Genomic Characterization of Fluoroquinolones Resistance Determinants in *Pseudomonas aeruginosa* Clinical Isolates

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A thesis submitted in partial fulfillment of the requirement for the degree of Master of Science (MS) in Industrial Biotechnology

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I, **Mariam**, declare that this research work titled "**Genomic characterization of fluoroquinolones resistance determinants in *Pseudomonas aeruginosa* clinical isolates**" is my own work. The work has not been presented elsewhere for assessment. The work here in was carried out while I was a post-graduate student at Atta-Ur-Rahman School of Applied Biosciences, NUST under the supervision of **Dr. Saadia Andleeb**. The material that has been used from other sources has been properly acknowledged / referred.

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Dr. Saadia Andleeb

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Dedicated to:

My beloved Parents, Mumtaz Ahmed and Bushra Mumtaz

for their endless support, endearment, care & encouragement.

My little angel, Waliya Zeeshan

who was a constant source of happiness for me in this time and endured my time away from her while I was busy with my work.

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who supported me in every way possible way and motivated me throughout this time.

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GLOSSARY OF ABBREVIATIONS

P. aeruginosa	*Pseudomonas aeruginosa*
WHO	World Health Organization
WGS	Whole Genome Sequencing
ARGs	Antibiotic Resistance Genes
Mbp	Megabase Pair
HAI	Hospital Acquired Infections
CF	Cystic Fibrosis
VAP	Ventilator Associated Pneumoina
UTI	Urinary Tract Infections
BSI	Blood Stream Infections
SNPs	Single Nucleotide Polymorphism
gyrA	DNA gyrase subunit A
gyrB	DNA gyrase subunit B
parC	DNA topoisomerase IV subunit A
parE	DNA topoisomerase IV subunit B
HGT	Horizontal Gene Transfer
CIP	Ciprofloxacin
LEV	Levofloxacin
iTOL	Interactive Tree of Life
AMRFinder	Antimicrobial Resistance Finder
QRDR	Quinolone Resistance Determining Regions
AST	Antibiotic Susceptibility Testing
ZOI	Zone of Inhibition

ABSTRACT

Pseudomonas aeruginosa has an increasing fluoroquinolones resistance rate globally and the surveillance for identifying the resistance mechanisms is low in countries like Pakistan. This study detects the genotypic mechanisms associated with fluoroquinolones resistance and evaluates if these genotypic mechanisms were associated with fluoroquinolones susceptibility. The Whole Genome Sequencing (WGS) of 77 isolates was performed by Illumina short reads sequencing, collected from three different hospitals in Pakistan during the time of 2016 and 2017. The antibiotic susceptibility testing (AST) was performed by Kirby-Bauer disk diffusion method in which fluoroquinolones resistance was high, with 71% isolates resistant to ciprofloxacin and 75% isolates resistant to levofloxacin. The genomes analysis revealed that the fluoroquinolone resistance was largely mediated by mutations in Quinolone Resistance Determining Regions (QRDRs) and regulatory genes of efflux pumps along with the possession of acquired genes. The resistance determinants identified were further compared with antibiotic susceptibility of ciprofloxacin and levofloxacin to check the association between phenotype and genotype. Moreover, comparison of zone sizes from AST with resistance mechanisms revealed that possession of multiple resistance mechanisms (mutations in QRDRs, efflux pumps upregulation and acquired fluoroquinolones resistance genes) collectively contribute to fluoroquinolones resistance suggesting that multiple resistance mechanisms adopted by these clinical isolates ensures their survival against same antibiotics.

INTRODUCTION

Pseudomonas aeruginosa is one of the opportunistic Gram-negative species distributed both abiotic and biotic habitats, including soil, water, and plant and animal tissues (Azam, Khan 2019). It is a pathogenic bacterium that infects plants and animals, including humans, and is a leading source of hospital-acquired infections (HAIs). It causes hospital-acquired pneumonia (HAP), ventilator-associated pneumonia (VAP), gastrointestinal infections, dermatitis, urinary tract infections (UTIs), skin infections like folliculitis and external otitis, bacteraemia, folliculitis, respiratory infections in patients with CF, bone and joint infections, and a variety of other infections, particularly in patients with severe burns and immunocompromised patients like those with cancer or AIDS (Tacconelli *et al.,* 2002). *P. aeruginosa* accounts for almost 10% of the total Hospital Acquired Infections (HAIs) (2017).

In hospitals and other clinical care settings, the prevalence of infections brought on by drug-resistant organisms is rising. The difficulties in diagnosing and treating infections brought on by these organisms with antibiotic resistance also raise morbidity and mortality (Pachori.P *et al.,* 2019). *Pseudomonas aeruginosa* is one of the most concerning pathogens involved in antibiotic resistance nowadays. As a result, it has been designated as an ESKAPE organism and is to be held responsible for a variety of ICU acquired infections in critically ill patients, along with other MDR pathogens like *E. coli, Klebsiella, and Acinetobacter baumannii* (Boucher *et al.,* 2009). The World Health Organization (WHO) has listed this species as one of the "critical" bacterial pathogens for which it is urgent need for new antibiotics (Tacconelli *et al.,* 2018)

P. aeruginosa genome size varies widely, from 5.5 to 7 Mbp (Subedi *et al.,* 2018). The so-called accessory genome is what causes the divergence in genome size (Klockgether *et al.,* 2011). Moreover, it has a dynamic genomic composition that keeps on changing due to constant exchange of accessory genetic elements in its genome which further leads to its evolution (Kung *et al.,* 2010). For harbouring virulence and acquired antibiotic resistance genes, accessory genomes are important. Drug resistant virulent strains are a result of the lateral transmission of such genes across strains. Additionally, chromosomal gene mutations can result in virulence and antibiotic resistance (Jeukens *et al.,* 2014). The reduced susceptibility of clinical *P. aeruginosa* strains to a variety of antibiotics has now become a global challenge and has been widely observed. This further makes it difficult to treat the infections caused by this bacterium (Treepong *et al*., 2018). The diverse genetic composition of P. aeruginosa has

allowed this pathogen to cause nosocomial infections globally such as chronic pneumoniae, ventilator associated pneumoniae, burn and wound infections etc (Subedi *et al*., 2018).

Strong antibiotics are used to treat bacterial infections and misuse of these medications has encouraged the spread of antibiotic resistance in most bacteria (Cassini *et al.,* 2019). Fluoroquinolones have been used for decades as an empirical therapy for treating *P. aeruginosa* infections and are widely prescribed (Khan *et al*., 2020). The fluoroquinolones resistance in various clinical isolates is constantly elevating. For instance, the fluoroquinolone resistant *P. aeruginosa* increased from 15% to 41% in ten years period in a single centre under study (Gasink et al., 2006). The reason for this increase has been associated with the misuse of these antibiotics. The fluoroquinolone resistance in isolates is highly dependent on the type of source from which the sample is isolated for example more fluoroquinolones resistance is observed in clinical isolates than those causing ocular infections (Smitha et al., 2005).

The resistance is *Pseudomonas aeruginosa* can be developed due to intrinsic, acquired or adaptive resistance mechanisms (Chevalier et al., 2017). The primary mechanisms for fluoroquinolones resistance in *P. aeruginosa* are by the target-site mutations in the Quinolones resistance determining Regions (QRDRs) and the upregulation of efflux pumps (Higgins *et al*., 2019, Rehman *et al.,* 2019). Four efflux pumps involved in fluoroquinolones resistance are MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY-OprM (Oh et al., 2003). The efflux pumps are controlled by regulatory genes and mutations in these genes result in overexpression of efflux pumps (Sun et al., 2014). For example, mutations in nfxB gene result in overexpression of MexCD-OprJ (Wong, Kassen, 2011). In wild-type the binding of nfxB with the promotor of MexCD-OprJ causes dimerization and restricts transcription while mutations stops dimerization and binding of DNA of nfxB hence causing overexpression of efflux pump leading to antibiotic resistance (Purssell, Poole, 2013). However, in some bacteria the presence of acquired fluoroquinolone resistance genes has been seen either on plasmids or on chromosome of *P. aeruginosa* also contribute to fluoroquinolones resistance. The presence of a *qnr* gene and its variants have been strongly associated with fluoroquinolones resistance. *qnr* is a pentapeptide protein which prevents the action of fluoroquinolones on DNA gyrase and DNA topoisomerase (Robicsek *et al*., 2005). Moreover, another enzyme CrpP encoded gene *crpP*, is a ciprofloxacin phosphorylating enzyme associated with increased fluoroquinolone resistance in *P. aeruginosa* (Chávez-Jacobo *et al*., 2018, de la Rosa *et al*., 2020).

The genomics and comparative genomics approaches have proved to be useful over the years in tracking the antibiotic resistance features of *P. aeruginosa*. The drug resistance in *P. aeruginosa* strains is primarily due to pathoadaptive mutations along with the acquired resistance genes by Horizontal gene Transfer (HGT) (Bianconi et al., 2019). For this reason, it is crucial to establish the knowledge of drug resistance, to further prevent or slow down the process of transfer of antibiotic resistance genes. For instance, after reporting the emergence of resistant isolates in a hospital setting, the changes are advised to be made to the use of commonly prescribed antibiotics, thus, reducing the frequency of isolation of drug-resistant isolates, ultimately allowing the temporarily ineffective antibiotics to be useful again in future (Johnsen *et al.*, 2009). Given the slow process, challenges, regulatory hurdles and high cost of developing novel and effective antimicrobials (Ventola, 2015), research efforts are required for surveillance of prevalent antibiotic resistance genes in drug-resistant bacteria to inhibit the emergence and transmission of these resistance genes amongst other bacteria and to maintain and control the efficacy of already available antibiotics.

The current study is conducted to identify the genetic determinants for fluoroquinolones resistance in 77 clinical isolates of *P. aeruginosa*, their phylogenetic relevance and comparison of genotypic determinants with the phenotypic Antibiotic Susceptibility Testing (AST) of fluoroquinolones (ciprofloxacin and levofloxacin). Here we utilized the Whole Genome Sequencing (WGS) to study the genotypic and phylogenomic patterns of 77 *P. aeruginosa* isolates previously collected from Islamabad and Rawalpindi regions of Pakistan. The sequences for both the resistant and susceptible isolates were used and we identified that whether the genotypic and phenotypic resistance has correlated or linked with phylogenetic background, as we observed fluoroquinolone resistant isolates in all phylogenetic clades. We found that isolates possessing multiple resistance mechanisms had higher levels of resistance to ciprofloxacin and levofloxacin than isolates having single or less resistance mechanisms. At first, we detected that isolates possess different fluoroquinolone resistance mechanism in our dataset. Then, we identified that phylogenetically correlated isolates have different fluoroquinolone resistance mechanisms (QRDR mutations, efflux pumps mutations, acquired gene). At the end we correlated the resistance mechanism and levels of resistance for CIP and LEV and found out that isolates with multiple resistance mechanisms have higher resistance to both fluoroquinolones.

THESIS OBJECTIVES:

- Identification of the genetic determinants of fluoroquinolone resistance in clinical *Pseudomonas aeruginosa* strains
- Investigation of relatedness of genotypic fluoroquinolone resistance mechanisms with phenotypic resistance in phylogenetic groups of *P. aeruginosa* strain

REVIEW OF LITERATURE

2.1 *Pseudomonas aeruginosa*: an opportunistic pathogen:

Pseudomonas aeruginosa is a Gram-negative rod-shaped bacterium present in wide variety of ecological environments from aquatic to soil habitats (Moradali *et al*., 2017). *P. aeruginosa* belongs to the family Pseudomonadaceae and they are aerobic, motile, non-spore forming, oxidase positive and lactose non-fermenters with a size ranging from 0.5 to 0.8 µm by 1.5 to 3 µm (Pachori *et al*., 2019). The first isolation of *P. aeruginosa* was done by Carle Gessard in 1885 from wounds of war patients whose bandages had blue green colour. The two primary soluble pigments produced by *P. aeruginosa* are pycocyanin, which produces blue colour and pyoverdin, which gives yellow-green or yellow-brown colour. The blue green colour of the *P. aeruginosa* colonies is due to production of these two primary pigments. When grown on sheep blood agar *P. aeruginosa* colonies display greenish shade due to beta-hemolysis and these pigments production which makes it easier to differentiate from other species (Moore and Flaws, 2011). *P. aeruginosa* can survive in humans, plants and animals (Getally *et al.,* 2013). It has minimal nutritional requirements and can digest a lot of environmental nutritional sources for its survival. It utilizes acetate and ammonia for fulfilling its carbon and nitrogen demand. Moreover, it can grow anaerobically without performing fermentation, instead it gets its energy from oxidizing sugars. These qualities allow its easy survival in marginal environments such as hospital equipment, operating rooms, medical appliances, sinks, showers etc (de Abru *et al.,* 2014). This increases the risk of transfer of *P. aeruginosa* from hospital settings to immune-compromised patients and due to this reason, it is considered as a major source for nosocomial infections (Basseti *et al.*, 2018).

P. aeruginosa is an opportunistic pathogen causing a wide range of acute and chronic infections (Burrows *et al.*, 2018). World Health Organization (WHO) has declared *P. aeruginosa* in the "critical" category for which there is urgent requirement for research and development of new antibiotics (Tacconelli *et al*., 2018). P. aeruginosa has also been included in ESKAPE pathogens; *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, P. aeruginosa* and *Enterobacter species* due to its capacity to cause a diverse range of diseases and its ability to escape the antibiotics used against it (Boucher *et al*., 2010).

2.2 Disease burden of *Pseudomonas aeruginosa*:

It has been reported in several studies that *P. aeruginosa* is a common cause for nosocomial infections (Pittet *et al.*, 1999, Kim *et al.*, 2000, Zhanel et al., 2008). The rate of nosocomial infections caused by *P. aeruginosa* is reported to be 13.8%. However, in Intensive Care Units (ICUs) the rate is around 13.2-22.6% (Erbay *et al*., 2003). Moreover, it caused infection in all major organs and parts of body. Ranging from respiratory tract infections (cystic fibrosis), eye infections (keratitis), ear infections (otitis media), central nervous system infection, GIT infections, blood infections (bacteraemia) and urinary tract infections. As it effects individuals with weakened immunity, it can infect patients with HIV, cancer, burn and cystic fibrosis (Morita *et al*., 2014). A study highlighted the primary acute infections by *P. aeruginosa* in different body organs. The infections in soft tissues, burns, ear and different body organs are displayed in figure 2.1 (Proctor *et al*., 2021). The primary type of infections caused by *P. aeruginosa* are discussed below.

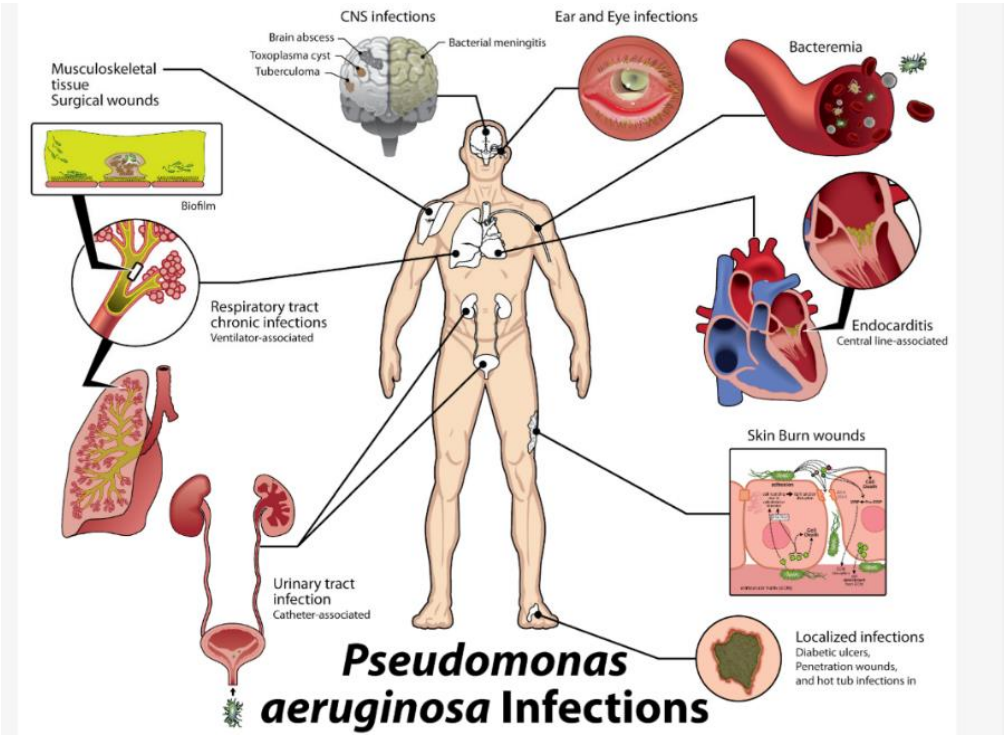

Figure 2.1 Acute *P. aeruginosa* infections in soft tissues, burns, wounds, ear and primary organs such as CNS infections and Urinary Tract Infection (UTIs) (adapted from Proctor *et al*., 2021)

2.2.1 Hospital Acquired Infections (HAIs):

P. aeruginosa has been a major pathogen in hospital acquired infections as compared to other pathogens due to its morbidity and mortality. In terms of nosocomial infections, it is reported to be the fourth most common pathogen and it is responsible for 10% of HAIs (Afshari *et al.*, 2012). These infections always pose a serious challenge due to organisms' ability to develop high intrinsic resistance to antibiotics (Tsao *et al.*, 2019). In Northern Ireland in 2014, it was reported that neonates were detected with bacteraemia caused by *P. aeruginosa* in critical care units and four deaths occurred due to this colonization (Walker *et al.*, 2014). HAIs caused by *P. aeruginosa* impose serious financial burdens on hospitals in terms of spending on antibiotics. In a healthcare associated infections study conducted in Serbia, hospital spent 11.2% of their drug budget on the antimicrobials included in study (Folic et al., 2021). In a recent study conducted in a hospital of Europe to check the prevalence of *P. aeruginosa* in HAIs for 8.5 years showed that P. aeruginosa infections were increased two times among Gram-negative infections and among HAIs, the infections increased by three times as depicted in figure 2.2 (Litwin *et al.*, 2021). One of the most important HAIs is Ventilator Associated Pneumoniae (VAP) which is discussed in detail below.

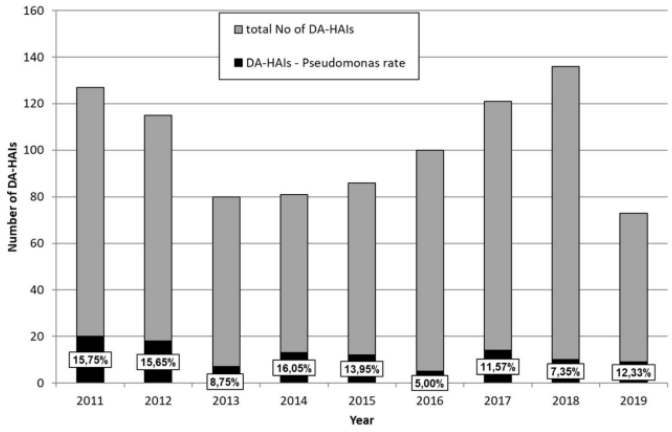

Figure 2.2 Graphical representation of P. aeruginosa HAIs in a research study conducted in a hospital in Europe over a period of 8.5 years (adapted from Litwin *et al.*, 2021)

2.2.1.1 Ventilator Associated Pneumoniae (VAP):

In ICUs the most common HAI is Ventilator Associated Pneumoniae (VAP), and the most prevalent ones are induced by mechanical ventilation (Tumberallo *et al.*, 2013). VAP by *P. aeruginosa* also depends on time because generally it is associated

with late onset and making it harder to treat. When compared with other VAP causing pathogens, *P. aeruginosa* displayed higher mortality (Raineri *et al*., 2014). The colonization of *P. aeruginosa* in tracheobronchial regions of the ventilated patient increases the fatality rates. For instance, the Multi-Drug Resistant (MDR) strains increase the fatality rate by 12% as compared to susceptible strains (Horcajada *et al*., 2013). The infections caused by *P. aeruginosa* are posing serious risks as it is harder to treat them with common antibiotics or a single drug because of their ability to develop intrinsic resistance and acquire resistance mechanisms by additional genes in bacterial genome or on plasmids (Pachori et al., 2019). It has also been reported in several studies that patients with chronic diseases (e.g cystic fibrosis), are at greater risk of *P. aeruginosa* infections (Inglis et al., 1993).

2.2.1.3 Nosocomial Urinary Tract Infections (UTIs):

P. aeruginosa accounts for causing UTIs as the third most common pathogen. The primary factor for inducing UTIs in hospitalized patients is catheterization. The installed catheter is sometimes damaging the mucous layer which allows the colonization of *P. aeruginosa* by passing through the natural barrier (Mittal et al., 2009). The possible outcomes of infection are discomfort, pyelonephritis and in some sever cases death may occur (Obritsch *et al*., 2005). *P. aeruginosa* has been held responsible for causing 9% UTIs in hospital patients and the percentage is even higher for ICU patients (16.3%) (Gaynes *et al*., 2005, Driscoll et al., 2007).

2.2.1.4 Hospital Acquired Blood Stream Infections (BSIs):

14-20% of Blood Stream Infections (BSIs) caused by *P. aeruginosa* has been reported in ICU patients as compared to other nosocomial BSI which is significantly low (4-6%) (Osman *et al*., 2004). The respiratory tract and venous catheters have been reported to be the major sources of BSI (Pachori et al., 2019). The major factors inducing BSI by *P. aeruginosa* are lungs cancer, pneumoniae, inappropriate and prolonged antimicrobial treatment and multi drug resistant strains (Vidaur et al., 2005).

2.2.2 Other infections:

The most common eye infection caused by *P. aeruginosa* is keratitis in cornea. Wearing contact lens can increase the risk for corneal infections. However, other risk factors include ocular surgery or eye infections (Khan et al., 2020). Other infections include folliculitis on skin, otitis externa in ear due to tissue injury, soft tissue infections

in post-surgery wounds and burns etc. Moreover, it can result in diabetic foot in diabetes patients (Gellaty and Hancock, 2013).

2.3 Diverse genome of *Pseudomonas aeruginosa*:

The high adaptive capacity of *P. aeruginosa* in different environments is due to its genome plasticity and high metabolic capacity. *P. aeruginosa* genome size is 5.5 to 7 Mbp displaying a highly variable nature (Stover et al., 2000). The genome variability is due to possession of accessory genome, which are the DNA sequences specific to a particular strain. According to a study, the accessory genome comprises 20% of *P. aeruginosa* genome (Klockgether *et al.*, 2011). The accessory genome majorly consists of elements acquired in the genome by Horizontally Gene Transfer (HGT). It constitutes prophages, Genomic Islands (GIs), transposons, Insertion Sequences (IS) and plasmids (Kung *et al*., 2010). The core genome is rather conserved and constitutes major part (90%) of the genome as these genes are common among all isolates representing their similar metabolic and pathogenic profile (Wolfgang *et al*., 2003). The considerable part of the accessory genome is Mobile Genetic Elements (MGEs) and some MGEs have retained their positions in genomes while others retain their mobility in other genomes (Qiu *et al*., 2006). The presence of accessory DNA is at highly specific loci for genomic islands rather than at random sites. The segments of DNA specific to only one kind of genome and differentiates a genome from other are called as " Regions of Genome Plasticity" (RGPs) by Mathee et al (Mathee *et al*., 2008). The pangenome is the total number of genes of the core and accessory genome specific to a particular specie and pangenome of *P. aeruginosa* is expanding due to increase in gene families in accessory genome (Jeukens *et al*., 2014). Thus, the total sequencing data available shows the total pangenome of specie. The pangenome of *P. aeruginosa* is always extending and with each addition of gene pool, it will expand the overall gene pool (Klockgether *et al*., 2011). Accessory genomes harbour acquired genes and virulence factors that are responsible for inducing antibiotic resistance in *P. aeruginosa*. These horizontal transferable elements along with chromosomal mutations in the core genes play vital role in antibiotic resistance (Ashish *et al*., 2012). The size of accessory genome ranges from few hundred to 200 kbp in P. aeruginosa genome. In a study of completely sequenced P. aeruginosa genomes, 38-53 of these accessory genomic elements were identified (Klockgether *et al*., 2011). With a higher G+C content of 66.6%, when *P. aeruginosa* genome was compared with 22 other bacterial genomes,

the closest relevance was shown to the genome of *Escherichia Coli* which has a genome size of 4.6 Mbp (Stover et al., 2000).

2.4 Phylogenetically Significant *P. aeruginosa* Strains:

The phylogenetically significant strains that span the phylogeny of *P. aeruginosa* are PAO1, UCBPP-PA14 (generally known as PA-14), PA-7, LESB58, DK2 and VRFPAO4 (Subedi *et al*., 2018b). The most widely used reference strain for *P. aeruginosa* is PAO1 first isolated in 1950s. PAO1 has the smallest accessory genome with inserts of 14kbp or smaller (Klockgether *et al*., 2011). PA-7 is considered as a taxonomic outlier, and it was first isolated from respiratory sources in Argentina. Then genomic identity of PA-7 with other *P. aeruginosa* strains is 95% (Roy et al., 2010). Notable strains such as DK2 and LESB58 isolated from cystic fibrosis patients have been reported in various studies (Rau *et al*., 2012, Weihalmann et al., 2015). LESB58 can also infect non-cystic fibrosis patients and severe infections have been observed (McCallum *et al*., 2002). The identified accessory genome elements in LESB58 are five genomic islands and prophages od 14-111 kbp in size (Klockgether et al., 2011). PA-14 is considered as a highly pathogenic and virulent strain as compared to PAO1. The genomic comparison of both of these genomes shows a slightly larger genome of PA-14 than PAO1. 58 extra gene clusters that were absent in PAO1 were detected in PA-14 and are associated with pathogenicity and virulence including PAPI-1 and PAPI-2 pathogenicity islands (Lee et al., 2006). VRPAO4 is an ocular pathogen isolated from keratitis patients in India. It has the largest genome of 6.8 Mbp reported to be the third largest bacterial genome available at Genbank (Murugan et al., 2016).

2.5 Commonly used antibiotics against *Pseudomonas aeruginosa*:

There are eight classes of antibiotics used primarily as antipseudomonal drugs. Aminoglycosides are the antibiotics with bactericidal effect employed to treat cystic fibrosis related infections. Gentamicin, amikacin, tobramycin and netilmicin are commonly aminoglycosides used in all areas of world (Poole K., 2005). Their primary action is inhibition of protein synthesis by binding with the 30S ribosomal subunit thus halting the translational process and causes cell death (Morita *et al.*, 2014). The entry of aminoglycosides in the bacterial cell is a complex process involving binding to the lipopolysaccharide layer and then it passes through cytoplasmic membrane followed by

binding with ribosomal subunit and production of mistranslated peptides (Krahn *et al*., 2012). β- lactams is a broader category which further includes penicillins, cephalosporins, monobactams and carbapenems. The mechanism of action of β-lactams is that they halt the transpeptidation process, which is considered as the last step in peptidoglycan (bacterial cell wall component) formation (Aurilo *et al*., 2022). Among β-lactams, carbapenems are considered as the most reliable option and trusted as the last resort for treating *P. aeruginosa* induced infections (Silva *et al*., 2020). They are the most used among antipseudomonal drugs. Carbapenems have extra stability to bacterial enzyme action due to their stable molecular structure (Aurilo *et al*., 2022). Commonly employed carbapenems are imipenem, doripenem, meropenem (Botelho *et al*., 2019). Cephalosporins such as ceftazidime and cefipime are frequently employed for treating *P. aeruginosa* infections and their mechanism of action on *P. aeruginosa* is similar to other β- lactams. Penicillins such as ticarcillin-clavulanic acid and piperacillin-tazobactam along with monobactams such as aztreonam have frequently been put into use (Pang *et al*., 2019). Fosfomycin and polymyxins such as colistin and polymyxin B have also generally used. Macrolides such as azithromycin have been employed for treating respiratory infections and their primary action is by binding to 50S ribosomal subunit and inhibiting protein synthesis (Giani *et al*., 2018). Fluoroquinolones such as ciprofloxacin and levofloxacin have been used for decades to treat a wide range of infections by *P. aeruginosa.* The general mechanisms of action of

main groups of antipseudomonal drugs are depicted in figure 2.3.

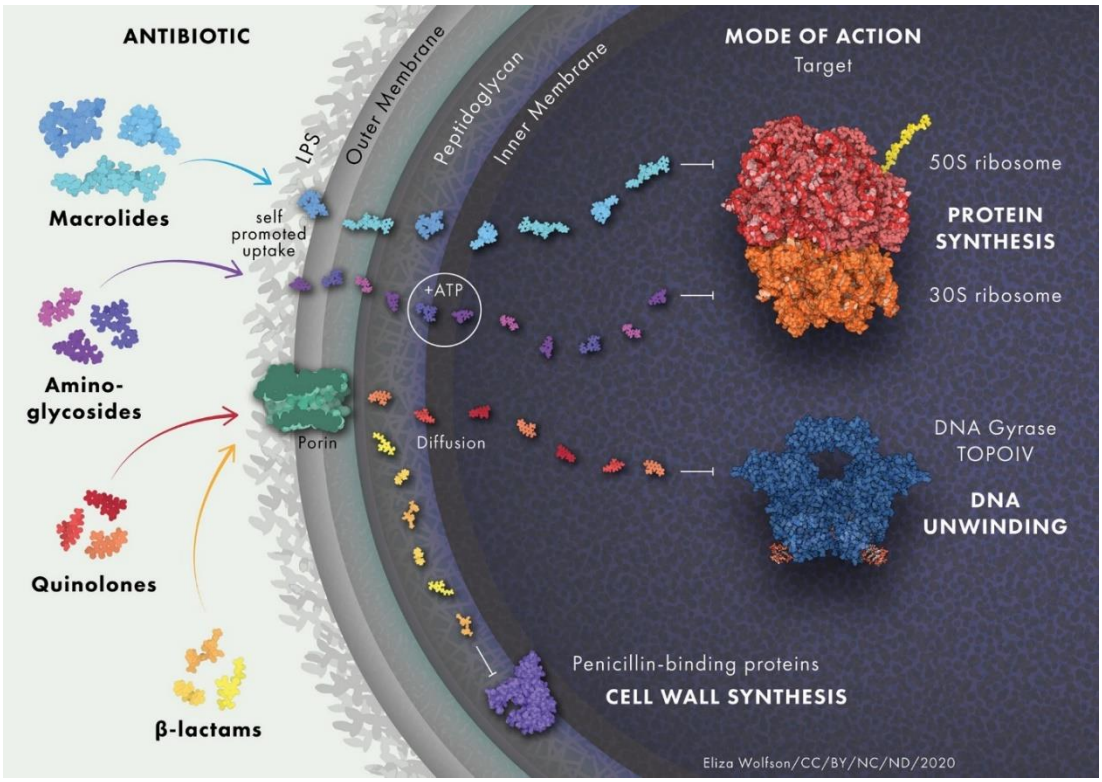

Figure 2.3 Mechanism of action of commonly used antipseudomonal drugs. Aminoglycosides and macrolides interact with lipopolysaccharide (LPS) to enter in the cell while quinolones and β- lactams gain entry by porin channels to cross cell membrane (adapted from Langendonk, R. Fredi et al., 2021).

2.5.1 Fluoroquinolones for treating *Pseudomonas aeruginosa* infections:

Fluoroquinolones are broad spectrum antibiotics, widely prescribed, used as an empirical therapy for treating a wide range of *Pseudomonas aeruginosa* infections for decades (Khan *et al*., 2020). The main target for fluoroquinolones is two bacterial DNA target proteins: DNA gyrase and DNA topoisomerase IV (Hooper *et al.,* 1998). DNA gyrase further constitutes two subunits gyrA and gyrB. They take part in transcription initiation, chromosome condensation and replication by cleaving the DNA backbone and re-ligate it by an ATP dependent reaction after introduction of supercoils (Wang *et al*., 2002). Each of these two subunits have their individual function as gyrA cleaves the DNA backbone and gyrB is involved in the catalysis of ATP for supercoiling process (Bush *et al*., 2015).

DNA topoisomerase IV is a tetramer molecule consisting of two subunits: two parC and two parE subunits. The primary action of topoisomerase IV is after replication process as it resolves daughter chromatids after the process of replication and also it is involved in the relaxation of supercoils in DNA (Kato *et al*., 1990, Levine et al., 1998). The main target of fluoroquinolones is that it inhibits or halts the re-ligation step during DNA processing by these enzymes which results in DNA double stranded breaks that stop further DNA replication. The overall effect of fluoroquinolones is bacteriostatic which can result in killing of bacteria as it directly inhibits DNA replication process (Drlica *et al*., 2009). Ciprofloxacin and levofloxacin are commonly employed fluoroquinolones for decades. The detailed structure for both fluoroquinolones and mechanisms of action are described below.

2.5.1.1 Ciprofloxacin:

Ciprofloxacin are the fluorinated quinolones having the structural similarity to nalidixic acid (Campoli-Richards *et al*., 1998). It is a quinolin- 4(1H)- one having cyclopropyl, carboxylic acid, fluoro and piperazin-1-yl substituents at positions 1, 3, 6 and 7, respectively (PubChem).

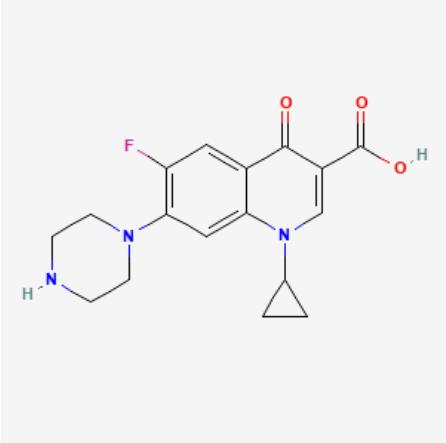

Figure 2.4 Chemical structure of ciprofloxacin (adapted from PubChem)

Since its discovery in 1980s, ciprofloxacin has been used to treat Gram-negative bacterial infections and they are highly susceptible to it (Davis *et al*., 1996). A study by A. Rehman *et al* involved the construction of homology model of ciprofloxacin interaction DNA gyrase based on DNA gyrase- ciprofloxacin complex of *Mycobacterium tuberculosis*. As the gyrA from *P. aeruginosa* had shown 43% sequence similarity to gyrA from *M. tuberculosis*, so based on this similarity homology model was constructed. The model revealed threonine 83 and aspartate 87 as two key

binding residues for on DNA gyrase for ciprofloxacin. These residues were present at the site of DNA binding for process of DNA replication but the binding of ciprofloxacin to these residues restricted the process of DNA binding and introduced double stranded DNA breaks as shown in figure 2.5 (Rehman et al., 2019).

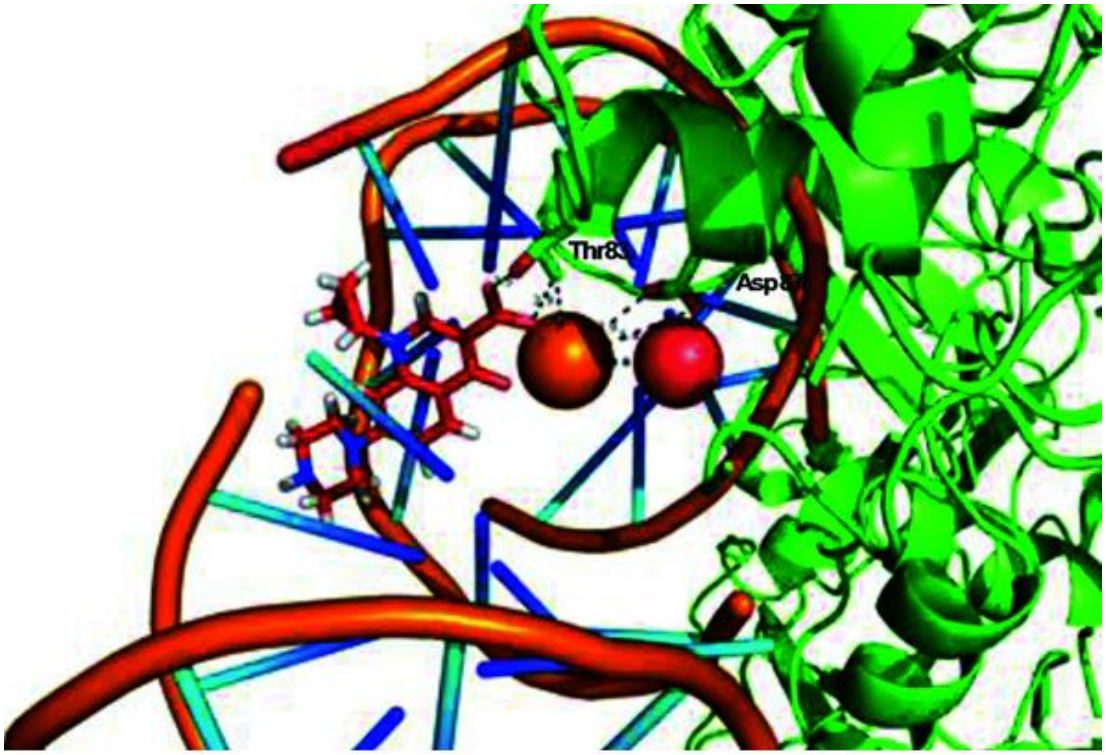

Figure 2.5 Homology model of *P. aeruginosa* DNA gyrase interaction with ciprofloxacin. The ciprofloxacin binding site in DNA gyrase is displayed in green, DNA is orange and ciprofloxacin are displayed as blue sticks. The interaction of ciprofloxacin by hydrogen bonding with residues Thr83 and Asp87 are displayed (adapted from A. Rehman *et al*., 2019).

2.5.1.2 Levofloxacin:

Levofloxacin is a broad-spectrum fluoroquinolone employed for the treatment of various respiratory and urinary tract infections by *P. aeruginosa*. Levofloxacin is an L-isomer of ofloxacin. The basic structure of levofloxacin is displayed in figure 2.6 (PubChem). Several studies have been conducted to compare the *invitro* activity of levofloxacin to ciprofloxacin. Some studies supported the better activity of levofloxacin against clinical *P. aeruginosa* isolates while other studies such as involving cystic

fibrosis isolates displayed lower activity results for levofloxacin (Segatore et al., 1999, Gesu *et al*., 2003, Marchetti *et al.*, 2003, Golini *et al*., 2001).

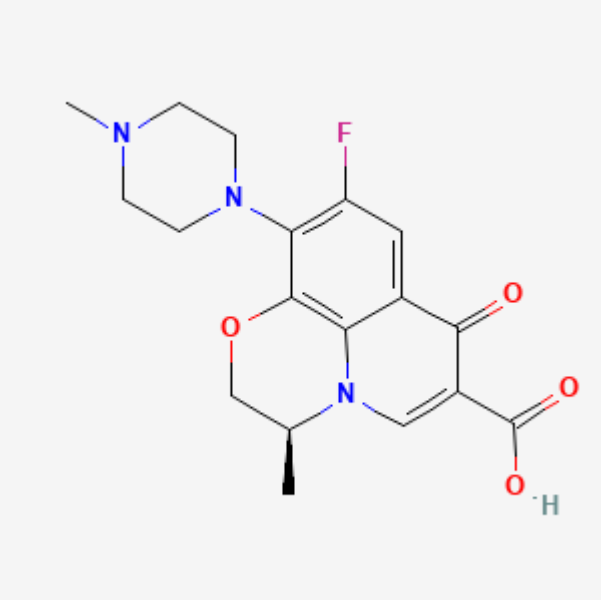

Figure 2.6 Chemical structure of levofloxacin (adapted from PubChem)

2.6 Antibiotic resistance mechanisms in *Pseudomonas aeruginosa*:

P. aeruginosa has evolved various resistance mechanisms to antibiotics to ensure its survival. As discussed previously, with extraordinary survival capacity and adaptation of different resistance mechanisms, it ensures its presence in ubiquitous environments causing more infections hence making the available treatments impractical (Azam *et al.*, 2019). There are various resistance mechanisms that *P. aeruginosa* has either developed intrinsically within its bacterial cell, acquired different genes to adapt new survival characteristics against antibiotics or adapted to the stress environment by biofilm formation according to its need (Pang et al., 2019). The intrinsic resistance mechanisms include restriction of antibiotics entry from the outer membrane by mutations in the porin genes (OprD, OprM), over expression of efflux pumps pumping excess antibiotics out of cells and by producing antibiotics inactivating enzymes inside cell such as phosphatases, lipases etc (Breidenstein et al., 2011).

The acquired resistance mechanisms are adopted by some chromosomal mutations or by acquiring some resistance genes by Horizontal gene Transfer (HGT). The chromosomal mutations involve target site modifications where the targets for

antibiotics are modified by mutations or mutations in efflux pumps genes (Munita *et al*., 2016). The adaptive resistance is attained by biofilm formation in stress conditions such as pH, temperature, antibiotics etc. The biofilm is basically formed by the group of microorganisms which adhere or stick together in a biotic or abiotic environment and wrap themselves either in self-produced matrix or Extra Polymeric Substances (EPS) (Donlan *et al*., 2002). Moreover, persister cells formation contributes to the development of adaptive resistance. This is achieved by the genetically identical bacterial population when they collectively resist to the higher concentrations of antibiotics hence rendering them ineffective (Balaban *et al*., 2013).

2.6.1 Fluoroquinolones resistance mechanisms in *P. aeruginosa*:

The main mechanisms contributing to fluoroquinolones resistance in *P. aeruginosa* are by target site modifications in the DNA gyrase and DNA topoisomerase IV subunits, by overexpression of efflux pumps and by acquiring fluoroquinolones resistance genes by Horizontal gene transfer (HGT). Each of the mechanisms is discussed in detail below.

2.6.1.1 Target site mutations:

Target site modifications occur in the DNA gyrase encoding genes *gyrA* and *gyrB* and in DNA topoisomerase IV subunit genes *parC* and *parE*. Mutations in these genes reduce the affinity for fluoroquinolones rendering them ineffective. The first mutation was identified in the *gyrA* gene where isoleucine at position 83 replaces threonine (T83I) and leucine replaces serine at position 87 in *parC* gene(S87L). Both of these sequence alterations are the most commonly observed mutations in fluoroquinolones resistant isolates (Lee et al., 2005, Pasca et al., 2016). The next common alteration is at position 87 in *gyrA* gene where aspartate is replaced by asparagine, tyrosine or glycine. these different antibiotics present at the attachment site of ciprofloxacin reduce the affinity of fluoroquinolones for DNA gyrase or DNA topoisomerase IV hence rendering high resistance to these antibiotics (Aldred *et al*., 2014). The mechanisms of target site alterations are depicted in Figure 2.7 where *gyrAB* and *parCE* mutations render the fluoroquinolones ineffective (Rehman *et al*., 2019)

2.6.1.2 Efflux pumps overexpression:

Efflux pumps are involved in the secretion of small molecules to the outside of cell such as antibiotics, disinfectants, dyes etc (Poole, 2011). The intracellular

concentration of fluoroquinolones is reduced by the over expression of efflux pumps which effluxes extra antibiotic out of the cell leaving a small concentration inside which is almost ineffective. This happens by the mutations in the regulatory genes of efflux pumps which upregulates their expression and hence the over expulsion of fluoroquinolones is performed (Wong et al., 2011). The efflux pumps in *P. aeruginosa* belongs to Resistance Nodulation Division (RND) (Pang *et al*., 2016). Efflux pumps further constitutes three parts, a cytoplasmic membrane-spanning substrate-proton antiporter protein, an outer membrane channel-forming protein and a periplasmic membrane fusion protein for bridging (Zgurskaya *et al*., 2009). Efflux requires ATP reaction in the process carried out by ATPase present in the cytoplasmic membrane. In total, there are twelve efflux pumps in *P. aeruginosa* but four are known to be linked with the efflux of fluoroquinolones (Poole, 2005). These four pumps are MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY-OprM (Goli *et al*., 2016).

The regulatory genes with mutations responsible for overexpressing MexAB-OprM pump are *mexR, nalC* and *nalD* (Díaz-Ríos *et al*., 2019). Several missense mutations such as V126E where valine is replaced by glutamic acid in *mexR* gene, G71E where glycine is replaced by glutamic acid and S209R where serine is replaced by arginine in *nalC* gene, have been previously linked with overexpressing MexAB-OprM efflux pump (López-Causapé *et al*., 2019). Some missense and frameshift mutations are linked with MexCD-OprJ regulator *nfxB* Figure 2.7 Resistance mechanisms of P. aeruginosa against fluoroquinolones. Target site alterations in *gyrAB* and *parCE* genes and over expression of efflux pumps (Rehman et al., 2019), that result in overexpression of this efflux pump (Purssell and Poole, 2013, Olsson *et al*., 2020). Genes *mexE, mexT* and *mvaT* are held responsible in several studies for overexpressing MexEF-OprN efflux pump. *mexT* is the regulator involved in upregulation of this efflux pump (Llanes *et al*., 2011). Previously, presence of missense mutation such as F172I and a deletion of eight nucleotides responsible for a frameshift mutation have been linked with overexpressed MexEF-OprN efflux pump (Jalal *et al*., 2000). Some studies have reported that missense mutations in gene *amgS* resulted in overexpressing MexXY-OprM efflux pump which is known to efflux fluoroquinolones (López-Causapé *et al*., 2018). The resistance mechanism of P. aeruginosa efflux pumps is depicted in figure 2.7 (Rehman *et al*., 2019).

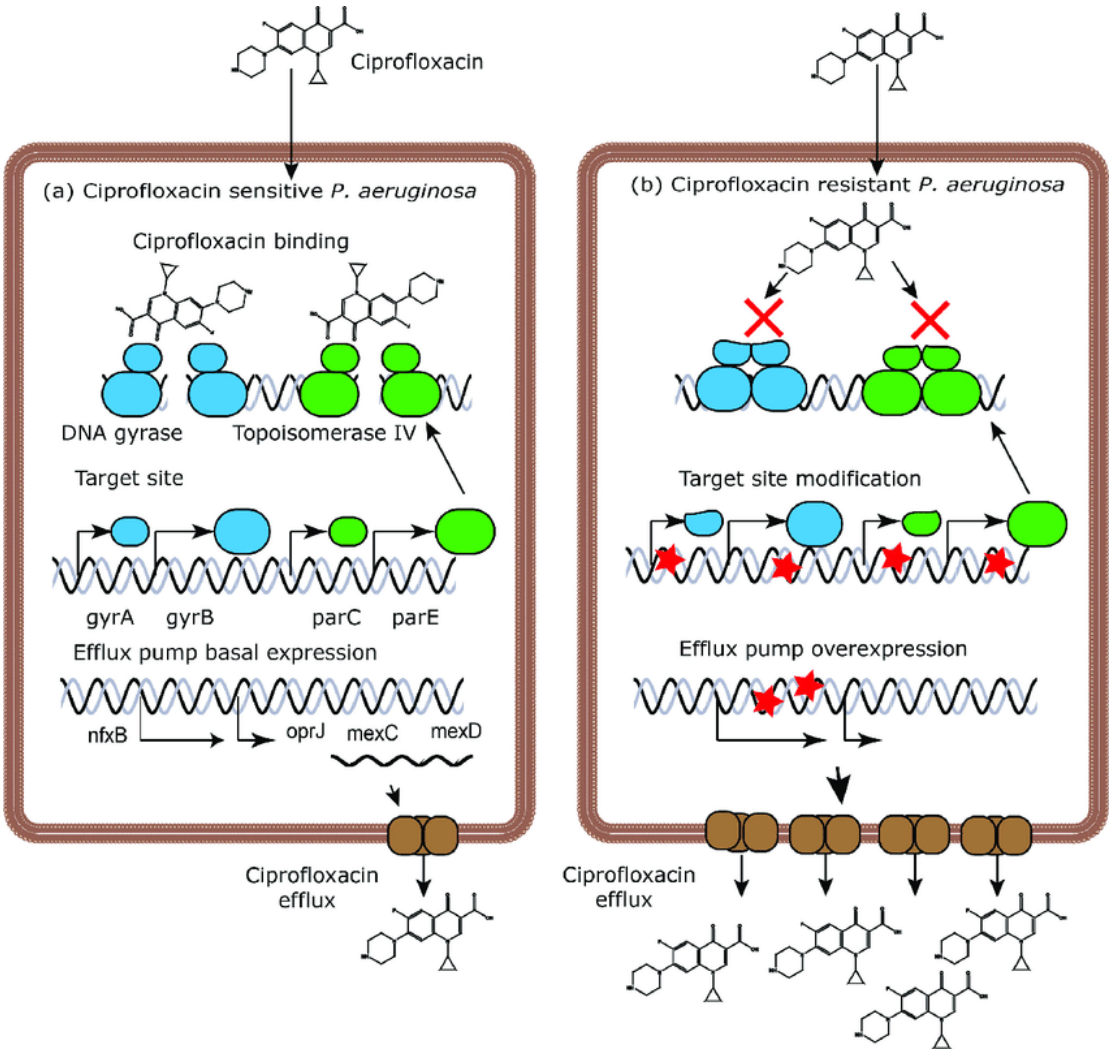

Figure 2.7 Resistance mechanisms of P. aeruginosa against fluoroquinolones. Target site alterations in *gyrAB* and *parCE* genes and over expression of efflux pumps (adapted from A. Rehman et al., 2019).

2.6.1.3 Acquired genes:

The genes can be acquired by bacteria from different sources to develop resistance against antibiotics. These horizontally transferable genes can be acquired on plasmids, transposons, integrons or prophages (Breidenstein *et al*., 2011). Three main mechanisms for Horizontal gene Transfer are conjugation, transformations and transduction (figure 2.8) (Von Wintersdorff *et al*., 2016). Acquisition of fluoroquinolone resistance genes have been reported in various studies (Botelho et al., 2020). *crpP* is a fluoroquinolones resistance gene which is a ciprofloxacin modifying protein involved in the phosphorylation of the antibiotic in an ATP dependent reaction. *crpP* was identified on plasmid pUM505 for the first time (Chávez-Jacobo *et al*., 2020) but there are no more studies supporting its presence on plasmid and various studies

have shown its association with chromosome rather than plasmid (Khan et al., 2019). *crpP* has its associated Mobile Genetic Elements (MGEs) which carry it from one genome to another. MGEs related to *crpP* also have association with Integrative Conjugative Elements (ICEs) such as PAPI-1 which is involved in virulence genes transfer (Khan et al., 2019, Subedi et al., 2019). Besides *crpP*, quinolone resistant gene (*qnr*) has also been associated with fluoroquinolones resistance and is acquired horizontally (Botelho et al., 2020). The *qnr* gene is carried on plasmid pBM413(Liu *et al*., 2018) and it is a pentapeptide protein providing its mechanism of resistance by preventing the fluoroquinolones to perform action on DNA gyrase and DNA topoisomerase (Robicseck *et al*., 2006). Although, the presence of *qnr* on plasmids is also rare (Cayci *et al*., 2014).

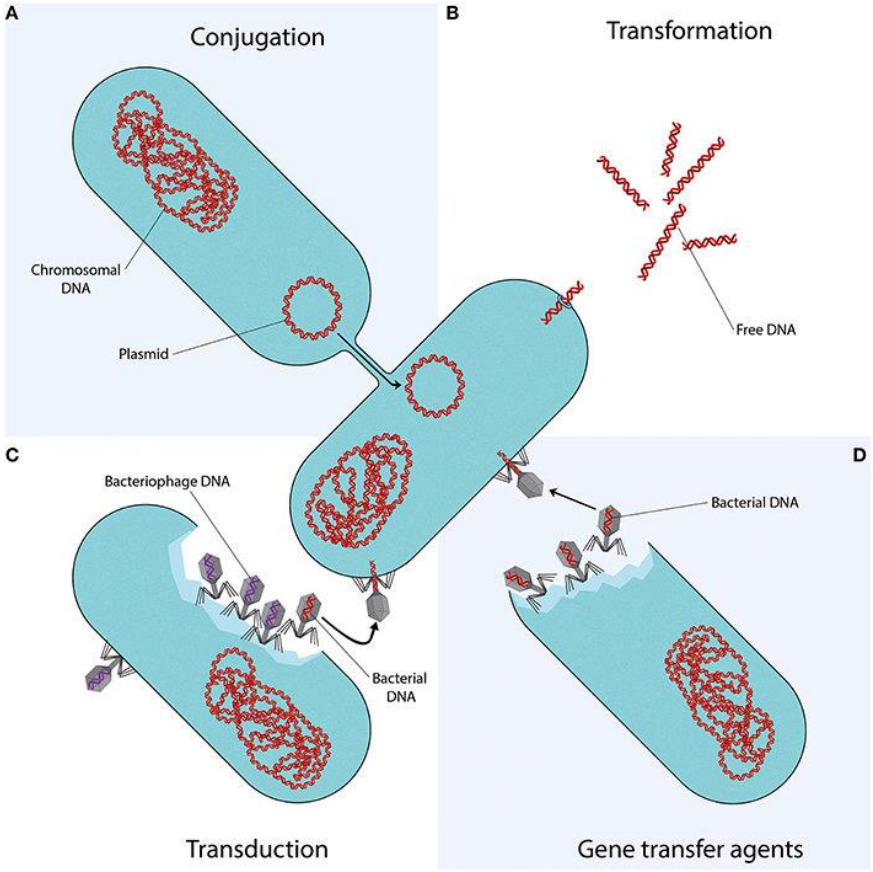

Figure 2.8 Mechanisms of Horizontal Gene Transfer (HGT) in *P. aeruginosa*. HGT mechanisms involve conjugation, Transformation and transduction. A) Conjugation directly transfers DNA between bacterial cells by direct contact. B) Transformation involves taking up free DNA in environment by bacteria. C) Transduction involves transfer of DNA from one to another bacteria by viruses which mediate the whole process (adapted from CJH Von Wintersdorff *et al*., 2016).

2.7 Global emergence of fluoroquinolones resistance in *Pseudomonas aeruginosa:*

The fluoroquinolone resistance in *P. aeruginosa* occurs at different rates across the world (Figure 2.9). Centre of Disease Dynamics, Economics and Policy (CDDEP) provides antibiotics resistance map for pathogenic bacteria against different classes of antibiotics. CDDEP includes 46 countries for antibiotic resistance data from 1999 to 2017. The highest fluoroquinolones resistance was shown in Russia (43%) and India (34%) along with small countries such as Belarus (87%) along with Czech Republic (100%). Canada and United States of America (USA) represented 19% fluoroquinolones resistance. South Africa represented 34% resistance and Australia showed only 5% resistance to fluoroquinolones. However, no resistance data for Pakistan was available.

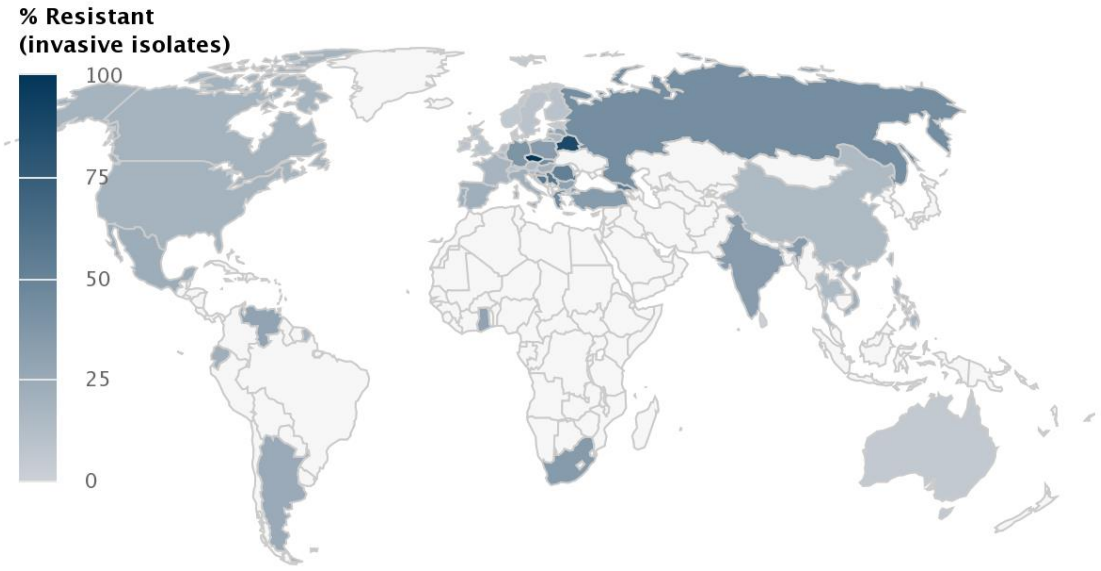

Figure 2.9 Global pattern of fluoroquinolone resistance in *Pseudomonas aeruginosa.* Resistance Map by Centre for Disease Dynamics, Economics and Policy (https://resistancemap.cddep.org/AntibioticResistance.php) from 1999 to 2017 including the data from 46 countries for resistance in *P. aeruginosa* for fluoroquinolones.

2.8 Phenotypic methods to detect antibiotics resistance in *Pseudomonas aeruginosa*:

The phenotypic detection of antibiotic resistance in *P. aeruginosa* can be done by performing Antibiotic Susceptibility Testing (AST). This can either be performed by Disk Diffusion method or by Minimum Inhibitory Concentrations (MICs) method (Abbas et al., 2018). Phenotypic testing is still considered as a primary choice for antibiotics resistance testing, and it has been used for decades. The Clinical Laboratory Standards Institute (CLSI) guidelines are used for standard breakpoints for susceptible, resistant and intermediate. The breakpoints for ZOI are represented in mm and breakpoints for MICs are represented in µg. For fluoroquinolones, ZOI breakpoints for ciprofloxacin are given as follows: ≥25 is considered susceptible, 19-24 mm is intermediate and ≤18 is considered resistant. ZOI breakpoints for levofloxacin are as follows: ≥22 is susceptible, 15-21 mm is considered intermediate and ≤14 is considered resistant (Hackel et al., 2019).

The phenotypic diagnosis of a pathogenic microbe is a laborious and extensive process, and it has certain setbacks. The treatment plan after this long diagnosis is offered after a sufficient time has passed. Moreover, the growth of pathogenic bacteria in different environment and parameters does not totally match with the growth at infection site (Yu et al., 2019).

2.9 Modern approaches to detect antibiotics resistance in *P. aeruginosa*:

Owing to the limitations of phenotypic methods for detecting antibiotics resistance, several modern approaches have been employed to overcome the shortcomings. Omics approaches has significantly helped in exploring the resistome of *P. aeruginosa* (Botelho et al., 2019). For instance, genomics has helped in the complete sequencing of PAO1 genome almost 50 years ago. The complete genome of *P. aeruginosa* somewhat painted a picture for its diverse antibiotic resistant nature and extraordinary survival capacity (Mathee et al., 2008). The genomics approach has further revealed that *P. aeruginosa* has higher number of acquired antibiotic resistance genes, virulence genes and genes linked to adhesion and motility when compared with other similar genomes (Chevalier *et al*., 2017). Comparative genomics revealed that P.

aeruginosa comprises a genome with mosaic nature wherelarge part of genome is ‘core genome’ and small strain specific regions are observed in the genome which has been named as accessory genome (Fischer *et al*., 2016, Freschi *et al*., 2018). The transcriptomics and proteomics approach has been applied to assess the production of molecular products by different genes (Chandler *et al*., 2019). Phylogenomics approach have been used to find the evolutionary relationship of *P. aeruginosa* and understanding the acquisition of resistance genes and mechanisms by its strains over time (Subedi *et al*., 2019).

METHODOLOGY

3.1 Data collection:

3.1.1 Sample collection and culturing:

The study included 350 *Pseudomonas aeruginosa* strains obtained from three different hospitals in Islamabad and Rawalpindi from 2016 to 2017. The random sampling was done from pus, sputum, tissue, tracheal secretions, urine, soft tissue and blood sources. After culturing, sub culturing and stocking in glycerol, the samples were sent to United States for further species identity and isolates purity confirmation(Richter *et al.*, 2013). 77 isolates were selected from the bulk of data for further analysis.

3.1.2 Antibiotic susceptibility testing:

All *P. aeruginosa* strains resistant profile was established against 15 antibiotics among which the following fluoroquinolones were used: ciprofloxacin (10 µg), levofloxacin (5 µg). Antimicrobial susceptibility testing was performed using Kirby-Bauer Disk Diffusion method and interpreted according to criteria from the CLSI guidelines M100-S30 (Wayne, 2011). The *P. aeruginosa* ATCC 27853 strain was used as a quality control strain.

3.1.3 Genomic DNA isolation and quantification:

A suspension of ~10 colonies from overnight incubated blood agar plates were used for genomic DNA isolation using the QIAamp BiOstic Bacteremia DNA Isolation Kit (Qiagen, Hilden, Germany). 1 ml of nuclease free (NF) water was added into eppendorf tubes, and few freshly cultured colonies were suspended in NF water. The supernatant was discarded, and palette was kept intact. After centrifugation a warm MBL solution was added to tubes. The lysate was transferred into 2 mL PowerBead tube Garnet 100 and closed. After heating the PowerBead tubes were centrifuged at 10,000 x g for 1 min. to pellet the debris and supernatant was transferred to a new 2 mL collection tube without disturbing pellet. Then, 100 µL of solution IRS was added and vortexed to mix. After vortexing the PowerBead tubes were re-centrifuged for 1 min. at 19,000 x g to pellet the debris (containing lipids and proteins) and supernatant (containing DNA) was transferred to a new 2 mL collection tubes. The 650 µL of vortexed mix was transferred onto MB spin column and recentrifuged. The supernatant was discarded as the surface of the filter entrapped all the extracted DNA. The remaining 600-650 µL supernatant was again added onto MB spin column and centrifuged again and again. The dried MB spin column were transferred to a new collection tube. During elution 50 µL of

solution EB was added directly in the center of the MB spin column membrane. The collection tubes with inserted MB spin columns were centrifuged at 10,000 x g for 1 min. MB spin columns were discarded and 2 mL collection tubes containing genomic DNA were caped.

3.1.4 Illumina Whole Genome Sequencing:

The sequencing libraries were prepared with the Nextera kit (Illumina, San Diego, CA, USA) using a modified protocol (Baym *et al.*, 2015). The reads adapters were removed with Trimmomatic v0.38(Bolger *et al.*, 2014b) and human reads contamination was removed using Deconseq v0.4.3 (Schmieder and Edwards, 2011). Processed reads were *de-novo* assembled into draft genomes with Unicycler v0.4.7 (Wick *et al.*, 2017b) using default settings and the assembly. fasta file was used for all downstream analysis. Assembly quality was verified using QUAST v4.5(Bolger *et al.*, 2014a; Chuang *et al.*, 2012).

The extracted DNA was quantified using Quant-iT PicoGreen dsDNA assay (Thermo Fisher Scientific, Waltham, MA, USA). After quantification the quality of the extracted DNA samples was checked by gel electrophoreses using 1% agarose gel. 1 gram of agarose gel was added into 100 mL of 1 x TAE buffer. 10 µL of Ethidium Bromide was added in to 100 mL gel solution and poured into a gel tray with the well comb in place. The gel was placed at 4 °C for 10-15 min. or until completely solidified.

3.1.5 Reference Genomes:

To strengthen the rooting and overall structure of the phylogenetic tree, eight reference genomes were selected from the collection described by Subedi *et al.*(Subedi *et al.*, 2018b). Four genomes were selected from each of the two major phylogroups described in that study, with the goal of representing a global pangenome. To ensure only high-quality reference genomes were used, we selected assemblies marked as “complete” or “gapless chromosome” in the Pseudomonas Genome Database (Winsor *et al.*, 2016b).

3.2 Genes selection for fluoroquinolones resistance:

Twelve genes were selected from core genome for fluoroquinolones resistance based on the literature review. The main factor to select genes was the resistance mechanism by which the mutations occurred in genes. Four genes (*gyrA, gyrB, parC and parE*) had the resistance mechanism of target site modifications in the quinolone resistance determining regions (QRDRs). Eight other genes were selected (*mexR, mexT, mexE, nfxB, mvaT nalC, nalD, amgS*)

which are regulatory genes of efflux pumps, involved in the upregulation of efflux pumps **(Table 3.1).**

Table 3.1: Genes selected from core genome for fluoroquinolones resistance in *P. aeruginosa* according to literature review.

Gene	Function	Resistance mechanism
gyrA	DNA gyrase subunit A	Target site mutations
gyrB	DNA gyrase subunit B	Target site mutations
parC	DNA topoisomerase IV subunit A	Target site mutations
parE	DNA topoisomerase IV subunit B	Target site mutations
mexR	mexAB-OprM efflux pump operon repressor	Efflux pumps overexpression
nalC	mexAB-OprM efflux pump operon repressor	Efflux pumps overexpression
nalD	mexAB-OprM efflux pump operon repressor	Efflux pumps overexpression
nfxB	mexCD-OprJ efflux pump operon repressor	Efflux pumps overexpression
mexE	mexEF-OprN efflux pump operon repressor	Efflux pumps overexpression
mexT	mexEF-OprN efflux pump operon repressor	Efflux pumps overexpression
mvaT	mexEF-OprN efflux pump operon repressor	Efflux pumps overexpression
amgS	mexXY-OprM efflux pump operon repressor	Efflux pumps overexpression

3.3 Variant calling:

Several types of non-synonymous variations were called using Snippy 4.6.0 (Jaton *et al.*, 2016). The reference genome used was PAO1. Complete manual inspection of QRDR and efflux pump regulators was performed. Only high quality non- synonymous SNPs were used for analysis and further interpretation. Snippy is a tool for core genome alignment and performs rapid haploid variant calling. This tool helps to find SNPs (substitutions, insertions and deletions) in your sequence reads after comparison with the reference genome. The reference strain should be in FASTA or GENBANK format, the reads in FASTQ or FASTA format and the reads should be paired end. Snippy uses one tool for aligning reads to the reference genome and then uses another tool to call if the discrepancies are real variants.

3.4 Acquired genes identification for fluoroquinolone resistance:

The acquired antibiotic genes for fluoroquinolone resistance were identified in the genomes by AMRFinderPlus v3.9.8 (Feldgarden *et al.*, 2021). AMRFindePlus is a tool developed by NCBI for detecting antimicrobial resistance genes, point mutations related to resistance and also detect other gene classes on the basis of assembled genomes or annotated

sequences. This tool is also used in Pathogen Detection Pipeline and later on the data is displayed on NCBI's Isolate Browser.

3.5 Phylogenomic analysis:

Protein-coding sequences were predicted previously with Prokka v1.14.5(Seemann, 2014). Coding sequences were clustered at 95% identity with Roary v3.12.0(Page *et al.*, 2015b), and the 4,509 core genes were used to create a core genome alignment with PRANK v1.0(Page *et al.*, 2015b). The core genome alignment file (ALN) was used as an input file for FastTree v2.1.10 and converted to an approximate maximum-likelihood tree (Price *et al.*, 2010) and viewed and annotated on iToL(Letunic and Bork, 2021).

3.6 Phenotypic and genotypic data correlation:

The phenotypic data of antimicrobial susceptibility testing of fluoroquinolones (ciprofloxacin, levofloxacin and delafloxacin), phylogenomic analysis and non- synonymous SNPs were viewed and annotated using iTol (Letunic and Bork, 2021). iTol stands for interactive tree of life and it is a tool for visualising, annotating and managing phylogenetic trees. The phylogenetic tree was uploaded in the newick. format. Phylogenetic groups were identified and AST data of both fluoroquinolones i.e Ciprofloxacin and Levofloxacin, were annotated first and then SNPs identified in the selected nine genes with sequence variations were also annotated.

3.7 Comparison of genotypic resistance mechanisms with phenotypic resistance profile:

The presence or absence of three fluoroquinolones resistance mechanisms i.e mutations in QRDRs and efflux pumps regulatory genes along with *crpP* presence, were compared with the Zone of Inhibition (ZOI) of both the fluoroquinolones. The comparison was done to check that either presence or absence of the resistance mechanisms has any role on increasing or decreasing the level of ZOI of both fluoroquinolones.

3.8 Relatedness of collection and transmission clusters in *Pseudomonas aeruginosa* isolates (Hospital collection data):

The hospital, ward and source of 77 isolates included in study were thoroughly studied to examine the transmission clusters. The was further correlated with phylogenetic analysis and

AST profile of isolates to better understand the transmission clusters and their role in antibiotic resistance.

RESULTS

4.1 Fluoroquinolones AST of 77 *Pseudomonas aeruginosa* isolates:

142 *Pseudomonas aeruginosa* were collected previously from different sources such as respiratory, blood, soft tissue, abdominal sources. The samples were collected from three main hospital settings in Islamabad and Rawalpindi, Pakistan: Pakistan institute of Medical Sciences (PIMS), Railway General Hospital (RGH) and Military Hospital (MH). Out of 142, 77 isolates were selected for this study. Most of the patients were from Medical Intensive Care Units (29/77, 37%), out-patient clinics (17/77, 22%), emergency rooms (15/77, 19%), medical wards (15/77, 19%). The antibiotic susceptibility testing for fluoroquinolones was previously performed for two antibiotics, Ciprofloxacin and Levofloxacin by Kirby-Bauer Disk Diffusion method according to Clinical Laboratory and Standards Institute (CLSI) guidelines. According to the Zone of Inhibition (ZOI) data, ciprofloxacin resistance was observed in 55/77 (71.4%) isolates, 19/77 (24.6%) isolates were susceptible and 2/77(2.5%) were intermediate. The levofloxacin resistance was observed in 59/77 (76.6%) isolates, 17/77 (22%) isolates were susceptible and 2/77 (2.5%) isolates were intermediate. The resistant isolates has the ZOI of 6mm to 13mm, the intermediate isolates had ZOI of ≥ 15mm and susceptible isolates with ZOI of ≥ 21mm

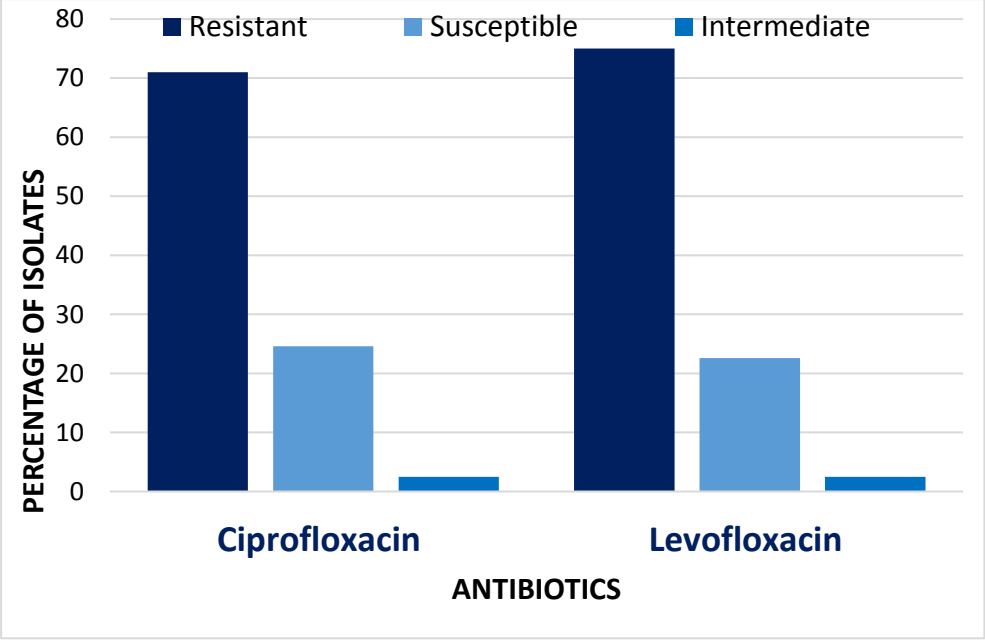

Figure 4.1: Antibiotic susceptibility testing for fluoroquinolones in 77 *P. aeruginosa* clinical isolates

4.2 Variant calling in the selected genes for fluoroquinolone resistance:

To identify the determinants responsible for fluoroquinolones resistance, twelve genes involved in resistance mechanisms from core genome of *Pseudomonas aeruginosa* were selected from literature review. Four genes selected from Quinolone Resistance Determining Regions (QRDR) were *gyrA, gyrB, parC, parE.* Eight genes from the regulatory genes of efflux pumps causing the upregulation of efflux pumps were *mexR, mexT, mexE, mvaT, nfxB, nalC, nalD, amgS.* Snippy v4.6.0 was used for identifying Single Nucleotide Polymorphism (SNPs), indels and stop gained variants in these selected genes. Using PAO1 as a reference genome, four types of variants were detected in the sample genomes: Missense variants, Frameshift variants, Conservative In-frame Insertion (CII), Conservative In-frame Deletion variants (CID) and Stop Gained variants.

In QRDR, a typical missense mutation T83I was detected in *gyrA* gene in 50/77 isolates which has often been detected in fluoroquinolone resistant *P. aeruginosa* (Kao *et al*., 2016). Interestingly, a conservative in-frame deletion S912-E913del, was also found in 10/77 isolates which has not been previously reported for fluoroquinolones resistance. Further, amino acid substitutions E468D was detected in *gyrB* gene. Mutations were also detected in DNA topoisomerase IV subunits *(parC* and *parE).* Missense mutation S87L in 44/77 isolates in *parC* gene has been a well-known target for mutation in fluoroquinolones (Khaledi *et al*., 2016). Also, D533E mutation in *parE* was present in 44/77 isolates and possibly related with fluoroquinolone resistance. To our knowledge, P752T and H262Q in *parC* and E215Q in *parE* have not been reported previously **(Table 4.1)**. Further analysis is needed whether these sequence variations are directly related with fluoroquinolone resistance.

The RND efflux pumps known to efflux fluoroquinolones are MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM (Goli *et al*., 2016). The alteration observed in regulators of MexAB-OprM pump were in the *mexR* gene, V126E in 57/77 isolates. Another regulatory gene *nalC* displayed a missense mutation G71E in all isolates and S209R in 79% (61/77) isolates. Other missense variants Q182K, E153Q and A145V were also observed in *nalC* gene. *nalD* gene mutations also contribute in overexpressing MexAB-OprM efflux pump (Haenni *et al*., 2017). It displayed a frameshift variant V133fs, conservative in-frame deletion variant D80-D83del, stop gained variant Q64*and missense variant S8C and S32N and L153Q.

Missense variant H109Y and frameshift variant F144fs were observed in the MexCD-OprJ regulator gene *nfxB.* The positive regulator of MexEF-OprN pump, *mexT* gene displayed missense mutation F172I and a deletion of 8 nucleotides causing frameshift mutation Q80fs. Missense variants in *mexE* gene detected were E2V, S8F, E368R, A79G and A231T. Only one missense mutation in one isolate was observed in MexEF-OprN regulator *mvaT* gene **(Table 4.1).** Only one missense mutation I260V in *amgS* gene related to MexXY-OprM efflux pump was shown in the isolates. The contribution of MexXY-OprM pump in fluoroquinolones resistance is rather less (Lau et al., 2015).

Table 4.1: List of variants in the selected genes for fluoroquinolones resistance

Gene	Type of Modifications	No. of isolates	Modifications
QRDRs			
gyrA	Missense	50	T83I
	Conservative in-frame deletion	10	S912-E913del
	Conservative in-frame insertion	1	E913-Pro914insSE
gyrB	Missense	2	H148N
	Missense	2	E468D
parC	Missense	44	S87L
	Missense	12	H262Q
parE	Missense	11	E215Q
	Missense	44	D533E
MexAB-OprM regulators			
mexR	Missense	57	V126E
nalC	Missense	77	G71E
	Missense	61	S209R
nalD	Frameshift	12	V133fs
	Conservative in-frame deletion	1	D80-D83del
	Stop gained	1	Q64*
	Missense	13	S8C
MexCD-OprJ regulators			
nfxB	Missense	6	H109Y
	Frameshift	1	F144fs
MexEF-OprN regulators			
mexT	Missense	75	F172I

	Frameshift	77	Q80fs
	Missense	36	P60S
mexE	Missense	18	E2V
	Missense	36	S8F
	Missense	18	E368R
	Missense	11	A79G
	Missense	11	A231T
mvaT	Missense	1	A71V
MexXY-OprM regulator			
amgS	Missense	31	I260V

4.3 Acquired fluoroquinolones resistance gene *crpP* was identified in isolates:

All of 77 genomes were analyzed for the presence of acquired antibiotic resistance genes for fluoroquinolones resistance by AMRFinderPlus tool by NCBI. A recent study identified the association of presence of *crpP* gene in elevated ciprofloxacin resistance (Chávez-Jacobo *et al*., 2018). Although *crpP* was identified on plasmid but its presence in our strains as an acquired gene represented its chromosomal origin. Moreover, there is no other study supporting the evidence of *crpP* presence on plasmid pUm505. *crpP* gene was identified in 68% (53/77) of our isolates after comparison of the sample genomes with PAO1 **(Figure 4.2)**. The presence of *crpP* in large percentage of isolates show it plays a role in inducing genotypic resistance to *P. aeruginosa* clinical isolates.

Isolate	*crpP*	Isolate	*crpP*	Isolate	*crpP*	Isolate	*crpP*
PA_1	■	PA_23	■	PA-103	■	PA_127	■
PA_3		PA_24	■	PA_104	■	PA_128	
PA_5		PA_25	■	PA_105		PA_129	■
PA_6	■	PA_26		PA_106	■	PA_130	■
PA_7	■	PA_27	■	PA_107		PA_131	
PA_8	■	PA_28	■	PA_108		PA_133	■
PA_9	■	PA_29	■	PA_110	■	PA_134	
PA_10		PA_30	■	PA_111	■	PA_135	■
PA_11		PA_31	■	PA_112	■	PA_136	
PA_12		PA_32		PA_113	■	PA_137	
PA_13		PA_33	■	PA_114		PA_139	■
PA_14	■	PA_34	■	PA_115	■	PA_140	■
PA_15	■	PA_35	■	PA_116		PA_141	
PA_16	■	PA_36	■	PA_119	■	PA_142	■
PA_17	■	PA_37	■	PA_120	■	PA_144	■
PA_18	■	PA_38	■	PA_121		PA_145	■
PA_19	■	PA_39	■	PA_122	■	PA_146	■
PA_20	■	PA_101	■	PA_124		PA_147	
PA_21		PA_102		PA_125	■	PA_148	■
						PA_149	■

Figure 4.2 Presence and absence of *crpP* acquired fluoroquinolone resistance gene in 77 *Pseudomonas aeruginosa* isolates after comparison with PAO1.

4.4 Phylogenomic analysis of *Pseudomonas aeruginosa* isolates:

To study the phylogenetic diversity of 77 isolates, the reads which were previously whole-genome short-read sequenced (Illumina) and assembled, were used to construct the core genome phylogenetic tree using Roary which used PRANK as an alignment tool which aligned core genes shared by most of the genomes used in the study and eight reference genomes (Page *et al.*, 2015b). The alignment file (ALN) generated was used as an input for FastTree to construct a core phylogenetic tree (Price *et al.*, 2010). 8 high quality reference genomes were included to ensure proper rooting of tree, including commonly studied genomes in *Pseudomonas aeruginosa* phylogeny, PAO1, PA-14 and LESB58 (Kiewatz et al., 2000, Lee *et al*., 2006) **(Appendix Table 1).** The isolates were divided in to two phylogenetic subgroups A and B. PAO1, DK2 and LESB58 were present in subgroup A and PA14 and VRPAO4 in subgroup B **(Figure 4.1)**. 45% (35/77) isolates were included in the group A, 48% (37/77) in group B and a minor group C with 6.5% (5/77) isolates, as mentioned in the previous phylogenetic analysis studies of *P. aeruginosa* (Ozer *et al.*, 2019).

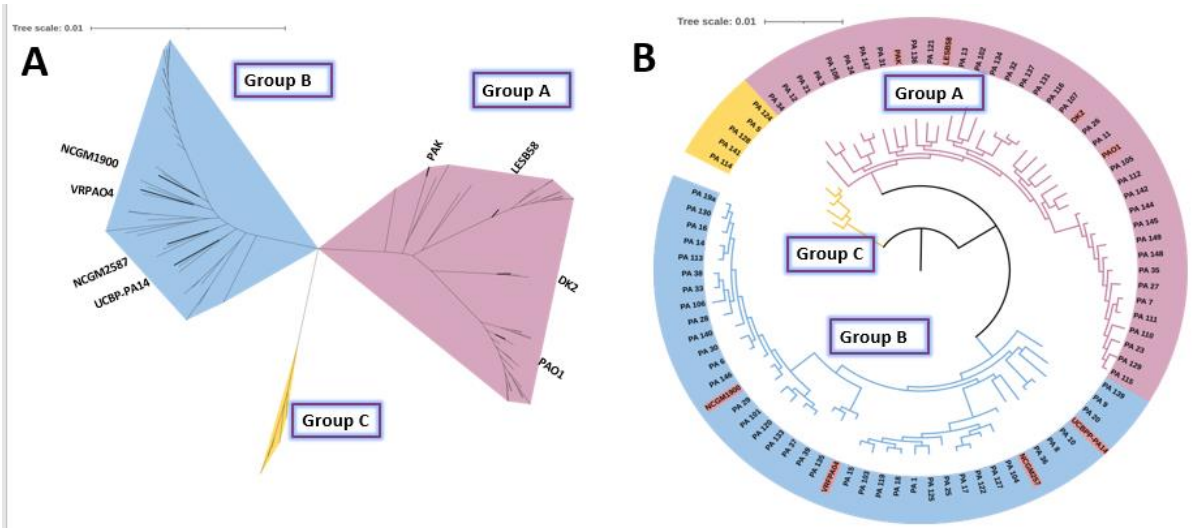

Figure 4.3: Phylogenetic analysis of *Pseudomonas aeruginosa* isolates displayed subdivision in three major subgroups. A) Unrooted maximum likelihood phylogenetic tree based on core-genome alignment 77 *Pseudomonas aeruginosa* genomes from this study and 8 reference genomes. Tree scale is substitution per site. Colored branches show group A in purple, group B in blue and group C in yellow. B) Circular representation of same tree in figure 4.1 (A). Reference genomes are highlighted and branch colors represent subgroups based on phylogeny. Tree scale is substitution per site.

4.5 Phenotypic and genotypic data correlation for fluoroquinolone resistance:

Fluoroquinolones resistance in *Pseudomonas aeruginosa* can be both due to acquired resistance gene *crpP* and mutations in QRDRs (*gyrA, gyrB, parC, parE*) and efflux pumps regulatory genes (*mexR, mexT, mexE, nfxB, mvaT, nalC, nalD, amgS*) (Khan et al., 2020). In order to explore fluoroquinolones resistance mechanisms in *Pseudomonas aeruginosa* the AST for both fluoroquinolones ie Ciprofloxacin and Levofloxacin was compared with the variants identified in the genomes included in this study. Furthermore, the presence of acquired gene *crpP* was also compared with AST profile of the isolates **(Figure 4.4).** In phylogenetic group A, it is clear that most of the resistant isolates display missense mutations in *gyrA, parC, parE, mexR, mexT, nalC and nalD* along with the presence of *crpP* acquired gene. The isolates which possessed intermediate AST to fluoroquinolones only displayed missense mutations in *gyrA, mexT* and *nalC* gene and *crpP* gene was absent. Susceptible isolates in group A displayed missense mutations in only *mexT* and *nalC* gene along with the absence of *crpP* gene. Group B resistant isolates mostly possessed missense mutations in *gyrA, parC, parE, mexR, mexT,*

mexE, *nalC* gene and also showed presence of *crpP* gene. Interestingly, 11/77 resistant group B isolates also displayed frameshift mutation in *nalD* gene and a missense mutation in *amgS* gene which is not observed in any other isolates or groups. Susceptible isolates showed missense mutations in *gyrA*, *parE*, *mexT* and *nalC*. Unlike group A isolates, group B susceptible isolates harbored *crpP* gene. Small group C isolates either resistant, susceptible or intermediate, all 5 isolates conferred mutations in *gyrA*, *parE*, *mexR*, *nfxB*, *mexT*, *nalC*, *amgS*. This analysis suggests that resistant isolates harbor mutations in both QRDRs and efflux pumps regulatory genes and possess acquired fluoroquinolone resistance gene *crpP*.

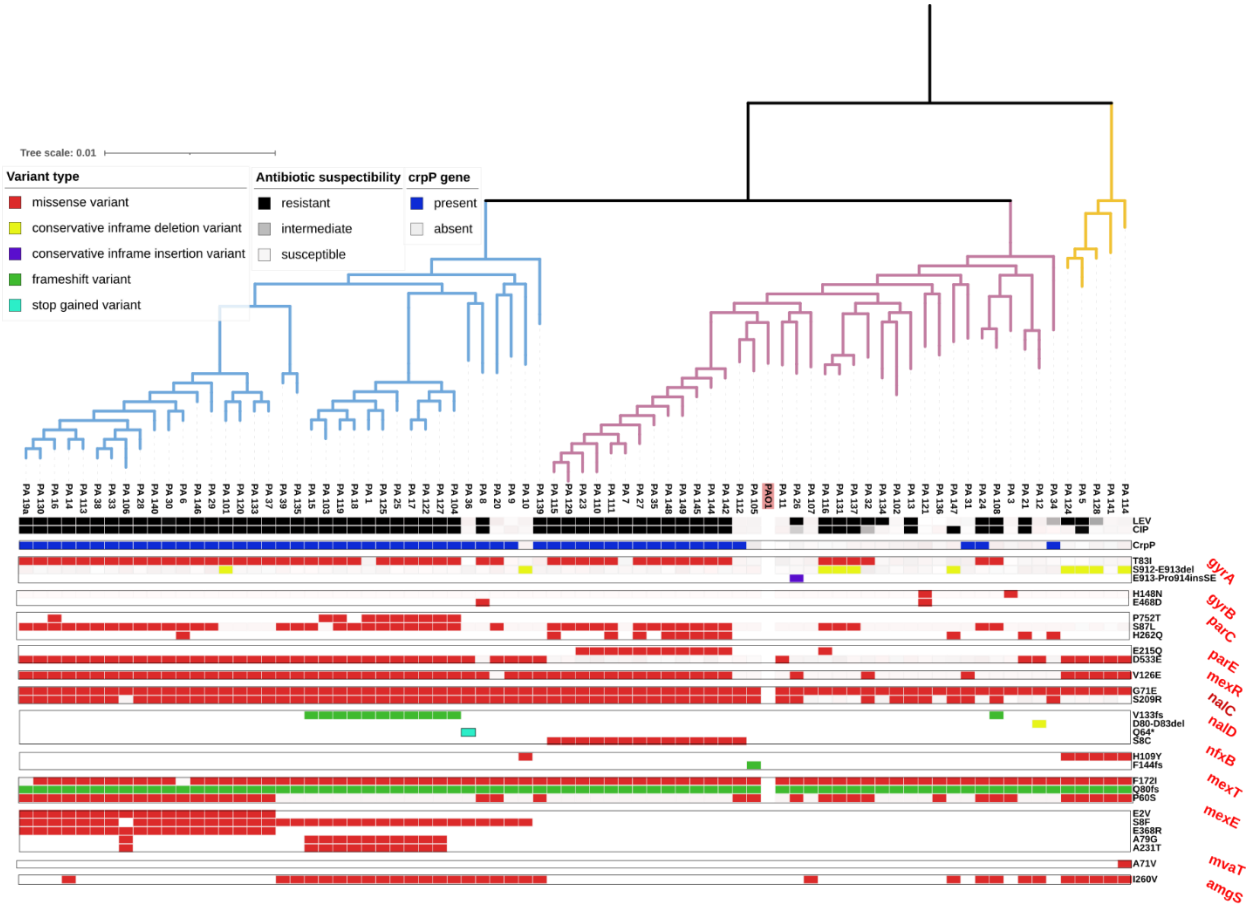

Figure 4.4 Phenotypic resistance and genotypic resistance determinants correlation off 77 *Pseudomonas aeruginosa* isolates. Rectangular phylogenetic tree representation of the same tree displayed in Figure 4.1. Tree scale is substitution per site. The AST data for both fluoroquinolones ie CIP = ciprofloxacin and LEV = levofloxacin, presence or absence of *crpP* acquired fluoroquinolone resistance gene and determinants in 12 selected genes data annotated by iTOL. Reference genomes are highlighted.

4.6 Effect of genotypic fluoroquinolone resistance on level of phenotypic resistance:

There were three fluoroquinolone resistance mechanisms identified in our isolates; mutations in QRDRs, efflux pumps regulatory genes and the presence of acquired gene *crpP*. The correlation of these resistance mechanisms on the phenotypic resistance profile was done to identify the effect of these resistance mechanisms on the level of phenotypic resistance of ciprofloxacin and levofloxacin. For ciprofloxacin, the isolates displaying presence of all three resistance mechanisms had a smaller zone of clearance of 6mm , while isolates with missing QRDR mutation or a missing *crpP* acquired gene had larger zone of clearance of 18mm to 35mm **(Figure 4.6)**. Similarly, for levofloxacin, the isolates possessing all three resistance mechanisms displayed a relatively smaller zone of clearance of 6mm, while the isolates with absence QRDR mutation or *crpP* gene displayed a relatively larger zones of clearance from 16mm to 32mm. This means that presence of three different resistance mechanisms collectively contribute to fluoroquinolones resistance where each mechanism is showing an additive effect on resistance of these drugs.

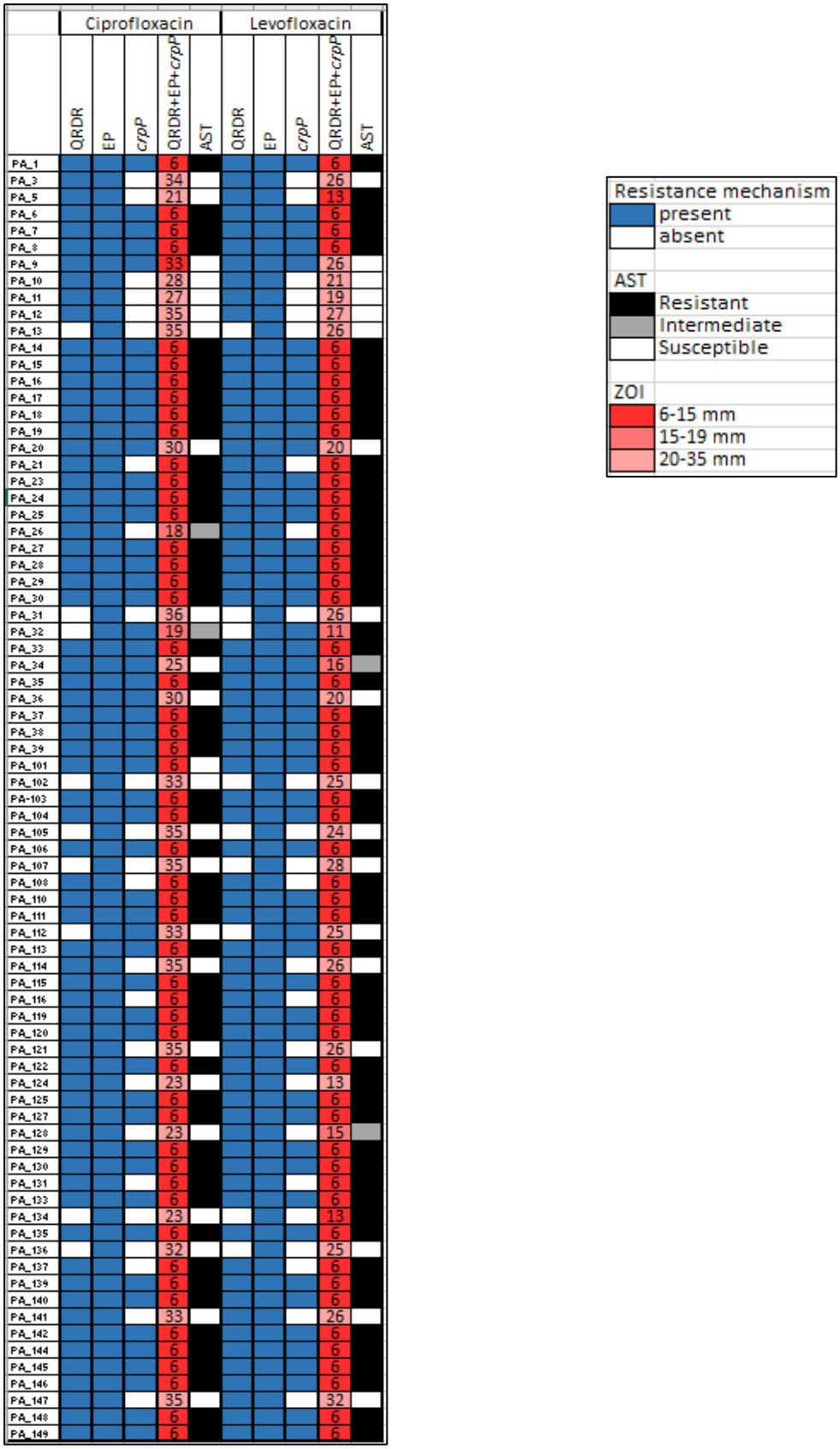

Figure 4.5 Effect of genotypic resistance mechanisms on level of phenotypic resistance (ZOI) of *P. aeruginosa* isolates. The presence or absence of each mechanism is displayed in the figure

along with their effect on the level of zone of clearance for each antibiotic ciprofloxacin and levofloxacin. The level of zone of clearance for each antibiotic is also displayed in different colors to show distinct variation. The Antibiotic Susceptibility Testing profile has also been displayed to clearly show the fluoroquinolones resistance profile of isolates.

4.7 Relatedness of collection and transmission clusters of isolates (hospital collection data):

Each isolate source, hospital and ward was thoroughly examined and annotated with AST profile to check the relatedness in collection and transmission clusters **(Figure 4.2).** There was no significant transmission clusters observed for example in group B isolates where the similar isolates PA_24, PA_147 and PA_108 were collected from different hospital and different wards. These isolates are also phylogenetically relevant but their different sources and sites of collection show that isolates are independent of the source of collection and there are no significant transmission clusters observed. Another example is the similar kind of fluoroquinolone resistant group B isolates PA_142, PA_144, PA_145 and PA_149 but again their source of isolation is different. This indicates that the population structure of our P. aeruginosa isolates are independent of sample source and sample type.

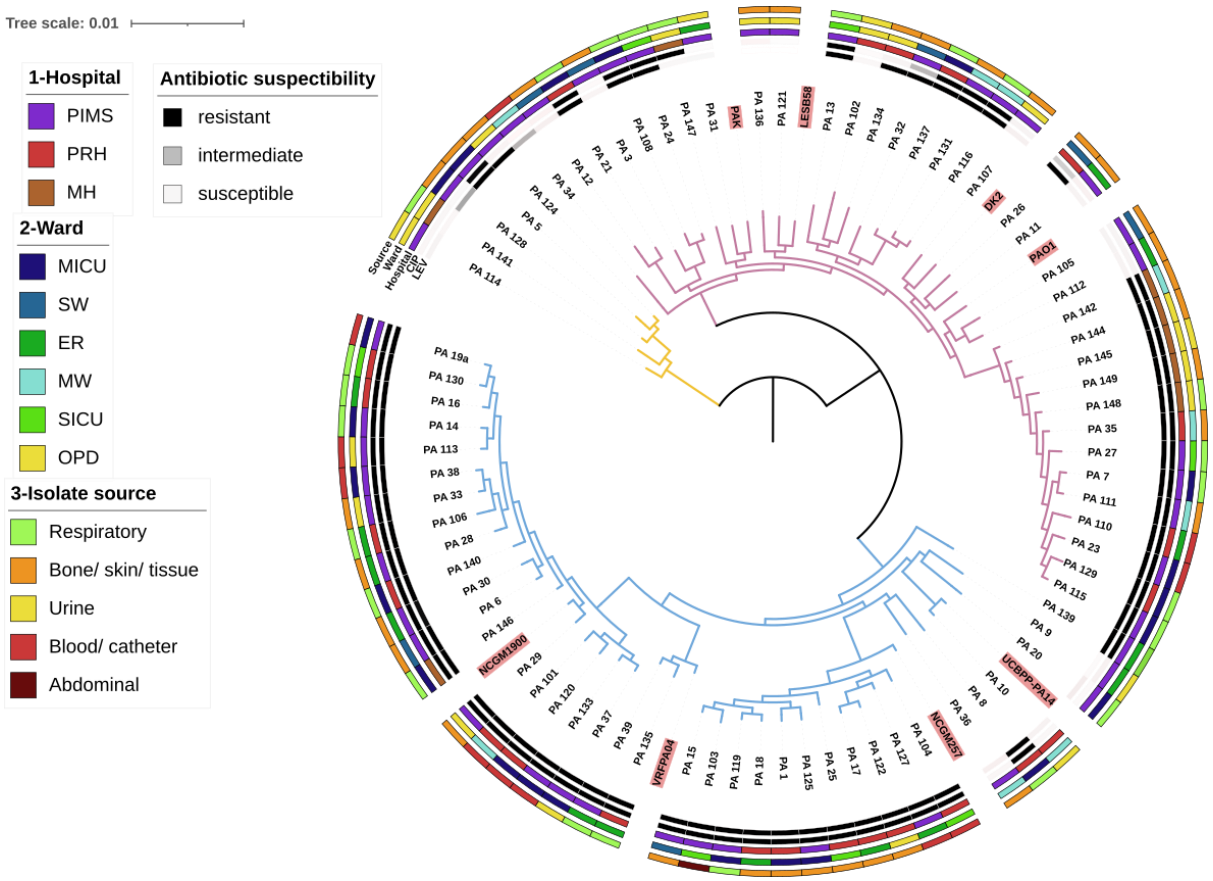

Figure 4.6 Phylogenetic tree annotated with fluoroquinolones AST, hospital, ward and source of isolates for relatedness. Circular representation on phylogenetic tree and branch colors are same as mentioned in figure 4.1.Tree is annotated using iTOL with the hospital, ward and source of each isolate along with fluoroquinolones (Ciprofloxacin and Levofloxacin) AST. CIP = ciprofloxacin, LEV = levofloxacin.

4.8 Summary of Correlation between Genotypic and Phenotypic Resistance:

The overall summary of the correlation between genotypic fluoroquinolone resistance mechanisms and phenotypic resistance profile is given in **Table 4.2.** The resistant isolates in phylogenetic subgroups were as follows; group A: 33/77, group B: 26/77,and group C: 1/77, which displayed mutations in *gyrA*, *parC*, *parE*, *mexR*, *mexT*, *mexE*, *nalC*, *nalD* and *amgS* genes. The identified variants which were also previously reported were T83I, S87L, D533E, V126E, F172I, Q80fs, G71E, S209R from respiratory, urine, bone/ skin/ tissue and blood resources and similar these variants were reported similarly from these resources in our isolates. The intermediate isolates were in group B: 3/77 and group C: 1/77, which displayed

mutations in *gyrA, nalC and mexT* genes displaying previously reported variants, T83I, G71E, S209R, F172I and Q80fs in isolates from bone/ skin/ tissue and respiratory sources also previously reported in these samples. The susceptible isolates in phylogenetic subgroups were as follows; group A: 4/77, group B: 9/77 and group C: 2/77, which displayed mutations in *parC, mexR, nalC, mexT, mexE* and *amgS* genes. The previously reported variants were D533Q, V126E, G71E, S209R, F172I and Q80fs from respiratory, bone/ skin/ tissue resources. The column representing the new identified variants are the novel variants reported in this study which have not been previously reported in clinical *P. aeruginosa* clinical isolates. Among all the phylogroups, the phenotypically resistant isolates to fluoroquinolones displayed the presence of *crpP* acquired gene while intermediate and susceptible isolates did not possess this gene.

Table 4.2 Summary of genotypic resistance profile with phenotypic resistance of 77 *P. aeruginosa* isolates

Strain type	Sources	Subgroups	Mutated genes	Reported sequence variations	New sequence variations	*crpP* presence
Resistant	Respiratory, Urine, Bone/ Skin/ Tissue, Blood	GroupA:33/77 Group B:26/77 Group C:1/77	*gyrA*	T83I	S912-E913del	Present
			parC	S87L	H262Q, P752T	
			parE	D533E	E215Q	
			mexR	V126E		
			mexT	F172I, Q80fs	P60S	
			nalC	G71E, S209R		
			nalD		Q64*, S8C, V133fs	
			amgS		I260V	
Intermediate	Bone/ Skin/ Tissue, Respiratory	Group A: 0/77 Group B:3/77 Group C:1/77	*gyrA*	T83I	E913-Proins914insSE,	Absent
			nalC	G71E, S209R		
			mexT	F172I, Q80fs	P60S	
Susceptible	Respiratory, Bone/Skin/ Tissue	Group A: 4/77 Group B: 9/77 Group C: 2/77	*parC*	D533Q	H148N, E468D	Absent
			mexT	F172I, Q80fs	P60S	
			mexR	V126E		
			nalC	G71E, S209R		
			amgS		I260V	

DISCUSSION

The rates of infections caused by *Pseudomonas aeruginosa* has elevated to threatening levels that significantly increases morbidity and mortality (Poole, 2011). This increasing threat is due to the acquisition of several resistance mechanisms against antibiotics such as chromosomal mutations and acquiring antibiotic resistance genes (del Barrio-Tofiño et al., 2019). In recent years, Whole Genome Sequencing (WGS) of pathogenic bacteria for the analysis of epidemiology of hospital acquired infections has played its vital role in public health (Yu *et al*., 2019). Fluoroquinolones have been used as an empirical and primary therapy for treating infections caused by *Pseudomonas aeruginosa* for decades (Neuhauser *et al*., 2003, Linder *et al*., 2005). Phenotypic fluoroquinolone resistance identification in *P. aeruginosa* pose limitations and hence use of modern methods such as WGS to explore the genotypic basis of fluoroquinolone resistance is required. Furthermore, the comparison of phenotypic fluoroquinolones resistance profile and resistome provides insights in understanding resistance mechanisms (Kos *et al.*, 2015).

The primary factors for fluoroquinolones resistance in *P. aeruginosa* are target site chromosomal mutations in *gyrA, gyrB, parC and parE* genes in QRDR and efflux pumps upregulation by mutations in their regulatory genes such *as mexR, nfxB, mexT* and *mexE* (Higgins *et al*., 2003, Li *et al*., 2015). The horizontally acquired genes such as crpP also contribute to the elevated fluoroquinolones resistance levels (Chávez-Jacobo *et al*., 2018). In this study, we conducted a genomic characterization of identified determinants in the genes for fluoroquinolones resistance in Whole Genome sequenced genomes and compared it with the phenotypic antibiotic susceptibility profile of 77 clinical isolates. The importance of determinants in resistance genes has been previously reported to have a prime role in fluoroquinolones resistance determination (Rehman *et al*., 2019, Zhao *et al*., 2020).

High resistance (75%) to both of fluoroquinolones ie ciprofloxacin and levofloxacin was observed in 77 selected *Pseudomonas aeruginosa* isolates. The phylogeny of fluoroquinolone resistant *P.aeruginosa* isolates was executed by core genome analysis and we observed two distinct phylogroups and a small phylogroup as mentioned in previous studies (Rau *et al*., 2012 , Kos *et al.*, 2015, Subedi *et al.*, 2018b). The SNPs analysis of isolates identified several distinct variants in fluoroquinolone resistance genes as according to literature review. The most prominent missense variants in QRDRs were T83I in *gyrA* gene and S87L in the *parC* gene which are consistently being reported for fluoroquinolone resistance (Díaz-Ríos

et al., 2021, Higgins *et al*., 2003). Although insignificant, but a small number of isolates also displayed missense variant E468D in *gyrB* gene which is found significantly in other studies (Lee et al., 2005, Bruchman *et al*., 2013). Other missense variants D533E in *parE* gene have also been linked previously with fluoroquinolones resistance (Varga *et al*., 2015).

The SNPs analysis of efflux pumps regulatory genes displayed interestingly elevated levels of resistance determinants resulting in fluoroquinolones resistance. For instance, missense variant V126E in *mexR* and G71E and S209R in *nalC* gene have been previously linked with over expression of MexAB-OprM efflux pump (Horna *et al*., 2018, Díaz-Ríos *et al*., 2021). Previous studies confirm that an active *mexT* variant for overexpression of MexEF-OprN efflux pump manifested the presence of missense mutation F172I and frameshift mutation Q80fs (Maseda *et al*., 2000).

The prevalence of *crpP* gene in our isolates was also observed in notable number of isolates and we believe it has contributed equally for acquiring fluoroquinolones resistance in our isolates agreeing with previous studies (Chávez-Jacobo *et al*., 2018). The comparison of phenotypic fluoroquinolone AST of isolates with the identified determinants and *crpP* gene presence/ absence presented an insightful image of the FQ resistance in these isolates. The resistant isolates in phylogroup A exhibited mutations in both QRDRs and efflux pump regulators with additional presence of acquired *crpP* gene suggesting that high resistance in fluoroquinolones has developed due to the presence of all three factors with ZOI=6 (Khan *et al.,* 2020). Overall *crpP* gene was absent in intermediate and susceptible isolates rather an irregular presence of *crpP* gene in PA_31 was observed which is susceptible to both fluoroquinolones according to AST.

As evident from the analysis, highly fluoroquinolone resistant isolates were clustered in phylogroup B displaying mutations in QRDRs and efflux pump genes along with exhibiting *crpP* gene. An irregular pattern of *crpP* presence was observed in the susceptible isolates PA_9, PA_20 and PA_36 which is unusual as *crpP* presence has been associated with fluoroquinolone resistance (Chávez-Jacobo *et al*., 2019). This suggests that these isolates might develop resistance to fluoroquinolones in future. Phylogroup C isolates presented an irregular behaviour where resistant isolates (PA_5 and PA_124) did not have *crpP* gene but possess variants as are present in group A resistant isolates. But interestingly susceptible isolates PA_114 and PA_141 also showed the QRDR and efflux pump regulator determinants. Also, intermediate isolate

PA_128 showed all the variants as were present in resistant isolates which suggests that these isolates might develop resistance **(Figure 4.4).**

When the sources of each isolate were compared with AST profile and genotypic resistance determinants, we found some concordance with previous studies. For example: in phylogroup A, isolate PA_110 and PA_23 was isolated from blood/catheter source and also displayed missense mutations T83I in *gyrA*, P60S and F72I in *mexT* and G71E in *nalC* gene and interestingly they are resistant to both fluoroquinolones. Also, group B isolates such as PA_19, PA_38, PA_101, PA_104, PA_120, PA_127, PA_133 showed similar results. Similar findings have been reported before (Cabot et al., 2016). The considerable number of isolates were from respiratory sources in group A and group B also displaying missense mutations in *gyrA, gyrB, parC* and *parE* along with *crpP* gene as mentioned in the previous study with isolates from broncho-alveolar sources (Madaha *et al.,* 2020, Varga *et al*., 2015).

The comparison of the resistance mechanism included in our study with the level of phenotypic resistance or Zone of Inhibition (ZOI) signifies that combination of fluoroquinolones resistance mechanisms are not only possible but prevalent in our area as they are present in the pathogenic clinical isolates from our region.

The limitation of this study is that we used short reads Illumina sequencing which could not suggest the presence of fluoroquinolone resistance genes on plasmids and associated mobile genetic elements (MGEs) as they are often detected by long reads sequences (Khan *et al*., 2020, Wick *et al*., 2017). Also, in this study we used disk diffusion method for AST so we could not obtain Minimum Inhibitory Concentrations (MICs) for fluoroquinolones.

CONCLUSION AND FUTURE PROSPECTS

In conclusion, we have demonstrated the utility of WGS for detecting and correlating the resistance mechanisms with phenotypic resistance in clinical *P. aeruginosa* isolates. We identified a unique relation by comparison of genotypic determinants for fluoroquinolones resistance with phenotypic resistance. The key findings of this study are the identification of genotypic determinants for fluoroquinolones resistance revealed presence of large number of mutations in the core genes (QRDRs and efflux pumps genes), along with the presence of acquired fluoroquinolone resistance gene *crpP*. The correlation between genotypic determinants and AST revealed that the large burden of resistance determinants was present in the highly resistant fluoroquinolones isolates in phylogenetic group A, B and C. The comparison of genotypic resistance profile with phenotypic profile showed that these resistance determinants may have combinatory effects to overall fluoroquinolone resistance in *P. aeruginosa.* This study drives to develop alternative therapeutic strategies against highly FQs resistant *Pseudomonas aeruginosa.* Moreover, surveillance and close monitoring methods are needed to be adopted at national level to control the outspread of fluoroquinolones resistance in *Pseudomonas aeruginosa*.

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