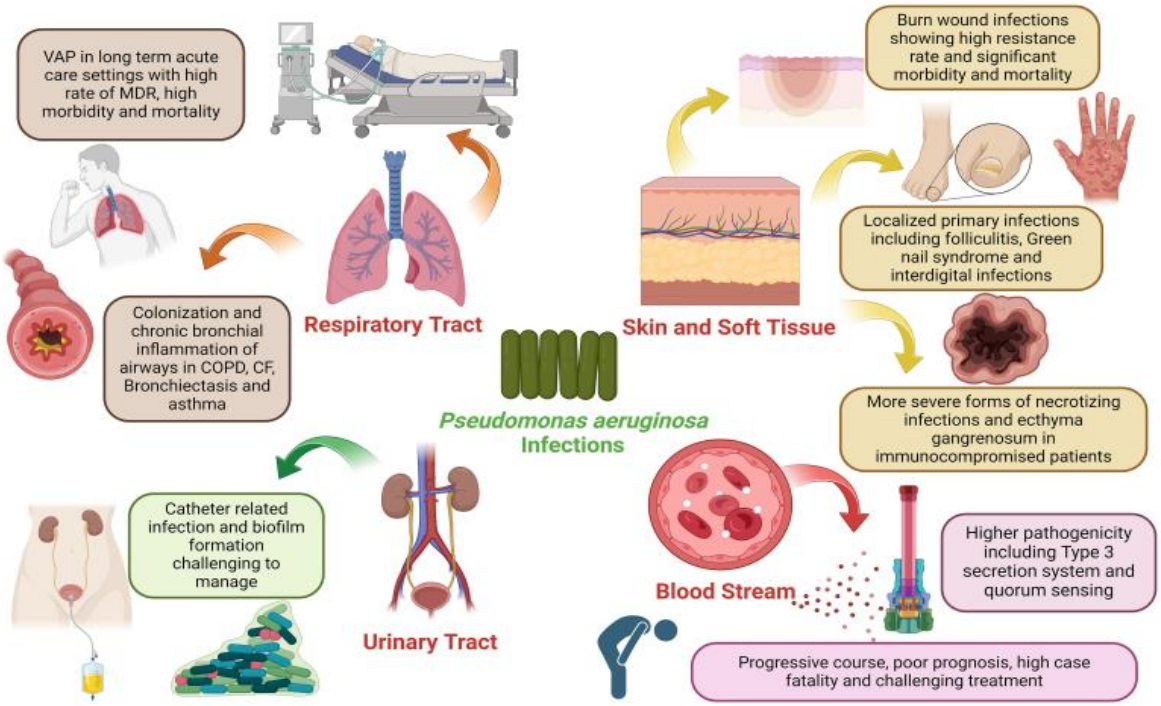

Figure 1.1. *P. aeruginosa* infection in various organs of the human body (Adapted from Elfadadny *et al*., 2024)

Figure 1.1 shows major infection sites and disease outcomes of Pseudomonas aeruginosa, including respiratory (VAP, chronic airway colonization), urinary (catheter-related, biofilm-associated), skin/soft tissue (folliculitis, green nail syndrome, necrotizing infections), and bloodstream infections characterized by high virulence, poor prognosis, and high mortality.

a) Blood Stream Infection (BSI)

P. aeruginosa is among the most common bacterial pathogen associated with bacteremia (Schechner *et al*., 2011). A recent study showed that mortality associated with *P. aeruginosa* bacteremia was greater than patients with members of other gram negative rod bacteria (Walters *et al*., 2019). Factors associated with *P. aeruginosa* blood stream infections include recent hospitalization, septic shock at admission, respiratory or unknown sources of bacteremia, breaking natural barriers (venous or urine catheters), disruption of a healthy microbiota and duration of hospital stay (Schechner *et al*., 2011). Initial site of infection,

septic shock, and immunosuppression at the time of *P. aeruginosa* blood stream infections are among the host factors associated with increased mortality (Walters *et al*., 2019). Skin lesions with distinct morphology may be present in *P. aeruginosa* bacteremia, a potentially fatal condition. Ecthyma gangrenosum, gangrenous cellulitis, and subcutaneous nodules are among the skin manifestations of systemic *P. aeruginosa* infections *(Peña et al*., 2013)..

b) Respiratory Tract Infection (RTI)

P. aeruginosa is among bacterial pathogens most frequently associated with trigging lower respiratory tract infections (Trinh *et al*., 2017). It is more common in cystic fibrosis, where thick layer of mucus is formed on airway surfaces due to mutation in the cystic fibrosis trans membrane conductance regulator (CFTR) gene (Cohen and Prince, 2012; Garcia-Clemente *et al*., 2020). The formation of mucus on airway limits lung epithelial cells' ability to internalize bacteria, inhibits the antimicrobial peptides and mucociliary clearance (Scotet *et al*., 2020). CFTR is important for chloride ion transport across epithelial tissue apical membranes, which results in viscous mucus in the airways (Scotet *et al*., 2020). Mutation in CFTR lead defect in water and sodium transport resulting in dehydrated airway, high salt concentration, abnormal mucociliary clearance and altered bacterial adherence and internalization that inhabit antibacterial peptide and immune response (Cohen and Prince, 2012).

Three bacterial pathogens *P .aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumoniae* were account for 80% ventilator-associated pneumonia (Sandiumenge and Rello, 2012).

c) Urinary Tract Infection (UTI)

P. aeruginosa is responsible for around 10% of UTIs diagnosed among hospital admitted patients (Djordjevic *et al*., 2013). This is associated with catheterization as a biofilm develop on catheters represent a major risk to patient (Bouza *et al*., 2001). *P. aeruginosa* is able to adapt, endure, and proliferate in fluctuating environment of urine osmolarity by using valve-

like channels to remove osmolytes to the outside of the bacterium (Paz-Zarza *et al.*, 2019). *P. aeruginosa* UTIs is a main cause of *P. aeruginosa* bloodstream infection and is associated with higher mortality than other bacterial bloodstream infections especially in patients with chronic renal failure, liver disease or diabetes mellitus (Horcajada and Shaw, 2012). Significant predictors of MDR *P. aeruginosa* infections includes recent history of isolation of an ESBL-producing Enterobacteriaceae strain, previous antibiotic therapy with carbapenems and indwelling urinary catheter (Tumbarello *et al*., 2020).

d) Skin and Soft Tissue Infection

P. aeruginosa is the most common bacteria isolated from chronic wounds (de Almeida Silva *et al*., 2017). It expresses various virulence factors affecting wound healing (Haghi *et al.*, 2018). The devascularization of the wound environment caused by severe burn trauma inhibits the migration of neutrophils and other innate effector cells into the wound that in turn inhabit eradication of bacterial infection (Everett *et al*., 2017). Because the burn wound is avascular, there is less oxygen and other vital nutrients getting to the damaged tissue, which increases the risk of ischemia. *P. aeruginosa* has the ability to thrive inside the burn wound and spread rapidly through the surrounding tissue and into the bloodstream *(*Everett *et al*., 2017; Haghi *et al*., 2018).

Cutaneous *P. aeruginosa* infection can be divided into two groups: those resulting from secondary *P. aeruginosa* bacteremia, or those resulting from initial infection following cutaneous inoculation (Sathe *et al*., 2023). Green nail syndrome, toe web infections, external otitis as well as hot tub folliculitis and hot hand-foot infections were among the most frequent skin infections associated with *P. aeruginosa* (Sathe *et al*., 2023; Wu *et al*., 2011). In immunocompromised individuals necrotizing skin infections , *Ecthyma gangrenosum* and subcutaneous nodules are mainly associated with patients with BSIs (Wu *et al*., 2011).

e) Otitis media (OM)

Pseudomonas aeruginosa can cause infections of the middle ear, particularly in children, patients with chronic ear disease, or immunocompromised individuals (Spagnolo et al.,

2021). The bacterium's ability to form biofilms contributes to persistent and recurrent infections in the ear canal and middle ear space (Verdial et al., 2023).

1.3.7. Treatment of *P. aeruginosa* Infections

There are many family of antibiotics that are used for *P. aeruginosa* infections treatment (Halawa *et al*., 2023; Saeed *et al*., 2018) The aminoglycoside and quinolone class of antibiotics inhibit the synthesis of bacterial proteins and prevent replication of DNA, respectively. β-lactam antibiotics inhibit bacterial cell walls synthesis, while polymyxins is associated with increasing the permeability of the cell membrane by binding to LPS (Halawa *et al*., 2023).

a. β-Lactam antibiotics

β-Lactam antibiotics are one of the most important drug classes of antibacterial agents (Gauba and Rahman, 2023). They have β-lactam ring that mimics with D-Ala-D-Ala structure (Lima *et al*., 2020). This structural similarity allows β-Lactam antibiotics binding to penicillin-binding proteins (PBPs) (Kim *et al*., 2023). These β-Lactam antibiotics are penicillins, cephalosporins, carbapenems, and monobactams (Kim *et al*., 2023). In penicillin and cephalosporins, the β-lactam ring is attached to a five and six membered sulfur-containing ring, respectively, while there is no ring attached to β-lactam ring of monobactam (Lima *et al*., 2020) .

Aztreonam-Monobactam is a best treatment options especially in penicillin-allergic patients and can be used for the treatment of MBL producing *P. aeruginosa* isolates (Lucena *et al*., 2014). In addition, cefepime (fourth generation) and ceftazidime (third generation) are antipseudomonal cephalosporins, while meropenem and imipenem are antipseudomonal carbapenems used for treatment of *P. aeruginosa* infections (Ibrahim *et al*., 2020). Cefiderocol is another newer cephalosporin antibiotic and potential choice for nosocomial MDR *pseudomonas* infections (Ghazi *et al*., 2018). Cefiderocol, as an iron chelator antibiotic, enters the periplasmic space through porins and active iron transporters where it

binds to PBPs. Cefiderocol has shown an increased susceptibilities against isolate harboring serine and metallo-β-lactamases and extended spectrum β-lactamases (Kocer *et al*., 2024).

Carbenicillin, the first antipseudomonal penicillin has several limitations including weak activity, susceptibility to many beta-lactamases, and a high sodium load, that could cause risk to patients (Riu *et al*., 2022). The second generation of carboxypenicillin, ticarcillin was demonstrated improved potency against *Pseudomonas* than that of Carbenicillin. It is combination with clavulanate improved enhanced it activity against *P. aeruginosa (Zobell et al*., 2010). The ureidopenicillins, including mezlocillin and piperacillin were developed with enhanced potency and spectrum than carboxypenicillin. These drugs contain ureido side chain that has improved activity against *P. aeruginosa* (Riu *et al*., 2022).

b. Combination of β-lactam Antibiotics with β-lactamase inhibitors

Combination of piperacillin with β-lactamase inhibitor tazobactam increased its activity against β-lactamase producing isolates (Ibrahim *et al*., 2020). Ceftolozane-tazobactam has been approved for the treatment of UTI, hospital-acquired pneumonia and intra-abdominal infections. It is also showed that showed better activities against MDR *P. aeruginosa* strains (Walkty *et al*., 2018). Ceftazidime-avibactam is a combination of new β-lactamase inhibitor, avibactam with cephalosporin, ceftazidime. Avibactam has inhibitory activity against class C, class A, and certain class D β-lactamases and lack of activity against metallo-β-lactamases (Sy *et al*., 2017).

Other new antimicrobial available for treatment of MDR *P. aeruginosa*, is called imipenem-cilastatin-relebactam. It is combination of carbapenem with relabactam, which is inhibitor class C cephalosporinase and class A carbapenemases (Titov *et al*., 2021). Meropenem-vaborbactam is an additional combination carbapenem-β-lactamase inhibitor, with activity against MDR *P. aeruginosa* (Doi, 2019).

c. Quinolones and Aminoglycosides

Quinolones including ciprofloxacin, levofloxacin, and prulifloxacin are used for treatment of *P. aeruginosa* infections. They are the only group of orally administered antipseudomonal

agents used to treat *P. aeruginosa* infections (Ibrahim *et al*., 2020). Aminoglycosides such as tobramycin, amikacin, and gentamicin despite their effectiveness against *P. aeruginosa*, should not be used alone, with the exception of treating urinary tract infections and can be used in combination with other antimicrobials for treatment severe infection. Tobramycin in particular has been shown to be effective in treating and preventing infections in people with long-term lung conditions such as cystic fibrosis and bronchiectasis (Horcajada *et al*., 2019; Walkty *et al*., 2018).

d. Colistin

Colistin has significant bactericidal and older antimicrobial with anti-pseudomonas activity against MDR-isolates. In medical therapy, it has been reintroduced as a last resort for treating serious infections caused by MDR and XDR stains (Doi, 2019; Reynolds and Kollef, 2021). It is one of the members of a family of cationic polypeptides known as polymyxins and considered as the last resort antibiotics for the treatment of severe infections caused by multidrug resistance bacteria strains (Reynolds and Kollef, 2021). Two-step antibacterial activities of colistin are initial binding of antibiotics to bacterial cell components and permeabilization of outer membrane of bacterial cell. It attaches with negatively charged structural components in bacterial cells and displaces the divalent cations of magnesium (Mg2+) and calcium (Ca2+). This will lead to impairing the LPS three-dimensional structure and damage to the outer membrane which can cause leakage of cytoplasmic content and bacterial death (Bolla *et al*., 2011).

1.3.8. Mechanisms of Antibiotic Resistance in *P. aeruginosa*

P. aeruginosa is one of the ESKAPE pathogens and prioritized for the development of new antibacterial (Jesudason, 2024). Mechanisms of antimicrobial resistance in *P. aeruginosa* are classified into three categories, including intrinsic, acquired, and adaptive resistance (Arzanlou *et al*., 2017) as shown in Figure 1.2. Intrinsic resistance mechanisms are limited permeability of the outer membrane, efflux pump overproductions and the synthesis of antibiotics inactivating enzymes (Table 1.1) (Bassetti *et al*., 2018) (Figure 1.2)

A. Intrinsic resistance

P. aeruginosa, have an asymmetric bilayer of phospholipid and LPS embedded with porins that serve as a selective barrier to limit antibiotic penetration (Henderson *et al*., 2016). The non-specific porins is used to facilitate the slow diffusion of small hydrophilic molecule, while specific porins have specific sites to bind a specific set of molecules (Chevalier *et al*., 2017).

Compared to other gram-negative bacteria, *P. aeruginosa* has much lower outer membrane permeability, because of its nonspecific porin, OprF with most of its part closed, that limit membrane permeability of *P. aeruginosa* (Chevalier *et al*., 2017). *P. aeruginosa* specific porins include OprB, OprD, OprE, OprO, and OprP; and the efflux pump associated porins are OprM, OprN, and OprJ. OprD has binding sites for the β-lactam antibiotic class known as carbapenems, and its absence promotes resistance to this class of antibiotics(Table 1.1) (Gauba and Rahman, 2023).

Efflux pump system is a tripartite system that is composed of a protein transporter through the inner membrane, a periplasmic connective protein, and an outer membrane protein component (Meletis *et al*., 2012). Resistance-nodulation-division (RND) families of efflux pump proteins are important for antibiotic resistance in *P. aeruginosa*. Four of RND family efflux pumps which are implicated in antibiotic resistance *P. aeruginosa* are MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM, with MexAB-OprM is the most common in antibiotic resistance *P. aeruginosa (Table 1.1).* Mutation in repressor genes (*mexR, nalC,* and *nalD*) lead overexpression of MexAB-OprM, which is associated with resistance to most of antibiotics used for treatment of *P. aeruginosa* infections (Gauba and Rahman, 2023). Mutation of mexZ gene induces overexpression of MexXY–OprM that enhances resistance to fluoroquinolones, aminoglycoside, β-lactam in *P. aeruginosa*. Overexpression of MexCD–OprJ is associated with mutation in nfxB gene that led antibiotic resistance in *P. aeruginosa* (Gauba and Rahman, 2023).

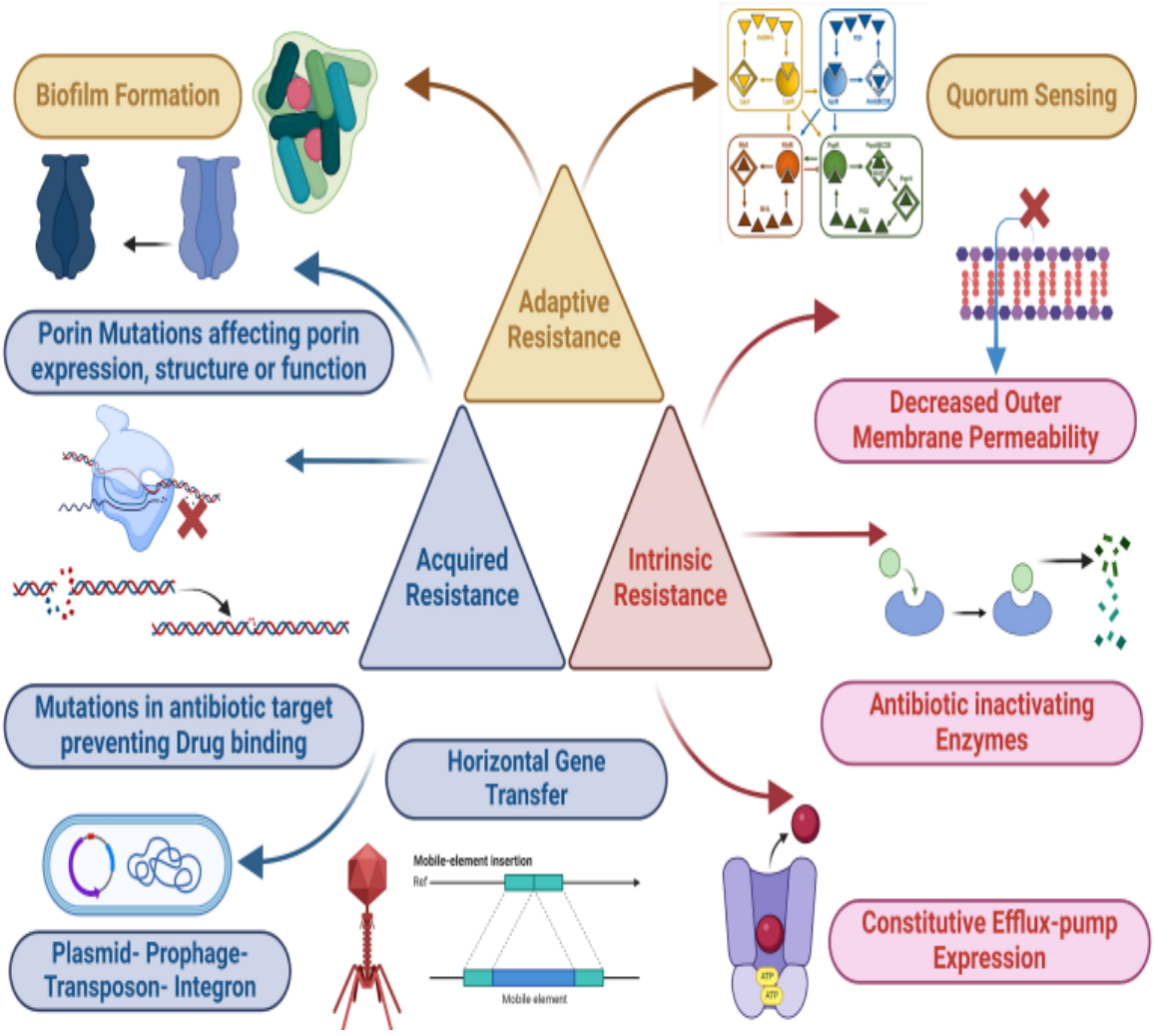

Figure 1.2. Mechanism of antimicrobial resistance of *P. aeruginosa* (Adapted from Elfadadny *et al*., 2024)\

Figure 1.2 showsthree primary resistance pathways: Intrinsic mechanisms: including low outer-membrane permeability, constitutive expression of efflux pumps (e.g., RND family), and chromosomally-encoded β-lactamases (e.g., AmpC). Acquired mechanisms: involving horizontal gene transfer of resistance determinants (e.g., carbapenemases, ESBLs) and porin modifications or loss (e.g., OprD), contributing to carbapenem resistance. Adaptive mechanisms: encompassing biofilm formation, quorum-sensing regulation, which enhance persistence and multidrug resistance.

B. Acquired Resistance

Bacteria can acquire antibiotic resistance genes through horizontal gene transfer from either the same or the distinct bacterial species (Arzanlou *et al*., 2017). These genes can be carried on plasmids, transposons, integrons, and prophages. The spread of antibiotic resistance among *P. aeruginosa* strains has been studied to be significantly aided by integrons, which are genetic elements that use site-specific recombination to insert mobile gene cassettes into a particular genomic location (Meletis *et al*., 2012). Genetic components such as integrons and plasmids have been found to carry *P. aeruginosa* MBL genes (Botelho *et al*., 2019).

C. Adaptive resistance

Bacteria in a biofilm exhibit a major differences in protein and gene expression from planktonic bacteria (Johnston *et al*., 2023). The cell in biofilm are highly tolerant to different chemical, physical stress and antibiotics because of their metabolically dormant and protective matrix (Flemming & Wingender, 2010).

Antibiotic absorption into the biofilm may be restricted by the biofilm matrix. Some antibiotic can form complexes with matrix components or being broken down by enzymes, which reduces the concentration of antibiotics that reach the bacterial cells (Goel *et al*., 2021). Formation neutrophil extracellular trap that form to prevent dissemination of bacteria can also prevent access of antibiotics to biofilm (Thanabalasuriar *et al*., 2019). The higher density of bacterial cell found in biofilm or inoculum size can overcome antibiotics activities and impact susceptibilities (Lichtenberg and Jakobsen, 2022). In order to preserve or alter the characteristics of the biofilm, enzymes present in the matrix can convert complex sugars into fermentable polysaccharides that can be utilized as a source of nutrients and alter the matrix's structure (Flemming and Wingender, 2010).

Cell in biofilm can be lysed and release DNA that could serve as a source of genes for HGT. Because the cells in a biofilm are tightly packed together, high level of cell-to-cell interaction makes it better environment for HGT and the spread of AMR genes (Flemming and Wingender, 2010). Study showed that outer membrane vesicles of biofilm can induce the HGT of AMR genes in *P. aeruginosa* (Johnston *et al*., 2023).

Within a biofilm, there are cells present at various phases of the growth cycle, with metabolically active cells generally being found at the surface of the biofilm and dormant, including 'persister' cells, found in the deeper layers (Olsen, 2015). Many antimicrobials target cells that are actively proliferating and replicating, therefore persister cells can tolerate a broad spectrum of antimicrobials (Melander and Melander, 2015)

Table 1.1. Main mechanisms of antimicrobial resistance in *P. aeruginosa*

Mechanisms	**Genetic event**	**Antimicrobials**	**References**
Beta-lactamases Induced AmpC cephalosporinase	Chromosomal mutation	Penicillins (with or without beta-lactamase inhibitors), cephalosporins, aztreonam	(Akpaka *et al.*, 2009; Ruppé *et al.*, 2015)
Penicillinases[a]	MGE acquisition	Penicillins	
Extended-spectrum beta-lactamases[b]		Penicillins, cephalosporins, aztreonam	
Carbapenemases[c]		Penicillins, cephalosporins, carbapenems	
Loss of OprD	Chromosomal mutation	Imipenem	
Active efflux pumps MexAB-OPrM	Chromosomal mutation	Ticarcillin, cephalosporins, aztreonam, meropenem, fluoroquinolones	
MexEF-OPrN		Cefepime (±penicillins), aminoglycosides, fluoroquinolones	
MexCD-OprJ		Meropenem, fluoroquinolones	
MexXY-OprM		Cefepime, aztreonam, (+/− penicillins), fluoroquinolones	
Lipid A modifications	Chromosomal mutation	Polymyxins	

Aminoglycoside-modifying enzymes[d]	MGE acquisition	Aminoglycosides	
16S rRNA methylases	MGE acquisition	Aminoglycosides	
Topoisomerases modification	Chromosomal mutation	Fluoroquinolones	

MGE mobile genetic element (plasmid or transposon)
Most common enzyme types: [a](*PSE* and *OXA); [b](PER, SHV, GES and OXA); [c](VIM and IMP (SIM, GIM and SPM)* types are less common*); [d](AAC(3)-I, AAC(3)-II, AAC(6')-I, AAC(6')-II and ANT(2')-I).*

1.3.9. Mechanism of β-lactam resistance in *P. aeruginosa*

A. β-lactamases Production and classification

β-lactamases are enzymes found in gram-negative bacteria that protect the bacteria against the lethal actions of β-lactam antibiotics by breaking down amide bond of β-lactam ring and limiting antimicrobial activity of the drug (Gauba and Rahman, 2023). They have the ability to hydrolyze several antibiotics, penicillin, cephalosporins, carbapenems and monobactam (Tooke *et al*., 2019). Bush–Jacoby (functional) and Ambler (amino acid sequences) are the two classification schemes of β-lactamases (Bush and Jacoby, 2010). Among four disparate molecular classes β-lactamases based on amino acid sequences, class B enzymes require zinc as a metal cofactor whereas other class use a serine residue for inactivation of antibiotics (Tooke *et al*., 2019). Based on function β-lactamases can be classified as cephalosporinases, broad-spectrum extended-spectrum β-lactamases, serine carbapenemases and metallo-β-lactamases (Sawa *et al*., 2020). Carbapenemases and ESBLs are found in all classes of the Ambler classification system (Bush and Jacoby, 2010). β-lactamases Classifications scheme derived from Sewa *et al.*(2020) is shown in Figure 1.3.

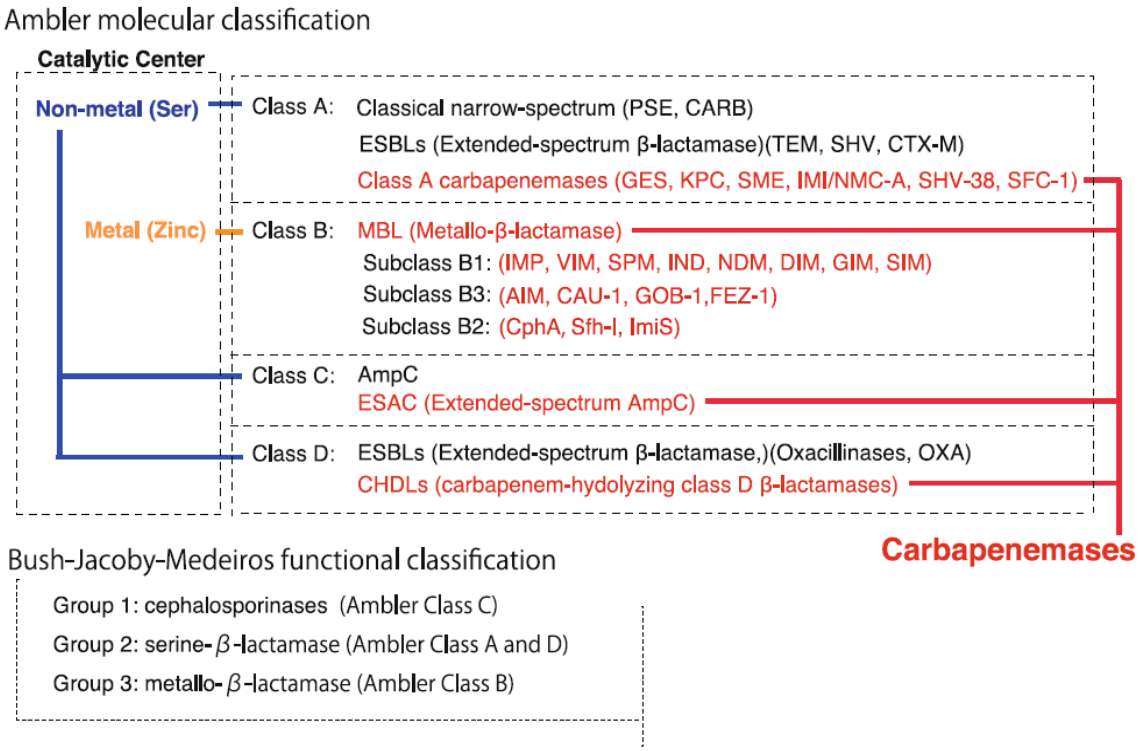

Figure 1.3. The classification of β-lactamases using the Ambler and Bush-Jacobi-Medeiros methods (Adapted from Sewa *et al*., 2020).

β-lactamases are grouped into Ambler Classes A–D based on molecular structure: serine-β-lactamases (A, C, D) and metallo-β-lactamases (B, zinc-dependent). Carbapenemases occur in Classes A, B, and D. The Bush–Jacoby–Medeiros system classifies them functionally as Group 1 (cephalosporinases, Class C), Group 2 (serine-β-lactamases, Classes A and D), and Group 3 (metallo-β-lactamases, Class B).

B. Extended-Spectrum β-lactamases (ESBLs)

ESBL are functionally and structurally mutated versions of β-lactamases and exhibit resistance to monobactam and extended spectrum cephalosporins. The most commonly reported ESBLS include several types of Class A Temoniera β-lactamases and Cefotaxime-M (CTX.M) β-lactamases, and Sulfhydryl Variable (SHV) β-lactamases (Castanheira *et al.*, 2021).

I. TEM Families

TEM enzymes are one of the widely distributed ESBL families in *P. aeruginosa*. There are 214 different TEM variants from different bacteria species have been described, but not all of them are ESBL (https://www.ncbi.nlm.nih.gov/pathogens/refgene/#TEM). In *P. aeruginosa*, TEM-42, TEM-24 and TEM-12 are exhibited extended spectrum activity against third generation cephalosporins (Chen *et al*., 2015), while others TEM-1 and TEM-2 are mainly associated with hydrolyzing penicillin and early cephalosporins (Castanheira *et al*., 2021).

II. CTX-M Families

CTX.M enzymes are another widely distributed class of ESBLs including in *P. aeruginosa*. There are 266 different CTX-M variants from different bacteria species have been described (https://www.ncbi.nlm.nih.gov/pathogens/refgene/#CTX-M). Many variants of CTX-M have been identified in *P. aeruginosa* including CTXM-1,CTXM-2 and CTXM-3 (Chen *et al*., 2015), and CTXM-15 (Ozsolak and Milos, 2010).

III. SHV Families

SHV families that are known for their hydrolytic activities against extended spectrum cephalosporin in *P. aeruginosa* include SHV-5 and SHV-12 (Chen *et al*., 2015). There are 211 different SHV variants that have been described from different bacterial species (https://www.ncbi.nlm.nih.gov/pathogens/refgene/#SHV).

IV. OXA-type ESBLs

OXA-type ESBLs are belong to molecular class D β-lactamases and are known by their hydrolytic activity against extended cephalosporin. There are 1335 different OXA variants have been described from different bacterial species, although not all of them are OXA-type ESBLs (https://www.ncbi.nlm.nih.gov/pathogens/refgene/#OXA). Most ESBLs are found in Enterobacteriaceae like *E. coli* and *K. pneumoniae*, while OXA-type ESBLs are mainly found in *P. aeruginosa*. Several variants of OXA-type ESBLs including OXA-11, OXA-13, OXA-14, OXA-15, OXA-16, OXA-17, OXA-18, OXA-19 and OXA-28 have been identified in *P. aeruginosa* (Polse *et al*., 2023).

V. Other ESBLs

While the majority of ESBLs are derived from CTX-M, TEM or SHV- β-lactamases and others were reported from new ESBL families. These less frequently reported β-lactamases include VEB, PER, GES, BEL, and PSE families. There are 20 different VEB variants, and 18 different PER variants, 66 different GES variants, 4 different BEL variants, and 18 different PSE variants have been described from different bacterial species (https://www.ncbi.nlm.nih.gov/pathogens/refgene/).

C. Carbapenemases

Carbapenemases are beta-lactamases with flexible hydrolytic capacities. Carbapenemases in *P. aerugonosa* are represented almost in class A, B and D in the Ambler classification system (Bush and Jacoby, 2010). The class A carbapenemases are partially inhibited by clavulanic acid and actively hydrolyze carbapenems. It contains serine at the active site and Glu-166, as the general base for the catalytic site (Chen *et al*., 2008). Six types of class A carbapenemases have been reported, these include KPC (*Klebsiella pneumoniae* carbapenemase), GES (Guiana extended spectrum β-lactamase), SHV (sulfhydryl variable lactamase), SFC (*Serratia fonticola* carbapenemase), SME (*Serratia marcescens* enzyme) and IMI/NMC-A (imipenemase/non-metallocarbapenemase-A). Class A carbapenemase enzymes revealed a broad hydrolysis spectrum that includes the penicillins, early cephalosporins, aztreonam, and carbapenems (Chen *et al*., 2008). IMI and SME are chromosomally encoded, and the GES gene is inserted into the class 1 integron in a plasmid of *P. aeruginosa* (Sawa et al., 2020). KPC-2, GES-1 and GES-2 variants have been reported in *P. aeruginosa* isolates (Cuzon *et al*., 2016; Falahat *et al*., 2016).

There are several types of MBLs identified in CRPA. *P. aeruginosa* strains carrying MBL inhibit all β-lactam drugs except monobactams (Sawa *et al*., 2020). These include the verona integron-encoded metallo-β-lactamase (VIM), Imipenemase (IMP), Sao Paulo metallo-β-lactamase (SPM), Florence imipenemase (FIM), Seoul imipenemase metallo-β-lactamase (SIM), New Delhi metallo-β-lactamase (NDM), and German imipenemase (GIM) families

(Hong *et al*., 2015). There are 93 different VIM variants, 1 SPM variant, 3 different FIM variants, and 3 different GIM variants have been described from different bacterial species (https://www.ncbi.nlm.nih.gov/pathogens/refgene/). Among carbapenemases identified in *P. aeruginosa*, IMP- and VIM-type MBL followed by GES variants are globally predominant (Hong *et al*., 2015).

Carbapenem-hydrolyzing class D β-lactamases (CHDLs) are OXA enzymes with carbapenem-hydrolyzing activity mainly identified from chromosome of *Acinetobacter baumannii*, while OXA-23 and OXA-48 were plasmid mediated identified from enteric bacteria strains (Antunes *et al*., 2014). Class D β-lactamases, namely OXA-40 has been reported in carbapenem resistant *P. aeruginosa* isolates (Garch *et al*., 2011). The $bla_{\text{OXA-396}}$ is belongs to OXA-50 family and reported in CRPA isolates in several studies (Fang *et al*., 2022). OXA-type family can exhibit the range of carbapenem hydrolyzing activities and significant activity might be achieved when combine with other resistance mechanisms. Kinetic study conducted to investigate carbapenem-hydrolyzing activities of OXA-families showed varying efficiencies in hydrolyzing meropenem and imipenem, while some variants have showed increased activities over specific carbapenem (Walther-Rasmussen and Høiby, 2006).

D. AmpC beta lactamase

P. aeruginosa produces low level of AmpC beta lactamase and determines resistance to many penicillins and most of early cephalosporins. However, its production can be increased in presence of inducing antibiotics like imipenem and overproduction of AmpC beta lactamase can contribute to resistance in cephalosporin (Chalhoub *et al*., 2018). Mutations dependent over expression of AmpC β-lactamase in certain *P. aeruginosa* clinical isolates is considered as the main resistance mechanism to cephalosporins (Berrazeg *et al*., 2015). This can be induced by mutation in regulator genes including *ampR, ampG* or *ampD, ampR*, encodes a positive transcriptional regulator; *ampG*, encodes a transmembrane protein that acts as a importing muropeptides; while *ampD* encodes a cytosolic amidase and act as repressor of *ampC* expression (Berrazeg *et al*., 2015; Pachori *et al*., 2019).

E. Non-Enzymatic Mechanism of Resistance to B-Lactam Antibiotics

In addition to β-Lactamase production, *P. aeruginosa* exhibit several mechanisms of resistance to β-lactam antibiotics. Resistance to carbapenems in *P. aeruginosa* can be from overexpression of MexAB-OprM efflux pump, which resulted from gene alterations of transcriptional regulators, *mexR, nalB, nalC, or nalD*. The *NfxB* mutants in MexCE-OprJ efflux pump system are more susceptible to imipenem and some β-lactams (Pai *et al*., 2001). *P. aeruginosa* with a defect in OprD exhibits substantial carbapenem resistance, particularly against imipenem, while low expression of outer membrane proteins is associated with resistance in *P. aeruginosa* (Chevalier *et al*., 2017). Both deletion and insertion mutations of OprD2 gene have been reported to affect the expression of OprD2 and the resistance of *P. aeruginosa* to carbapenem (Meletis *et al*., 2012).

Mutation in *MexR* (V126E substitution) has been associated with an increase resistance to carbapenem, while mutation in PBPS affects β-Lactam antibiotics target affinity and can be associated with increasing resistance to other β-Lactam antibiotics (Hujer *et al*., 2023).

1.3.10. Mechanisms of Resistance to Colistin

Colistin resistance in *P. aeruginosa* can be raised from several mechanisms including lipid A modification, efflux pump activity and regulatory genes mutation (Puja *et al*., 2020). Primary mechanism of colistin resistance in *P. aeruginosa* is reducing binding affinity of lipid A of LPS by addition of 4-amino-4-deoxy-L-arabinose molecules. Additionally, *P. aeruginosa* developed enhanced polymyxin resistance because of mutations in the two-component regulatory systems of PhoPQ and PmrAB, which promoted modification of aminoarabinose addition to lipid A. Because of these mutations, the arnBCADTEF operon is up regulated, which causes the cationic 4-amino-4-deoxy-L-arabinose to replace the phosphate groups of lipid A in the LPS structure (Pang *et al*., 2019). Over expression of the MexXY/OprM efflux pump and impermeability of the outer membrane is important in driving resistance *Pseudomonas aeruginosa* isolates to colistin (Puja *et al*., 2020). Further research has demonstrated that *P. aeruginosa* possesses the *mcr* gene, an enzyme that

converts lipid A found in LPS into a phosphoethanolamine metabolite and inhibits its binding to colistin (Abd El-Baky *et al*., 2020).

1.3.11. Mechanisms of Resistance to other Class of Antibiotics

a. Aminoglycosides

In *P. aeruginosa* resistance to aminoglycosides is mediated by low outer membrane permeability, aminoglycosides modifying enzymes, active efflux and, rarely target modification (Pachori *et al*., 2019). Resistance to aminoglycoside of antibiotics is mostly caused by the enzymatic alteration of the amino and glycoside groups in the aminoglycoside chemical structure. It has been found that bacteria contain three different forms of aminoglycoside-modifying enzymes: aminoglycoside phosphotransferase (APH), aminoglycoside acetyltransferase (AAC), and aminoglycoside nucleotidyltransferase (ANT) (Ruppé *et al*., 2015). *P. aeruginosa* APHs inhabit aminoglycosides such as neomycin and streptomycin through transferring a phosphoryl group to the 3′-hydroxyl of aminoglycosides. *P. aeruginosa* AACs inactivate gentamicin and tobramycin by transferring an acetyl group to the amino group at positions 3′ and 6′ of aminoglycosides, and ANTs of *P. aeruginosa* confer resistance to amikacin by attaching an adenylyl group to the amino or hydroxyl group of this aminoglycosides (Verdial *et al*., 2023).

Efflux mediated aminoglycoside resistance is mainly because of over expression of the MexXY- OprM efflux pump (Gauba and Rahman, 2023). Target alteration by methylation of 16S rRNA can result in resistance to aminoglycoside. Various 16S rRNA methylases have been identified in *P. aeruginosa,* those include RmtB, ArmA, and RmtD. RmtA confers resistance to all aminoglycosides such as amikacin, tobramycin, isepamicin, kanamycin, and gentamycin (Pachori *et al*., 2019).

b. Fluoroquinolones

Resistance to fluoroquinolones in *P. aeruginosa* can be caused by overexpression of MexAB-OprM and MexEF-OprN which resulted from gene alterations of transcriptional regulators, *mexR, nalB, nalC, or nalD* (Gauba and Rahman, 2023). Mutations in the genes

encoding DNA gyrase (*gyrA* and *gyrB*) and/or topoisomerase IV (*parC* and *parE*), confer resistance in *P. aeruginosa* by lowering the binding affinity of the encoded proteins to quinolones (Pachori *et al*., 2019).

1.3.12. Trends and Burden of MDR *P. aeruginosa*

A Global and Regional Overview

The prevalence of MDR *P. aeruginosa* infection has increased over the last few decades in multiple geographical areas. According to data from the SENTRY Antimicrobial Surveillance Program spanning 20 years, Latin America had the largest percentage of MDR phenotypes (41.1%), followed by Europe (28.4%) (Shortridge *et al*., 2019). The study on global distribution of *P. aeruginosa* isolate among diabetic foot infections showed 37.9% isolates were MDR strains (Garousi *et al*., 2023). According to estimates from the US Centers for Disease Control and Prevention (CDC), the rate MDR *P. aeruginosa* infections in hospital setting was increased by 32% as compared to 2019 (CDC, 2022). As reported from USA, 13% of severe HAIs is due to MDR *P. aeruginosa* (Horcajada *et al*., 2019). The high rate of 30 day mortality rate associated with carbapenem resistant strains were reported from Middle East and south and central America (Reyes *et al*., 2023)

The EARS-Net reported, 19.7%% of *P. aeruginosa* isolates were resistant to at least two antibiotics tested during surveillance (ECDC, 2023). The number of antibiotic-resistant *P. aeruginosa* infections in Europe increased by nearly three times in year 2007 to 2015 , with an average of 4155 deaths among 72,000 illnesses (Cassini *et al*., 2019).

High treatment cost (US$99,672 vs. US$69,502) in-patient with resistance strain was reported as compared to susceptible isolates (Nathwani *et al*., 2014). The study from Spain showed 26% of *P. aeruginosa* were MDR, of which 65% of were XDR (Del Barrio-Tofinõ *et al*., 2019).

The multicenter study in Asia and Africa reported, 19.6% and 46% of XDR and MDR *P. aeruginosa* strains respectively (Salleh *et al*., 2025). A study conducted in China reported

34% of CRPA and 26.32% MDR of *P. aeruginosa* were among the isolates from blood stream infections (Shuzhen *et al*., 2023). The study from Middle East and North Africa region reported varying prevalence of MDR *P. aeruginosa* strains across the regions, with the highest in Egypt (75.6%), followed by Saudi Arabia (7.3%) and Qatar (8.1%) (Al-Orphaly *et al*., 2021). Increased antibiotic resistance and high MDR proportion in *P. aeruginosa* has been observed in a previous study conducted in Ethiopia (Addis *et al*., 2021; Asmare *et al*., 2024).

1.3.13. Risk Factors Associated with MDR *P. aeruginosa* Infections

Several risk factors has been associated with development of MDR *P. aeruginosa* infections (Folic *et al*., 2021; Zhang *et al*., 2018). Association between history of antibiotics use and drug resistance in the *P. aeruginosa* infection was reported (Newman *et al*., 2017). History of aminoglycosides, quinolones and carbapenems use were reported to be associated with MDR *P. aeruginosa* infections (Raman *et al*., 2018). The factors including mechanical ventilation, length of hospital stay, and history of chronic disease were found as independent risk factors for infection with carbapenem-resistant *P. aeruginosa* (Zhang *et al*., 2018). Meta-analysis study showed intensive care unit admission, history of medical device usage, having underlying diseases, length of hospital stays and priors use of carbapenem were significant risk factors for CRPA in multivariate analysis. The same study revealed prior use of carbapenem (OR = 7.09; 95% CI= 5.43 to 9.25) and medical device usages (OR = 5.11; 95% CI = 3.55 to 7.37) were independent risk factors for CRPA *(*Voor *et al*., 2014). Study conducted in Korea indicated prior urinary tract infections, underlying cerebrovascular accidents, and indwelling urinary catheter were all contributing factors for MDR *P. aeruginosa* infections (Kang *et al*., 2021).

In order to manage this increasing MDR *P. aeruginosa* in hospital setting, studies have shown different infection prevention and control strategies. The antibiotic stewardship program, strict adherence to hand hygiene and proper disinfection and sterilization of medical devices have been shown to reduce prevalence of MDR *P. aeruginosa* (Sid Ahmed *et al*., 2020).

1.3.14. Molecular Epidemiology Multidrug-Resistant *P. aeruginosa*

Molecular typing has identified several sequencing types that linked to MDR/XDR global clones, referred to as "high-risk" clones, which disseminated worldwide (Woodford *et al*., 2011). This high-risk clone are associated with significant morbidity and mortality worldwide (Botelho *et al*., 2019). As writing of this thesis (effective date April 9, 2025), PubMLST database for *P. aeruginosa* has collected 10,733 isolates containing 39,392 unique alleles with 3309 distinct STs (https://pubmlst.org/organisms/pseudomonas-aeruginos). Based on global prevalence of strain types and association with antimicrobial resistance, top 10 *P. aeruginosa* high-risk clones include ST233, ST244, ST235, ST111, ST357, ST308, ST175, ST277, ST298 and ST654. These clones are associated with MDR and XDR phenotypes and they are growing as a global threat in the health care system (Del Barrio-Tofiño *et al*., 2020). ST235 is the most common high-risk clone distributed globally and frequently associated with MDR and XDR strains (Del Barrio-Tofiño *et al*., 2020). In Brazil, *P. aeruginosa* MDR strain carrying CTXM and GES variants were reported (de Almeida Silva *et al*., 2017).

Across Europe, studies have shown different ESBL gene associated with MDR in *P. aeruginosa*. In Spain, ST175 was the most common STs associated with MDR phenotypes and linked to O4 serotypes (Adelantado Lacasa *et al*., 2022). Study conducted in Poland revealed several ESBL genes including *GES-1, GES-5, OXA-2, OXA-10* and *VEB-9* from patients admitted at hospital (Laudy *et al*., 2017). In Germany, several high-risk clones including ST235 followed by ST244, ST175, and ST233 were identified, while *GES-7* variants is among the most common ESBL identified (Weber *et al*., 2022).

In China, the most frequently reported ESBLs was $bla_{\text{CTX-M-15}}$ (32%) followed by other variants $bla_{\text{SHV-11}}$ (27%), $bla_{\text{TEM-1}}$ (14%) and $bla_{\text{CMY-2}}$ (Chen *et al*., 2015). The study from China found plasmids from the incompatibility groups (*Inc)* were shown to be present in ESBL carrying *P. aeruginosa* (Chen *et al*., 2015). Study conducted on clinical isolates from Asia and Africa revealed that the prevalence rates of *P. aeruginosa* that produces extended-spectrum β-lactamases were 33.4% (Salleh *et al*., 2025). In India, 15% *P. aeruginosa*

isolates were carried ESBLs genes, with the most common *bla*$_{VEB}$ followed by *bla*$_{PER}$ and *bla*$_{TEM}$, while the most frequent STs were ST233, ST244, ST235, and ST35 *(Pragasam et al.,* 2018). Finding from Iran showed, 42% of the isolates carried *bla*$_{OXA-10}$ (Mohammadnezhad *et al.,* 2024). The most common reported β-lactamases genes include *bla*$_{OXA-10}$ (59.26%), *bla*$_{PER-1}$ (44.44%) , and *bla*$_{SHV}$ (11.11%) in Iraq (Polse *et al*., 2023).

In Africa, different ESBLs genes were reported among MDR isolates in studies conducted in different countries. Studies conducted in South Africa showed high rate of *bla* $_{SHV}$(93%) harboring isolates followed by *bla*$_{TEM}$ (40%) and *bla*$_{CTX-M}$ (20%) (Hosu *et al*., 2021b), while other South African's study indicated 31.7% of the isolates were carried *bla*$_{CTX-M}$ genes (Hosu *et al.*, 2021a). The study conducted among burn patients hospitalized to the Military Hospital of Algiers in Algeria revealed the most prevalent ESBL genes were *bla*$_{CTX-M2}$ (82.14%) and *bla*$_{PER}$ (34.04%), with five (17.9%) co-harboring *bla*$_{CTX-M2}$, *bla*$_{TEM}$, and *bla*$_{PER}$ genes (Tchakal-Mesbahi *et al*., 2021).

1.3.15. Molecular epidemiology and genetic diversity of CRPA

Carbapenem resistance in *P. aeruginosa* is associated with increased mortality rates (Peña *et al*., 2013). Currently emergence of carbapenem-resistant strains has been becoming a global concern and limiting treatment options of *P. aeruginosa* infections (Bassetti *et al*., 2018). Carbapenemase genes, commonly carried on mobile genetic elements, have the potential for rapid dissemination (Walters *et al*., 2019). The two most prevalent MBL genes in *P. aeruginosa*, IMP and VIM, are often linked to high-risk clones and multiple outbreaks and mortality (Del Barrio-Tofiño *et al*., 2020; Persoon *et al*., 2019). Other carbapenemases, such as SPM, GIM, and FIM, are uncommon and usually associated with certain genetic lineages with restricted regional spread (Dos Santos *et al*., 2023). Most KPC producers are Enterobacteriaceae and they are mainly associated with nosocomial systemic infections (Pesesky *et al*., 2015). KPC harboring *P. aeruginosa* MDR clones were reported from several countries including in Trinidad and Tobago (Akpaka *et al*., 2009), Brazil (De Araújo Jácome *et al*., 2012) and Iran (Falahat *et al.*, 2016).

In Europe, the prevalence of carbapenemase producing *P. aeruginsa* has been increasingly reported with regional variability. Study conducted in Norway found high prevalence of *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{VIM-1}}$ genes in XDR and MDR strains, with 72% of isolates linked to ST235, ST773 and ST111 high risk clones (Haldorsen *et al*., 2025). Additionally, *P. aeruginosa* isolates harboring *bla*$_{\text{NDM-1}}$ were reported from France (Bobo & Dawson, 2005), Serbia (Jovcic *et al*., 2011), Poland (Urbanowicz *et al*., 2019) and Portugal (Fortunato *et al*., 2023). There have been report of *P. aeruginosa* isolates harboring *GES-1* and *GES-5* that linked to clone ST235 and *bla*$_{\text{VIM-2}}$ carrying isolate in Spain (Cuzon *et al*., 2016; Perez-Vazquez *et al*., 2020; Viedma *et al*., 2009), while isolates harboring *bla*$_{\text{VIM-2}}$ that linked to ST111 and ST233 was reported in Portugal (Fortunato *et al*., 2023). OXA-40 and OXA-198, associated CRPA isolates were reported in Spain (Bassetti *et al*., 2018) and Belgium (Garch *et al*., 2011), respectively.

In North and South America, studies have shown increasing prevalence of CRPA that often linked to high-risk clones. It has been shown to differ across different regions, with *bla*$_{\text{KPC}}$ and *bla*$_{\text{VIM}}$ representing the prevalent groups in South and Central America (Reyes *et al*., 2023). In Brazil, 48.90% of the isolates were found to be HRCs, with the two most prevalent being ST277 harboring *bla*$_{\text{SPM-1}}$ (33.55%) and ST244 harboring *bla*$_{\text{KPC}}$ (9.73%) (Dos Santos *et al*., 2023). In Chile, the KPC-producing clone ST654 is predominant *(*Opazo-Capurro *et al*., 2024). From 2018 to 2022, 2% of CRPA isolates submitted to antimicrobial resistance network lab in USA carried at least one carbapenemase gene with the most common *bla*$_{\text{VIM}}$ (57%) followed by *bla*$_{\text{KPC}}$ (24%) (Sabour *et al*., 2024).

In Asia, particularly a study carried out in China revealed that the two most common clones were ST244 and ST1076 , while carbapenemase genes were found for *bla*$_{\text{GES-1,}}$ *bla*$_{\text{GES-14}}$, and *bla*$_{\text{VIM-2}}$ (Hu *et al*., 2024), with the most predominant KPC-producing isolates (Reyes *et al*., 2023). According to the study conducted in India, CRPA was associated with high prevalence of *bla*$_{\text{NDM-1}}$, loss-of-function, and mutations in *oprD* (Menon *et al*., 2024). Additional research from India revealed that 40% of *P. aeruginosa* isolates have

carbapenamse genes, with *bla*$_{VIM}$ and *bla*$_{NDM}$ being the most common (Pragasam *et al*., 2018). *P. aeruginosa* isolates harboring *bla*$_{NDM-1}$ were reported from Singapore *(Prakki et al*., 2023). In Middle East, *bla*$_{VIM}$ and *bla*$_{GES}$ variants were the two most common genes associated with CRPA (Reyes *et al*., 2023). In addition, CRPA strain ST316 was reported in countries of the Gulf Cooperation Council (Zowawi *et al*., 2018).

In several African countries, CRPA has been increasingly reported among hospital admitted patients. A meta-analysis research carried out in Sub-Saharan Africa revealed that a number of genes were linked to CRPA, including *bla*$_{NDM}$, *bla*$_{VIM}$, *bla*$_{IMP}$, *bla*$_{OXA-48}$, *bla*$_{SIM-1}$, *bla*$_{OXA-181}$, *bla*$_{KPC}$, and *bla*$_{OXA-23}$, with the two most common genes were *bla*$_{VIM}$ and *bla*$_{NDM}$ (Arowolo *et al*., 2023). Study from south Africa revealed the emergence of *bla*$_{NDM-1}$-positive ST773 clade that acquired ICEs and integrons carrying ARGs that confer resistance to several antibiotics (Jung *et al*., 2024). In Kenya, isolate harboring serine carbapenemase were mostly belongs to high-risk clones, ST235, ST111, ST357, and ST463 (Musila *et al*., 2021). In Ethiopia, *P. aeruginosa* isolates producing *bla*$_{NDM-1}$ that belong to ST2948 were reported in a study conducted in Jimma, Southwest Ethiopia (Sewunet *et al*., 2022). According to other previous studies, carbapenem resistance was significantly caused by inactivation of the oprD gene and overexpression of efflux mechanisms (Khalili *et al*., 2022).

1.3.16. Molecular Epidemiology and Genetic Diversity of CoRPA

As *P. aeruginosa* has become more resistant to carbapenem, colistin has been proposed as a last-resort treatment option (Reynolds and Kollef, 2021). The recent increase in colistin resistance in *P. aeruginosa*, may be due to overuse and abuse of antibiotics in clinical and agricultural settings, which has increased the selective pressure on bacterial populations (Bostanghadiri *et al*., 2024). Colistin resistance has developed intermittently around the world, despite the fact that the rate is currently low. Although it is not more common in *P. aeruginosa*, plasmid-mediated colistin resistance gene *mcr-1* has been reported, that shows its potential for horizontal spread of colistin resistance (Abd El-Baky *et al*., 2020). Several

insertions and deletions were found in the sequences pmrAB and phoPQ of colistin-resistant *P. aeruginosa* with no mcr genes identified in a study carried out in Turkey (Wi *et al*., 2017). According to the study conducted in China several non-synonymous mutations were identified in *P. aeruginosa* pmrB including S27R, Y345H, V15I, and while additional substation in parR and cprS was identified in one isolate (Lin *et al*., 2019).

1.4. Conventional and Molecular Methods for the Detection of Carbapenemase-Producing and Colistin-Resistant *P. aeruginosa*

I. Phenotypic Detection of Carbapenemase-Producing *P. aeruginosa*

The carbapenem susceptibility in *P. aeruginosa* is usually screened with reduced susceptibility to meropenem, imipenem or doripenem according to Clinical Laboratory Standard Institute (CLSI, 2021) and European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2017). Carbapenem-resistant is defined as resistance to at least one of meropenem, imipenem or doripenem drugs based on standard interpretation criteria provided by CLSI (2021) or EUCAST (2017). Several phenotypic methods have been developed for detection of carbapenemase in *P. aeruginosa*. Currently, more commonly used phenotypic assays in clinical practice for *P. aeruginosa* include, growth-based assays, which measure resistance based on growth in the presence of an antibiotic (e.g., modified carbapenem inactivation method (mCIM) and hydrolysis methods, which detect the product of hydrolysis catalyzed by carbapenemase enzymes (Akhi *et al*., 2017).

a. Modified Carbapenem Inactivation Method (mCIM)

When compared to other techniques, mCIM is a very specific and affordable method for phenotypic detection of carbapenemase (Akhi *et al*., 2017). This test is based on the idea that a 10-µl loop of carbapenemase-producing isolate will hydrolyze a 10-µg meropenem disk when it is incubated for two hours (Jean *et al*., 2022). Then, the meropenem disk is taken out and put on a Mueller-Hinton agar plate that has been streaked with susceptible *E. coli* strain. The zone of inhibition is assessed after an overnight incubation. No zone of inhibition detected indicates the presence of Carbapenemase. Conversely, a zone of

inhibition shows that the isolate is not generating a carbapenemase, meaning meropenem in the disk has maintained its activity (CLSI, 2021). The mCIM was reported to be high sensitivity and specificity for detection of carbapenemase (Tamma and Simner, 2018).

b. Carba NP method

It measures hydrolysis of imipenem in bacterial extracts through carboxylic derivative production. This in turn lowers pH and change color from red to yellow (Akhi *et al*., 2017). It has high sensitivity and specificity in identifying carbapenemase *P. aeruginosa* isolates (Tamma and Simner, 2018).

II. Phenotypic detection of colistin resistant *P. aeruginosa*

The following methods are acceptable for the detection of colistin resistance in *P. aeruginosa*. Broth micro dilution, agar dilution and broth disk elution are all suitable MIC methods for colistin. It is not recommended to use the disk diffusion, automated systems or gradient strip diffusion methods (E-test) (CLSI, 2021), because of the high probability of misleading antibiogram results (Hindler and Humphries, 2013).

a. Broth micro dilution method

Broth micro dilution (BMD) is currently the accepted standard technique for assessing colistin susceptibility in Gram-negative bacteria (EUCAST, 2017). Despite its reliability, this process is challenging to use practically and consistently in the clinical laboratory because of its various technical issues like inaccurately weighing of colistin powder or potential colistin adhesion to certain plastic plates.

b. Colistin Broth Disk Elution test

The colistin disk elution test is based on elution of specific concentration of colistin from antibiotic disk into adjusted Muller-Hinton broth (CAMHB) (Simner *et al*., 2019). It is important to allow the tube to sit at room temperature for 30 minutes and vortex to elute colistin from the discs. Each tube of is then added with 50-µL McFarland standardized inoculum. The minimum inhibitory concentration (MIC) is determined by incubating the test

isolate at 35°C for 16–20 hours. Resistance and intermediate to colistin was considered if the MIC is ≥4μg/mL and ≤ 2μg/mL respectively as per of CLSI breakpoint (CLSI, 2021).

c. Colistin Agar Test

It works by principle of colistin resistant *P. aeruginosa* can grow on media containing colistin while susceptible isolates do not (Humphries *et al*., 2019). Briefly, a loop of a 1:10 dilution of a 0.5 McFarland suspension of isolate is used to inoculate each agar plate (0 to 4 μg/ml). After that, plates are incubated for 16 to 20 hours at 35°C, the lowest concentration at which no observable growth occurred is identified as the minimum inhibitory concentration (MIC); any discernible growth is interpreted as positive (CLSI, 2021).

III. Molecular Methods for detection of ESBLs, carbapenemase and colistin resistance in *P. aeruginosa*

There are different commercially available molecular methods for detection of ESBLs and carbapenemase genes from bacterial isolates. Molecular techniques include WGS applications, microarrays, and automated and manual nucleic acid amplification testing (Simner *et al*., 2024).

WGS used to determine drug-resistance profiles, virulence factors of the isolates and reveal evolutionary relationships between the strains (Madaha *et al*., 2020). WGS has the benefit of being able to identify the set of AMR genes of the bacterial isolate; however, it is constrained by the cost of the test, the molecular expertise needed to run it and the bioinformatics expertise needed to interpret the sequencing results (Simner *et al*., 2024). There are several high-throughput WGS techniques that are available such as pyro sequencing, Nano pore technology, Illumina sequencing and Ion Torrent sequencing (Schoumans *et al*., 2010).

To characterize ESBL genes and/or variations, several studies exclusively employ molecular tests, such as DNA microarray assays, PCR, sequencing, and WGS. However, because of

the intricacy of the procedures, they are not widely used in clinical laboratories (Castanheira *et al*., 2021).

DNA microarray was used to identify the clinically significant genes for carbapenemase and cephalosporinase encoded by plasmids as well as to differentiate between non-ESBL and ESBL genes (Cuzon *et al*., 2012).

The multiplex real time PCR assay can be used for identification of carbapenemases (KPC, GES, IMP, VIM, and NDM-1) in a single reaction (Monteiro *et al*., 2012). The PCR-based assay can be used to detect plasmid mediated the *mcr-1* gene in colistin resistant isolates (von Wintersdorff *et al*., 2016). For the surveillance and epidemiological research multiplex PCR method that can be used to detects multiple variants of *mcr* gene was described (Rebelo *et al*., 2018). In general, for accurate and rapid detection of gene associated with resistance and guiding antibiotic treatment molecular technique is crucial.

1.5. Alternatives Therapeutic Strategies for *P. aeruginosa* Infections

In order to overcome sophisticated resistance mechanisms in *P. aeruginosa* several strategies have been studied (Patil *et al*., 2024). Antimicrobial peptides, phage based therapy, monoclonal antibodies, and vaccines are emphasized as therapies that target particular *P. aeruginosa* (Yaeger *et al*., 2021) .Efflux pump inhibitors and biofilm disturbing agents have shown promising in restoring efficacy of antibiotics and enhancing antibiotic penetration (Patil *et al*., 2024). Antimicrobial peptides (AMP) usually cause bacterial membranes to rupture, allowing cellular contents to spill out and killing the bacteria and disrupt biofilm formation. Although AMP is growing as a promising therapeutic strategy, concern is raising due to their potential for human toxicity (Yaeger *et al*., 2021).

Bacteriophages are reemerging as therapeutic option, because of their strong anti-biofilm action and a safer safety profile than antibiotics. Some phage-derived products might increase the effectiveness of antibiotics by decreasing pathogenicity through the modification of fundamental bacterial structures (Anastassopoulou *et al*., 2024).

Over the past 50 years, a large number of vaccine candidates and monoclonal antibodies have been studied; however, because of bacterium antigenic diversity no licensed vaccine is yet available (Yaeger *et al*., 2021). Vaccine approaches that target lipopolysaccharides and O-antigens have showed better promise (Hoggarth *et al*., 2019). Recombinant flagella and pili that target TLR5 have been used to prevent adherence and motility. The exotoxin *PopB* and the needle-like structure *PcrV* are examples of components of the type 3 secretion system that could be targets for vaccination. *OprF* and *OprI*, two more recent examples of outer membrane proteins, are potential vaccine candidates (Killough *et al*., 2022). Anti-virulence strategies that specifically disrupt bacterial virulence pathways have been investigated and shown promising against *P. aeruginosa* isolates (Mudgil *et al*., 2024).

Future research needs to be directed at integrative genomic investigations to unravel the complex mechanisms of multidrug resistance strains. The One Health strategy is necessary to monitor high-risk clones and environmental reservoirs to enable more effective treatment strategies. Developing rapid molecular diagnostic tools for early detection and resistance profiling is crucial for evidence based clinical decision-making. Additionally, future effort should be placed on integration of antibiotic stewardship program with molecular surveillance for early detection and targeted control and treatment strategies

1.6. Significance of the Present Study

The present study generated data on AMR profile of *P. aeruginosa*, which will help public health policies in compacting raising threat of AMR. This study also showed variability in resistance pattern with some showing better sensitivity and others high resistance which can help clinical practices in guiding healthcare professionals in choosing appropriate treatment options.

Genomic-based analysis showed the presence of ESBL and carbapenemase producing *P. aeruginosa* strains. The present study showed novel and local circulation of global high-risk clone (HRC) of *P. aeruginosa* that poses a significant threat to health care. This finding provides a valuable data for public health surveillance and informing policy makers for

better management and infection control measures to prevent further dissemination of resistant strains. The finding of novel *P. aeruginosa* strains in present study is highly significant as it shows the isolate is evolving rapidly that contribute to better understanding of emerging strains and evolution of antimicrobial resistance.

Phylogenetic analysis revealed the presence of closely related *P. aeruginosa* strains, suggesting intra-hospital transmission. This finding is significant as it shows the need for improving infection control and effective monitoring system to prevent transmission.

In addition, this study revealed data on serotypes of *P. aeruginosa* and their association with MDR and as well as a number of virulence genes linked to pathogenicity. It revealed that the presence of highly virulent strain in Ethiopia and emphasized the importance of ongoing molecular surveillance to track the further spread of such strains.

1.7. Hypothesis

In this study, we have hypothesized: -

- *P. aeruginosa* isolates that are phenotypically multidrug resistance carry corresponding resistance genes and mutations
- High-risk clones and genetically related *P. aeruginosa* isolates are presents among the study isolates

1.8. Objectives of the Study

GENERAL OBJECTIVE

- To determine the phenotypic and molecular characterizations of *P. aeruginosa* isolated among patients admitted at Tikur Anbessa Specialized Hospital (TASH) and Yekatit12 Hospital Medical College (Y12HMC), Addis Ababa, Ethiopia.

SPECIFIC OBJECTIVES

- To determine the prevalence of *P. aeruginosa* isolates in different clinical samples.
- To determine antibiotic resistance profile of *P. aeruginosa* isolates from different clinical samples.
- To evaluate the biofilm-forming capacity of the isolates and its association with antimicrobial resistance phenotypes
- To determine factors associated with MDR *P. aeruginosa* infections
- To characterize serotypes and virulence factors of *P. aeruginosa* isolates
- To determine resistance mechanisms and genetic relatedness of MDR *P. aeruginosa* isolates

CHAPTER TWO: MATERIALS AND METHODS

2.1. Study design, Setting and Period

A cross-sectional study was conducted in Tikur Anbessa Specialized Hospital (TASH) and Yekatit 12 Hospital Medical College (Y12HMC) from August 2022 to September 2025.

TASH is the largest teaching referral hospital in Ethiopia and has more than 700 beds. TASH was selected because it represents Ethiopia's healthcare landscape, receiving patients from various regions. The hospital has well established laboratory facility, which is built to perform different laboratory tests. Its bacteriology laboratory is well established and performs culture and susceptibility testing from different clinical samples.

Y12HMC is one of the hospitals in the Addis Ababa City Administration and has been giving routine health services for Addis Ababa as well as referral cases from health centers in Addis Ababa and its surroundings and also from different regional states of the country. The hospital has more than 265 beds. Y12HMC was chosen due to its dedicated burn center, where *P. aeruginosa* is a key bacterial isolate associated with burn patients.

2.2. Source of Population and Sample Size

All patients admitted to TASH and Y12HMC during the study period were a source of population for sampling. The sample size was calculated by employing a single population proportion formula by taking prevalence (P=0.5) (Naing *et al*., 2006), using a 95% confidence interval with a 5% margin of error and a 10% non-response rate, the total sample size was 422.

$$N = \frac{z^2\, p\,(1-p)}{D^2} = \frac{(1.96)^2 * 0.5(1-0.5)}{(0.05)^2} = 384 + 10\% = 422$$

Total sample size was proportional allocated to TASH and Y12HMC based on average monthly admission data obtained from each hospital. TASH has 282 admissions and Y12HMC has 214 admissions. The proportion of admissions at each hospital serves as the basis for the sample distribution.

TASH: (282/496)*422 = 240 and Y12HMC: (214/496)*422 = 182

Therefore, in order to maintain the proportionate representation of each hospital's admissions, 240 patients were recruited from TASH and 182 patients were from Y12HMC.

2.3. Eligibility and sampling techniques

Patients of all ages admitted to TASH and Y12HMC for more than 48 h during the study period and diagnosed with suspected Urinary Tract Infection (UTI), Blood Stream Infection (BSI) and burn wound and surgical site infections, who volunteered to participate and gave informed consent (Annex II and III)**,** and were able to give clinical samples were included in the study.

Patientswho had a history of antibiotic uses within the last 15 dayswere excluded from the study.

A purposive sampling approach was used to enroll eligible patients. Physicians identified patients suspected of HAI, and data collectors assigned to each ward screened them for eligibility. Samples were collected from wards where *Pseudomonas aeruginosa* infections are commonly isolated: TASH — medical, orthopedic, surgical, pediatric, urology, obstetrics and gynecology (OB/GYN), neonatal ICU (NICU), and surgical ICU; Y12HMC — medical, surgical, and burn wards. Samples from respiratory infections were not planned during proposal development, as the study was designed during the COVID-19 pandemic. Recruitment and data collection were conducted concurrently at both hospitals during the study period.

2.4. Study Variables

Dependent variables were MDR in *P. aeruginosa*, while independent variables were age, sex, residence, history of hospitalization, history of surgery, history of invasive medical procedures, previous exposure to antibiotics, previous history of surgery, source of isolates and duration of hospitalization.

2.5. Data Collection

The demographic characteristics were obtained through interviews with the participants using standardized questionnaire prepared for the study (Annex IV). Clinical profiles and antibiotics related information were obtained from the participant's medical record.

2.6. Sample Collection and Transport

a. Blood Sample

. Blood samples were collected from adult and pediatric participants clinically suspected of bloodstream infection (BSI). Approximately 10 mL of blood was drawn from adult patients and 1–5 mL from children, following standard aseptic procedures. .For the volume of blood collection from children, age and weight-based blood sampling approach was considered as recommended by different researchers (Gaur *et al*., 2003; Huber *et al*., 2020; Revell, 2017).

Aseptic method implemented during blood collection by the attending physician/nurse using tincture iodine (70% alcohol and 2% iodine) and put into a blood culture bottle containing Tryptic Soy Broth (TSB) (Oxoid, England). A 1:5 and 1:10 blood to blood culture broth dilution was made for children and adult, respectively. Inoculated broth was incubated aerobically at 35–37 °C for seven days and inspected for turbidity daily.

b. Urine sample

Urine samples (5 mL each) were collected aseptically by the attending physician or nurse from participants clinically suspected of urinary tract infection (UTI). For catheterized participants, samples were obtained directly from the catheter port, and for non-catheterized participants, clean-catch midstream urine was collected using standard aseptic techniques.

c. Wound sample

Surgical and burn wound swab samples were collected aseptically by the attending physician/nurse from participants suspected of infected burn wounds and surgical site wound infections. A sterile cotton wool swab was used to collect swabs from the depths of infected sites. The swab was placed in a bottle with normal saline and then inoculated on appropriate culture media (Bonham, 2009).

2.7. Culture and Identification of *P. aeruginosa*

Culture and identification were done using conventional standard microbiological methods at TASH and Y12HMC microbiology laboratory. All blood culture bottles were incubated aerobically at 35-37°C for seven consecutive days and inspected daily for signs of bacterial growth. Blood cultures that showed turbidity were sub-cultured on blood agar and MacConkey agar (BioMérieux, Craponne, France). Urine and wound swab samples were directly inoculated on blood agar and MacConkey agar. All plates were incubated at 35–37 °C for 16–48 h for bacterial growth. All positive cultures were identified based on colony characteristics (non-lactose fermenter on MacConkey agar) and further identification at the species level was done through biochemical tests including oxidase, catalase, triple sugar iron, and growth at 42°C (Monica Cheesbrough, 2005).

Urine cultures from non-catheterized patients that grew $\geq 10^5$ CFU/ml of urine and from catheterized patients that grew $\geq 10^2$ CFU/ml were taken as significant bacteriuria that indicates UTI (Karah *et al.*, 2020). All *P. aeruginosa* isolates were kept in STGG media (Skim milk-Tryptone-Glucose Glycerin) at -80°C for further antibiotic susceptibility testing and molecular characterizations.

2.8. Antibiotic Susceptibility Testing

a. Disk Diffusion Method

Antibiotic susceptibility testing (AST) was performed at each hospital microbiology laboratory and the test repeated at Biomedical center of Uppsala University, Sweden using the Kirby-Bauer disk diffusion method according CLSI guideline (CLSI, 2021). All *P. aeruginosa* isolates were sub-cultured on cystine lactose electrolyte deficient (CLED) agar to confirm viability and purity. Briefly, 3-5 pure colonies were picked using sterile wire loop, mixed well in nutrient broth and adjusted to 0.5 McFarland using densitometer. Using cotton swab adjusted suspension was then swabbed to Muller-Hinton agar and then using antibiotic disc dispenser 11 antibiotics discs were dispended onto swabbed MHA. After

incubation for 16-18hrs at 37°C, zone of inhibition was measured in millimeter and interpreted based on CLSI (2021).

Susceptibility test was performed against 11 antibiotics discs grouped into four classes obtained from Oxoid:

Beta-lactam Antibiotics: Imipenem (IMP, 10µg), Meropenem (MEM, 10µg), Aztreonam (ATM, 30µg), Ceftazidime (CAZ, 30µg), Cefepime (FEP, 30µg)

Beta lactam antibiotics with beta lactamase inhibitor: Piperacillin-Tazobactam (PTZ, 100/10µg) and Ceftazidime-avibactam (CZA, 30/20µg)

Aminoglycosides: Gentamicin (CN, 10µg) and Netilmicin (NET, 30µg)

Fluroquinolones: Ciprofloxacin (CIP, 5µg) and Levofloxacin (LEV, 5µg)

b. Broth Micro Dilution Method

Isolates found to be carbapenem non-susceptible (19 MEM/IMP-R and 10 MEM/IMP-I) using disk diffusion method were further subjected to minimum inhibitory concentration (MIC) testing at each hospital microbiology labortaory. The MIC was performed with concentration of Imipenem (0.5-32 µg/mL), and Meropenem (0.5-32µg/mL) using broth micro dilution method. Resistance and intermediate to carbapenem was considered if the MIC is ≥8µg/mL and 4µg/mL, respectively (CLSI, 2021).

P. aeruginosa was categorized as MDR, Drug resistant (DR), and susceptible isolates based on the criteria described by Magiorakos *et al* (2012). DR *P. aeruginosa* is defined as resistant to more than one antimicrobial agent in less than three antimicrobial categories. MDR is defined as non-susceptibility to at least one agent in three or more antimicrobial categories. XDR is defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (Magiorakos *et al*., 2012).

c. Modified carbapenem inactivation method for carbapenemases production

At Uppsala University, the modified carbapenem inactivation method (mCIM) was performed to detect carbapenemase production among the 29 isolates (19

meropenem/imipenem-resistant and 10 meropenem/imipenem-intermediate), following CLSI guidelines(CLSI, 2021). Accordingly, a 10-µL loopful of colonies form overnight blood agar was mixed in 2 mL Tryptic Soy Broth (TSB) and 10-µg meropenem disk was added and incubated for 4 hours at 35–37°C. As the routine disk diffusion procedure, standardized inoculum of 25922 susceptible *E. coli* strain was prepared and inoculated onto Mueller-Hinton agar (MHA) plate and meropenem disk from the TSB-meropenem suspension was placed on it and incubated at 35–37°C for 18–24 hours. Zone of inhibition diameter 6–15 mm or pinpoint colonies within 16–18 mm was considered as positive for carbapenemase enzyme production, and a zone of inhibition ≥19 mm was considered to be negative for carbapenemase enzyme production according to CLSI guideline (CLSI, 2021; Verma *et al*., 2019).

d. Colistin broth disk elution test for colistin susceptibility

At Uppsala University, all 29 carbapenem-non-susceptible isolates were tested for colistin susceptibility using the colistin broth disk elution (CBDE) method, following CLSI guidelines. Accordingly, after warming cation-adjusted Muller-Hinton broth (CAMHB) and colistin disks at room temperature, four tubes with 10 ml of CA-MHB were taken for each isolate. Final concentrations of 0 (growth control), 1, 2, and 4 g/ml were obtained in the CA-MHB tubes using colistin discs (10 µg; BD, USA). To ensure that the colistin was sufficiently eluted from the discs, the tubes were vortexed and allowed to sit at room temperature for 30 minutes. The inoculum was standardized to match the McFarland 0.5 standard, and each tube added with a 50 µL aliquot of the standardized inoculum (7.5×10^5 CFU/mL). The MIC was determined by incubating the test isolate at 35°C for 16–20 hours. Resistance and intermediate to colistin was considered if the MIC is ≥4µg/mL and ≤ 2µg/mL, respectively as per of CLSI guideline (Abd El-Baky *et al*., 2020; CLSI, 2021).

2.9. Biofilm Formation Assay

At Uppsala University, a microtiter plate assay was employed to determine biofilm formation, following previously described methods(Karami *et al*., 2019). The overnight

cultures of tested isolates in Mueller-Hinton broth were adjusted to match a 0.5 McFarland standard. Then, 180 µl of TSB (Oxoid) and 20 µl of bacterial suspension were added into a flat bottom 96-well polystyrene microtiter plate to a final volume of 200 µl. The plate was incubated overnight at 37^{o}C for 24 h. Following overnight incubation, planktonic cells were washed off with water; the plate was dried and stained with a 1% crystal violet for 15 min. Afterwards, 250 µl of 33% acetic acid was added to each well, and the biofilm formation was quantified by measuring optical density (OD) at 595 nm. Biofilm assays were performed in triplicates, the mean absorbance was determined, and the OD cut-off value was calculated as described previously (Feifei *et al*., 2021). Biofilm phenotypes were categorized as no biofilm and weak biofilm as well as moderate and strong biofilm phenotypes (Karami *et al*., 2019).

2.10. Molecular Characterizations

I. DNA Extraction

Genomic DNA from Pseudomonas aeruginosa isolates was extracted at Uppsala University using the QIAGEN DNA extraction kit (QIAGEN, Germany) following the manufacturer's protocol. Extractions were done by taking 3-5 pure colonies that grew on CLED agar at 37^{o}C for 24 hours. After extraction, Qubit$^{TM3.0}$ (Thermo Scientific, Waltham, MA, USA) was used to measure the concentration of DNA. All extracted DNA (30–50 ng/µL) samples were kept at -20^{o}C until they were submitted for whole genome sequencing (WGS).

II. Whole Genome Sequencing of Isolates

From each DNA samples, 25µL was transferred into 96 well WGS plate and sent for WGS at Science for Life laboratory, Solna, Sweden. The Sequencing libraries were created using Nextera XT illumina kits, and short read sequencing was performed via 150 bp insert size paired-end sequencing protocol on an Illumina HiSeq 2500 system (illumina, San Diego, CA, USA).

III. Genome Assembly and Quality Assessing

Before assembly, fastq v0.12.1 was used to evaluate the quality of the paired-end short reads from Illumina sequencing (Andrews, 2010). Adapters, unreliable reads and low-quality sequences were trimmed using fastp v0.23.4 (Chen *et al*., 2018). SPAdes version 3.15.0 was used to perform de novo assembly of raw readings (Bankevich *et al*., 2012). Assembly quality was assessed using QUAST (Gurevich *et al*., 2013). Completeness and contamination was checked using CheckM v1.2.2 (Parks *et al*., 2015). The genome annotation was performed using Bakta v1.6.1 (Schwengers et al., 2021).

IV. Species Identification

Average Nucleotide Identity (ANI) was calculated against reference genomes (GenBanK accession: SAMN02603714) using JSpeciesWS v3.2.7 (Richter *et al*., 2016). An ANI value ≥95% was used as the species delineation threshold. Following the completeness and contamination assessment (Parks *et al*., 2015), 64 *P. aeruginosa* isolates with high quality genome (≥ 95% completeness and <5% contamination) were selected for further genomic analysis **Error! Reference source not found.**

V. Virulence Genes Detection and Serotyping of *P. aeruginosa*

Virulence genes were detected using abricate v1.0.1 (Seemann, 2023) with searches performed against Virulence Factor Database (VFDB) (Liu *et al*., 2022). Serotyping of *P. aeruginosa* isolates was performed using Past v1.0 software (CGE Server (dtu.dk) (Thrane *et al*., 2016).

VI. Antimicrobial Resistance Genes Detection

Resistance genes for *P. aeruginosa* were identified from *de novo* assemblies using abricate v1.0.1 (Seemann, 2023) with searches performed against Resfinder v4.5.0 (Bortolaia *et al*., 2020).

VII. Mutational Analysis of Resistance Associated Genes

To investigate resistance-associated mutations, assembled genomes were analyzed for mutation in regulatory genes, specifically for *ampD, mexR* and *nalC* genes. In Colistin

resistant isolates, *pmrB* gene was analyzed. The *ampD* (Locus tag PA4522, NC_002516.2: 5064774–5065340), *mexR* (Locus tag PA0424, NC_002516.2: 471306–471749), *nalC* (Locus tag PA3721, NC_002516.2:4166518–4167159) and *pmrB* (Locus tag PA4777, NC_002516.2: 5364760–5366193) genes from *P. aeruginosa* PAO1 were used as the reference sequence for BLASTn based extraction. Extracted sequences were aligned to reference sequences using MAFFT v7 (Katoh and Standley, 2013). The mutations were detected and translated using BioPython script (Cock *et al*., 2009). Finally, identified mutations were compared with previously published data.

VIII. Detection of the Presence of Biofilm related Genes

The *pelA* (Locus tag PA3064, NC_002516.2: 3431045 –3433891) and *pslA* (Locus tag PA2231, NC_002516.2: 2453667 –2455103) genes were retrieved from *P. aeruginosa* reference genome, while *algD* and *pilA* were identified through VFDB. The presence of *pelA* and *pslA* was confirmed based on alignment identity of ≥95 % and ≥90% coverage.

IX. Multi-Locus Sequencing Typing (MLST) and Novel ST Identification

Multi-locus sequencing typing (MLST) of was identified using internal fragments of seven housekeeping genes with tool at PubMLST database. The alleles number at each of the seven loci define the allelic profile or sequence type (ST) (https://pubmlst.org/) (Jolley *et al.*, 2018). Isolates with new allele profiles were flagged as novel STs. New allele's combinations were submitted to PubMLST database for official designation of assigned new STs.

X. Phylogenetic Analysis

The parsnp v2.0.6 tool was used for phylogenetic analysis (Kille *et al*., 2024). The output file (. xmfa file) was used to generate multi-fasta format using harvest tools (Treangen *et al.*, 2014). The phylogenetic tree was constructed from core alignment fasta using IQ-Tree v2.0.7, and branch support was assessed using 1,000 ultrafast bootstrap replicates (Minh *et al*., 2020). The resulting tree (.treefile) was visualized and annotated using Chiplot (Xie *et al*., 2023). To assess pairwise SNP distances, core-genome SNPs were extracted form

parsnp output (parsnp.ggr file) using harvest tools (Treangen *et al.*, 2014) and pairwise distances were generated using a custom Python script (Cock *et al*., 2009). Cutoff value for SNP threshold was selected based previously conducted studies (Romano-Bertrand et al., 2025; Watt et al., 2025). Accordingly , ≤25 SNPs threshold was used to define close genetic relatedness (Romano-Bertrand *et al*., 2025). The summary of molecular characterizations of *Pseudomonas* spp. (I to X) is outlined in Figure 2.1.

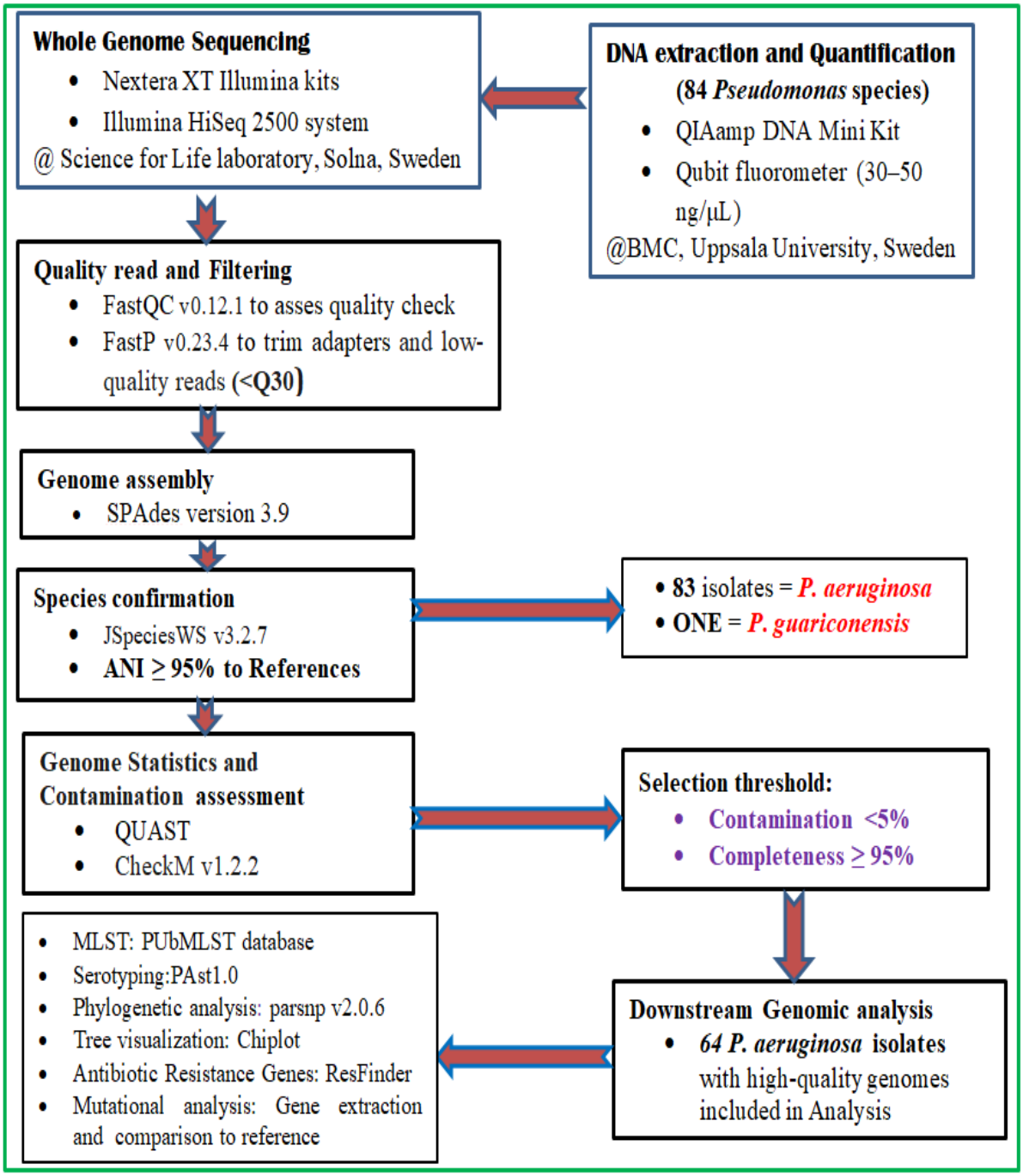

Figure 2.1. Flow chart for molecular characterizations of *Pseudomonas* isolates

2.11. Quality Control

Several QC procedures were followed to ensure the quality of the laboratory procedures implemented in this study. The media were prepared in accordance with the manufacturer's instructions, and non-inoculated plates were incubated at 37°C for 24 to 48 hours to verify sterility. The pH of media was checked to make sure it was within the suggested range. *Staphylococcus aureus* (ATCC 25923) was used to evaluate the performance of Blood Agar and catalase test. *Escherichia coli* (ATCC 25922) was used to confirm the appropriate differentiation of fermentation on MacConkey Agar. *P. aeruginosa* (ATCC 27853) was used to evaluate performance of oxidase test Triple Sugar Iron (TSI) agar test. *P. aeruginosa* (ATCC 27853) was used as the positive control in the biofilm formation assay, and sterile media without bacteria was added as a negative control to confirm that there was no contamination. The inoculum density was standardized to a 0.5 McFarland standard for antibiotic susceptibility testing, and all the culture plates were incubated at 37 °C for 16–18 h to allow for optimal bacterial growth.

2.12. Statistical Analysis

The data was prepared using a Microsoft Office Excel sheet and imported to SPSS version 25 for analysis. Descriptive statistics were used to describe relevant variables in terms of percentage and frequency. The bivariate and multivariable logistic regression analyses were used to identify the associated factors of multidrug resistance. Variables with $p<0.25$ were taken as a candidate for multivariable logistic regression on bivariate. Then, those with $p<0.05$ on multivariable logistic regression were considered statistically significant risk factors of multidrug resistance. Spearman correlation (rs) was performed to evaluate the relationship between biofilm formations and drug resistance. A clustered percentage bar chart was used to show the distribution of biofilm phenotypes among gene-positive isolates.

2.13. Ethical Consideration

The research project was ethically approved by the Department Research Ethics Review Committee (DRERC) of Microbiology, Immunology and Parasitology (DRERC 002/2022, May 20, 2022), Institutional Review Board (IRB) of College of Health Sciences of Addis Ababa University (Protocol number: 054/22/DMIP, June 22. 2022) and then by National Ethics Review Committee (NERC) (ref no: 17/152/845/23, January 30, 2023) with Material Transfer Agreement. Before being recruited for the study, the purposes of study were explained to participants or participants' parents or guardians (Annex IA and IB). The children whose parents or guardians provided informed consent as well as the study participants who provided written informed consent were included to this study (Annex IIA, Annex IIB and Annex III). Permission was obtained from TASH and Addis Ababa City Health Bureau. An informed consent and assent were obtained accordingly from study participants.

CHAPTER THREE: RESULTS

3.1. Socio-Demographic Characteristics of Study Participants (Paper I)

During the study period, a total of 422 participants were recruited, of which 170 (40.3%) were female and 252 (59.7%) were male. Out of all the participants, 268 (63.5%) were from urban areas, 188 (44.1%) were from 16-40 years age group, 240 (56.9%) were from TASH and 182 (43.1) were from Y12HMC. The majority of the patients, 182 (43.1%), had length of stay in hospital for 3-10 days (Table 1).

Table 3.1. Socio-demographic characteristics of patients at TASH and Y12HMC, Addis Ababa, Ethiopia

Variables	**Categories**	**Frequency (no. %)**
Age in years	0-15	98 (23)
	16-40	188 (44.1)
	41-60	74 (17.4)
	>60	62 (14.6)
Sex	Male	252 (59.7)
	Female	170 (40.3)
Residence	Urban	268 (63.5)
	Rural	154 (36.5)
Hospitals	TASH	240 (56.9)
	Y12HMC	182 (43.1)
Hospital stay time	3-10 days	182 (43.1)
	11-20 days	113 (26.7)
	21-30 days	52 (12.3)
	>30 days	75 (17.8)
Clinical samples	Blood	90 (21.3)
	Urine	140 (33.2)
	Wound	192(45.5)

3.2. Prevalence of *Pseudomonas aeruginosa*

Out of 422 clinical samples cultured, 83 (19.6%) were positive for *P. aeruginosa* isolates. Among the 83 *P. aeruginosa* isolates, 52 (62.7%) were from male, and 36 (43.4%), were from age group of 16 to 40 years old as outlined in Table 3.2.

Table 3.2. Characteristics of patients with positive culture for *P. aeruginosa* at TASH and YK12HMC, Addis Ababa, Ethiopia

Variables	**Categories**	**Frequency (no. %)**
Age in years	0-15	21(25.3)
	16-40	36 (43.4)
	41-60	13 (15.7)
	>60	13 (15.7)
Sex	Male	52 (62.7)
	Female	31 (37.3)
Residence	Urban	56 (67.5)
	Rural	27 (32.5)
Hospitals	TASH	47 (56.6)
	Y12HMC	36 (43.4)
History of Hospitalization	Yes	39 (47.0)
	No	44 (53%)
History Surgery	Yes	11(13.3
	No	72 (86.7)
History of invasive medical procedure usages	Tracheostomy	11(13.3)
	Urinary Catheter	19 (22.9)
	Indwelling gastric tube	4 (4.8)
	None	49 (59.0)
Hospital stay time	3-10 days	36 (43.4)

	11-20 days	22 (26.5)
	21-30 days	10 (12)
	>30 days	15 (18.1)

3.3. Frequency of *Pseudomonas aeruginosa* in Different Clinical Samples

Samples were collected from 8 different wards in TASH — medical, orthopedic, surgical, pediatric, urology, obstetrics and gynecology (OB/GYN), neonatal ICU (NICU), and surgical ICU; three different wards in Y12HMC — medical, surgical, and burn wards. Blood and urine samples were mainly collected from medical and surgical wards, while wound swabs were obtained mostly from burn and surgical ICU wards. Out of 83 *P. aeruginosa* isolates, 27 (32.5%) were detected from urine, 23 (27.7%) were from surgical wound, 19 (22.8% were from blood and 14 (16.9%) were from burn wound as shown in Figure 3.1a. All BSI were from TASH, and all burn wound infections were from YK12HMC. Out of the 83 isolates, 47 (56.7%) were from TASH and 36 (43.3%) were from YK12HMC. The distribution of infections and frequency of *P. aeruginosa* isolates among two hospitals are presented in Figure 3.1b.

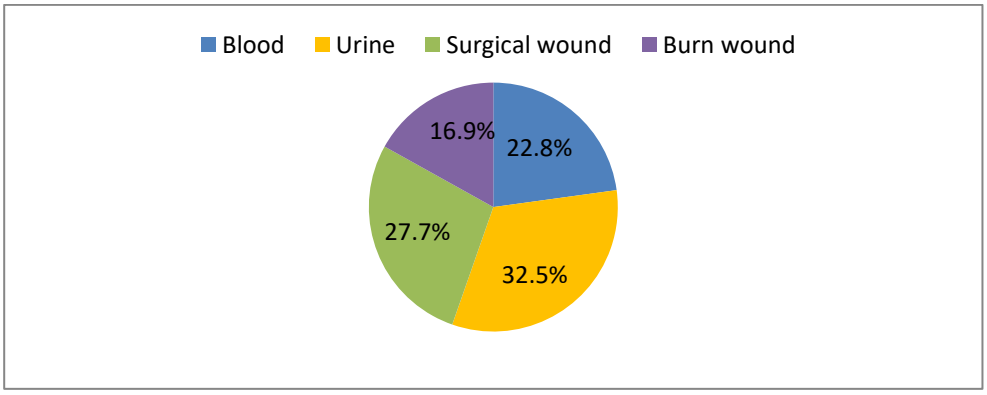

Figure 3.1a. Distribution of *P. aeruginosa* in different clinical samples

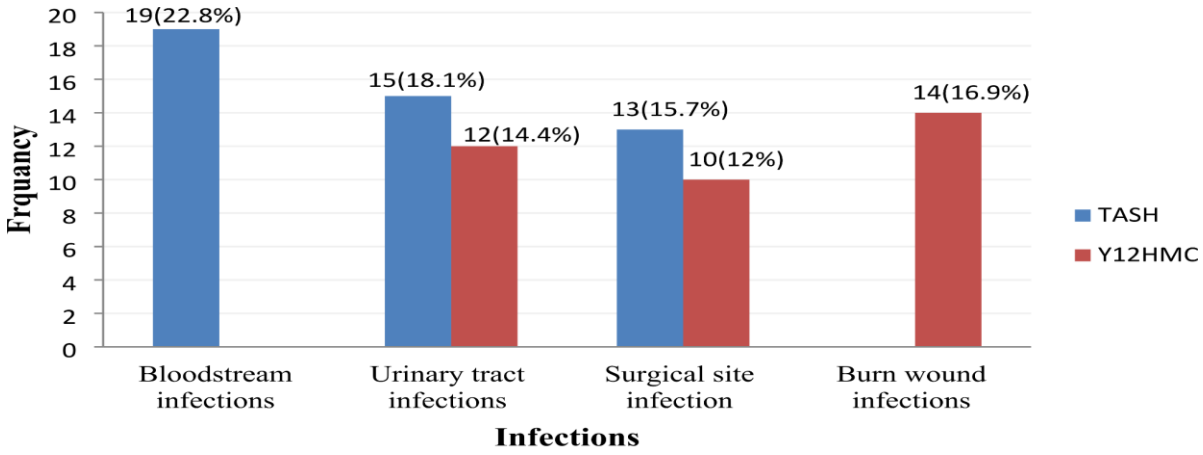

Figure 3.1b. The distribution of infections and frequency of *P. aeruginosa* isolates in TASH and Y12HMC, Addis Ababa, Ethiopia.

3.4. Antibiotic Susceptibility Patterns of *P. aeruginosa* (Paper I)

Out of 83 *P. aeruginosa*, 69 (83.1%) were resistant to at least one of the tested drugs. The highest level of resistance of *P. aeruginosa* was observed against ciprofloxacin 43 (51.8%), followed by ceftazidime 42 (50.6%), and cefepime 40 (48.2%). A low level of resistance was observed to ceftazidime-avibactam 4 (4.8%). The resistance levels of *P. aeruginosa* to various antibiotics are shown in Table 3.3.

Out of 83 *P. aeruginosa* isolates, fourteen 14 (16.9%) were sensitive to all antibiotics, 22 (26.5%) isolates were drug resistance (DR) isolates and 47 (56.6%) were multidrug resistance (MDR) isolates.

Table 3.3. Antibiotic susceptibility patterns of *P. aeruginosa* isolated from patients at TASH and Y12HMC, Addis Ababa, Ethiopia

Antibiotics Tested	***P. aeruginosa* susceptibility pattern (n=83)**		
	Resistant	**Intermediate**	**Susceptible**
MEM	19 (22.9)	10 (12)	54 (65.1)
IMP	14 (16.9)	3 (3.6)	66 (79.5)

CN	36 (43.4)	0 (0.0)	47 (56.6)
NET	22 (26.5)	2 (2.4)	59 (71.1)
CIP	43 (51.8)	10 (12)	30 (36.1)
LEV	31(37.3)	3 (3.6)	49 (59)
CAZ	42 (50.6)	13(15.7)	28(34.9)
FEP	40(48.2)	14(16.9)	28(34.9)
PTZ	19 (22.9)	1 (1.2)	63 (75.9)
CZA	4 (4.8)	0(0.0)	79 (95.2)
ATM	35 (42.2)	0 (0.0)	48 (57.8)

MEM: meropenem; IMP: imipenem; CN: gentamicin; NET: netilmicin; CIP: ciprofloxacin; LEV: levofloxacin; CAZ: ceftazidime; FEP: cefepime: PTZ: piperacillin-tazobactam; CZA: ceftazidime-avibactam; ATM: aztreonam.

3.5. Carbapenems and Colistin Resistance

The level for resistance for meropenem and imipenem were 19 (22.9%) and 14 (16.9%), respectively according to disk diffusion test (Table 3.3). The broth micro dilution method was performed for 29 carbapenem-non susceptible isolates and confirmed susceptibility profile observed by disk diffusion method. Accordingly, nineteen isolates were resistant to meropenem (MIC ≥8 μg/mL) and fourteen isolates were resistant to imipenem (MIC ≥8 μg/mL). The rest were found to be at least intermediate resistant (4 μg/mL) to one of carbapenem (Imipenem or Meropenem) drugs tested.

Carbapenem inactivation method (CIM) was performed for 29 carbapenem-non susceptible isolates and four isolates were found to be CIM-positive. Colistin susceptibility testing was performed for all carbapenem-non susceptible isolates and four isolates demonstrated resistance to colistin. Based on standard definition, these four colistin isolates were classified as extensively drug resistant isolates.

3.6. Biofilm Profiles and Association with Drug resistance (Paper I)

Out of 83 *P. aeruginosa* isolates tested for biofilm formation, 79 (95.2%) were biofilm producers (strong (n=23), moderate (n=33) or weak (n=23) and four (4.8%) were non-biofilm-producing isolates as shown in Figure 3.2. According to spearman correlation analysis, there was a weak positive correlation between biofilm-forming capacity and antibiotic resistance in *P. aeruginosa*, and the association was statistically significant (n = 83; rs = 0.266; p-value = 0.015). The average optical densities (OD) of isolates increased from susceptible isolates (0.45) to drug resistance isolates (0.52) and MDR isolates (0.61). The OD value increased with the level of resistance. This indicates most of the isolates with high OD values were MDR isolates. Sixteen (69.6%) of strong biofilm-producing isolates and 19 (57.6%) of moderate biofilm-producing isolates were MDR *P. aeruginosa* isolates (Figure 3.2 and Table 3.4).

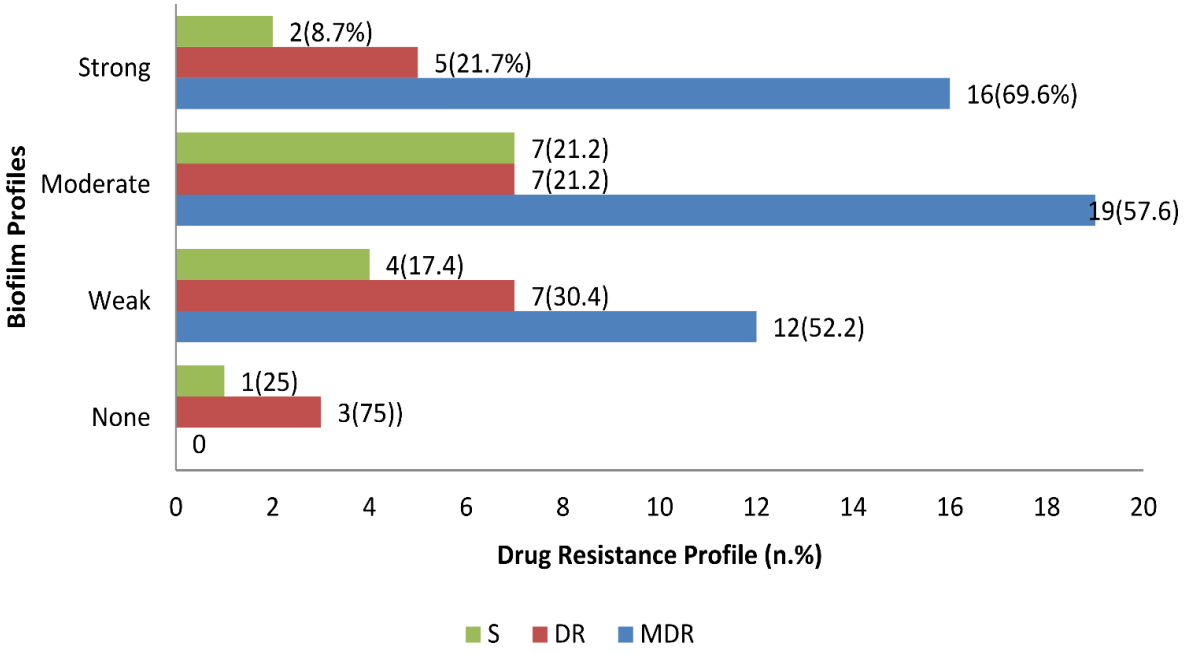

Figure 3.2. Distribution of resistance among *P. aeruginosa* isolates with different biofilm formation capacities. S, sensitive to all; DR, Drug Resistant; MDR, Multidrug Resistant

Table 3.4. Correlation between biofilm formation capacities and drug resistance profiles of *P. aeruginosa* isolates

Resistance profile	Susceptible isolates	DR-isolates	MDR- isolates	Correlation coefficient	P-value
(n. %)	14(16.9)	22(26.5)	47(56.6)	0.266	0.015
OD average	0.45	0.52	0.61		

Optical density (OD) was determined at 595nm; DR, drug resistant; MDR, Multidrug-resistant

3.7. Analysis of Factors Associated with MDR in *P. aeruginosa* (Paper I)

Variables in different categories were entered into bivariate and multivariable logistic regression for the risk analysis of MDR in *P. aeruginosa* (Table 3.5). Among the variables, including the 41-60 age group, urban residence, types of antibiotics used in the last three months (cephalosporin, ciprofloxacin, gentamicin, and meropenem), history of invasive medical procedure, and isolates from blood and urine showed significance ($p<0.25$) in bivariate logistic regression and were analyzed together for multivariable logistic regression. A few patients had underlying diseases, although the number was small and not analyzed further. In multivariable logistic regression, only one variable, previous usage of ciprofloxacin, was statistically associated with MDR in *P. aeruginosa* (OR: 5.1; 95%CI: 1.12- 24.7; p-value: 0.032). Multivariable logistic regression showed that *P. aeruginosa* isolated from those with previous exposure to ciprofloxacin were about five times more likely to develop MDR as compared to those without previous exposure to ciprofloxacin (Table 3.5).

Table 3.5. Bivariate and multivariable analysis of risk factors associated with multidrug-resistant *P. aeruginosa* isolates

Variables	**MDR (%)**	**Bivariate logistic regression**	**Multivariable logistic regression**

		COR (95% CI)	p-value	AOR (95% CI)	p-value
Age in Years					
0-15	52.4	2 (0.48-8.77)	0.335	1.9 (0.31-10.4)	0.444
16-40	61.1	1.4 (0.37-5.55)	0.604	0.8 (0.17-3.74)	0.763
41-60	38.5	3.6 (0.71-18.25)	**0.122**	2.62(0.41-15.9)	0.296
>60*	-				Ref
Residence					
Urban	63.6	2.3 (0.92- 5.90)	**0.074**	0.28 (0.68-1.15)	0.077
Rural*	-				Ref
Types of antibiotics used in last three months (Yes/No*)					
Cephalosporin	66.04	2.92 (1.15- 7.4)	**0.023**	2.6 (0.81- 8.1)	0.111
Gentamicin	88.9	7.17(0.85- 60)	**0.069**	3.9 (0.369-41.4)	0.258
Ciprofloxacin	68	1.9 (0.74- 5.3)	**0.173**	5.1(1.12- 24.7)	0.032
Meropenem	83.3	4.16 (0.47-37.9)	**0.202**	3.1(0.23- 39.9)	0.392
History of invasive medical procedures usage (Yes/No*)					
Yes	47.1	0.52(0.212-1.26)	0.145	0.37(0.09 -1.5)	0.272
Source of isolates (Clinical samples)					
Blood	42.1	2.9 (0.92-8.97)	0.071	1.5 (0.356- 6.4)	0.355
Urine	51.8	1.9 (00.69-5.37)	0.206	0.9 (0.21-3.53)	0.858
Wound*	-				Ref

"*"reference categories; "CI" confidence interval; "OR" odds ratio; "**bold**," significant; Cephalosporin", replaced for at least one from ceftriaxone, cefepime or ceftazidime). Significant variables in the binary logistic analysis (p <0.25) were entered into a multiple-variable analysis. Significant variables in multivariable analysis (p< 0.05) were identified as independent risk factors in this study.

3.8. Molecular Characterizations of *Pseudomonas aeruginosa*

3.8.1. WGS and isolate Selection

All 83 *Pseudomonas* isolates were subjected to WGS at Science for Life laboratory, Solna, Sweden. Of total isolates, 83 (98.8%) were confirmed as *P. aeruginosa*, showing ANI value ≥95 to *P. aeruginosa PAO1*. Following the quality assessment, 64 *P. aeruginosa* isolates with high quality genome were selected for further molecular characterization for serotype,

virulence factors and resistance determinants to ensure high-quality and informative sequencing results.

3.8.2. Serotypes of *Pseudomonas aeruginosa* (Paper II)

Eight different serotypes were identified amongst the 64 isolates. Overall, the most prevalent serotypes were O6 (50%), O11 (14.1%), O3 (10.9%), O5 (9.4%), O1 (7.8%), O2 (3.1%) and O4 (3.1%), with O9 (1.6%) being the least common. In terms of infections, serotype O6 was the most frequently found serotype, accounting for 47.4%, 56.3%, 38.5% and 56.3% of isolates from urine, surgical wounds, burn wounds and blood, respectively (Figure 3.3). Serotype O1 was the second most prevalent serotype among the urine isolates (15.8%), serotype O3 was the second most prevalent serotype among the blood isolates (18.8%), and O11 was more common among the surgical wound (12.5%) and burn wound (38.5%) isolates (Figure 3.3).

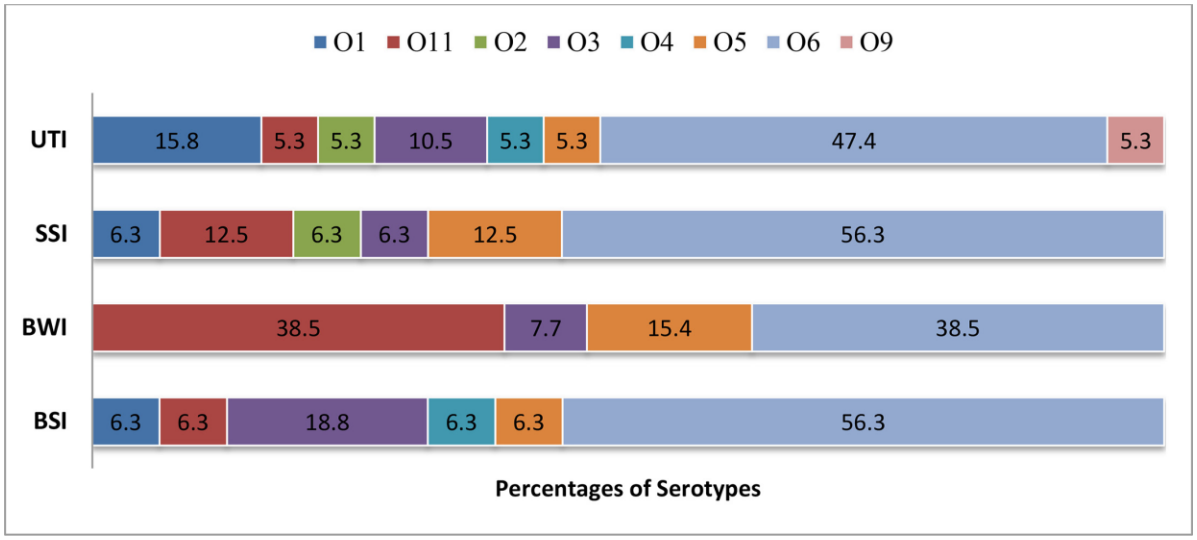

Figure 3.3. Percentages of *P. aeruginosa* serotypes amongst different types of infections. BSI, Blood Stream Infection; BWI, Burn Wound Infection; SSI, Surgical Site Infection; UTI, Urinary Tract Infection.

3.8.3. Serotypes and MDR phenotypes (Paper II)

Certain serotypes had high percentages of MDR, whereas others did not, indicating varying resistance levels across serotypes. Serotype O11 had the highest percentage of MDR strains (88.9%), followed by serotype O5 (66.7%). Serotypes O6 and O2 had equal distributions (50%) of MDR and non-MDR percentages, whereas serotypes O4 and O9 had 100% non-MDR isolates (Figure 3.4).

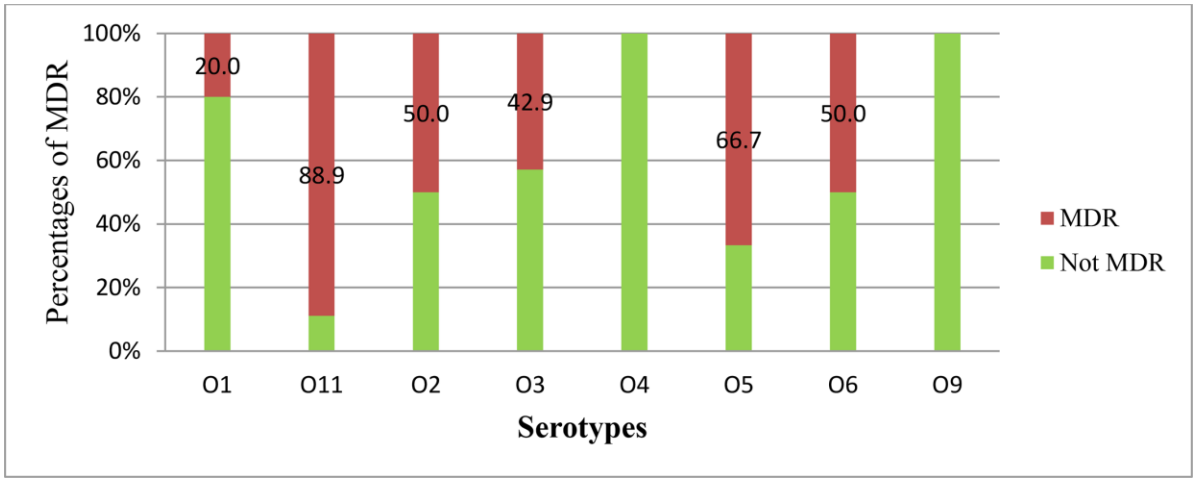

Figure 3.4. Percentages of MDR phenotypes amongst *P. aeruginosa* serotypes

3.8.4. Distribution of MDR and *exoU* toxin genes across serotypes

Phylogenetic analysis revealed that most isolates of the same serotype clustered under the same clade and exhibited similar MDR profiles, indicating genetic relatedness. However, some serotypes in the same clade exhibited different MDR profiles, indicating possible variation in resistance acquisition amongst serotypes. Our study revealed that all isolates with the presence of *exoU* toxin were MDR isolates, and all belonged to serotype O11 (Figure 3.5). The identification of both *exoU*-positive and MDR strains in the O11 serotype shows the potential of this serotype in terms of virulence and resistance.

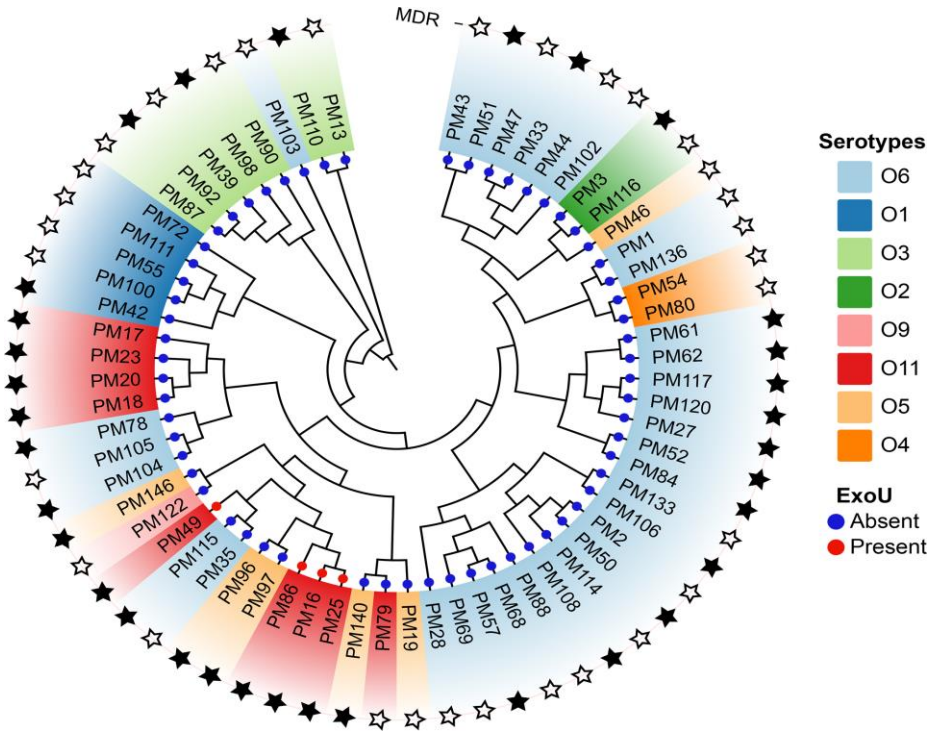

Figure 3.5. Phylogenetic clustering and distribution of MDR and *exoU* toxin genes in *P. aeruginosa* serotypes.

The black stars represent MDR strains, the white stars represent non-MDR strains, the red circles represent *exoU*-positive strains and the blue circles represent *exoU*-negative strains with different bands of similar color, indicating strains with the same serotypes.

3.8.5. Prevalence of virulence genes (Paper II)

A total of 241 virulence genes were identified. Amongst the 241 virulence genes, 201 (83.4%) were present in nearly every isolate. Examples of these frequent virulence genes were flagella-related genes (*fliF* and *fliQ)*, T2SS system components (*xcpZ* and *xcpR*), T3SS components (*pscN* and *pscP)*, T6SS-related genes (*clpV1* and *vgrG1a*) and secreted factors (*pvdG* and *phzm*). The most common toxin genes identified were *exoY* (96.9%), *exoT* (96.8%), *exoS* (95.3%) and *toxA* (93.8%). The least common toxin identified was *exoU*

(6.3%). A comprehensive list of the virulence genes is available in the supplementary file (Annex V).

3.8.6. Distribution of virulence genes across serotypes (Paper II)

The majority of genes associated with pilus and flagella were found in every isolate, whilst the prevalence of *fleP*, *fliS*, *fleI/flag*, *fliC* and *flgL* was 35.9% (Figure 3.6). These genes were present in all O2, O3 and O4 serotypes, but only in 6.3% of O6 and 22.2% of O11 serotypes. The rare virulence factors include *pvdI*, which was found in one isolate of the O5 serotype, and *pvdJ*, which was found in three isolates, two of which were O11 and one of which was O6. The *exoU* toxin gene was found in four isolates. Of these, one showed co-presence of *exoU* and *exoS*, and all belonged to serotype O11. All other isolates carried the *exoS* gene. Only eight (12.5%) isolates contained *wzz* and *wzy* genes, which encode enzymes involved in the biosynthesis and modification of LPSs. Amongst the eight isolates having *wzz* and *wzv* genes, five (62.5%) were serotype O5, two (25%) were serotype O2 and one (12.5%) was serotype O11. Only 11 (17.8%) isolates contained *pilA* genes, with 63.6% in O11 and 36.4% in O6. Other virulence genes present in a few isolates were *vgrG1b* in 11 (17.8%) and *phzA1* in 18 (28%), and these were more frequently found in O6 serotypes, with 54.5% and 61.1%, respectively. Virulence genes for biofilm formation, *algP/algR3*, was detected in 48 isolates (75%) and was present in almost all of the identified serotypes, indicating its broad distribution among various serotypes.

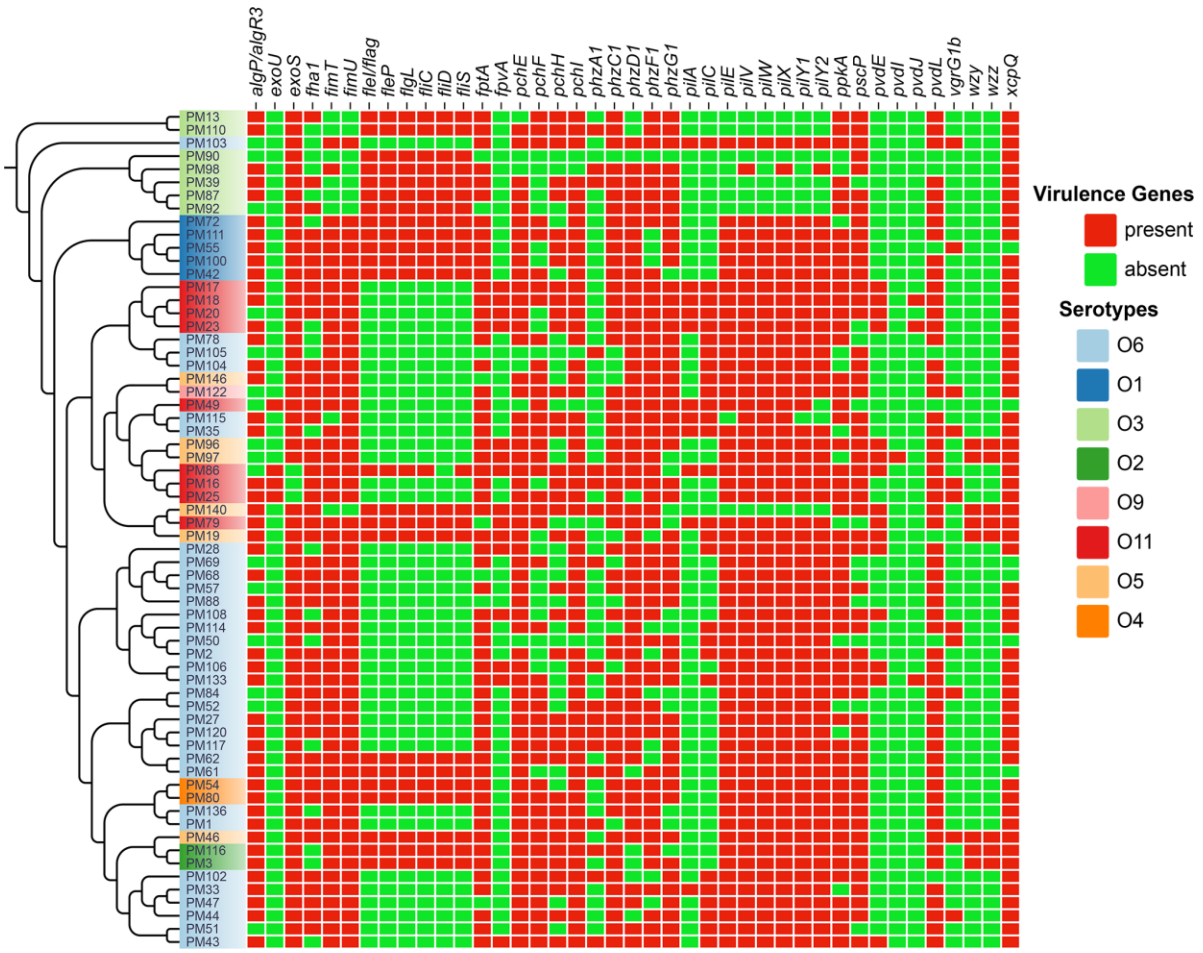

Figure 3.6. Phylogenetic clustering and distribution of variable virulence genes in 64. *P. aeruginosa* strains.

Isolates of the same serotypes are highlighted with the same color. The presence and absence of virulence genes are shown in red and green, respectively.

3.8.7. Biofilm associated genes in *Pseudomonas aeruginosa*

Out of 64 isolates retained for downstream analysis, 60 (93.4%) isolates were biofilm-forming isolates. Of which 19 (29.7%) were strong biofilm producers, 24 (37.5%) were moderate biofilm producers, 17(26.3%) were weak biofilm producers and four (6.3%) were none biofilm producers. The presence of biofilm related genes including *pelA, pslA, algD*

and *pilA* were analyzed for each biofilm categories. All 60-biofilm forming (strong, moderate and weak) isolates contain *pelA*, *pslA*, and *algD* genes. In addition, *pelA*, *pslA* and *algD* were also identified in non-biofilm forming isolates. Out of 11 *pilA* genes, six (54.5%) were detected in moderate and 5 (45.5%) were detected in strong biofilm forming isolates (Figure 3.7).

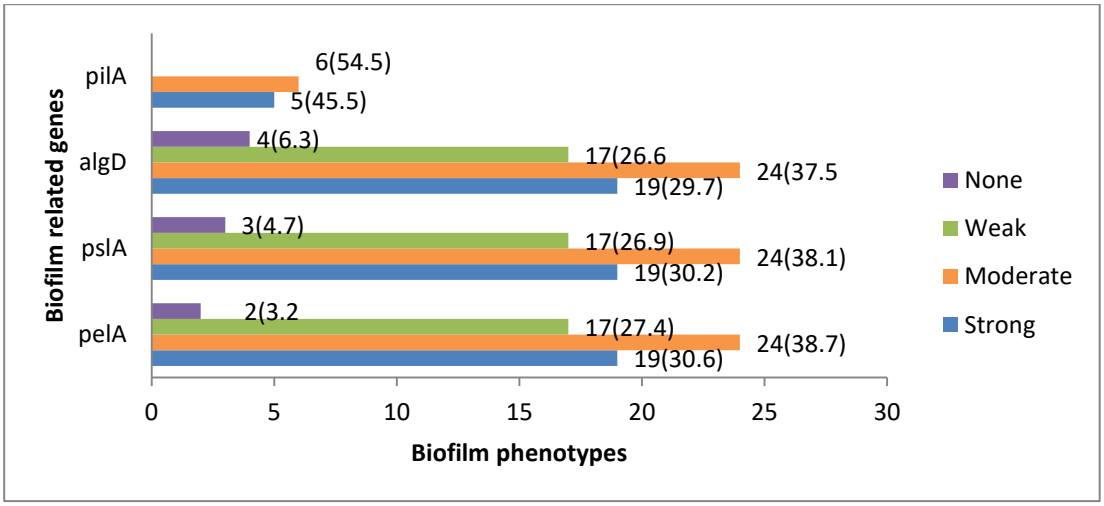

Figure 3.7. Distribution of biofilm-related genes among 64 *P. aeruginosa* isolates

3.8.8. Antibiotics resistance genes in MDR *P. aeruginosa*

Out of 64 isolates, 33 (51.6%) were MDR isolates. Comprehensive WGS based analysis revealed a diverse range of resistance genes for the major classes of antibiotics and mutation associated with MDR phenotypes (Figure 3.8, Annex VI).

Aminoglycosides

At least a one-aminoglycoside gene (AMGs) was identified in all MDR isolates (Figure 3.9). More commonly identified AMGs were *aph(3')-Iib* in 19 (57.6%), *aph(3'')-Ib* in 16 (48.5%) and *aph(6)-Id* in 14 (42.4%) MDR isolates. In addition, *ant (2'')-Ia* and *ant (3'')-Ia* were found in 12 (36.4%) and 10 (30.3%) MDR isolates, respectively. The less commonly AMGs were *aac(3)-Id and aac(6')-II* present in 2 and 3 MDR isolates respectively. The presence of

these several AMGs highlights the genetic basis for the high-level resistance to aminoglycosides observed among MDR isolates.

Fluroquinolones

The *crpP* and *qnrVC1* genes associated with decreased susceptibility to fluoroquinolone resistance were identified in 26 (78.8%) and four (12.1%) MDR isolates, respectively (Figure 3.9). Although the precise genetic context of *crpP* was not investigated, its presence with others resistance mechanisms highlights high-level ciprofloxacin resistance observed in present finding.

Beta-Lactam Antibiotics

The most frequently identified ESBLs was $bla_{\text{CTX-M-15}}$ that present in 10/33 (30.3%) MDR *P. aeruginosa* isolates. Non-classical ESBLs identified were $bla_{\text{VEB-1}}$, $bla_{\text{VEB-4}}$ and $bla_{\text{PER-7}}$, each identified in single MDR isolate. Non-ESBL TEM variants, $bla_{\text{TEM-1B}}$ was detected in 13 (39.4%) of the MDR isolates. The most prevalent OXA genes identified were $bla_{\text{OXA-396,}}$ $bla_{\text{OXA-494}}$ and $bla_{\text{OXA-50}}$ present in 17(51.5%), 9 (27.3%) and 7 (21.2%) MDR *P. aeruginosa* isolates, respectively. The less commonly found OXA genes were $bla_{\text{OXA-395}}$ and $bla_{\text{OXA-10}}$ present in two and three isolates, respectively. All $bla_{\text{OXA-10,}}$ $bla_{\text{VEB-4}}$ and $bla_{\text{OXA-395}}$ were identified from TASH, while $bla_{\text{PER-7}}$ was from Y12HMC (Figure 3.8).

Other classes of antibiotics

Resistance genes for macrolides, tetracycline, rifampin, chloramphenicol, trimethoprim and sulfonamide were also detected in MDR *P. aeruginosa* (Annex VI).

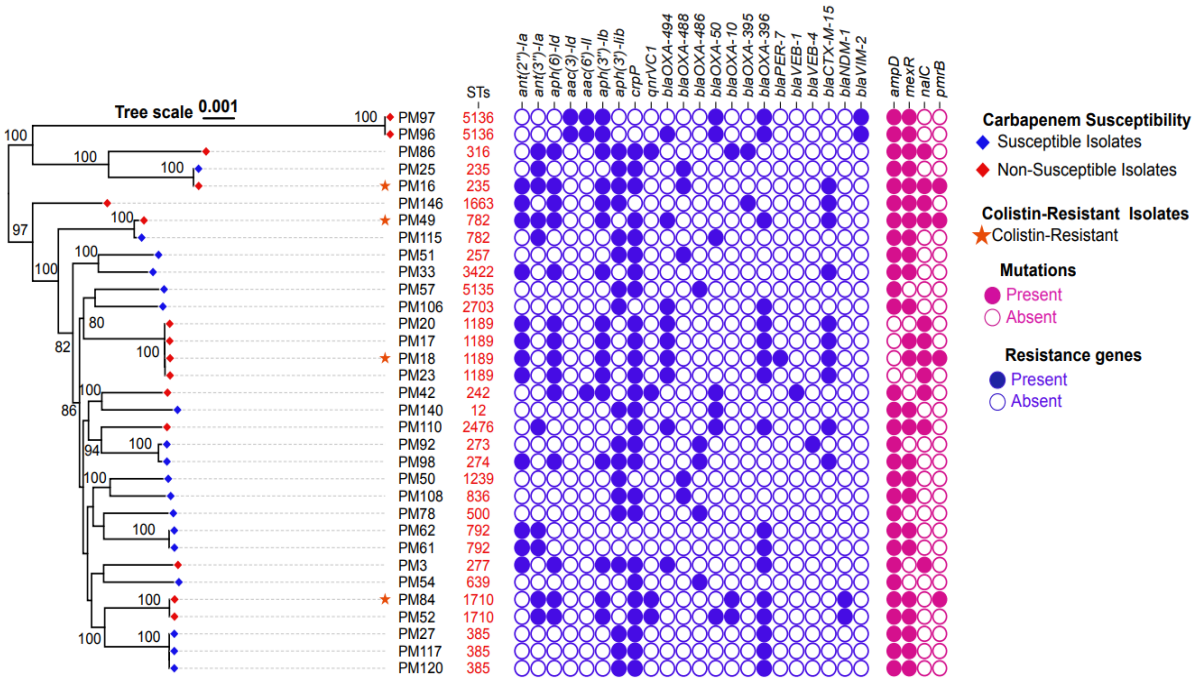

Figure 3.8. Distribution of antimicrobial resistance genes and mutations in regulatory loci among 33 MDR *P. aeruginosa* isolates

P. aeruginosa isolates (n=33) were shown. SNPs clusters are shaded in colored block of isolates. Susceptible and non-susceptibility to carbapenems are indicated using blue and red diamond, respectively. Red star represent colistin resistant. Presence and absence of antimicrobial resistance genes and mutations were shown in filled and empty circle. Bootstrap values are shown at major nodes

3.8.9. Mutational analysis of *mexR, nalC* and *ampD* in MDR *P. aeruginosa*

All MDR *P. aeruginosa* isolates were investigated for mutations profile of regulatory gene associated with resistance. A mutation of *mexR* was found in 25(75.8%), *nalC* was found in 11(33.3%), while *ampD* was in 29(87.9%) MDR isolates. The most frequent identified *mexR* mutations were R21L, L13Vand A15V present in 21 (63.3%) MDR isolates. The *nalC* mutations identified were G71E in 11(33.3%) isolates, the S209R in 6(18.2%) isolates and A186T in 4(12.1%) carbapenem non-susceptible isolates. The most frequently detected

ampD mutations were G148A, D183Y and S175L present in 29(87.9%), 8(24.2%) and 6 (18.2%) MDR *P. aeruginosa* isolates respectively (Figure 3.9).

Figure 0.10

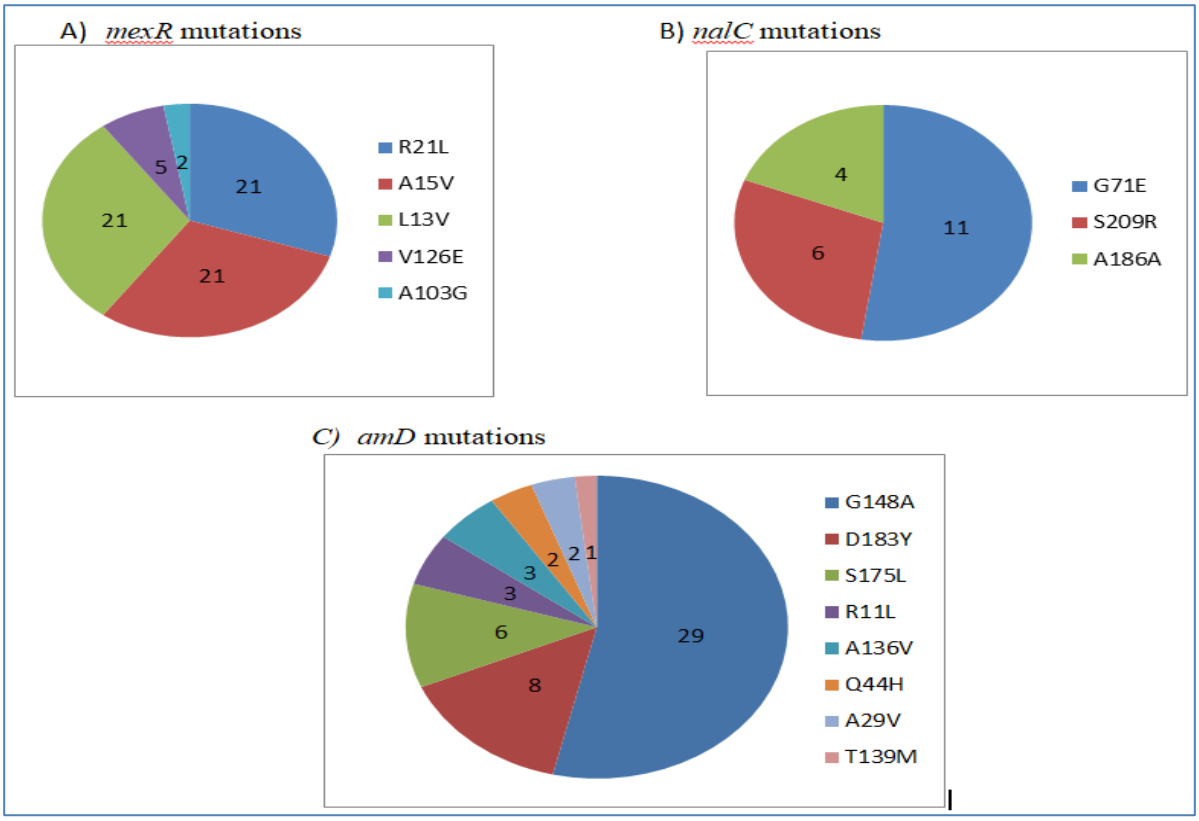

Figure 3.9. Frequency of mutations in *mexR* (A), *nalC* (B) and *ampD* 9(C) genes among MDR *P. aeruginosa* isolates

3.8.10. Carbapenem resistance determinants in *P. aeruginosa*

Out of 33 MDR isolates, 15 were carbapenem non-susceptible isolates. Two different carbapenemase genes were identified in four of 15 carbapenem non-susceptible isolates. Two of $bla_{\text{VIM-2}}$ genes were identified in novel ST5136 strains and two $bla_{\text{NDM-1}}$ genes were identified in ST-1710. Out of 10 $bla_{\text{CTX-M-15}}$ identified, 8 were found in carbapenem non-

susceptible isolates. In addition, 76.5% of carbapenem non-susceptible isolates carried the *bla*$_{\text{OXA-396}}$ gene, indicating its contribution to resistance; synergistically with other mechanisms. Carbapenemase genes lacking isolates were contained mutations in either *mexR* or *nalC*, with other resistance genes. This combination can mediate resistance through enhancing antibiotics efflux and β-lactam hydrolysis (Figure 3.8).

3.8.11. Colistin resistance determinants in *P. aeruginosa*

Out of 29 carbapenem non susceptible isolates, four isolates were found to be colistin resistant isolates. Three of these isolates were identified from Y12HMC with only one isolate from TASH. Mutation based analysis revealed all CoRPA isolates were contained at least one mutation in *pmrB* gene. The Y345H observed in all CoRPA isolates, while D70N and H340R were observed in single isolate. Besides *pmrB* mutations, no known *mcr* genes were identified by molecular analysis. Colistin resistant isolates were distributed across different sequencing types, while one is HRC ST235, the rest were ST1189, ST1710 and ST782 (Figure 3.8).

3.9. Multi Locus Sequence Types: Frequency and allelic profile of novel STs

WGS revealed high genetic diversity with distinct STs among 22/33 (66.7%) MDR and 11/15 (66.7%) CRPA isolates, respectively (Figure 3.8 and Figure 3.10). The most frequently identified ST was ST1189 in 4/33 (12.1%), followed by ST385 in 3/33 (9.1%) isolates. Two global high-risk clone types identified were ST235 in two (6.1%) isolates and ST277 in one (3%) isolate. Fourteen (42.4%) STs were represented by a single isolate (Figure 3.10).

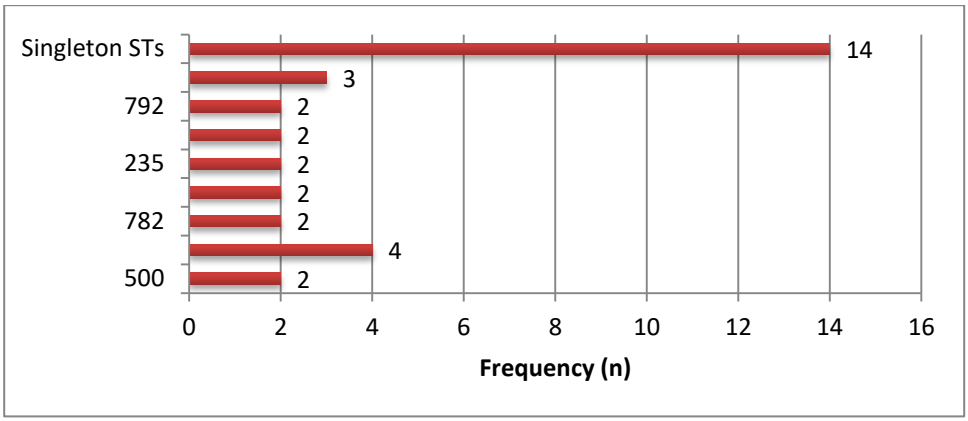

Figure 3.10. Frequency of sequencing types in 33 *P. aeruginosa* isolates

Singleton STs: ST12, ST277, ST242, ST257, ST273, ST274, ST316, ST836, ST1239, ST1663, ST2476, ST2703, ST3422, and ST5135

Sixteen different STs (ST2476, ST274, ST273, ST500, ST12, ST1663, ST782, **ST5136**, ST316, **ST5135**, ST2703, ST836, ST1710, ST792, ST385 and ST257) were identified in TASH, and eight different STs (ST242, **ST1189**, ST782, ST235, ST1239, ST385, ST277 and ST3422) were identified in Y12HMC, indicating diversity in the distribution of STs across hospitals (Figure 3.10). ST385 and ST782 were identified in both hospitals. All ST1189 were identified from burn wound infection from patients at Y12HMC. The two novel STs identified were ST5135 in one isolate and ST5136 in two isolates and all three novel STs were from TASH. The allelic profile of novel STs were shown in Table 3.6.

Table 3.6. Allelic profiles of novel *P. aeruginosa* ST types

STs	**Allelic profiles of the housekeeping genes**							Isolates
	acsA	*aroE*	*guaA*	*mutL*	*nuoD*	*ppsA*	*trpE*	
5135	1	5	11	5	4	15	10	PM57
5136	89	30	64	26	48	24	96	PM96 and PM97

3.10. Genetic relatedness analysis

Phylogenetic construction was performed using SNP based alignment of the isolates using parsnp bioinformatics tool. Genetic relatedness was assessed using ≤25 SNPs threshold. There were four distinct clusters identified. Isolates within the same clusters were shared similar STs and frequently identified from the same hospital.

Two clusters were identified at Y12HMC. A major cluster (**cluster B**) contain four isolates belonged to ST1189 and all from hospital burn center, which could indicate transmission with in hospital. The second cluster (**cluster A**), contain two isolates belonged to global high-risk clone ST235 and were identified from different wards (Medical vs. Surgical). Similar to Y12HMC, two clusters were identified at TASH. The first cluster (**cluster C**) contains two isolates identified from different wards (Medical vs. Surgical). Both isolates were harbored blaNDM-1, and belonged to ST1710. The other cluster at TASH (cluster D) contains two isolates identified from different wards (Pediatric vs. Medical). The isolates were assigned to novel ST5136 carrying blaVIM-2. This isolates (**cluster D**) were clustered separately on the tree with long distances form the rest of isolates (Figure 3.11). This indicates clonal unrelated to rest of isolates and newly emerging local high-risk clone. Despite from different wards, close relatedness of some isolates shows likely transmission events due to shared healthcare staff, medical equipment's or contamination of common hospital environments.

In contrast, some appeared on distinct branch from the rest of isolates, indicating genetically diverse and unrelated lineage. Phylogenetic analysis among CoRPA isolates showed divergence in STs (ST235, ST1189, ST1710 and ST782), and SNPs clusters. This distinct suggest that colistin resistance in this study is likely due to independent acquisition rather than clonal spread of single resistant lineages (Figure 3.11).

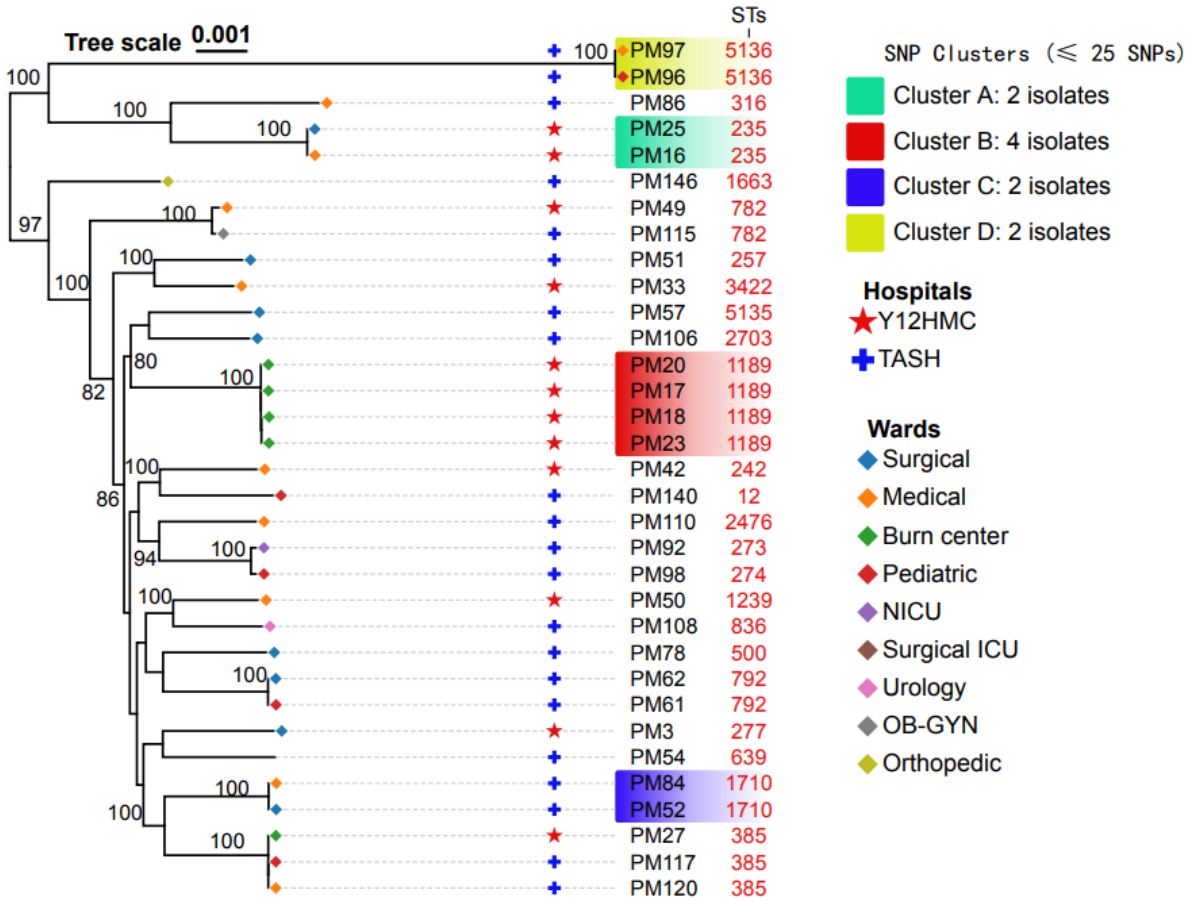

Figure 3.11. Genetic clustering of P. aeruginosa based on core SNP differences

P. aeruginosa isolates (n=33) were shown. SNPs clusters are shaded in colored block of isolates. TASH and Y12HMC are indicated using blue cross and red star, respectively. Diamond symbols represent different hospital wards. Bootstrap values are shown at major nodes.

CHAPTER FOUR: DISCUSSION

P. aeruginosa is the most common gram-negative bacterium associated with HAIs (Reynolds and Kollef, 2021). It is one of the most common opportunistic pathogen associated with acute and chronic infections, primarily in compromised hosts (Juan *et al*., 2017). Its mortality rate was reported in the range of 17.9% to 44.6% in health care associated infections (de Matos *et al*., 2018; Rolo *et al*., 2022; Shuzhen *et al.*, 2023).

In the present study, the prevalence rate of *P. aeruginosa* was 19.6%. Similar findings have been reported in previous studies conducted in Ethiopia (19.3%) (Asamenew *et al.*, 2023), Iran (21.3%) (Karami *et al*., 2019), China (19.8%) (Wang *et al*., 2021), and Pakistan (21.2%) (Saeed *et al*., 2018). However, it is a slightly higher than studies reported among burn wound patients in Ethiopia (12.86%) (Abdi *et al*., 2024), and Italy (13.4%) (Migliara *et al*., 2019), but lower than that reported among catheterized patients at Jimma University Teaching Hospital (49.3%) (Bekele *et al*., 2015), Tanzania (24.2%) (Shangali *et al*., 2023), Egypt (28.3) (Farhan *et al*., 2019), China (34.5%) (Yang *et al*., 2021), and Sweden and Norway (25.8% to 48.9%) (Knudsen *et al*., 2009).

While our methodology aligns with some studies, differences in prevalence may arise from variations in population characteristics, sample type, and healthcare setting. Within Ethiopia, Bekele et al. (2015) reported a higher prevalence among catheterized patients, who are at increased risk of *P. aeruginosa* infection. In contrast, Abdi et al. (2024) reported a lower prevalence in burn wound patients, likely due to the small number of burn wound samples in their study. Our study included a larger number of samples across multiple infection types, including surgical site infections, burn wounds, urinary tract infections, and bloodstream infections, providing a more representative estimate of overall prevalence.

In present study, 32.5% *P. aeruginosa* isolates were detected from urine, 27.7% were form surgical wound, 22.8% from blood, and 16.9% were from burn wound samples. Previous study conducted in Ethiopia showed that similar finding has been reported isolates from

wound (29.2%), and higher and lower findings from urine (41.4%) and blood (10.4%), respectively (Asamenew *et al.,* 2023). A study conducted in Nepal showed a relatively similar finding of isolates from urine (29.4%), but low prevalence from surgical wound (19.11%) and blood (11.76%) (Maharjan, 2022). Lower percentages of isolates have been also reported from surgical wound in Egypt (12.3%) (Farhan *et al*., 2019) and burn wound isolates in Iran (11.8%) *(*Karami *et al*., 2019). The variation in *P. aeruginosa* distribution across sample types and studies may be explained by several factors. Differences in patient populations, including the proportion of hospitalized, catheterized, or immunocompromised patients, can affect isolate frequency (Maharjan, 2022). Underlying conditions, such as burns, diabetes, or surgical interventions, influence susceptibility to specific infections (Farhan *et al*., 2019). Additionally, sample size, study setting, and infection control practices can contribute to these differences.

The present study revealed resistance to ciprofloxacin was 51.8% among *P. aeruginosa* isolates. Comparable findings have been reported from India (50%) (Radhika *et al.,* 2022) and Uganda (50%) (Aruhomukama *et al*., 2019). Lower level of resistance to ciprofloxacin have also been reported in previous studies conducted in Ethiopia (18% to 36%) (Asamenew *et al*., 2023; Motbainor *et al*., 2020), Iran (29%) (Fazzeli *et al*., 2012) and Spain (38%) (Del Barrio-Tofinõ *et al*., 2019). However, high-levels resistance to ciprofloxacin have been reported from Ethiopia (61.1%) (Mekonnen *et al*., 2021), China (80.4%) (Feifei *et al*., 2021), Brazil (94.3%) (de Almeida Silva *et al.*, 2017), and Egypt (70%) (Rania *et al*., 2020).

Resistance to levofloxacin in present study was 37.3%, which was lower than the study reported from India (67%) (Gill *et al*., 2016) and higher than the study reported from Ethiopia (24%) (Addis *et al*., 2021).

Resistance to ceftazidime and cefepime in present study was 50.6% and 48.2% respectively. Higher resistance to ceftazidime and cefepime was reported from the study conducted in Uganda (69% vs. 55 %) (Kateete *et al*., 2016), whereas lower resistance rates to were

reported in studies from China (11.3% vs. 5.1 %) (Bai *et al*., 2024) and Iran (37.7% vs. 40.6%) (Mohammadnezhad *et al*., 2024).

In this study, resistances towards the aminoglycoside drugs (Gentamicin and Netilmicin) were observed among 43.4% and 26.5% of the isolates, respectively. A wide range of resistance against gentamicin was reported from previous studies conducted in Ethiopia (7-62.97%) (Abdi *et al*., 2024; Addis *et al*., 2021). Higher levels of resistance against both gentamicin and netilmicin were reported in India (81.03% vs. 58.6%) (Biswal *et al*., 2014).

The level of resistance against aztreonam in the present study was 42.2%. Higher rates of resistance against aztreonam were reported from studies in Egypt (69%) (Rania *et al.*, 2020), and India (81.8%) (Kumari *et al*., 2022), while lower resistance against aztreonam was reported from Iran(12.3%) (Mohammadnezhad *et al*., 2024).

Various studies reported that the combination drug ceftazidime-avibactam has a better effect against MDR *P. aeruginosa* isolates (Weber *et al.*, 2022; Yin *et al*., 2019). The present study revealed better sensitivity to ceftazidime-avibactam (95.2%) and piperacillin-tazobactam (75.9%) in *P. aeruginosa* isolates, which was relatively higher than previous findings conducted in three European countries (Greece, Italy, and Spain) (Pérez *et al*., 2019). The superior activity of these drugs can be explained by their mechanisms of action: both inhibit bacterial cell wall synthesis and include β-lactamase inhibitors (avibactam or tazobactam), which protect the antibiotics against the most common resistance enzymes in MDR strains (Weber *et al.*, 2022; Yin *et al*., 2019).

Carbapenems (Meropenem and Imipenem) are well known β-lactam antibiotics with broad spectrum activity and are used in treatment of severe gram-negative bacterial infections (Nicolau, 2008). However, the use of carbapenems is threatened by the global emergence of carbapenem-resistant strains (CRPA) as one of WHO's priority pathogen (Elshafiee *et al*., 2020). The present study revealed 22.9% of *P. aeruginosa* isolates were meropenem

resistant. Comparable findings have been reported from Ethiopia (19.4%) (Abdeta *et al*., 2021) and India (18.2%) (Kumari *et al*., 2022).

Resistance to imipenem was observed in 16.9% of *P. aeruginosa* isolates in the present study, which was in agreement with the studies conducted in Ethiopia (18%) (Addis *et al*., 2021) and India (18.2%) (Kumari *et al*., 2022).

However high level of resistance to meropenem and imipenem were reported in Poland (61.6% vs. 41.1 %) (Ratajczak *et al*., 2021), India (80% vs. 78%) (Gill *et al*., 2016), Saudi Arabia (42.4% vs. 36.4%) (Yamani *et al*., 2021), and Iran (30% vs. 70%) (Gholami *et al*., 2017). This varation may reflect differences in antimicrobial stewardship, local infection prevention practices, prevalence of carbapenemase-producing strains, and the use of carbapenems in clinical practice. These multifactorial influences highlight that carbapenem resistance varies geographically due to both microbial and healthcare system factors (Parcell *et al*., 2018; Tooke *et al*., 2019).

In current study, out of 29 carbapenem non-susceptible isolates, 4 (13.8%) were found to be colistin resistant isolates. A study conducted in Egypt reported 21.3% of colistin resistance, which was more frequently observed in XDR isolates (Abd El-Baky *et al*., 2020), while lower level of colistin resistant in CRPA isolates were reported in Iran (1.3%) (Farajzadeh Sheikh *et al*., 2019), and China (3.45%) (Liang *et al*., 2023). Furthermore varying level of colistin resistance in *P. aeruginosa* were reported in Ethiopia (6%) (Addis *et al*., 2021), India (82%) (Suresh *et al*., 2024) and China (5.6%) (Zhao *et al*., 2025). This variation might be due to difference in infection prevention and control, source and type of clinical specimens, variation in use of colistin in livestock and agriculture among countries.

The discrepancies in resistance rates between countries may reflect variations in antimicrobial stewardship, infection prevention and control, and antibiotic use in livestock and agriculture. Stewardship programmes have been shown to reduce MDR *P. aeruginosa*

rates (Tacconelli *et al*., 2018).Variations in specimen types, local healthcare practices, and diagnostic methods may further explain discrepancies in resistance rates across settings.

Infection caused by multidrug-resistant *P. aeruginosa* strains has become a significant public health problem globally (Jean *et al*., 2022). In the present study, 56.6% of *P. aeruginosa* isolates were MDR, which was within a range of the previous studies conducted in Ethiopia *(*4.6– 84%) (Addis *et al*., 2021; Mekonnen *et al*., 2021; Motbainor *et al*., 2020) and relatively lower than the findings reported in Brazil (76.2%) *(*de Almeida Silva *et al*., 2017), Iraq (76.06%) (Polse *et al*., 2023), and Egypt (70%) (Rania *et al*., 2020). Other studies demonstrated low rates of MDR isolates from Iran (16.5%) (Mirzaei *et al*., 2020), Ghana (43.6%) (Odoi *et al*., 2021), Kenya (31%) (Kiyaga *et al*., 2022), Germany (34%) (De Rosa *et al*., 2019), China (22.3%) (Qin *et al*., 2022) and from three European countries (Greece, Italy, and Spain) (30.2%) (Pérez *et al*., 2019).

The observed variation in MDR between countries may reflect differences in antimicrobial stewardship strategies, definitions of MDR, antibiotic use in agriculture, and local epidemiological trends, as reported in previous studies (Magiorakos *et al*., 2012; Tacconelli *et al*., 2018). The present study revealed that 95.2% of *P. aeruginosa* were biofilm producers, which was relatively consistent with findings published in previous studies conducted in Iran (70%) (Gholami *et al*., 2017), and South Korea (92.7%) (Cho *et al*., 2018). In this study, 34.04% and 40.42% of MDR isolates were strong and moderate biofilm-forming isolates, respectively which is in agreement with study conducted in Saudi Arabia (Yamani *et al*., 2021). The present study found a significant weak positive correlation (rs = 0.266; p-value = 0.015) between biofilm production and drug resistance, which is comparable to those studies carried out in Iran (Karami *et al*., 2019) and Ethiopia (Diriba *et al*., 2020).

In this research, multivariable logistic regression showed that isolates from patients with previous exposure to ciprofloxacin were about five times more likely to develop MDR compared to isolates from those without previous exposure to ciprofloxacin (OR: 5.1;

95%CI: 1.12–24.7; p-value: 0.032). Similarly, we found the highest resistance rate to ciprofloxacin. Widespread use and higher prescribing rate of ciprofloxacin may be a cause of increasing resistance against this drug, and is driving the selection of resistance genes that lead to multiple drug resistance in *P. aeruginosa.* This observation corresponds with findings from a previous study that identified recent antibiotic therapy as an independent factor for MDR in *P. aeruginosa*, although our study specifically emphasized previous ciprofloxacin exposure as the only significant factor (Hernández-Jiménez *et al*., 2022).

P. aeruginosa is a dynamic pathogen with extensive genetic variability (Feinbaum *et al*., 2012). Given that antibiotic resistance in *P. aeruginosa* is complicating treatment options, a comprehensive understanding of its molecular features including serotypes, virulence potential and resistance determinants are crucial for effective therapeutic strategies and control measures.

In our study, O6 (50%) and O11 (14.1%) were the most common serotypes. Similarly, O6 and O11 were among the most common in China (29.13% vs. 23.3%) (Hu *et al.*, 2024), Spain (17.8% vs. 13.3%) (Del Barrio-Tofiño *et al*., 2019), and France (26% vs. 23%) (Lu *et al*., 2014). In present study, serotypes O6, O11 and O3 accounts 75% of the isolates, comparable with the previous study that revealed 70% of the isolates were O3, O6, O11, or O12 (Thrane *et al*., 2016). Geographical differences, serotyping techniques, the number of isolates tested, and genetic adaptation can all contribute to variations in the number and type of serotypes. Generally, the predominant serotypes of *P. aeruginosa* isolates in the present study were not significantly different from the predominant serotypes reported in other countries.

Our finding showed that serotypes O3 (18.8%) and O11 (38.5%) were more prevalent in bloodstream infections and burn wound infections, respectively, whereas O6 was the most common of all infections. Previous serotyping data indicate that serotypes O6 (29%) and O11 (23%) are the most common in *P. aeruginosa* nosocomial pneumonia (Lu *et al*., 2014) and other types of infections (Hu *et al.*, 2024). Serotype O9 was a rare serotype identified in

only one isolate, which is comparable with previous findings (Thrane *et al*., 2016). A study revealed that serotype O9 is significant in the clinical setting, as it can induce a good immune response and is a promising candidate for vaccine development, although this has been challenged by the acid-labile nature of its polysaccharides (Moustafa *et al*., 2023). Some *P. aeruginosa* serotypes may be more adaptable to specific tissues because of differences in their O-specific antigen, which could increase their persistence in particular infections.

Serotype O11 was the most common among MDR isolates in this study, similar to finding of previous study (Maatallah *et al*., 2011). Inconsistence with the present study, serotype O4 (57.3%) was the most common in MDR isolates in study conducted in Spain (Del Barrio-Tofiño *et al*., 2019). In addition, serotype O12 has been reported as the common serotype in clinical settings and MDR isolates (Del Barrio-Tofiño *et al*., 2019; Maatallah *et al*., 2011). Serotypes O11, O12, and O4 were accounted for the majority of serotypes in MDR phenotypes; however, variations among studies can be linked to differences in strain distribution, geographic location and the dynamic nature of *P. aeruginosa*.

P. aeruginosa has a number of virulence factors that could be involved in its pathogenicity (Feinbaum *et al*., 2012). Using data from hundreds of Pseudomonas genomes, the Pseudomonas Genome Database (http://www.pseudomonas.com) has documented over 320 virulence genes (Winsor *et al*., 2016). In the present study, 241 virulence genes were identified. Although our results are consistent with those of previous studies (Kiyaga *et al*., 2022), possibly because both studies used the same methodology (WGS-based), virulence genes still vary because of the methodological variation and genetic diversity of *P. aeruginosa* strains. This diversity and variation can be associated with diverse in colonization, tissue damage and infection severity, influencing its pathogenicity (Berre *et al*., 2011).

The present study revealed the highest frequencies of *exoY* (96.9%) and *exoT* (96.8%), followed by *exoS* (95.3%) and *toxA* (93.8%). Comparable results were reported regarding

the frequency of *toxA* (97.8%) and *exoY* (93.1%) in Iran (Haghi *et al*., 2018), whereas a higher prevalence rate for *exoT* (83%) was reported in another study (Javanmardi *et al*., 2019). In general, the frequency of virulence genes identified in the present study was not significantly different from the frequency of virulence genes reported in other studies (Kiyaga *et al.*, 2022). However, minor variations across the studies may be due to variability in the laboratory techniques employed, such as PCR-based or genome sequencing approaches (Almaghrabi *et al*., 2024; Benie *et al*., 2017).

In the current present study, *exoU* toxin gene was detected in 6.3% of the *P. aeruginosa* strains and all e*xoU*-positive strains were serotyped as O11, which is comparable with the results of a previous study (Berre *et al*., 2011). Previous study revealed that 32% of *P. aeruginosa* isolates carried *exoU* genes (Javanmardi *et al*., 2019), whereas a study carried out in Spain reported that 31.1% of clinical strains produce *exoU* toxins (Alonso *et al*., 2020). Furthermore, *exoU* toxin was reported in *P. aeruginosa* strains from food products (2.5%) in Côte d'Ivoire (Benie *et al*., 2017) and drinking water (7.6%) in China (Wei *et al*., 2020).

Our study revealed that all O11 strains harboring *exoU* toxin were MDR isolates. Similarly, a relatively high *exoU* frequency in multidrug-resistant strains was reported in a previous study (Urzedo *et al*., 2020). The co-occurrence of *exoU* with MDR traits in serotype O11 strains suggests the convergence of virulence and resistance, which poses serious therapeutic challenges (Papa-Ezdra *et al.*, 2024). Such strains not only have an enhanced capacity to cause severe infections but also lead to limited treatment options and clinical outcomes (Song *et al*., 2023).

ExoU is the most intensely cytotoxic of the four effector proteins and has phospholipase activity; it is capable of rupturing host cell membranes and compromising the innate immune response to infection (Hardy *et al*., 2022). *ExoU* induces strong inflammatory responses (Lima *et al*., 2012) and has been studied as a potential therapeutic target (Wood *et al*., 2019). When the *exoU* gene is expressed in *P. aeruginosa* infections, the prognosis is

worse, and it is associated with increased virulence, tissue injury (Berre *et al*., 2011), disease severity and mortality rate (Recio *et al*., 2020). Although studies have reported greater *exoU* presence in O11, further molecular investigation is needed to clarify the mechanism behind the connections between *exoU* and O-antigen types. Furthermore, although serotype O6 is the most prevalent in this study, infections due to serotype O6 strains often result in lower severity and better clinical outcomes as compared to serotype O11(Hu *et al*., 2024; Lu *et al.*, 2014). Notably, serotype O6 strains are often linked to the presence of the *exoS* gene and lack of *exoU* toxin, that may contribute to the variability in clinical outcomes(Lu *et al*., 2014).

In this study, one strain carried both *exoU* and *exoS*; similarly, single hypervirulent strains carrying both toxins were reported in previous studies (Hu *et al*., 2024; Song *et al*., 2023). It is uncommon for one bacterial strain to possess both *ExoU* and *ExoS* (Shaver and Hauser, 2004). This is hypothesized to be due to the deletion of *exoS* in strains that acquire the *exoU/spcU* locus resulting from a targeted deletion event caused by a product of a gene linked to the *exoU/spcU* region at the time of acquisition, even if the exact mechanism is not clear (Kulasekara *et al.,* 2006). Despite the rarity of finding both toxins in a single strain, it has been documented, which can be attributed to the genetic complexity of *P. aeruginosa*. According to previous findings, the co-expression of *exoU* and *exoS* toxins enhances the cytotoxicity and pathogenicity of *P. aeruginosa* strains, and it is likely that horizontal gene transfer of the pathogenicity island PAPI-2 promotes *exoU* acquisition by *exoS+ P. aeruginosa* (Song *et al*., 2023). Our findings of a *P. aeruginosa* strain that possesses both *exoU* and *exoS* show the existence of a highly virulent strain that might cause serious infections.

The present study found that majority of flagella genes were found in every serotype, which align with the previous study highlighting the role of flagella as immune activator *(*Hassan *et al*., 2018). However, *fleP, fliS, fleI/flag, fliC,* and *flgL* were found in 6.3% of O6 and 22.2% of O11 serotypes. This low prevalence of flagella genes in these serotypes can be used as immune evasion mechanisms, contributing in their high prevalence among clinical isolates

(Lu *et al*., 2014). According to our finding, 63.6% of *pilA* were found in O11, while 36.4% were in O6. The *pilA* gene was important for initial adhering of bacterial to host cell and surfaces, which is important for biofilm formation (Bleves *et al*., 2010). High prevalence of *pilA* gene in O11 serotype might be linked to severity and persistent infections in this serotype (Recio *et al*., 2020). These variations of virulence factor genes across serotypes can be linked to differences in gene regulation, O-antigen structures, clonal lineages, and genetic adaptations.

Our finding showed that most of the biofilm forming *P. aeruginosa* isolates were positive for *pslA* gene, comparable to study conducted in Iran (Abootaleb *et al*., 2020), and Egypt (Farhan *et al*., 2023). In present study *algD, pslA and pelA* genes were also detected in non-biofilm forming isolates, comparable to previous studies conducted elsewhere (Hou *et al*., 2012; Samira and Fereshteh, 2015). This suggests that all isolates producing *psl* and *pel* genes are not actively secrete exopolysaccharide for biofilm formation (Colvin *et al*., 2012). This might be due to factors such as mutation in regulatory region that may make *pslA or pelA* genes silence (Byrd *et al*., 2015), or some strains may need specific conditions like temperature, nutrient limitations or may differ in triggering biofilm formation in vivo and laboratory condition (Ma *et al*., 2009). The present study revealed that the frequency of *pilA* was 17.2%, which is relatively comparable with the study conducted in Iran (24.7%) (Haghi *et al*., 2018). All *pilA* gene in present study was detected in strong and moderate biofilm forming isolates, consistent with the previous study conducted in Iraq (Al-Mohammed and Mahmood, 2024). In general, while *pelA, algD and pslA* genes are essential for formation of biofilm matrix, their presence alone may not sufficient for biofilm formation, indicating other factors including environmental condition, gene regulation and mutations may play role in biofilm formation.

In this research, several antibiotic resistance genes were identified among 33 MDR *P. aeruginosa* isolates. The beta lactamase genes, $bla_{\text{OXA-10}}$ was found in three (9.1%) and $bla_{\text{CTX-M-15}}$ in 10 (30.3%) MDR isolates. Higher percentage of $bla_{\text{OXA-10}}$ (42%) and $bla_{\text{CTX-M}}$ (31.7%) were reported in Iran (Mohammadnezhad *et al*., 2024) and South Africa (Hosu *et*

al., 2021a), respectively. Comparable with this study, *P. aeruginosa strains* carrying *bla*$_{\text{VEB-1}}$, *bla*$_{\text{OXA-50}}$, *bla*$_{\text{OXA-395}}$, *bla*$_{\text{OXA-396,}}$ *bla*$_{\text{OXA-485}}$ and *bla*$_{\text{OXA-486}}$ have been documented in several studies (Bai *et al.*, 2024; Shahbazzadeh *et al*., 2020).

In present study, genes coding for fluroquinolones resistance *crpP* and *qnrVC1* were detected and 78.8% of MDR strains contain *crpP* genes, while *qnrVC1* was found in 12.1% of the MDR isolates. A previous study revealed that the *qnrVC1* and *crpP* genes were present in 12% and 63% of *P. aeruginosa* clinical strains, respectively (Khan *et al*., 2020). Other studies have reported that 65.4% of *P. aeruginosa* strains possess *crpP* variants (Hernández-García *et al*., 2021). The increased non-susceptibility of the *crpP*-positive isolates in the present study to ciprofloxacin implies that the *crpP* gene with other resistance mechanisms may contribute to susceptibility to this antibiotic.

In our study, aminoglycosides (AMGs) resistance genes were detected such as *aph(3')-Iib*, *aph(3'')-Ib* and *aph(6)-Id* were present in 19 (57.6%), 16 (48.5%) and 14 (42.4%) MDR isolates, respectively. According to the study conducted in Australia, high rate of *aph(6)-Id* and *aph(3'')-Ib* were reported among MDR clinical isolates (Khan *et al*., 2021). In our study, *ant(2'')-Ia* was identified in 12 (36.4%) AMG non-susceptible MDR isolates. Similar rate of *ant(2'')-I (*13.95%) was reported in Iran (Ahmadian *et al*., 2021). Higher rate of *ant(2'')-Ia* (72%) was detected in AMG-non-susceptible isolates in Thailand (Poonsuk *et al*., 2013). The presence of these several AMGs highlights the genetic basis for the high-level resistance to aminoglycosides observed among MDR isolates. These genes have been reported among AMG-susceptible isolates in different studies (Ahmadian *et al*., 2021; Poonsuk *et al*., 2013). These might be due to low-level expression of these genes, which could contribute to resistance but not active in a typical laboratory setting. This shows that environmental factors, the presence of other resistance mechanisms, or the regulation of gene expression may all be important in the development of AMG resistance.

In present study, carbapenem resistance genes were identified. Two carbapenemase genes identified were *bla*$_{\text{VIM-2}}$ in ST5136 and *bla*$_{\text{NDM-1}}$ in ST1710. Similarly, *bla*$_{\text{NDM-1}}$ in ST2948

P. aeruginosa were reported from Ethiopia (Sewunet *et al*., 2022), whereas the $bla_{\text{NDM-1}}$ belonging to different STs (ST38, ST773, ST235, ST357 and ST654) and $bla_{\text{VIM-2}}$ genes belonging to ST111 and ST233 were reported in several studies (Fortunato *et al*., 2023; Kiyaga *et al*., 2022). Moreover, *P. aeruginosa* carrying $bla_{\text{VIM-2}}$ were reported in Singapore (Prakki *et al*., 2023) and Spain (Pérez-Vázquez *et al*., 2020). The presence of these genes in present study indicates a potential threat to the effectiveness of carbapenem and strict monitoring need to be emphasized to prevent the spread of such resistant variants.

Similar to the present study, several studies have shown $bla_{\text{OXA-396}}$ presence in CRPA isolates. A study conducted in China showed, the presence of $bla_{\text{OXA-396}}$ in all CRPA isolates and indicated its association with resistance in *P. aeruginosa* (Bai *et al*., 2024). Similarly, the $bla_{\text{OXA-396}}$ was reported in CRPA isolates in Chile (Opazo-capurro, 2022). In our study, $bla_{\text{OXA-396}}$ was also identified in high-risk clone ST235 and ST277. Study conducted in China revealed all CRPA isolates were harbored $bla_{\text{OXA-396}}$ in ST2444 and ST292 (Fang *et al*., 2022). Furthermore, $bla_{\text{OXA-396}}$ has been detected in high-risk clone ST654 and ST938 in the strain obtained from human and Pig in Italy (Bonardi *et al*., 2022). Generally, report of $bla_{\text{OXA-396}}$ genes in CRPA from several studies, particularly in high-risk clones, highlights its association in carbapenem hydrolyzing activities. However, knowledge gap remain its specific enzymatic activities as compared to other well-known carbapenemases. Future research should prioritize characterization of its specific enzymatic activities in CRPA.

In current study found that several regulatory genes mutations in MDR isolates. The MexAB-OprM efflux pump is capable of expelling a wide variety of antibiotics that are essential for treating *P. aeruginosa* (Avakh *et al*., 2023). In present study, *mexR* mutations including R21L, L13Vand A15V were present in 63.3% of MDR isolates. Although the specific amino acid differs, the report of mutations at the same residue position such as R21W and L13M in *mexR* suggested the importance of these specific positions (Anandapadamanaban *et al*., 2016; Andrésen *et al*., 2010). Some of mutations observed in this study in *mexR* were not previously reported. This shows additional substitution that may

affect repression of efflux pump; however, research needed to identify the impact of these mutations. The finding of these unique mutations contributes to the growing evidence of diverse mechanisms underlying efflux-mediated resistance

In current study the most common *ampD* mutation detected in MDR *P. aeruginosa* isolates were G148A (87.9%), followed by D183Y (24.2%) and S175L (18.2%). A study conducted in US showed the link between mutations D183Y and G148A and *ampC* overexpression (Rubio *et al*., 2021). Study from Spain indicated G148A, T139M and D183Y mutations in high-risk clone isolates and several other mutations including G156S, P41L, H157R and R11L in AmpC ß-lactamase overproducing isolates (Cabot *et al*., 2012). Mutation in *ampD* can lead to over expression of ampC ß-lactamase, which causes hydrolysis of ß-lactam antibiotics (Pachori *et al*., 2019). Study showed that overproduction of AmpC ß-lactamase when combined with other resistance mechanism can enhance ß-lactam resistance (Lister *et al*., 2009). Additionally, MexAB-OprM and AmpC β-lactamase overexpression at the same time increases *P. aeruginosa's* resistance to a variety of antipseudomonal β-lactams in a synergistic manner, demonstrating the complex interactions between these mechanisms (Grosjean *et al*., 2021).

In our study, *mexR* mutations including R21L, L13Vand A15V were present in 60% of carbapenem non-susceptible isolates. Comparable to our study, 57% and 89.6% of carbapenem non-susceptible isolates contain mutations in *mexR* and *nalC* gene respectively (Yonggang *et al*., 2023). Similar to the present study, V126E and A103G mutation in *mexR* and G71E, S209R and A186T in *nalC* among carbapenem non-susceptible isolates were reported in previous study (Yonggang *et al*., 2023).

Molecular analysis among colistin resistant isolates in present study revealed several mutations in *pmrB* gene with the most frequent being Y345H, identified in 3 out of 4 colistin resistant isolates. The Y345H amino acid change in *pmrB* was reported from Chinese Tertiary Teaching Hospital (Xiao *et al*., 2023). Similar to the present study, amino acid

changes D70N, H340R, Y345H was also reported in *pmrB* of colistin resistant in a study conducted in China (Lee and Ko, 2014; Liang *et al.*, 2023).

Several specific mutations including V51I, G68S, R155H, R135Q and R259H in *pmrB* of colistin resistant *P .aeruginosa* were reported in other studies (Moskowitz *et al*., 2012; Xiao *et al*., 2023). No plasmid mediated mcr variants were observed in present study. The plasmid-mediated colistin resistance gene *mcr-1* has been identified in pervious study, although being uncommon in *P .aeruginosa* (Abd El-Baky *et al*., 2020).

In present study, the most frequently identified ST was ST1189. Comparable to ST1189 reported in Indonesia (Pelegrin *et al*., 2019), and Australia (Madden *et al*., 2022). According to previous studies, ST274 and ST357 were the most frequently identified STs in Ethiopia (Sewunet *et al*., 2022), and Kenya (Kiyaga *et al*., 2022), respectively. The two global high-risk clones (ST235 and ST277) and two novel STs **(ST5135 and ST5136)** were identified in present study. The detection of high-risk clones known globally and distributed across different countries, including Kenya (Kiyaga *et al*., 2022) and India (Pulusu *et al*., 2022), suggests the importance of local surveillance, as it indicates that these clones may spread further across the country. Novel strains have been also detected in different countries, including Kenya (Kiyaga *et al.,* 2022) and countries of the Gulf Cooperation Council (Zowawi *et al*., 2018), and repeated identification of novel strains across studies have revealed that the emergence of new variants and isolates is evolving rapidly across countries.

In this research, we used ≤25SNP threshold for indicative of transmissions cluster. This threshold is aligning with the previous threshold used to define transmission cluster (Romano-Bertrand *et al.,* 2025). We identified four distinct clusters by using ≤25SNP threshold. Except these four clusters, all other isolates were differing by >100SNP in pairwise SNP analysis, which is beyond accepted threshold for genetic relatedness. Similar clusters were reported with≤25 SNPs in previous study (Romano-Bertrand *et al.,* 2025),

while other thresholds were used in different studies including, <10 SNPs (Stribling *et al.*, 2023) and <37 SNPs (Moloney *et al*., 2020).

The finding of these closely related isolates within hospital settings, might be due to movement of patients, contaminated hospital environments from which patients are colonized or healthcare worker mediated transmission (Moloney *et al*., 2020). This showed possible intra-hospital transmission suggesting the need for strict infections prevention measures.

Limitation of the study

The study included clinical isolates from hospitalized patients in only two hospitals in Addis Ababa, Ethiopia, which may limit the generalizability of the findings to other regions of the country.

CONCLUSION

- The findings of this study showed high proportion of MDR *P. aeruginosa* isolates, suggesting a complex resistance landscape. Therefore, to create efficient management strategies and monitoring the spread of MDR strains, continuous surveillance is essential.
- In this study we found association between resistance to multiple drugs and the level of biofilm formation. In addition, biofilm related genes were detected in both biofilm forming and non-biofilm forming isolates. This shows the presence of biofilm related genes alone might not sufficient for biofilm formation, indicating a complexity of biofilm formation in *P. aeruginosa*.
- WGS analysis revealed several serotypes and virulence genes among the present *P. aeruginosa* isolates. Serotype O6 was the most frequently found serotype. We found highly virulent strains that harbored *exoU* toxin and both *exoU* and *exoS* in the O11 serotype.
- Molecular based analysis revealed a diverse range of resistance genes, along with multiple mutations of regulatory genes in *mexR, nalC* and *ampD* in MDR isolates. The most frequently identified ESBL gene was $bla_{\text{CTX-M-15.}}$ Other resistance genes were also identified. Such findings showed that a combined effect of mutations and resistance genes were contributed in MDR phenotypes. In addition, we identified unique mutations that may contribute to the growing evidence of diverse resistance mechanisms in *P. aeruginosa.*
- Carbapenem-resistant isolates were consistently exhibited mutations in either *mexR* or *nalC* genes. The $bla_{\text{NDM-1}}$and $bla_{\text{VIM-2}}$ genes were identified among four CRPA isolates. These finding suggest that both carbapenemase genes and regulatory genes mutations contributes to the development of CRPA phenotypes. The presence of $bla_{\text{OXA-396}}$ genes in most of CRPA isolates, suggesting its association in carbapenem hydrolyzing activities. The occurrence of colistin resistant *P. aeruginosa* is very serious and poses a problem for the treatment CRPA.

- In present isolates high-risk clones and novel MDR strains were identified. ST1189 and ST385 were the most common STs identified. Analysis of genetic relatedness suggests the presence of transmission within hospital. Moreover, the presence of novel STs with high antimicrobial resistance profile suggests ongoing genetic evolution and posing serious challenges.
- The result of the present study can be used as baseline data for further studies and provide basic information to the ongoing current national initiatives implemented by the Ministry of Health to fight antibiotic resistance.

RECOMMENDATIONS

- A finding of high MDR proportion shows the need for monitoring antibiotic resistance patterns, optimizing antibiotic use, and proper implementation of antibiotic stewardship program.
- Identification of new mutation in regulatory gene contributes to the growing evidence of diverse mechanisms underlying efflux-mediated resistance. However, further researches needed be conducted for better understanding the impact of this novel mutation on antimicrobial resistance.
- Knowledge gap remains the specific enzymatic activities of $bla_{\text{OXA-396}}$ and future research should prioritize characterization of its specific enzymatic activities in CRPA.
- Finding of genetically related isolates shows potential clonal spread within hospital. This study calls for enhanced molecular surveillance and infection prevention strategies in healthcare settings to monitor spread of resistant strains.
- Future studies could include environmental isolates to explore potential links between human and environmental sources of antimicrobial resistance.

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Annexes

Annex IA: Information Sheet for Parents/Guardians (English version/Amharic Version)

Title of the Study:

Title of the Study: Phenotypic and Molecular Characterizations of *Pseudomonas aeruginosa* isolated among patients admitted at Tikur Anbessa Specialized Hospital and Yekatit 12 Hospital Medical College, Addis Ababa, Ethiopia

Background:

Pseudomonas aeruginosa is a bacterium that can cause infections in hospitalized patients. Some types become resistant to important antibiotics such as carbapenems and colistin. This study aims to understand how resistance develops and spreads.

What will happen if your child participates?

- A health professional will ask questions to confirm eligibility.
- If eligible, a small sample (blood, urine, or wound swab) may be collected.
- The sample will be tested in the laboratory.

Risks:

Sample collection may cause mild discomfort, similar to routine hospital tests. No serious risks are expected.

Benefits:

There is no direct payment or benefit. However, if results help in your child's treatment, they will be shared with the doctor. The findings may also help prevent and manage infections in the future.

Confidentiality:

Your child's name will not appear in any records. Each sample will have a code known only to the research team and your child's healthcare provider.

Rights:

- Participation is voluntary and refusing will not affect your child's care.
- You may withdraw your child at any time without losing any rights.

Contact:

- Matifan Dereje (Principal Investigator): 0913162344, maathy4@gmail.com
- Prof. Daniel Asrat (Supervisor): 0911223019, asratdan@gmail.com
- IRB Office, Addis Ababa University: +25111896139

Annex IB: Information Sheet for Adult Participants (English version/Amharic Version)

Title of the Study: Phenotypic and Molecular Characterizations of *Pseudomonas aeruginosa* isolated among patients admitted at Tikur Anbessa Specialized Hospital and Yekatit 12 Hospital Medical College, Addis Ababa, Ethiopia

1. Background:

Pseudomonas aeruginosa is a common bacterium that can cause infections in hospitalized patients. It can develop resistance to important antibiotics, including carbapenems and colistin. This study aims to investigate the molecular patterns of resistance in *P. aeruginosa* infections.

2. Study Procedures:

If you agree to participate, a trained health professional will ask some questions to determine your eligibility. If eligible, we will collect information through a brief interview and may take samples such as blood, urine, or wound swabs for laboratory analysis.

3. Risks:

Sample collection may cause minimal discomfort, similar to routine diagnostic procedures. No serious risks are expected.

4. Benefits:

There is no direct benefit or payment for participation. However, results that may help in clinical management will be shared with your attending physician. The findings may also guide future public health measures and infection prevention strategies.

5. Confidentiality:

Your information will be kept strictly confidential. Samples and data will be identified only by a code number, not by your name. Only the investigators and treating health professionals will have access to this code.

6. Rights of Participants:

Participation is voluntary. You have the right to refuse or withdraw at any time without affecting your healthcare or rights at the hospital.

7. Contact Information:

For questions about this study, you may contact:

- **Matifan Dereje (Principal Investigator):** 0913162344, maathy4@gmail.com
- **Prof. Daniel Asrat (Supervisor):** 0911223019, asratdan@gmail.com
- **IRB Office, Addis Ababa University:** +251118961396

Amharic version of Information sheet (Parents/Guardians and Adults) (የተሳታፊዎች መረጃ ቅፅ)

የምርምር ርዕስ- "በቲኩር አንበሳ ልዩ ሆስፒታልና የየካቲት 12 ሆስፒታል የሕክምና ኮሌጅ ተያይዞ የተገኘው የPseudomonas aeruginosa ፈኖቲፒክ እና ሞለኪውላር ባህሪ ላይ መየሚደረጊ ምርምር"

የድርጅት ስም - የጤና ሳይንስ ኮሌጅ፣ አዲስ አበባ ዩኒቨርሲቲ

መግቢያ፡ ስዲምኖስ እሮጂኖሳ የሆስፒታል እንፈክሽን ከምየመቱ ባክተረያ ዝርያዎቺ መካከል አንዱ እና ዋነኛዉ ነዉ።ይህ ባክተረያ የተለላዩ መዳንቶቺን አንዳዉም ካረባፐነምና ኮልሲትን መዳንቶቺን ጨምሮ መቁቃም አቂሙ ከፈተኛ ነዉ።የጥናቱ ዓላማ መዳንቶቺን መቁቃም የሚቸሉ የስዲምኖስ እሮጂኖስ የባክተረያ ዝርያዎቺ ዓይነትና ሲሪጭት ለማወቅ የሚደረጊ ምርምር ይሆናል።

የጥናቱ ሂደት

በዚህ ጥናት ለመሳተፈ ፍቃደኛ ከሆንክ/ከሆንሽ (ልጆ በዚህ ጥናት የምሳተፈ ከሆነ/ከሆነቺ) በሆስፒታሉ የጠና ባለሙያ ኣሲፈላጊዉ ምሪመራ ያደሪጎሎታል ።በጥናቱ የምሳተፉ ከሆነ ኣንዲንዲ ጥያቀዎቺ ይጠይቃሉ።በመቀጠልም የደም ፤የሽንት ወይም የቁስል ናሙና ተወስዶ ምሪመራ ይደረጎሎታል።

ሊከሰቱ የሚችሉ አደጋዎች (በናሙና ስብሰባ ወቅት)፡ በጥናቱ ላይ በመሳተፍዎ ልደረስ የምቺል ምንም ዓይነት ጉዳት የለም። ነገር ግን ናሙና ሲሰጥ የሚሰማው ሕመም ሊኖር ይቺላል።

ሊገኝ የሚችለው ጥቅም ፡በጥናት በመሳተፎ የሚያገኙት ቀጥተኝ የሆነ ጥቂም የለም። የምከፈል ቢርም የለም።በጥናቱ የምገኚ የምርመራ ዉጠት ለሚከታተሉ ሃክም ይሰጣል ። ከዚህ ጥናት የተገኙ ውጤቶች የጤና ሰራተኞችን እና ፖሊሲ አውጪዎችን ለማሳወቅ ጥቅም ላይ ይውላል።

ምሥጢራዊነት፡ በዚህ ጥናት ላይ ስተካፈለው ያተገኘውን መረጃ በጥቢቅ እንጠብቃለን። በማንኛውም ሪፖርት ላይ ስም አንጠቀምም ፤ እንዲሁም ከምርምር ቡድኑ ውጪ ካሉ ሰዎች ጋር ስለ ተሳትፎ አንወያይም።

በተሳታፊነት ያለው መብት፡ ፡ በዚህ ምርምር መካፈል ይፈልግ እንደሆነና እንዳልሆነ ለመወሰን ነፃ ናችሁ።ምንም ዓይነት መጥፎ ተጽዕኖ ሳያሳድር በማንኛውም ጊዜ ተሳትፎ እንዳያደርግ ልታደርጉ ትችላላችሁ። በጥናቱ እንዴት እንደሚስተናገድ ወይም ተሳትፎ በማድረግ ረገድ ማንኛውም ዓይነት ጥያቄ ካላችሁ ተመራማሪዎችን ልታነጋግሯቸው ትችላላችሁ።

የጥናቱ ተጠርዎቺ

1. ማቲፋን ደረጀ (ስልክ፡ 0913162344; ኢሜይል፡ maathy4@gmail.com)
2. ፕሮፈሰር ዳንኤል ኣስራት (ስልክ፡ 0911223019; ኢሜይል፡ asratdan@gmail.com)
3. IRB office (chs.irb@aau.edu.et;+251118961396)

Annex IIA: Informed Consent for Parents/ Guardians (English version/Amharic Version) (<12 years old)

I. **English version of Consent form (Parents/Guardians) for study participants**

Patient name_____

Patient ID**:** _____

Name of family or guardian_____

Relationship to participant_____

I have read (or it has been read to me) and understood the information sheet about the study entitled:

"Phenotypic and Molecular Characterizations of *Pseudomonas aeruginosa* Isolated Among Patients Admitted at Tikur Anbessa Specialized Hospital and Yekatit 12 Hospital Medical College, Addis Ababa, Ethiopia."

- The purpose of the study has been clearly explained to me. I understand that:
- There may be minimal discomfort to my child during sample collection (blood, urine, or wound swab).
- I will be asked some questions about my child's health, and samples will be collected for laboratory examination.
- All information and laboratory results related to my child will be kept strictly confidential.
- I have the right to ask questions at any time and have received clear answers to my questions.
- Participation in this study is voluntary, and I may withdraw my child at any time without affecting the health services provided at the institution.

I confirm that I was given sufficient time to consider participation before signing. With full understanding, I voluntarily provide my informed consent for my child to participate in this study.Participants signature: _____

Witness signature _____

Investigator/data collector signature: _____

Date_____

II. Consent form for parents and guardians (Amharic Versions)

የፈቃደኝነት መጠየቂያ ፎርም

የተሳታፍ ሙሉ ስም፡_____

የምስጥር ቁጥር፡_____

የቤተሰብ ወይም አሳዳጊ ሙሉ ስም:_____

ዝምድና፡_____

መረጃ ቅፅ ኣምቢበዋለዉ/ተነቦልኝል። "በቲኩር አንበሳ ልዩ ሆስፒታልና የየካቲት 12 ሆስፒታል የሕክምና ኮሌጅ ተያይዞ የተገኘው የPseudomonas aeruginosa ፈኖቲፒክ እና ሞለኪውላር ባህሪ ላይ መየሚደረጊ ምርምር" በሚል ርዕስ የሚካሄዲ ነዉ።

የጥናት ዋና አላማ ተብርሪቶልኛል። ልጀ ናሙናዜ የምሰጥብት/በምትሠጥበት ጊዘ ኣነስተኛ የሆነ ጉዳት ልኖር አንደምቺል ተነግሮኝል።ጥየቀም ጠይቀ ማባራሪያም ተሰጦኛል። በዚ ጥናት ልጀ የደም ፤ የሽንት ወይም የቁስል ምሪመራ እንደምደረጊልት/አንደምደረጊላት ተነግሮኝል።ልጀ በጥናቱ በምሳተፍ የሚያስከትለውን ጉዳት ፣ ጥናት ውስጥ በመሳተፍ ቀጥተኛ የሆነ ጥቅማ ጥቅም የሌለው መሆኑን ፣ ፣ ጥናት ውስጥ መሳተፍ ያለብኝ በእኔ ፈቃደኝነት ላይ ብቻ የተመሰረተ እንደሆነ ፣ልጀ ጥናት ውስጥ መግባት ባለመቻሉ ምንም አይነት ቅጣት እንደሌለው፣ ከጥናቱ በማንኛውም ሰአት ማቋረጥ እንደምቻል፣ በሚገባ ተነግሮኝል። በተጨማሪም በዚህ ጥናት የተገኘውን መረጃ ከምርምር ቡድኑ ውጪ ላሉ ሰዎች ካለኔ ፈቃድ ተላልፎ እንደማይሰጥ ተነግሮኝል።

የመረጃ ቅጹን አንብቤና ተረድቼ በዚህ ጥናት ውስጥ ልጀ እንዲሳተፍ ፈቃደኛ መሆኔን በፊርማዬ አረጋግጣለሁ።

ፊርማ ---------- ቀን----------

Annex IIB: Informed Consent for Adult Participants (English version/Amharic Version) (⩾18 years old)

I. **English version of Consent form for adult participants**

Patient name_____

Patient ID**:** _____

I have read (or it has been read to me) and understood the information sheet about the study entitled:

"Phenotypic and Molecular Characterizations of *Pseudomonas aeruginosa* Isolated Among Patients Admitted at Tikur Anbessa Specialized Hospital and Yekatit 12 Hospital Medical College, Addis Ababa, Ethiopia."

The purpose of the study has been explained to me. I understand that:

- There may be minor discomfort during the collection of samples (urine, blood, or wound swab).
- I will also be asked some questions related to my health.
- The study might benefit me based on the clinical and laboratory findings.
- All laboratory results and personal information about me will be kept strictly confidential.
- I have the right to ask questions at any time and have received clear answers.
- Participation is voluntary, and I can withdraw at any stage without affecting the medical services I receive.

I confirm that I was given enough time to consider participation before signing. With full understanding, I voluntarily give my informed consent to take part in this study.

Participants signature: _____

Witness signature _____

Investigator/Data Collector Signature:: _____

Date_____

II. **Amharic version of Consent form for Adult participants**

የፈቃደኝነት መጠየቂያ ፎርም

የተሳታፍ ሙሉ ስም፡_____

የምስጥር ቁጥር፡_____

እኔ (ቃለመጠይቁ የሚደረግልኝ) “በቲኩር አንበሳ ልዩ ሆስፒታልና የየካቲት 12 ሆስፒታል የሕክምና ኮሌጅ ተያይዞ የተገኘው የPseudomonas aeruginosa ፈኖቲፒክ እና ሞለኪውላር ባህሪ ላይ መየሚደረጊ ምርምር” በሚል ርዕስ የሚካሄደውን ጥናት ዋና አላማ እና የሚያስከትለውን ጉዳት እንደለለዉ ተነጊሮናል። ጥየቀም ጠይቀ ማባራሪያም ተሰጦኛል። ኣንዲንዲ ጥያቀዎቺ እንደምጠይቂ ና የደም ፤የሽንት ወይም የቁስል ምሪመራ እንደምደረጊልኝ ተነግሮኝል።እንዳዉም ፣ ጥናት ውስጥ በመሳተፍ ቀጥተኛ የሆነ ጥቅማ ጥቅም የሌለው መሆኑን ፣ እኔ የምሰጠው መረጃ በመንግስት እና በከተማው ጤና ቢሮ አስፈላጊ መሆኑን ፣ ጥናት ውስጥ መሳተፍ ያለብኝ በእኔ ፈቃደኝነት ላይ ብቻ የተመሰረተ እንደሆነ ፣ ጥናት ውስጥ መግባት ባለመቻሌ ምንም አይነት ቅጣት እንደሌለው፣ ከጥናቱ በማንኛውም ሰአት ማቋረጥ እንደምችል፣ በሚገባ ተነግሮኝል። በተጨማሪም በዚህ ጥናት የተገኘውን መረጃ ከምርምር ቡድኑ ውጪ ላሉ ሰዎች ካለኔ ፈቃድ ተላልፎ እንደማይሰጥ ተነግሮኝል። የመረጃ ቅጹን አንብቤና ተረድቼ በዚህ ጥናት ውስጥ እንዲሳተፍ ፈቃደኛ መሆኔን በፊርማዬ አረጋግጣለሁ።

ፊርማ ---------- ቀን----------

Annex III: Assent Form (English/Amharic version) (12-17 years old)

I. English version of Assent form

It has been read to me and I understood the information sheet about the study entitled:

Study Title:
"Phenotypic and Molecular Characterizations of *Pseudomonas aeruginosa* Isolated Among Patients Admitted at Tikur Anbessa Specialized Hospital and Yekatit 12 Hospital Medical College, Addis Ababa, Ethiopia"

If you join:

- You will be asked some questions.
- A small sample may be taken from your blood, urine, or a wound.
- You might feel a little uncomfortable, but it should not hurt much.

You do not have to join if you do not want to.

- Saying **no** will not make anyone upset.
- Even if you say yes, you can change your mind and stop at any time.

If you agree to take part, please sign your name below.

Child's Name: _____
Child's Signature: _____
Date: _____

Investigator/Data Collector Signature: _____

II. Amharic version of Assent form

የፈቃደኝነት መጠየቂያ ፎርም

የተሳታፍ ሙሉ ስም፡_____

የምስጥር ቁጥር፡_____

እኔ (ቃለመጠይቁ የሚደረግልኝ) “በቲኩር አንበሳ ልዩ ሆስፒታልና የየካቲት 12 ሆስፒታል የሕክምና ኮሌጅ ተያይዞ የተገኘው የPseudomonas aeruginosa ፈኖቲፒክ እና ሞለኪውላር ባህሪ ላይ መየሚደረጊ ምርምር” በሚል ርዕስ የሚካሄደውን ጥናት ዋና አላማ እና የሚያስከትለውን ጉዳት እንደለለዉ ተነጊሮናል። ጥየቀም ጠይቀ ማባራሪያም ተሰጦኛል።

በመሳተፍህ ምክንያት:

- አንዳንድ ጥያቄዎች ይጠየቃሉ።
- ናሙና ወይም ደም ወይም ከቁስል ምርመራ ይወሰዳል።
- ትንሽ ህመም ሊሰማህ ይችላል፣ ነገር ግን ከፍተኛ ህመም አይሰማህም።

በተጨማሪም በዚህ ጥናት የተገኘውን መረጃ ከምርምር ቡድኑ ውጪ ላሉ ሰዎች ካለኔ ፈቃድ ተላልፎ እንደማይሰጥ ተነግሮኝል።

የመረጃ ቅጹን አንብቤና ተረድቼ በዚህ ጥናት ውስጥ እንዲሳተፍ ፈቃደኛ መሆኔን በፊርማዬ አረጋግጣለሁ።

ፊርማ ---------- ቀን----------

Annex IV: Questionnaire (English and Amharic Version)

I. English Version of questionnaire (Data collection format)

Questionnaire for investigation of socio demographic characteristics and associated factors among patients admitted at Tikur Anbesa specialized hospital in Addis Ababa, Ethiopia

I. Socio-demographic characteristics

1. Identification code: _____Date form completed: ____/_____/_____
2. Sex □ Male □ Female
3. Age in years :_____
4. Marital status : □ Single □ Divorced
   □ Married □ Others
5. What is the highest level of school you have completed or the highest degree that you have rece
   - ❑ Illiterate □ Elementary school
   - ❑ □ High school □ College diploma
   - ❑ □ Degree and above
6. What is your current working status:
   - ❑ House wife □ Farmer
   - ❑ Government employee □ Merchant
   - ❑ Unemployed □ If other specify
7. Residence □ urban □ Rural

II. Clinical data

8. Admission: Ward □ Name of ward: _____ Room No_____
   ICU □ Name of ICU: _____ Room No _____
9. Reason for admission:_____
10. Principal Diagnosis: □UTI □BSI □WI □ Burn Infection □If other specify_____

III. Risk Factors information

11. How many days have you stayed in hospital? _____
12. Have you had a previous history of hospitalization? □ Yes □ No
13. Have you had a previous history of surgery? □ Yes □ No
14. If, yes for question number 14, when did you make surgery? _____
15. If any underlying conditions, (tick that all apply)
    - □Diabetes □Renal diseases
    - □ Liver disease □Cardiovascular diseases
    - □Hypertension □Stroke
    - □Malignancy □coronary artery disease
    - □lung disease □Gastrointestinal diseases

□If other specify_____

16. If any previous history medical invasive procedures usage, (Check all that apply)
    □Indwelling gastric tube □Urinary catheter
    □Tube drainage □Tracheostomy
    □ Mechanical ventilation □Suprapubic catheter
    If other specify_____
17. If the patients have any history antibiotic usage in the last 3 months, check all that apply (to be filled from medical record)
    □First- and second-generation cephalosporin □Third-generation cephalosporin
    □Carbapenems □Penicillins
    □Aminoglycosides □Fluoroquinolones
    □Macrolides □Colistin
    □Metronidazole □Vancomycin
    □Clindamycin □Fosfomycin

II. **Amharic Version of Questionnaires** (የመረጃ መጤየቅ ቅፅ)

አዲስ አበባ ዩኒቨርሲቲ ; የጤና ሳይንስ ኮሌጅ

የህከምና ት/በት፣የማይክሮባዮሎጅ፤ኢምኖሎጅ ና ፓረስቶሎጅ ት/ክፍል

የመረጃ መጤየቅ ቅፅ

የምረምሩ ርዕሥ፤ <u>"በቲኩር አንበሳ ልዩ ሆስፒታልና የየካቲት 12 ሆስፒታል የሕክምና ኮሌጅ ተያይዞ የተገኘው የPseudomonas aeruginosa ፈኖቲፒክ እና ሞለኪውላር ባህሪ ላይ መየሚደረጊ ምርምር"</u>

የሶሾዮ ስነ ህዝቢ ባህርያት

1. መለያ ቁጥር፤
2. ጸታ □ ወንዲ □ ሰት
3. እድመ:_____
4. የጋብቻ ሁነታ፡ □ የላገባ □ የገባ □ የፈታ/ች □ ለሎች_____
5. የትምህርት ደረጃ: □ የልተማረ □ አንደኛ ደረጃ □ ሁለተኛ ደረጃ □ ዲፒሎማ □ ድግሪና ካዛ በላይ
6. የሥራ ሁነታ: □ የበት እመበት □ የመንጊስት □ ኣርሶአደር □ ነጋደ

□ ስራ አጥ □ ለሎቺ ይግለጹ_____

7. የመኖረያ ኣካባቢ: □ ከተማ □ ገጠር

I. ክሊንካዊ መረጃ

8.የመታከሚያ ዋርዲ፤ የዋርዱ ሥም: _____ የክፍሉ ቁጡር :_____

አይ ሲ ዩ ሥም:_____ የክፍሉ ቁጡር:_____

9. የተኙበት ሚክንያት:_____

10. ዋና ምርምራ: □ የኩላለት እንፈክሽን □ የቁስል እንፈክሽን □ የደም ዝዉዉርእንፈክሽን □ የቃጣሎእንፈክሽን □ ለላ ከሆነ ይግለጹ:_____

II. የቺግሩ መንስዎች

11. ሆስፒታሉ ለምን የህል ጊዘ ተኙ:_____

12. ከዝ በፍት ሆስፒታል ተኝቶ ዉ የቃሉ? □ አዋ □ ኣላዉቅም

13. ከዝ በፍት የቀዶ ህክምና ተደርጎለት የቃሉ □ አዋ □ ኣላዉቅም

14. የቀዶ ህክምና ተደርጎለት የምያቁ ከሆነ መቸ፡ _____

15. ከዝ በታቺ የተገለ ተጋዳኝ ቺግሮች አለቦት?

□ የሱካር በሽታ □ የጉበትበሽታ

□ የደም ግፍት □ የኩላለት በሽታ

□ የሳምባ በሽታ □ የካንሰር □ ለላ ከሆኔ ይግለጹ_____

16. ከዚህ በፈት የምከተሉትን የህክምና መሣርዎቺን ተጠቂሞ ያቃሉ?

□ መካንካል ቨንቲልተር □Indwelling gastric tube

□ ዩረነር ካታተር □ ተዉቢ ዲረነጅ

□ ለላ ከሆነ ይግለጹ:_____

17. በኣኑ ሠያት የምወስዱት መዳንት ኣለ ? □ አዋ □ የለም ኣዋ ከሆነ የመዳንቱ ኣይነት(ከመዲካል መዚገቢ የሚሞላ)_____

18. ከዝህ ቀድም የወሰዱት መዳንት ኣለ ? □ አዋ □ የለም ኣዋ ከሆነ የመዳንቱ ኣይነት(ከመዲካል መዚገቢ የሚሞ

Annex V: Supplementary Table for Virulence Genes

No	Virulence factors genes
Alginate biosynthesis & regulation (biofilm formation):	
1.	alg44
2.	alg8
3.	algA
4.	algB
5.	algC
6.	algD
7.	algE
8.	algF
9.	algG
10.	algI
11.	algJ
12.	algK
13.	algL
14.	algP/algR3
15.	algQ
16.	algR
17.	algU
18.	algW
19.	algX
20.	algZ
21.	mucA
22.	mucB
23.	mucC
24.	mucD
25.	mucE
26.	mucP
Secretion systems & effectors	
27.	**Type III secretion system (T3SS):** popB
28.	popD
29.	popN
30.	ppkA
31.	pppA
32.	pscB
33.	pscC
34.	pscD
35.	pscE

36.	pscF
37.	pscG
38.	pscH
39.	pscI
40.	pscJ
41.	pscK
42.	pscL
43.	pscN
44.	pscO
45.	pscP
46.	pscQ
47.	pscR
48.	pscS
49.	pscT
50.	pscU
51.	pcr1
52.	pcr2
53.	pcr3
54.	pcr4
55.	pcrD
56.	pcrG
57.	pcrH
58.	pcrR
59.	pcrV
60.	exsA
61.	exsB
62.	exsC
63.	exsD
64.	exsE
65.	**T3SS effectors and toxins:** exoY
66.	exoS
67.	exoT
68.	exoU
69.	toxA
70.	**Regulator of pseudotoxin:** ptxR
71.	**Type VI secretion system:** tse1
72.	tse2
73.	tse3

74.	vgrG1a
75.	vgrG1b
76.	hsiG1
77.	hsiH1
78.	hsiJ1
79.	icmF1/tssM1
80.	hsiA1
81.	hsiB1/vipA
82.	hsiC1/vipB
83.	hsiE1
84.	hsiF1
85.	*hcp1*
Secondary metabolites:	
86.	**Pyoverdine:** pvcA
87.	pvcB
88.	pvcC
89.	pvcD
90.	pvdA
91.	pvdE
92.	pvdF
93.	pvdG
94.	pvdH
95.	pvdI
96.	pvdJ
97.	pvdL
98.	pvdM
99.	pvdN
100.	pvdO
101.	pvdP
102.	pvdQ
103.	pvdS
104.	**Pyochelin:** pchD
105.	pchE
106.	pchF
107.	pchG
108.	pchH
109.	pchI
110.	pchR
111.	pchA

112.	pchB
113.	pchC
114.	**Pyocyanin:** phzA1
115.	phzB1
116.	phzC1
117.	phzD1
118.	phzE1
119.	phzF1
120.	phzG1
121.	phzH
122.	phzM
123.	phzS
124.	**Secondary metabolite biosynthesis :** mbtH-like
Type 2 secretion system (T2SS)	
125.	xcpA/pilD
126.	xcpP
127.	xcpQ
128.	xcpR
129.	xcpS
130.	xcpT
131.	xcpU
132.	xcpV
133.	xcpW
134.	xcpX
135.	xcpY
136.	xcpZ
Surface components & biosynthesis:	
137.	Type IV pili (T4P): pilA
138.	pilB
139.	pilC
140.	pilE
141.	pilF
142.	pilG
143.	pilH
144.	pilI
145.	pilJ
146.	pilK
147.	pilM

148.	pilN
149.	pilO
150.	pilP
151.	pilQ
152.	pilR
153.	pilS
154.	pilT
155.	pilU
156.	pilV
157.	pilW
158.	pilX
159.	pilY1
160.	pilY2
161.	chpA
162.	chpB
163.	chpC
164.	chpD
165.	chpE
166.	clpV1
167.	dotU1
168.	fimT
169.	fimU
170.	fimV
171.	**Other surface components & polysaccharide biosynthesis:** waaA
172.	waaC
173.	waaF
174.	waaG
175.	waaP
176.	tagF/pppB
177.	tagQ
178.	tagR
179.	tagS
180.	tagT
181.	rhlA
182.	rhlB
183.	rhlC
184.	rhlI
185.	wzy
186.	wzz

187.	fha1
Motility structures (Flagella):	
188.	fleQ
189.	fleR
190.	fleS
191.	flgA
192.	flgB
193.	flgC
194.	flgD
195.	flgE
196.	flgF
197.	flgG
198.	flgH
199.	flgI
200.	flgJ
201.	flgK
202.	flgL
203.	flgM
204.	flgN
205.	flhA
206.	flhB
207.	flhF
208.	fliA
209.	fliC
210.	fliD
211.	fliE
212.	fliF
213.	fliG
214.	fliH
215.	fliI
216.	fliJ
217.	fliK
218.	fliL
219.	fliM
220.	fliN
221.	fliO
222.	fliP
223.	fliQ
224.	fleI/flag
225.	fleN
226.	fleP

227.	fliR
228.	fliS
229.	fptA
230.	fpvA
231.	motA
232.	motB
233.	motC
234.	motD
235.	motY
Enzymes:	
236.	lasA
237.	lasB
238.	lasI
239.	lip1
240.	aprA
241.	plcH

Annex VI. Supplementary Table for AMR Genes

No	**Antibiotic Resistance genes**
Aminoglycoside resistance gene	
1.	*ant(2'')-Ia*
2.	*ant(3'')-Ia*
3.	*aph(6)-Id*
4.	*aac(3)-Id*
5.	*aac(6')-Il*
6.	*aph(3')-Iib*
7.	*aph(3'')-Ib*
Beta-Lactamase	
8.	*blaOXA-50*
9.	*blaOXA-10*
10.	*blaOXA-396*
11.	*blaOXA-486*
12.	*blaOXA-488*
13.	*blaOXA-49*
14.	*blaOXA-395*
15.	*blaVIM-2*
16.	*blaNDM-1*
17.	*blaCTX-M-15*

18.	*blaVEB-1*
19.	*blaVEB-4*
20.	*blaPER-7*
21.	*blaTEM-1B*
Tetracycline resistance gene	
22.	*tet(G*
23.	*tet(A)*
Chloramphenicol resistance gene	
24.	*catB7*
25.	*floR*
26.	*cmlA1*
Fluoroquinolone Resistance Gene	
27.	*crpP*
28.	*qnrVC1*
Trimethoprim resistance gene	
29.	*dfrB5*
Rifampicin resistance gene	
30.	*ARR-3*
Macrolide Resistance gene	
31.	*mph(F)*
32.	*msrE*
33.	*mphE*
Sulfonamide Resistance gene	
34.	*sul1*
Fosfomycin Resistance Gene	
35.	*fosA*

Annex VII: Published Papers