MULTIDRUG-RESISTANT PSEUDOMONAS AERUGINOSA – A PROBLEM IN HOT TUBS AND WHIRLPOOLS IN CANADA

by

Md Murad Mufty

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University of Alberta

Abstract

Objectives: *Pseudomonas aeruginosa* is one of the most problematic opportunistic pathogens. It is resistant to many currently used antibiotics, making it difficult to treat, and resistance may transfer to other *P. aeruginosa* strains. The organism can acquire resistance through horizontal gene transfer but is thought to be incompetent in natural transformation.

Free-living amoebae (FLA) are a critical group of the microbial protozoa community that influence biofilm associated bacteria (e.g., *P. aeruginosa*) in hot tubs and whirlpools. There appears to be no study relating to *P. aeruginosa*`s interaction with free-living amoebae and its general ecology from the water system in the hot tubs and whirlpools.

Therefore, this study aimed to investigate the antibiotic resistance profiles of *P. aeruginosa* from water in hot tubs and whirlpools and to demonstrate if the *P. aeruginosa* isolates are competent in the uptake of extracellular DNA leading to the transfer of antibiotic resistance, and whether FLA may promote early uptake of the extracellular DNA.

Methods: 45 *P. aeruginosa* isolates from water in hot tubs and whirlpools were assayed, against 36 antibiotic agents, including penicillins, cell wall inhibitors containing extended-spectrum beta-lactamases (ESBLs), protein synthesis inhibitors, carbapenems, fluoroquinolones, cephalosporins, folic acid synthesis inhibitors, nitrofurans, monobactams and aminoglycosides. The resistance was phenotypically assessed using Kirby-Bauer's disk diffusion method, and the results were interpreted according to the Clinical & Laboratory

Standards Institute (CLSI) guidelines. The prevalence and frequency of antimicrobial resistance genes were investigated by PCR.

The study also examined transformation of naked plasmid extracellular DNA carrying antibiotic resistance genes into recipient *P. aeruginosa* isolates from water in hot tubs and whirlpools. In addition, using fluorescent microscopy, the experiment examined in-situ interactions of *P. aeruginosa* with two FLAs - *Acanthamoebae polyphaga* and *Willaertia magna* on the transformation of the extracellular plasmid DNA to *P. aeruginosa* isolates.

Results: All the tested *P. aeruginosa* isolates were resistant to at least six antibiotics; and with all 45 strains possessing a common backbone of resistance to four antibiotics (ampicillin, cloxacillin, mecillinam and cephalothin). All strains were resistant to at least three classes of antibiotics, designating them as multidrug resistant strains. Some hot tub strains were resistant to as many as 10 antibiotics. Worryingly, five isolates were shown to possess carbapenem resistance (ertapenem and doripenem). Among the 36 antibiotics tested, 21 antibiotics are clinically relevant, and all the isolates were susceptible to those 21 antibiotics except for one carbapenem (doripenem), for which only one isolate was resistant to this drug.

All the tested *P. aeruginosa* isolates expressed genes present for porin (*oprL*, *oprD*), efflux pumps (*ampC*, *mexC1,2*, *mexC3,4*), QS (*lasI*, *lasR*), T3SS (*popB*), and T6SS (*tssC1*). The study found most of the *P. aeruginosa* isolates (89.13%) had effector protein gene *exoY*, and fewer had another two effector protein genes *exoS* (54.35%) and *exoU* (34.78%). The biofilm-

associated gene *ndvB* was identified in all *P. aeruginosa* isolates, but no isolate contained *NDM*, the β-lactam biofilm gene.

The research also demonstrated that *P. aeruginosa* were competent at taking up plasmid DNA containing antibiotic resistance genes from their environment, and stably incorporating this DNA into their cellular metabolism. Transformation was observed after 49 days of monoculture with plasmid DNA. In contrast, when FLA were present in the culture, transformation occurred in only nine to 14 days, albeit only a single experiment was performed, and therefore some uncertainty remains as to whether predatory amoeba may induce greater rates of transformation in *P. aeruginosa*.

Conclusions: This study's results indicate a high frequency of multi-class and multi-drug resistance in *P. aeruginosa* isolated from hot tubs in Alberta. The observation that carbapenem-resistant *P. aeruginosa* were found in hot tubs is of critical concern. Nevertheless, this study demonstrated that hot tub isolates were mostly susceptible to the currently used antibiotics recommended for treatment of *P. aeruginosa* infections. The study observed that the *P. aeruginosa* isolates from hot tubs and whirlpools could acquire extracellular plasmid DNA containing antibiotic resistance genes through transformation. The results also suggest that the presence of FLAs within the same niche as *P. aeruginosa* may potentially prompt extracellular plasmid DNA transformation in *P. aeruginosa*.

Keywords: *P. aeruginosa*, antibiotic resistance, hot tub, whirlpool, efflux pump, porin, amoebae, transformation, extracellular DNA.

Preface

Professor Nicholas J. Ashbolt and I designed the experimental procedures and research flow. Candis Scott was responsible for the collection of *Pseudomonas* isolates and performed VITEK™ strains identifications. Tania Nasreen helped with PCR analysis of antimicrobial-resistant *P. aeruginosa* isolates. The gene transfer experiment was successful with supports from Mohamed Shaheen. I was responsible for data collection, data analysis and writing. Professor Nicholas J. Ashbolt was the original primary supervisor but transitioned over to Dr. Norman Neumann upon resignation of Dr. Ashbolt from the University of Alberta.

This thesis is an original work by Md Murad Mufty. No part of this thesis has been previously published.

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List of Abbreviation

ABC	:	ATP-binding cassette	$NO^{\cdot}$	:	Nitrogen-based radicals
AMR	:	Antimicrobial resistance	TSA	:	Tryptic soy agar
ARG		Antimicrobial resistance gene	RNS		Reactive nitrogen species
ATP	:	Adenosine triphosphate	TAE	:	Tris-acetate-EDTA
CDC	:	Centers for disease control and prevention	ATCC	:	American Type Culture Collection
CLSI	:	Clinical & Laboratory Standards Institute	LB	:	Luria-Bertani
DNA	:	Deoxyribonucleic acid	PBS	:	Phosphate buffered saline
eDNA	:	Extracellular DNA	QS	:	quorum sensing
ESBLs		extended-spectrum beta-lactamases	MHA	:	Mueller-Hinton agar
FDA	:	Food & Drug Administration	CFU	:	Colony-forming unit
FLA	:	Free-living amoebae	SOS	:	Save our souls
GIM	:	Germany Imipenemase	PBPs	:	Penicillin-binding proteins
HGT	:	Horizontal Gene Transfer	Tet	:	Tetracycline
IMP	:	Imipenemase	$ONOO^{-}$	:	Peroxynitrite
MFS	:	major facilitator superfamily	PLIN	:	Perilipin
NADPH	:	Nicotinamide adenine dinucleotide phosphate	H_2O_2		Hydrogen peroxide
EDTA	:	Ethylenediaminetetraacetic acid	CF	:	Cystic fibrosis
NDM	:	New Delhi Metallo- β-lactamase	NRAMP	:	natural resistance-associated macrophage protein
PCR	:	Polymerase chain reaction	ROS	:	Reactive oxygen species
RNA		Ribonucleic acid	amp	:	Ampicillin
RND	:	resistance-nodulation-division	NaCl	:	Sodium Chloride
SPM	:	Sao Paulo metallo- β-lactamase	SCGYEM	:	Serum casein glucose yeast extract medium
ssDNA	:	single-stranded DNA	SI No	:	Serial Number
$\cdot OH$	:	Hydroxyl radical	T4P	:	Type IV pili
MATE	:	Multidrug and toxic compound extrusion	VIM	:	Verona integron-encoded metallo-β-lactamase
GFP	:	Green fluorescent protein	WHO	:	World Health Organization

1. Introduction

1.1 Biology of *Pseudomonas aeruginosa*

1.1.1 Pseudomonas

The genus *Pseudomonas* belongs to kingdom Bacteria, phylum Proteobacteria, class Gammaproteobacteria, order Pseudomonadales, and family Pseudomonadaceae. The genus includes over 216 species with 18 subspecies (Novik et al., 2015). Members of the genus *Pseudomonas* show significant metabolic and physiologic adaptability, allowing colonization in diverse terrestrial and aquatic habitats (Palleroni, 1992). Most of the pseudomonads are pathogenic to plants, although several strains are pathogenic to animals (Özen and Ussery, 2012). Twenty-five identified *Pseudomonas* species had been isolated from humans, including *P. aeruginosa*, *P. fluorescens*, *P. putida*, *P. cepacia P. stutzeri*, *P. maltophilia* and *P. putrefaciens*. *P. aeruginosa* and *P. maltophilia* are collectively cause approximately 80 percent of human pseudomonad infections (Iglewski, 1996).

Pseudomonads in environmental and pathogenic conditions are often attached to surfaces through biofilms (Mann and Wozniak, 2012) providing cell attachment to the substratum, i.e., the sediment-water interface on and in which organisms live (Walker, 1979) and that enhances stability under unfavorable environments (Ghafoor et al., 2011), e.g., can develop resistance where antibiotics are used widely (Lambert, 2002). Biofilms also perform a defensive role for *Pseudomonas* species, supporting increased resistance to disinfectants and antimicrobials when compared to planktonic bacteria (Wingender & Flemming, 2011).

Pseudomonads can produce diffusible pigments that fluoresce in short-wavelength (254 nm) ultraviolet light. The yellow-green pigment pyoverdine binds iron (III), and forms ferripyoverdine which is actively transported to bacterial cells (Meyer & Abdallah, 1978). Pyoverdine from *P. aeruginosa* is essential for virulence in animal models (Takase et al., 2000). *Pseudomonas* spp. produce other pigments including pyocyanin (*P. aeruginosa*, blue color), pyorubin (*P. aeruginosa*, red color), chlororaphin (*P. chlororaphis*, green color) and pyomelanin (*P. aeruginosa*, black color) (Blondel-Hill et al., 2007). Each species of *Pseudomonas* can be characterized by a specific pyoverdine (Meyer et al., 2002).

Pseudomonas is naturally prevalent in a wide range of environmental niches, including soil, water, plants, and animals, and is able to adapt to various adverse conditions such as high and low temperature growth (Brown, 1957), in osmotic stress (Iglewski, 1996), at low oxygen levels (Alvarez-Ortega and Harwood, 2007), and in the presence of antibiotics (Novik et al., 2015).

1.1.2 Pseudomonas aeruginosa

1.1.2.1 Physiology and environmental niches of P. aeruginosa

P. aeruginosa is the most clinically significant member of the Pseudomonadaceae family (Koenig, 2012). The widespread abundance of *P. aeruginosa* in the environment makes these sources potentially a risk to public health (Rado et al., 2017). The World Health Organization designated *P. aeruginosa* as a critical priority pathogen of concern because of its increasing resistance to common and available antimicrobials (WHO, 2017).

P. aeruginosa is a Gram-negative, aerobic, non-sporulating, rod-shaped bacterium of 0.65µm by 2.25µm in average size and motile by a single polar flagellum (Bergey's Manual of Systematic Bacteriology, 2001; Wu et al., 2015). The optimum growth temperature of *P. aeruginosa* is 37°C, but the organism is able to grow between 4 – 42°C (Wu et al., 2015), and its growth capabilities at 42°C distinguish it from many other clinically significant *Pseudomonas* species (Baron, 2001; Kiska and Gilligan, 2003), such as *P. putida* and *P. fluorescens* which are unable to grow at 42°C (Public Health England, 2015). *P. aeruginosa* is a glucose non-fermenting bacterium and produces the characteristic blue-green pigment, pyocyanin (Pitt and Simpson, 2006; O'Malley et al., 2004; Rada and Leto, 2013). The organism can be identified biochemically as being indophenol oxidase-positive, citrate-positive, and L-arginine dihydrolase-positive activity (Planet, 2018).

P. aeruginosa is ubiquitous in the environment (Jorgensen et al., 1999; Wolfgang et al., 2003), can persist in diverse habitats (Chatterjee et al., 2017), and is usually found in soil and water ecosystems, including the sea, rivers, freshwater lakes, streams, and potable water sources (Ashbolt 2015a; Garvey et al., 2018). The organism has been isolated from water, air, soil,

animals, and plants (Kominos et al., 1972; Kucera and Lysenko, 1968; Mahajan-Miklos et al., 1999). *P. aeruginosa* can inhabit and grow on different matrices and surfaces, including those found in hospitals and other healthcare settings (de Abreu et al., 2014), as well as hot tubs (Zichichi et al., 2000; Dulabon et al., 2009).

P. aeruginosa is an opportunistic pathogen because it rarely causes diseases among healthy individuals. However, whenever the integrity of the physical barrier to infection is lost, or for which the host has an underlying immune deficiency (e.g., immunosuppression), this pathogen takes the opportunity to infect (Merakou et al., 2018; Albasanz-Puig et al., 2019; Horcajada et al., 2019). The organism is primarily at risk for patients with cystic fibrosis (CF), AIDS, cancer, internal medical devices, burn and eye injuries, and non-curative diabetic wounds (Diggle et al., 2020). *P. aeruginosa* can also colonize the lungs of individuals who have inhaled steam from contaminated hot tub water (Crnich et al., 2003). Hydrotherapy tanks in hospital physical therapy burn units and pools are also reservoirs of *P. aeruginosa* outbreaks (Stone and Kolb, 1971; Tredget et al., 1992; McGuckin et al., 1981; Penny, 1991; Schaech et al., 1986). *P. aeruginosa* can also cause folliculitis outbreaks in whirlpool spas in hotels and indoor swimming pools (Rima et al., 1983; Sausker et al., 1978; Washburn et al., 1976; Ashbolt, 2015b).

1.1.2.2 P. aeruginosa identification

Numerous diagnostic approaches have been developed to identify *P. aeruginosa,* such as flow cytometry (Rüger et al., 2014), immunological detection methods (e.g., enzyme-linked immunosorbent assay [ELISA]) (Rao et al., 2005) and molecular biology-based detection methods (e.g., PCR technologies) (Choi et al., 2013). However, conventional culture methods are still the most applied methods in clinical practices (Tang et al., 2017). Conventional *P. aeruginosa* culture methods primarily target the bacterium's biological and biochemical characteristics, namely oxidase and acetamidase activity, and production of the fluorescent pigments pyocyanin and pyoverdine (Tang et al., 2017).

P. aeruginosa is a non-fermenting bacterium and there are various chemical assays for identification. The organism grows well on standard broth (e.g., Luria-Bertani broth) and solid

medium including blood agar, MacConkey agar, and Luria-Bertani (LB) agar (Bergeyès Manual of Determinative Bacteriology, 1964). Colonies isolated on these agar bases may be presumptively identified by a positive oxidase reaction (Paulsson, 2017). *Pseudomonas aeruginosa* contains a high concentration of cytochrome c oxidase that renders the colonies positive in an oxidase test, allowing for rapid differentiation from fermenting bacteria (Hamada et al., 2014). Another assay involves the catabolism of acetamide, where acetamide medium is used to determine the ability of non-fermenting Gram-negative bacteria (e.g., *P. aeruginosa*) to deaminate acetamide. The deamination of the acetamide produces ammonia, which increases the medium's pH and helps to distinguish *P. aeruginosa* from other Pseudomonads (Buhlmann et al., 1961). *P. aeruginosa* can produce at least six colony types after aerobic incubation on nutrient agar for 24h at 37°C. The most common, is that of colonies which are large, oval, convex and with rough edges (Public Health England, 2015).

P. aeruginosa produces the fluorescent pigments pyocyanin and pyoverdine (Paulsson, 2017). Pyocyanin is an antimicrobial pigment that is blue in its oxidized state (Vardakas et al., 2013). Pyoverdine is a yellow green siderophore (iron-chelating molecules) that is secreted from the cell to bind iron in the extracellular space, then reimported into the periplasm, where iron is harvested. Production of these pigment molecules is crucial for virulence and biofilm development (Bodey, 1985). The low phosphorous concentration, as well as the presence of magnesium chloride and potassium sulphate in *Pseudomonas* isolation agar, facilitate pyocyanin production. Cetrimide nalidixic acid (CN) agar is also often used for isolation of *P. aeruginosa,* as cetrimide inhibits bacterial cells other than *P. aeruginosa* due to the release of nitrogen and phosphorous, and nalidixic acid inhibits growth of other microbial flora, but not *P. aeruginosa*. Potassium sulphate and magnesium chloride enhances pigment production of *P. aeruginosa* (Goto and Enomoto,1970).

1.1.2.3 Virulence factors and pathogenicity of *P. aeruginosa*

P. aeruginosa possesses and produces a wide variety of both cell-associated and extracellular virulence factors. The pathogenicity of *P. aeruginosa* is not related to a single virulence factor, rather, the interaction between different factors (van Delden, 2004). Various virulence factors enable *P. aeruginosa* to adhere to and/or disrupt host cells (Antonio et al., 2019; Zeng, 2004).

The major virulence factors of *P. aeruginosa* include lipopolysaccharides, flagellum, pilus, exopolysaccharide alginates and type III secreted exotoxins (Antonio et al., 2019; van Delden, 2004) (Figure 1).

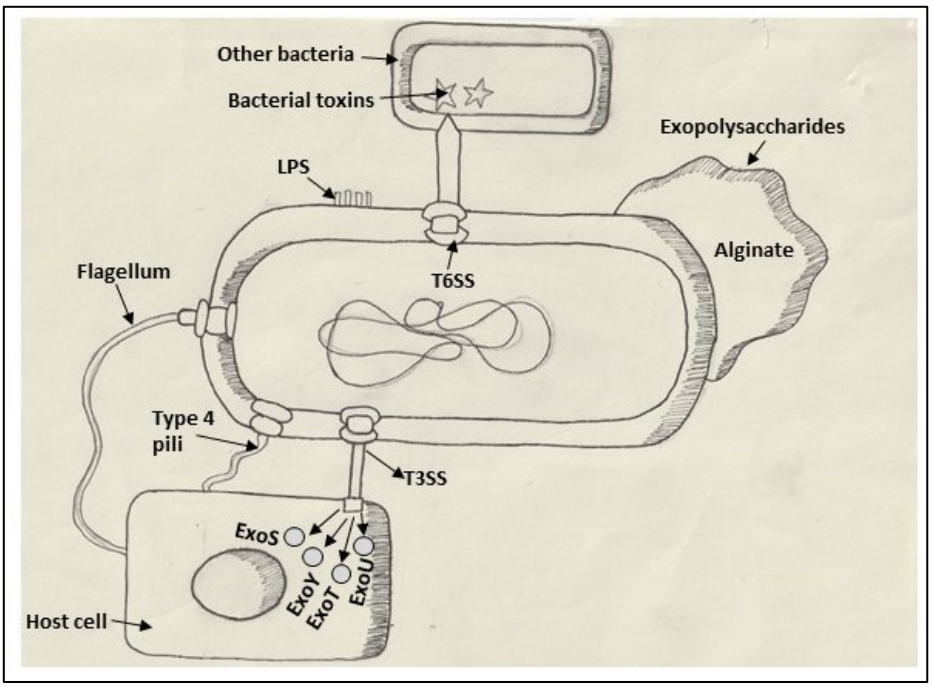

Figure 1: The major virulence factors of *P. aeruginosa*. Lipopolysaccharides (LPS) protects the cell from outer environmental threats. Flagella and type IV pili are the main adhesins, capable of binding to the host cell's epithelium. Once contact with the host epithelium has occurred, the T3SS (type III secretion system) is activated and injects cytotoxins (ExoS, ExoY, ExoT and ExoU) directly into the host cell. Extra cellular polysaccharide alginate supports biofilm structure and cell-to-cell substrate attachment (Adapted from Sainz-Mejías et al., 2020 and Gellatly and Hancock, 2013).

Lipopolysaccharide

Lipopolysaccharide is an essential component of the cell envelope of Gram-negative bacteria. The outer membrane of the cell envelope serves as the first line of defense against environmental threats (Bertani and Ruiz, 2018). Lipopolysaccharides consist of three parts- lipid A, a core oligosaccharide, and an O antigen of oligo- or polysaccharide chain (Alexander and Rietschel, 2001). Lipid A is known as endotoxin, which can interact with a host cell receptor to initiate a host cell inflammatory response. Lipid A is composed of a β (1 →6)-linked glucosamine disaccharide backbone that is phosphorylated at positions 1 and 4 of the

disaccharide and acylated at positions 2 and 3 of each monosaccharide (Steimle et al., 2016). Covalently attached to lipid A is the core oligosaccharide that links to the lipid through a 3-deoxy-D-manno-oct-ulosonic acid bond (Kdo) (Amor et al., 2000). The core oligosaccharide interacts with the host's epithelial cells, leading to bacterial innate immune resistance to the pathogens. It is located on the outermost part of lipopolysaccharide O-antigen sugar chain responsible for resistance to human serum, detergent, and antibiotics (Backhed et al., 2003; Hajjar et al., 2002; Roger et al., 2001).

Flagella

Each *P. aeruginosa* cell has a single polar flagellum and several short Type IV pili (Gellatly and Hancock, 2013). *P. aeruginosa* can swim in liquid using flagella and move across surfaces using type IV pili (Semmler et al., 1999). In addition to swimming motility, the role of *P. aeruginosa* flagellum in adhesion has been investigated in several studies (Feldman, 1998; Montie et al., 1982; Haiko et al., 2013), and mucin is thought to be important target for *P. aeruginosa* adhesion as the lung epithelium contains a thick mucus layer (Haiko et al., 2013). A nonflagellated mutant was shown to be less invasive than motile strains (Drake & Montie,1988; Fleiszig et al., 2001; Montie et al., 1982). The flagellar cap protein (FliD) of *P. aeruginosa* enables adherence to the human respiratory mucin and helps colonize the lung in CF patients (Arora et al., 1998).

Pilus

Type IV pili (T4P) are one of the most common forms of bacterial surface structure and are involved in adherence, motility, competence for DNA uptake and pathogenesis. Bacteria lacking T4P cannot adhere to the surfaces and are therefore avirulent. *P. aeruginosa* uses this fibre-like motorized appendages to sense initial contact with surfaces (Persat et al., 2015). The surface-associated twitching motility is driven by extension and retraction of type IV pili (Skerker and Berg, 2001). Three different types of T4P are found in *P. aeruginosa* – type IVa, type IVb, and Tad (Bernard et al., 2009; Carter et al., 2010; de Bentzmann et al., 2006). The type IVa and Tad encoded genes are common in all *P. aeruginosa* (Winsor et al., 2009), whereas type IVb are found in strains that have *P. aeruginosa* pathogenicity island 1 (PAPI-1) (Carter et al., 2010; Wurdemann and Tummler, 2007). Many type IVa pili expressing species,

including *P. aeruginosa* exhibit pilus mediated twitching motility (Burrows, 2012). Because of type IV pili help establish initial contact with a host, they are important virulence factors for many species including *P. aeruginosa* (Hahn, 1997).

In addition to adherence, type IV pili are involved in biofilm formation (Gellatly and Hancock, 2013). During biofilm formation by type IV pili, bacteria first assemble a fibrous protein – pilin at one or both cell poles to extend type IV pili. To generate motion, bacteria assemble type IV pili (Marathe et al., 2014) and tightly attached their tips to the surface (Lee et al., 1994). To retract the extended type IV pili, bacteria depolymerise the assembled pilins using PilB-like Adenosine triphosphatase (ATPase) with a typical retraction time of 1-3 s. By repeated cycles of extension, attachment, and retraction, type IV pili enable bacteria to move irregularly and twitch on the surface (Skerker and Berg, 2001).

Type III secreted exotoxins

P. aeruginosa can secrete toxins into eukaryotic host cells using type III secretion system (T3SS). The T3SS initially evolved to protect the organism from predators (Pukatzki et al., 2002), such as free-living or biofilm-associated amoebae (Matz et al., 2008; Abd et al., 2008). The toxins found in *P. aeruginosa* are ExoS, ExoT, ExoU and ExoY (Hueck, 1998). Toxins contribute to avoidance of phagocytosis by the host. ExoS is the major cytotoxin required for colonization and dissemination during infection (Kaufman et al., 2000). ExoU is more than 100-fold toxic than ExoS (Lee et al., 2005) and ExoU causes cell lysis (Finck-Barbançon et al., 1997). ExoT and ExoS share 76% amino acid identity (Yahr et al., 1996).

Biofilm formation

Biofilm formation is an important factor in the pathogenesis of *P. aeruginosa* and is involved in many chronic infections, including chronic lung infections of cystic fibrosis patients (Hoiby et al., 2011; Al-Wrafy et al., 2017). Biofilms are bacterial populations enclosed in a self-produced matrix comprising polysaccharides, extracellular deoxyribonucleic acid (eDNA), proteins and lipids (Ghafoor et al. 2011, Strempel et al., 2013), which function as a matrix holding bacteria cells together (Wei and Ma, 2013). *P. aeruginosa* synthesizes three exopolysaccharides, alginate, Pel, and Psl, which are the biofilm matrix's main components

(Ma et al, 2006, Ryder et al., 2007; Ghafoor et al., 2011). *P. aeruginosa* isolates collected from chronic pulmonary infections of patients with cystic fibrosis are often mucoid because of a large amount of alginate production (Franklin et al., 2011). Extracellular polysaccharide alginate, encoded by the *algD* gene, supports biofilm structure by enabling cell-to-cell substrate attachment, and serves as a barrier for *P. aeruginosa* cells against phagocytes and antibodies (Krieg et al., 1988; Meshulam et al., 1984; Zhulin et al., 1997). Non-mucoid *P. aeruginosa* strains produce Psl and Pel polysaccharides (Colvin et al., 2012), which act as the primary structural molecules for biofilm development (Colvin et al., 2011). The pslA gene encoded exopolysaccharide provides structural support during the primary stage of biofilm formation and facilitates both cell-to-cell and cell-to-substrate attachment (Asadpour, 2018; Colvin et al., 2012).

Psl polysaccharide contains D-mannose, L-rhamnose and D-glucose residues (Byrd et al., 2009). Psl forms a fabric-like matrix connecting the biofilm cells (Franklin et al., 2011). Psl can react with extracellular DNA to form a web of eDNA-Psl fibres, which appear like a biofilm skeleton to give bacteria structural support (Wang et al., 2015). Pel polysaccharide is a glucose-rich polysaccharide-like cellulose (Friedman and Kolter, 2004). This positively charged exopolysaccharide is composed of N-acetylgalactosamine and N-acetylglucosamine by which Pel cross-links extracellular DNA in the biofilm matrix (Jennings et al., 2015). Pel polysaccharide plays a protective role by enhancing resistance to aminoglycoside in biofilms (Colvin et al., 2011).

Biofilm development is a consecutive process beginning with the attachment of planktonic cells to a surface, followed by microcolony formation (Neidig et al., 2013). Biofilm formation of *P. aeruginosa* occurs in five stages – attachment and replication, microcolony formation, propagation, maturation, and mature biofilm dispersal (Engel, 2003). *P. aeruginosa* initiates the first stage of biofilm development with a reversible attachment to a surface via support of flagella and type IV pili. In the second stage, bacterial cells undergo the switch from reversible to irreversible attachment, which forms microcolonies in the extracellular polymeric matrix. In the third stage bacteria go through progressive propagation on attached surfaces through cell division called microcolony formation. During the fourth stage, microcolonies are developed

further into extensive three-dimensional mushroom-like structures. In the final stage, some bacteria cells become motile and disperse due to quorum-sensing and physical disruption followed by the transition from sessile to planktonic growth mode to spread and colonize to other substratum, allowing the biofilm cycle to repeat.

Extracellular DNA acts as an essential matrix in *P. aeruginosa* biofilms (Whitchurch et al, 2002; Allesen-Holm et al., 2006). The source of extracellular DNA in the bacterial biofilm may be from the bacteria themselves, from lysed cells of bacteria in the biofilm environment (Whitchurch et al, 2002), or may be from other than prokaryotic organisms. Extracellular DNA is abundant in many habitats, including soil, sediments, oceans and freshwater (Nagler et al., 2018), and can be released from prokaryotic and eukaryotic cells, from intracellular DNA by active or passive extrusion mechanisms or by cell lysis (Pietramellara et al., 2009). *P. aeruginosa* is known to release membrane vesicles (Muto and Goto, 1986; Kadurugamuwa and Beveridge, 1996) and extracellular DNA may also derive from membrane vesicles of *P. aeruginosa* containing DNA (Whitchurch et al, 2002).

Extracellular DNA has been observed in biofilms isolated from the lungs of cystic fibrosis patients in which the DNA was almost all of human origin (Lethem et al., 1990). Extracellular DNA plays a crucial role in the early development of *P. aeruginosa* biofilms and acts as a cell-to-cell connecting compound (Whitchurch et al, 2002; Flemming et al., 2010; Allesen-Holm et al., 2006; Whitchurch et al., 2006). The cell-to-cell and cell-to-matrix interactions help for the structural integrity of biofilms. DNA binding proteins are involved in the eDNA-cell and eDNA-matrix interaction. Type IV pili in *P. aeruginosa* plays a role in both bacterial attachment and eDNA-guided motility (Van et al., 2005). Non-proteinaceous compounds were identified as mediating the interaction of eDNA with the cell surface of *P. aeruginosa*. For example, pyocyanin on the surface of *P. aeruginosa* could intercalate with eDNA and thereby mediate cell-to-cell interactions (Das et al., 2013). Extracellular DNA helps the twitching mortality-mediated biofilm growth and comprises a nutrient source for bacteria during food shortage (Mulcahy, et al, 2014).

The adhesion of bacterial cells to a surface is the first stage in biofilm formation, and eDNA acts as an adhesive substance. The bacteria cells behave as colloidal particles that come into contact with surfaces, then after reversible interaction (attractive and repulsive) between bacteria cell and the surfaces, the adhesins (eDNA) acts as an anchor to attach the cell more firmly to the surface (Marshall et al., 1971). Extracellular DNA also influences the bacterial cell surface's hydrophobicity and promotes the bacterium to attach, depending on the relative hydrophobicity of the substratum (Okshevsky and Meyer, 2015).

Quorum sensing

Quorum sensing (QS) is a cell-to-cell communication system among bacteria that allows sharing of information about cell density and gene expression regulation (Rutherford and Bassler, 2012; Zhao et al., 2020). When the bacterial population reaches a certain density, bacteria communicate using autoinducers diffused by the bacteria cell, and with the increase of bacterial population density, the synthesis of autoinducers increases. Once the critical threshold concentration of bacterial population and autoinducers is reached, a response regulator is activated to facilitates the quorum-sensing dependent gene expression needed to support bacterial population adaptation (Paul et al., 2007). QS allows cells to communicate between the individual cells necessary to adapt and survive in bacterial communities. The QS systems regulate bacterial biofilm formation, virulence factors, toxin production, and drug resistance (Zhao et al., 2020; Dekimpe and Deziel, 2009). Quorum sensing is the mechanism whereby an individual bacterium produces small diffusible molecules that surrounding organisms can detect. In *P. aeruginosa*, these signal molecules are acyl homoserine lactones (AHLs). When the concentration of AHLs in the environment increases, due to increasing numbers of bacteria, these higher concentration of AHLs activate transcriptional regulators. This mechanism of communication enables bacteria to act as a community in the coordinated regulation of gene expression. This regulated expression of virulence genes is thought to give the bacteria a selective advantage over host defenses and thus is important for the pathogenesis of the organism (Smith and Iglewski, 2003). *P. aeruginosa* has two acyl-homoserine lactone (AHL)-based QS systems, Las and Rhl. The Las system is comprised of the transcriptional activator LasR and the autoinducer synthase enzyme Lasl. Lasl directs synthesis of the signal molecule N-(3-oxododecanoyl) homoserine lactone (3-oxo-C_{12}-HSL), synthesized by the AHL

synthase Lasl (Gambello and Iglewski, 1991; Passador et al., 1993). LasR requires 3-oxo-C_{12}-HSL in order to become an active transcription factor. In the presence of 3-oxo-C_{12}-HSL, LasR forms multimers, which can bind DNA and regulate the transcription of multiple genes (Kiratisin et al., 2002). Similarly, the Rhl system is comprised of the transcriptional activator RhlR and the autoinducer Rhll that synthesizes N-butyryl-homoserine lactone (C_4-HSL), (Ochsner et al.,1994; Latifi et al., 1995).

Whiteley et al. (1999) reported the first systematic investigation on the QS regulon of *P. aeruginosa*, it was observed that 47 genomic loci were transcriptionally activated by AHL. Thirty-nine quorum sensing-regulated genes were identified. The genes were classified into classes depending on the pattern of regulation. About half of the genes appear to be in seven operons, and some seem organized in large patches on the genome. Many of the QS-regulated genes code for putative virulence factors or production of secondary metabolites. Many of the genes identified showed a high level of induction by acyl-HSL signaling. Many identified QS-controlled genes were classified based on the temporal pattern of their response in cells grown in the presence of Las AHL signal and/or Rhl AHL signal, an important indication that additional regulation was superimposed on the QS control.

Research groups have used microarray experiments to analyze the QS-regulated transcriptome of *P. aeruginosa* (Wagner et al., 2003; Schuster et al., 2003). Both studies used independently derived mutant lasI/rhlI strains of *P. aeruginosa* PAO1. Gene expression levels were determined for this mutant bacterium when grown with or without exogenous 3O-C_{12}-HSL and C_4-HSL Wagner et al. (2003) found that 616 genes showed statistically significant differential expression ($P \leq 0.05$) in response to the exogenous autoinducers and were classified as QS regulator. A total of 244 genes were identified as being QS regulated at the mid-logarithmic phase, 450 genes were identified as being QS regulated at the early stationary phase and 222 genes were identified as being QS repressed. Schuster et al. (2003) identified 315 quorum-induced and 38 quorum-repressed genes, representing about 6% of the *P. aeruginosa* genome.

QS may affect the viulence of *P. aeruginosa* (Smith and Iglewski, 2003). Many studies indicated that the deletions of one or more QS genes results in reduced *P. aeruginosa* virulence

compared with wild-type *P. aeruginosa* **(**Rumbaugh et al., 1999; Smith et al., 2002; Pearson et al., 2000; Wu et al., 2001**).** These data indicate that both the Las and the Rhl QS systems are essential for *P. aeruginosa* to disseminate virulence (Smith and Iglewski, 2003). QS is also involved in early stage biofilm formation, and strains deficient in the production of the Las signal molecules, 3-oxo-C_{12}-HSL, formed a very thin biofilm that lacked regular three-dimensional biofilm architecture, suggesting that Las QS system is important for *P. aeruginosa* biofilm development (Smith and Iglewski, 2003; De Kievit et al., 2001; Davies et al., 1998).

1.1.2.4 Multidrug resistance *P. aeruginosa*

Antimicrobial resistance is one of the most severe global public health threats of this century (Wang et al., 2019; El-Banna et al., 2019) and one of the most significant challenges faced by healthcare (Hannan et al., 2010; IACG, 2019). Globally, it is estimated that antimicrobial-resistant infections kill 700,000 people each year, and by 2050 it is predicted that 10 million people could die from AMR infections, representing more deaths than from cancer (World Health Organization, 2019). Multidrug-resistance is defined as an acquired resistance to at least one agent in three or more antimicrobial classes (Magiorakos et al., 2012; Kumar & Khan, 2015; Basak et al., 2016). *P. aeruginosa* exhibits multidrug resistance to a range of antibiotics, including β-lactams, aminoglycosides, quinolones and polymyxins (Hancock and Speert, 2000; Hussein et al., 2020). The prevalence of multidrug-resistant strains of *P. aeruginosa* decreases treatment options and substantially increases the rate of morbidity (Chatterjee et al., 2016).

Structure and mechanisms of actions of antimicrobials

β-lactams

A major group of antibiotics, β-lactams, belong to a chemical class having a common four-member ring (three carbon and one nitrogen). If this ring is fused to a thiazolidine ring, the β-lactam is classified as penicillin (e.g., ampicillin). The thiazolidine ring is replaced by dihydrothiazine ring in cephalosporin (e.g., cephalothin). Monobactams contain a monocyclic ring structure, where the β-lactams ring stands alone and not fused to another ring (e.g., aztreonam), whereas carbapenems have a bicyclic ring with an associated five-membered ring structure (John et al., 2015).

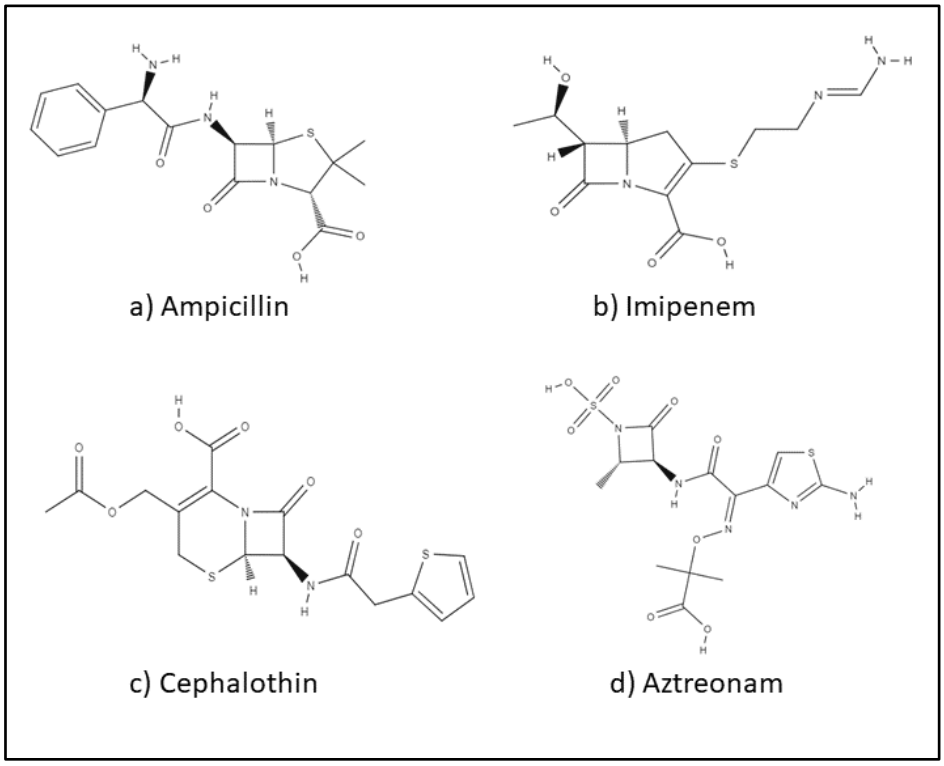

Figure 2: Chemical structures of β-lactams: a) Penicillin - Ampicillin, b) Carbapenem - Imipenem, c) Cephalosporin - Cephalothin and d) Monobactam – Aztreonam. The similarity between β-lactam structure with the 4-member β-lactam ring is shown in the red arrow (National Center for Biotechnology Information, 2021).

The peptidoglycan layer of the *P. aeruginosa* cell wall is composed of glycan chains made of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) disaccharide subunits. The NAM is cross-linked with tetrapeptide stems (l-alanine-d-glutamic acid-meso-diaminopimelic acid-d-alanine). The β-lactam antibiotics inhibit peptidoglycan synthesis by acylating the transpeptidase involving cross-linking peptides to form peptidoglycan (Pandey and Cascella, 2021).

Aminoglycosides

Aminoglycosides consist of an amino group (-NH_2) attached to glycoside (a derivative of sugar) (Britannica, 2019), with two or more amino sugars joined in glycosidic linkage to a hexose nucleus (Krause et al., 2016). Once inside bacterial cells, aminoglycosides exert their effects by binding to ribosomes (30S ribosome), subcellular organelles that are fundamental to protein synthesis. As a result, protein synthesis is inhibited, and the bacterial cell dies (Britannica, 2019). Aminoglycosides diffuse across the outer membrane of gram-negative

bacteria through porin channels. The bacterial cell wall inhibitors (e.g., β-lactam) enhance the entry of aminoglycosides and exhibit synergism (Krause et al., 2016).

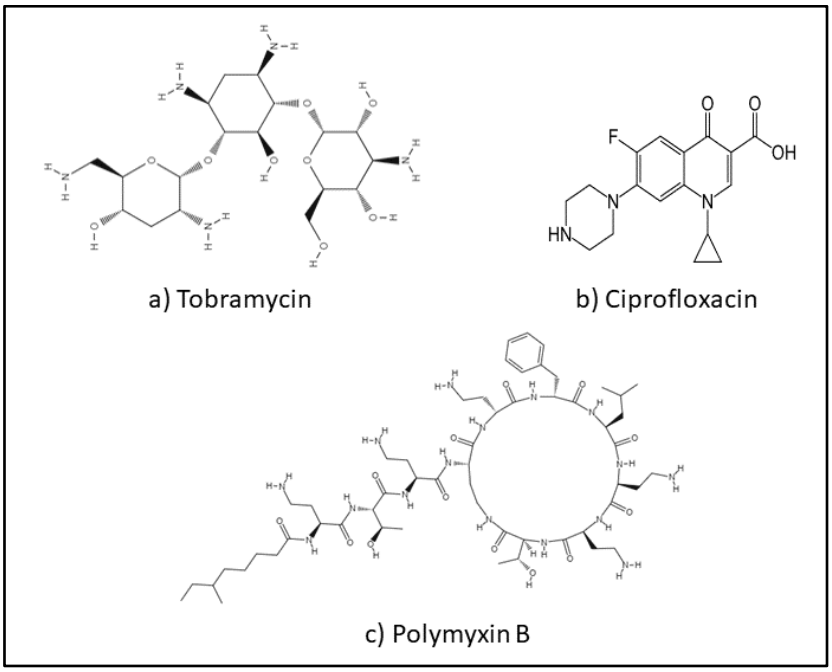

Figure 3: Chemical structures of Aminoglycosides (a. Tobramycin), Fluoroquinolones (b. Ciprofloxacin) and Polymyxin B (c) (National Center for Biotechnology Information, 2021).

Quinolones

Quinolones are strong antimicrobial agents with a basic chemical bicyclic ring structure, a naphthyridine core with a nitrogen molecule in positions 1 and 8. A fluorine atom at position C-6 and various substitutions on the basic quinolone structure yielded fluoroquinolones (Kocsis et al., 2016). Most quinolone antibiotics in use are fluoroquinolones. The target molecules of quinolones and fluoroquinolones are bacterial DNA gyrase (also known as topoisomerase II) (Kocsis et al., 2016). Quinolones inhibit DNA synthesis by promoting cleavage of bacterial DNA in the DNA-enzyme complexes of DNA gyrase and type IV topoisomerase, causing rapid bacterial death (Hooper, 2000; Hooper and Wolfson, 1993; Hooper, 1999).

Polymyxins

The basic structure of polymyxin is a cyclic heptapeptide (L-α-γ-diaminobutyric acid) with a tripeptide polar side chain where the amino terminus is acylated by a fatty acid tail (Li et al., 2005). Amino acid components in the peptide chain differentiate between the polymyxins, where all except polymyxin C contain leucine, polymyxins B and C contain phenylalanine, and only polymyxin D contains serine (Stansly et al., 1949; Poirel et al., 2017). The intramolecular cyclic heptapeptide loop is linked between the amino group of side chain on diaminobutyric acid (Dab) residue at position 4 and carboxyl group of C-terminal L-Thr (L-threonine) residue at position 10 (Yu et al., 2015). Polymyxin B is a mixture of polypeptides, polymyxins B1 and B2. Polymyxin B and Polymyxin E share an almost identical primary structure with a major difference present at position 6, where D-Phe (D-phenylalanine) in Polymyxin B is replaced by D-Leu (D-leucine) in Polymyxin E (Velkov et al., 2010).

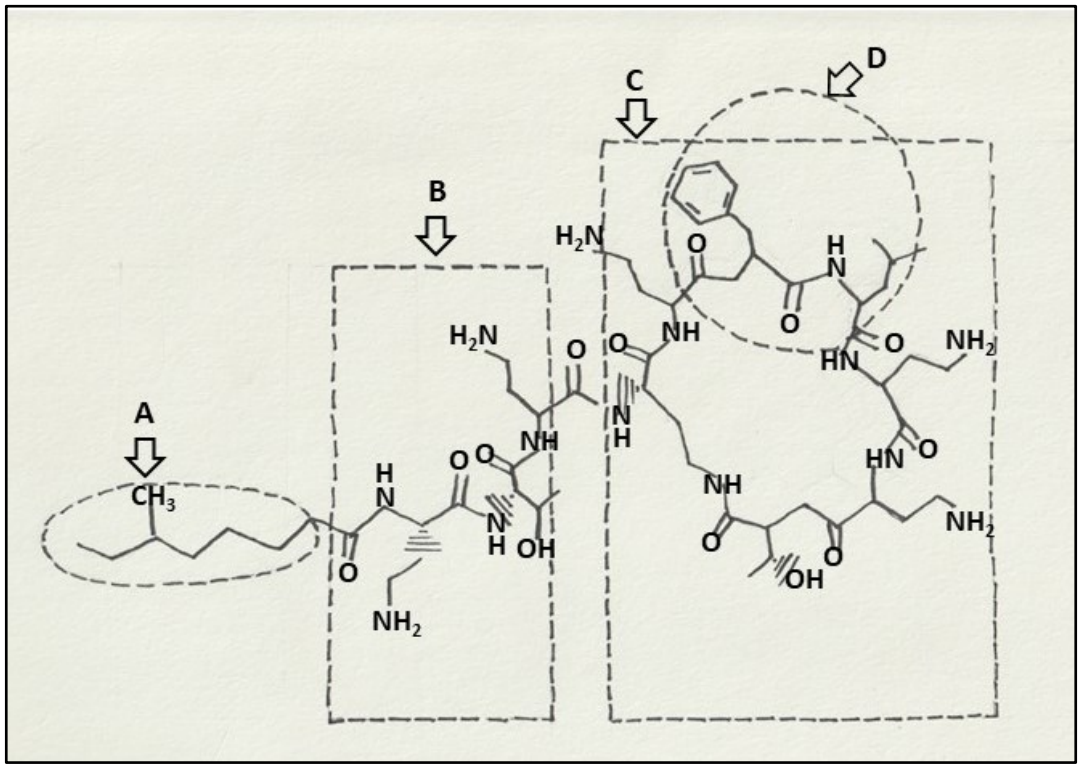

Figure 4: Chemical structures of polymyxin B. The functional segments of polymyxins. A) fatty acyl chain, B) linear tripeptide segment, C) the polar residues of the heptapeptide, D) the hydrophobic heptapeptide ring (Kumar et al., 2020).

Polymyxins increase cell membrane permeability of Gram-negative bacteria by displacing Ca^{2+} and Mg^{2+} from PO_4^{3-} in the bacterial cell membrane through electrostatic interaction between Dab of the polymyxins and PO_4^{3-} of the outer membrane of bacteria. The initial target of polymyxin is the lipopolysaccharide of the outer membrane of Gram-negative bacteria. Polymyxin exerts its antimicrobial action by permeabilizing the bacterial outer membrane via direct interaction with the lipid A component of the lipopolysaccharide. The amphipathicity of the polymyxins and their ability to form pore-like aggregates may be responsible for their outer membrane permeabilizing action (Hancock, 1997; Hancock and Lehrer, 1998; Clausell et al., 2007; Melo et al., 2009; Powers and Hancock, 2003). Polymyxins act like cationic detergents that bind with high affinity to the negatively charged lipopolysaccharide present in the outer membrane of Gram-negative bacteria resulting in disruption of the integrity of the outer membrane. The polar face of the peptide of Polymyxin B interacts with the polar face of lipid A, while the lipophilic face of the peptide inserts into the hydrophobic fatty acid acyl layer of the outer membrane (Hancock, 1997; Hancock and Lehrer, 1998; Clausell et al., 2007; Melo et al., 2009; Powers and Hancock, 2003). The positive charge of polymyxins allows for accumulation at the anionic bacterial membrane. The electrostatic interaction between the positively charged polymyxin Dab residue and the negatively charged lipid A phosphate displaces divalent cations (Ca2+ and Mg2+) that usually function to bridge and stabilize the lipopolysaccharide of the outer membrane (Nikaido et al., 2003; Hancock, 1997; Hancock and Lehrer, 1998; Clausell et al., 2007; Melo et al., 2009; Powers and Hancock, 2003). The disruption of the outer membrane subsequently allows polymyxins to interact with the inner cytoplasmic membrane, increasing permeability and causing the death of the bacteria (Ayoub, 2020).

Mechanism of antimicrobial resistance of P. aeruginosa

There are several mechanisms by which *P. aeruginosa* resist the action of antibiotics, which includes drug inactivation/alteration (e.g., production of β-lactamases), modification of drug binding sites/targets, changes in cell permeability, presence of numerous genes coding for different multidrug resistance efflux pumps and biofilm formation (biofilms role on AMR discussed in section 1.1.2.3) (Wright, 2005; Li et al., 2004; Wilson, 2014; Livermore, 2002; Henrichfreise et al., 2007; Lambert, 2002).

Antibiotic inactivation enzymes

P. aeruginosa produces enzymes that irreversibly modify and inactivate antibiotics, such as β-lactamases. β -lactamases hydrolyze the β-lactam ring, which is present in all β-lactams (Pang et al., 2019), included penicillins, cephalosporins, monobactams, and carbapenems (Jacoby and Munoz-Price, 2005). *P. aeruginosa* produces β-lactamases by cleaving the amide bond of the β-lactam ring (Korfmann and Wiedemann, 1988; Jacoby, 2009). Overexpression of β-lactamases inhibit the peptidoglycan-assembling transpeptidases and affects bacterial fitness (Lambert, 2002; Zeng and Lin, 2013). Some strains of *P. aeruginosa* can produce ESBLs. ESBLs are a rapidly evolving group of β-lactamases produced by certain Gram-negative bacteria (e.g., *P. aeruginosa*) that can hydrolyze the β-lactam ring of extended spectrum cephalosporin (third generation), penicillin and aztreonam (Livermore, 2008; Paterson and Bonomo, 2005; Wolter & Lister, 2013).

P. aeruginosa can also produce penicillin-binding proteins (PBPs), which involve peptidoglycan synthesis (Poole, 2004). These PBPs reduce the affinity of β-lactam antibiotics (Fuda et al., 2004). β -lactam rings block bacterial cell wall biosynthesis by crossing the outer membrane and interact with the penicillin-binding proteins because they are similar in chemical structure to the modular pieces that form the peptidoglycan (Nguyen-Distèche et al., 1982). When PBPs bind to penicillin, the β-lactam amide bond is ruptured to form a covalent bond with the catalytic serine residue at the PBPs active site and produces serine β-lactamases to inhibit β-lactams (Smith et al., 2013).

Alterations in membrane permeability

Antibiotics that can enter through the outer cell membrane are common for *P. aeruginosa* treatment (Lambert, 2002). The cell envelope of *P. aeruginosa* consists of a cell wall and an inner cytoplasmic membrane; the cell wall comprises an outer membrane and peptidoglycan layer (Beveridge et al., 1999). The poor permeability of the outer membrane protects *P. aeruginosa* from a wide range of antibiotics (Brown, 1975) and a key factor that enables *P. aeruginosa* to survive in different unfavourable environments (Paulsson, 2017).

The outer membrane of *P. aeruginosa* showed 100- to 400-fold lower permeability to glucose -6-phosphate, fructose-6-phosphate and *p*-nitrophenylphosphate than *Escherichia coli* (Yoshimura and Nikaido, 1982). The size of the pores is too small to allow efficient penetration of most antibiotics whose molecular weights are generally higher than 400 grams/mole. The outer membrane of *P. aeruginosa* constitutes porin channels that only allow the diffusion of saccharides with an M_r (molecular weight of the repeat unit) of less than roughly 350 to 400 Daltons (Caulcott et al., 1984; Yoneyama et al., 1986; Yoneyama and Nakae, 1986; Yoshihara et al., 1988) and allow the penetration of polysaccharides with an M_r of less than 3,000 to 7,000 (Decad and Nikaido, 1976; Hancock and Nikaido, 1978).

Most antibiotics used to treat *P. aeruginosa* infections must be able to penetrate the cell membrane to reach intracellular target (Lambert, 2002). For example, β-lactam antibiotics block bacterial cell wall biosynthesis by targeting the penicillin binding proteins that are enzymes involved in peptidoglycan synthesis (Poole, 2004). Polymyxins bind to the lipopolysaccharides on the outer membrane of the Gram-negative bacteria, leading to increased cell membrane permeability and enhance antibiotic uptake. To enter the bacterial cell, β-lactams and quinolones penetrate cell membranes through porin channels, whereas aminoglycosides and polymyxins promote their own uptake by interacting with bacterial LPS on the outer membrane of Gram-negative bacteria (Lambert, 2002). The outer membrane of Gram-negative bacteria, such as *P. aeruginosa*, which acts as a selective barrier to prevent antibiotic penetration, is an asymmetric bilayer of phospholipid and LPS, embedded with porins that form β-barrel protein channels (Delcour, 2009).

Antimicrobial resistance via efflux pumps

An efflux pump is a cell membrane protein channel that selectively eliminates antimicrobial toxic compounds from the cytoplasm before reaching their cellular targets (Routh et al., 2011), thereby decreasing the effectiveness of the drug (Levy, 1992; Bolhuis et al., 1997; Nikaido, 1994). Many bacteria produce multilayer protein efflux pumps that transport antimicrobial drugs out of the cell to prevent excessive accumulation of drugs (Sharma et al., 2019).

Efflux pumps consist of five families, including those within the i) RND (resistance-nodulation-division) family ii) MFS (major facilitator superfamily) iii) ABC (ATP-binding cassette) superfamily iv) small multidrug resistance (SMR) family, and v) multidrug and toxic compound extrusion (MATE) family (Sun et al., 2014). Among the five, the RND efflux pumps play a significant role in the antibiotic resistance of *P. aeruginosa* (Li et al., 2004). An RND pump comprises transporters (cytoplasmic membrane), porin channel proteins (outer membrane) and periplasmic linker proteins (Daury et al., 2016). *P. aeruginosa* expresses twelve types of RND family efflux pumps. Among them, four (MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM) contribute to antibiotic resistance (Dreier & Ruggerone, 2015). MexAB-OprM expels β-lactams and quinolones, MexCD-OprJ pumps out β-lactams, MexEF-OprN works on extruding quinolones, and MexXY-OprM expels aminoglycosides (Pang et al., 2019). Overexpression of the efflux MexAB-OprM may occur due to the mutation of transcriptional regulator genes *mexR*, *nalB*, *nalC* or *nalD*, resulting in resistance to β-lactam and fluoroquinolones (Braz et al., 2016; Tian et al., 2016).

Most studies on multidrug efflux pumps focus on their role as antibiotic resistance elements, even if they can confer resistance to other compounds, e.g., heavy metals and pyocyanin (Blanco et al., 2016; Dietrich et al., 2006; Nies, 2003). In addition to selecting antibiotic-resistant mutants, some studies have shown that biocides can also induce the expression of multidrug resistance efflux pumps (Blanco et al., 2016). For instance, pentachlorophenol and triclosan can induce the expression of the efflux pump mexAB-OprM in *P. aeruginosa* through the binding to nalC, one of the regulators of the expression of this efflux pump (Ghosh et al., 2011).

Antimicrobial resistance by horizontal gene transfer

Gene transfer is an essential microbial adaptation tool responsible for transferring various phenotypic traits between organisms, including virulence factors, metabolic capabilities, as well as antimicrobial resistance (Bello-López et al., 2019). In addition, gene transfer is essential in microbial adaptation to new environmental niches (Kamruzzaman & Iredell, 2019). Antimicrobial resistance gene transfer occurs by three distinct mechanisms: lateral, horizontal, and vertical transmission. Lateral transfer is the transfer of genetic elements among the same

species of microorganism. Horizontal gene transfer (HGT) occurs between different species, and vertical transfer follows by transmitting genetic materials from parent to offspring. Inter and intra-species DNA exchange via HGT is the most common mechanism by which Gram-negative bacteria acquire antimicrobial resistance (Bello-Lopez et al., 2019).

Bacteria living in the same ecological niche may lead to the spread of genetic materials and antibiotic resistance genes through HGT (Quintieri et al., 2019). Horizontal gene transfer between bacteria occurs by three different processes: conjugation (gene transfer through plasmids or conjugative and integrated elements), transduction (bacteriophage mediated gene transfer) and transformation (free DNA uptake). Conjugation is considered the primary recognized mechanism for genetic material transfer in bacteria and a major cause of multidrug resistance (Lerminiaux & Cameron, 2019). Conjugation is the process of DNA transfer through direct cell-to-cell contact between the donor and the recipient cells through a conjugative pilus. The donor bacterium carries a DNA sequence called the fertility factor, or F-factor. The F-factor allows the donor to produce a thin tube-like structure called a pilus, which the donor uses to contact the recipient. The pilus draws the two bacteria together, and eventually triggers to form a mating bridge, which establish direct contact and the formation of a controlled pore that allows transfer of genetic materials from the donor to the recipient bacterium. Typically, the recipient cells acquire the genetic and phenotypic characteristics in the form of a plasmid, or a small, circular piece of DNA (Zhou and Li, 2015, Ilangovan et al., 2015). The degree of plasmid transfer by conjugation in *in vivo* conditions is dependent on several biotic and abiotic factors: natural microbiota, the organic matter concentration, pH, and comparative *in situ* concentrations of donors and recipients (Sorensen et al., 2005).

Transduction is a transfer process of bacterial DNA from a donor to a recipient bacterium via an intermediate virus agent (Griffiths et al., 2000). Transduction does not require physical attachment between the cell donating the DNA and the cell receiving the DNA. The bacterial donor DNA is incorporated into the bacteriophage either through the lytic or lysogenic cycle. After infection with a bacteriophage, bacterial DNA is sometimes accidentally packaged in a bacteriophage capsid. A capsid containing bacterial DNA is fully capable of binding to a recipient cell and injecting the foreign donor DNA into the cytoplasm of the host bacterial cell.

If the transferred bacterial DNA is recombined into the recipient cell's genome, transduction has occurred (Lerminiaux & Cameron, 2019). Transduction is recognized as a potential contributor to the spread of antimicrobial resistance genes, especially between members of the same species (Dzidic and Bedekovic', 2003; Hens et al., 2006; Gillings et al., 2017). In Gram-negative bacteria, transduction has been observed to transfer multiple resistance genes, including ESBL genes, from *P. aeruginosa* hospital isolates to other *P. aeruginosa* strains in the Laboratory (Blahova et al., 2000). A *P. aeruginosa* transducing phage, φPA3, which can transduce chromosomal markers between *P. aeruginosa* strains with high frequency, was observed (Monson et al., 2010). Several other *P. aeruginosa* transducing bacteriophages have been described previously, though the transduction efficiency of these bacteriophages can be very low (Budzik et al., 2004; Kidambi et al., 1994; Ripp et al., 1994).

Transformation is the direct uptake of the environmental DNA by the recipient cells, where environmental DNA is recombined onto the recipient cell's genome. During transformation, the bacteria cell takes eDNA from its environment across its plasma membrane. eDNA that is not integrated may break down and be used as a nutrient source for DNA replication or repair. The ability of a cell to acquire DNA across the plasma membrane is called competency. Transformation competency can be induced naturally or artificially, but not all bacterial species can develop natural competency (Johnston et al., 2014). Bacteria must accumulate competence factors and produce important protein or structural components such as type IV pili, Rec2, comEC, comA, PilQ, PilE before uptake of the eDNA. Transformation requires deoxyribonuclease (DNase) sensitive extracellular naked DNA as the substrate (Blesa and Berenguer, 2015). Naked DNA can be naturally delivered from dead cells to nearby cells within the same habitat or microenvironment (Hasegawa et al., 2018). Two membrane protein complexes containing conserved proteins are responsible for acquiring DNA during natural transformation (Chen and Dubnau, 2004; Burton and Dubnau, 2010; Johnston et al., 2014; Cabezon et al., 2015; Ilangovan et al., 2015, 2017). In *P. aeruginosa*, three proteins, PilQ, PilE and ComA, support the creation of a transformation channel in the cell membrane. PilQ and PilE are from the family of the perilipin (PLIN) proteins, which supports formation of pili. Pili help create channels to facilitate the exchange of genetic materials between cells and support gliding motility. The extracellular DNA enters through the PilQ channel and interacts with

PilE within the cytoplasmic region. An enzyme nuclease (DNase) starts to breakdown one strand of the DNA, while the other strand is translocated into the cytoplasm by Rec2/ComEC protein. The new strand displaces the resident strand and recombines with a homologous sequence in the chromosome (Mell and Redfield, 2014).

Plasmids are extrachromosomal DNA molecules capable of autonomous reproduction, and antimicrobial resistance genes in plasmids are among the most significant challenges for counteracting the spreading of antimicrobial resistance. Plasmids facilitate the horizontal transfer of resistance factors among different bacteria species, genera and kingdoms (Thomas & Nielsen, 2005). Plasmids confer resistance to β-lactams, aminoglycosides, chloramphenicol, tetracyclines, trimethoprim, sulfonamides, macrolides, and quinolones (Carattoli, 2009). A significant quantity of extracellular plasmid DNA encoding antimicrobial resistance genes is present in clinical and environmental settings, which can be sampled by competent bacteria through natural transformation (Ibanez de Aldecoa et al., 2017). Some *P. aeruginosa* strains acquire plasmids encoding β-lactamases that confer resistance to penicillins and cephalosporins. *P. aeruginosa* have MBLs (metallo- β-lactamases) and seem to have acquired this through plasmids from other bacteria (Sacha, 2008). *P. aeruginosa* also carry bla_{NDM-1} gene, which exhibits resistance to imipenem and meropenem. Previous studies have revealed that the bla_{NDM-1} gene can be found on plasmids isolated from *Klebsiella pneumoniae*, *E. coli*, and *Raoultella ornitholytica* (Nagaraj et al., 2012; Bora and Ahmed, 2012; Khajuria et al., 2013a). Therefore, the ability of bla_{NDM-1} to transfer from *P. aeruginosa* to other *Enterobacteriaceae,* and vice-versa, is assumed to be significant (Khajuria et al., 2013b).

1.1.3 Ecology of P. aeruginosa

1.1.3.1 Environmental niches of P. aeruginosa

P. aeruginosa is a ubiquitous microorganism found in aquatic environments including recreational and hydrotherapy pools and can also be isolated from human skin, throat and stool (Iglewski, 1996; Moore et al., 2002). Although considered environmentally abundant, *P. aeruginosa* in water is directly linked to human pollution including that associated with agricultural and domestic wastewater contamination (Relai and Rosati, 1994, de Vicente et al., 1988; Warburton et al., 1994). Higher numbers of *P. aeruginosa* have been found near sites of

urban runoff in rivers compared to further downstream locations (Alonso et al., 1989). In lakes, the counts of *P. aeruginosa* are correlated with the number of human bathers (Seyfried and Cook, 1984). *P. aeruginosa* concentration in rivers and lakes has been found at concentrations ranging from 10/100 mL to >1,000/100 mL (Mena and Gebra, 2009). *P. aeruginosa* is one of the most routinely isolated opportunistic pathogens from water in hot tubs and whirlpools (Rice et al., 2012). The water supply for the hot tubs and whirlpools could be sources of *P. aeruginosa* (Rusin et al., 1997; Leoni et al., 2001) as *P. aeruginosa'*s persistence in low nutrient levels enables survival in tap water by existing in a dormant state (van der Kooij et al., 1982, de Vicente et al., 1988; Lewenza, et al., 2018). Both persister cells and starved stationary phase cells are dormant growth states (Lewis, 2007; Gefen et al., 2014), achieved by lowering metabolism (Lewenza, et al., 2018) and slowing protein production and cell replication machinery (Lewis, 2007; Lennon and Jones, 2011) strategies used by *P. aeruginosa* to survive within these environments.

Hot tub and whirlpool users may be symptomatic or asymptomatic carriers, and *P. aeruginosa* transmits to the hot tubs or whirlpool water from infected bathers (Fisher et al., 1985; Buttery et al., 1998; Tate et al., 2003). *P. aeruginosa*'s incident in hot tubs and whirlpools is correlated in its ability to colonize in plumbing fixtures (i.e., faucets, showerheads, sinks etc.) and materials (e.g., pipes) (Gerba and Gerba, 1998; Mena and Gebra, 2009). *P. aeruginosa* biofilms have been shown to form on whirlpools and hot tubs accessories (toys and tools), adjacent flooring, and plumbing filters (Uhl and Hartmann, 2005; Leoni et al., 1999; Hopkins et al., 1981; Tate et al., 2003). Excess production of the extracellular polymeric matrix produced by *P. aeruginosa* mucoid strains is often associated with biofilm formation (Goeres et al., 2004). Mucoid strains show enhanced survival at free chlorine concentration commonly used for the disinfection of pools (0.11-0.57 ml/L), and thus may account for *P. aeruginosa* even in those pools with an adequately maintained chlorine residual (Grobe et al., 2001). A hot tub's typical temperature is 40°C (Health Canada, 2015; CDC, 2020), suitable for *P. aeruginosa* proliferation (Zichichi et al., 2000; Dulabon et al., 2009). Biofilms-associated growth of *P. aeruginosa* in plumbing and the surrounding damp surfaces enables proliferation (Price and Ahearn, 1988) due to the dissipation of chlorine levels by warm temperatures (39°C to 40°C) (Muraca et al., 1987) and aeration (Price & Ahearn, 1988). Whenever disinfectant levels fall

below recommended levels in hot tubs, *P. aeruginosa* can re-grow in the water (Lutz & Lee, 2011).

Cross-resistant bacteria have developed survival mechanisms which are effective against different antimicrobial molecules with similar mechanisms of action. In the natural ecosystem, antibiotics produced by other microbes may enhance antibiotic resistance by the bacteria. Bacteria interact with numerous other organisms, and those organisms can produce antimicrobial (Colclough et al., 2019). Selection for resistance to this antimicrobial has the potential to result in resistance to clinical antibiotics when active compounds target the same bacterial pathways. In addition to acquiring antibiotic resistance from natural ecosystems, resistance may develop by other mechanisms, i.e., by chlorination, or induced by antibiotic like molecules, e.g., heavy metals. Bacteria tolerance to chlorine is often linked to antibiotic cross-resistance. A relation between chlorine resistance and antibiotic resistant genes was found among *Salmonella* spp. (Xiao et al., 2022). Chlorination may cause enrichment of ARGs abundance (Shi et al., 2013). A study by Rizzo et al. (2013) found that antibiotic resistant *E. coli* exhibits resistance to high concentrations of chlorine (240 mg/L). Antibiotic resistance bacteria tolerance to chlorine might be connected to the similar mechanisms (e.g., multidrug efflux pumps) of gene resistance to antibiotics and chlorine. This kind of co-resistance might result in the ineffectiveness of chlorination on bacteria containing ARGs (Yuan et al., 2015). The presence of heavy metal ions (e.g., cadmium or lead) in the environment may contribute to antibiotic resistance by *S. aureus* (Chudobova et al., 2014). The above cross-resistance may develop among *P. aeruginosa* isolates present in hot tubs and whirlpools ecosystems.

P. aeruginosa may infect through almost any exposed tissue, including skin, ears, eyes, urinary tract, and lungs (Mena and Gebra, 2009; Kerckhoffs et al., 2011). Two routes appear to carry the greatest health risk from contacting water contaminated with *P. aeruginosa*- skin exposure in hot tubs and lung exposure from inhaling aerosols (Gerba and Gerba, 1998).

1.1.3.2 P. aeruginosa interaction with free-living amoebae

Structure and development stages of free-living amoebae

Amoebae are unicellular, motile, eukaryotic protozoa. Most free-living amoebae are ubiquitous organisms that do not require a host to survive (Cateau et al., 2014), but in some cases intermittently invade a host and live as parasites within host tissue (Page, 1988; Schuster et al., 2004; Khan, 2006; Teixera et al., 2009). FLA have two developing stages- the vegetative trophozoite feeding form and the resting cyst form. During the metabolically active trophozoite stage, amoebae feed on bacteria and multiply by binary fission. Inside the amoeba, resistant bacteria can survive, multiply, and be protected from harmful toxicants (i.e., chlorine), especially when the host amoeba forms cysts along with the bacteria inside (Strassmann & Shu, 2017). Amoeba form cysts due to adverse environmental conditions such as unfavourable pH, osmotic pressure, and intolerable temperatures, but excyst again whenever environmental conditions become favourable (Greub & Raoult, 2004). As a food source amoebae consume bacteria by engulfing them during phagocytosis. and inside the phagosome, bacteria undergo acidification, oxidative stress, nutrient deficiency and encounter various antimicrobial molecules -potassium, sodium, calcium, iron, manganese, copper and zinc (Haas, 2007; Cosson and Lima, 2014; German, et al., 2013).

Pathogenicity of free-living amoebae

The study of pathogenic FLA has gained much interest throughout the world due to their possible health implications (Majid et al., 2017). FLA may cause several infectious diseases in humans, and genera such as *Acanthamoebae*, *Naegleria*, *Balamuthia*, and *Sappinia* are pathogenic to humans (Rodríguez-Zaragoza, 1994; Visvesvara et al., 2007). *N. fowleri*, *Acanthamoeba* spp., and *B. mandrillaris* cause fatal amoebic meningoencephalitis by acquiring different routes of entry to the brain and can pass the blood-brain barrier and evade the immune system. *Acanthamoeba* spp. cause keratitis, a common infection of contact-lens wearers. *Naegleria fowleri* infections have been found in recreational water users, including swimmers, divers, and water skiers, and among healthy children and adults, causing significant nerve damage and subsequent central nervous system tissue damage often resulting in death (Grace et al., 2015). *Acanthamoeba*, *Balamuthia* and *Naegleria* cause central nervous system infections in animals (Govinda et al., 2007).

Willaertia magna is a thermophilic and non-pathogenic FLA that can internalize both *Legionella pneumophila* and its putative protozoan vectors *Vermamoeba* (Hasni et al., 2019). Because of *W. magna*'s ability to digest the pathogenic bacterium *L. pneumophila,* this amoeba has been proposed for use as a natural biocide to control *L. pneumophila* (Hasni et al., 2020). *W. magna* has been isolated from thermal swimming pools (Hasni et al., 2020), and has the capacity to grow at high temperature (up to 44°C) (Hasni et al., 2020). The co-existence of pathogenic bacteria and amoebas in the aquatic environment can facilitate the horizontal gene transfers between these microorganisms (Hasni et al., 2019). *W. magna* has been shown to share genes with bacterial species isolated from these amoebae in environmental samples, such as *Mycobacterium* spp., *Pseudomonas* spp., *Acinetobacter* spp. (Pagnier et al., 2008). These results suggest a substantial amount of horizontal gene transfers between *W. magna* and amoeba-resistant bacteria (Hasni et al., 2019). *W. magna* is phylogenetically close to *Naegleria* species, including *N. fowleri*, an opportunistic pathogen that caused primary amoebic meningoencephalitis, a rare but deadly disease in humans (Baral and Vaidya, 2018; Stubhaug et al., 2016). A putative virulence gene of *N. fowleri* is shared with *W. magna* C2c (Hasni et al., 2019). Therefore, the non-pathogenic *W. magna* could be a carrier of antimicrobial resistant genes among pathogenic bacteria. To date there are no studies that have found interactions between *W. magna* and *P. aeruginosa*.

Ecology of free-living amoebae

Free-living amoebae are widely distributed in the environment, and are common inhabitants of freshwater microbial ecosystems (Rodriguez-Zaragoza et al., 1994; Khan, 2006). FLA have been isolated from water (seawater, fresh water, drinking water), soil, air, (droplets and aerosol) and sewage (Greub & Raoult, 2004; Sawyer, 1980; Trabelsi et al., 2012), and can survive and proliferate in artificial aquatic environments and engineering water systems including domestic water supplies (Jeong and Yu, 2005; Rohr et al.,1998; Thomas et al., 2006, Schuster & Visvesvara, 2004; Khan, 2006; Teixera et al., 2009). FLA can be found in hot tubs and cooling towers (Barbaree et al., 1986; Berk et al., 2006), swimming pools (Vesaluoma et al., 1995) and hydrotherapy baths (Scaglia et al., 1983). Free-living amoebae associated with bacterial biofilms are found in a broad range of habitats, including water pipes and filters,

dental unit waterlines and the oral cavity (Arndt et al., 2003; Marciano-Cabral & Cabral, 2003; Parry, 2004).

The FLA genera frequently found in water are *Hartmannella*, *Vahlkampfia*, *Paratetramitus*, *Naegleria*, *Adelphamoeba, Echinamoeba,* and *Acanthamoeba* (Kyle and Noblet, 1986). The survivability of FLA in water depends on surface availability, pH changes, temperature, organic matter contents and salinity of the water (Kyle and Noblet,1986; Rodríguez-Zaragoza, 1994). In water, amoebae may exhibit a flagella stage to swim or attach to the suspended particulate matter to feed (Rodriguez-Zaragoza,1994). Cyst-forming FLA can survive longer during periods of food shortage (Rodríguez-Zaragoza, 1994). Various FLA are resistant to environmental adversities and germicides by forming cysts (Guimaraes et al., 2016). FLA species typically tolerate temperatures between 10°C and 30°C, but below 4°C only cold-resistant strains may survive, such as *Acanthamoeba polyphaga*, which has been isolated from frozen Antarctic lakes (Brown, 1977; Moore, 1988). *Acanthamoeba* is the only known pathogenic amoebae that can survive in marine aquatic environment within deep ocean sediments (Sawyer, 1980; Sawyer et al., 1992; Sawyer et al., 1993). *Acanthamoeba* has been recovered from drinking water (Hoffmann and Michel, 2001; Michel et al., 1998), cooling towers (Barbaree et al., 1986), natural thermal water (Rivera et al., 1990; Rivera et al., 1989), swimming pools (De Jonckheere, 1979), hydrothermal baths (De Jonckheere, 1982; Scaglia et al., 1983) and hospital water systems (Rohr et al., 1998).

Interaction between free-living amoebae and bacteria

Many pathogenic bacteria (e.g., *Legionella* spp., *Listeria* spp., *Mycobacterium* spp.) have the capacity to resist free-living amoebae (Rubeniņa et al., 2017) via evolving pre- (extracellular) and post-(intracellular) ingestional adaptations to survive in food vacuoles of amoebae (Jürgens and Matz, 2002). Intracellular resistant bacteria, also called amoebae-resistant bacteria (ARB), use the amoebae as a "training ground" to test resistance (Greub and Raoult, 2004; Thomas et al., 2006).

To escape from protozoan predation, bacteria have developed various extracellular and cell-associated virulence factors (Sun et al., 2018; van Delden, 2004). In order to avoid

internalization by amoebae phagocytes, extracellular bacteria secrete toxins and form biofilm (Sun et al., 2018). Other extracellular anti-protozoal mechanisms are filament formation and increased swimming speed and surface masking (Matz and Kjelleberg, 2005). Many bacteria including *P. aeruginosa* have developed anti-digestion mechanisms that support survival inside protozoa (Gong et al., 2016; Paquet and Charette, 2016). In mature phagosomes, protozoa use the toxicity of Cu and Zn to kill bacteria. Some bacteria can avoid damage induced by these metals using efflux pumps (German et al., 2013). Free-living amoebae have been found to be a reservoir host for many pathogenic bacteria (Molmeret et al., 2005; Scheid, 2014; Strassmann & Shu, 2017). FLA act as "Trojan horses" for pathogenic bacteria (Cabrero-Martínez et al., 2018). The bacterial pathogen can enter a human host undetected when they are within the amoebae, protecting the pathogen from the first line human defence and then cause widespread infections (Greub and Raoult, 2004).

Free-living amoebae are natural amplifiers of a broad range of intracellular and opportunistic bacterial pathogens (Clarholm, 1981; Thomas et al., 2014; Rodriguez-Zaragoza, 1994; Huws et al., 2008; Evstigneeva et al., 2009; Dey et al., 2012). Microorganisms use amoebae as a suitable replication environment and spread in water and environment through their amoebal host (Greub and Raoult, 2004). FLAs' role in the bacterial community is a neglected factor in water regulatory guidelines and building codes (ASHRAE, 2015; Bartram et al., 2007). Opportunistic human pathogens *Stenotrophomonas maltophilia, P. aeruginosa* and *Burkholderia cepacia* were found coupled with amoebae (*Tetramitus, Micriamoeba*, *Willaertia*, *Acanthamoeba*) and demonstrated antibiotic resistance phenotype and were virulent (Denet et al., 2017).

Biofilm and Quorum sensing

P. aeruginosa can rapidly colonize and kill biofilm-associated amoebae by quorum sensing (the quorum sensing mechanism described in section 1.1.2.3) and type III secretion system (T3SS) (Matz et al., 2008). Biofilms and microcolonies have been found to enhance *P. aeruginosa* persistence in the presence of amoebae (Matz et al., 2004; Matz and Kjelleberg, 2005) and *P. aeruginosa* biofilms have been shown to impede *Acanthamoeba* spp. grazing (Weitere et al., 2005). Matz et al. (2008) demonstrated that *A. castellanii* can be killed by *P.*

aeruginosa PAO1 biofilm formation, and consequently, biofilm formation (described in section 1.1.2.3) increases *P. aeruginosa* resistance to amoeba (Mulcahy et al., 2014).

Anti-Phagolysosome Activity

During phagocytosis, both the phagocytic protozoa and the microorganism attempt to defeat each other (Uribe-Querol & Rosales, 2017). In the course of phagocytosis, the host cytoplasmic phagolysosome body is formed by the fusion of a phagosome with a lysosome. The membrane of the phagosome and lysosome interact, and the lysosome content, including hydrolytic enzymes, are discharged into the phagosome. The digestion process of microorganism possesses various complicated mechanism. The V-ATPase (Vacuolar H^+-ATPase) molecule on amoebae food vacuoles membrane continues to accumulate and the phagolysosome interior becomes acidic (pH <4.5), as more H^+ are translocated into the lumen of the phagosome (Uribe-Querol & Rosales, 2017). The low pH activity of hydrolytic enzymes (e.g., cathepsins, proteases, lysozymes, lipases and scavenger proteins) can kill bacteria, e.g., lactoferrin traps iron required by some bacteria to live. The phagolysosome also contains several lysosomal hydrolases that enable the digestion of microbial pathogens (Schröder et al., 2010). For example, lysozyme degrades peptidoglycan by breaking the bonds between N-acetylmuramic acid and N-acetyl-D-glucosamine and other enzymes complete the bacterial cell lysis (Ganz & Lehrer, 1997).

Digestion occurs, and digested materials (e.g., bacteria, dead tissue, and mineral particles) are moved to the cytoplasm. Any undigested materials are exported from the cell by exocytosis. The membrane fusion of phagosome and lysosome is regulated by the Rab5 protein, which allows the exchange of material between phagosome and lysosome and prevents a complete fusion of their membranes.

Bacteria possess a variety of anti-phagocytic strategies that enable them to resist and escape from phagocytes (Flannagan et al., 2009; Smith & May 2013). Some approaches include interfering with phagosome maturation resistance to phagolysosome contents and physical escape from the phagosome (Uribe-Querol & Rosales, 2017). Other bacteria can inactivate the host cell phagosome machinery surrounding the phagosome by preventing actin

polymerization, a process fundamental to form a phagocytic cup that extend the membrane projection around the target organism (Uribe-Querol & Rosales, 2017). Some pathogens can block phagosome maturation by blocking the acidification, reducing activation of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and preventing phagosome to lysosome fusion (Uribe-Querol & Rosales, 2017). For example, *Mycobacterium tuberculosis* inhibits acidification by preventing V-ATPase accumulation on the phagosome membrane (Sturgill-Koszycki et al., 1994).

Inside the phagosome, microbial growth can be limited by reducing the amount of essential nutrients by capture molecules (e.g., lactoferrin captures Fe^{2+}) (Ward et al., 2002; Ward et al., 2005) or transporters (natural resistance-associated macrophage protein1 (NRAMP-1) transports Mn^{2+}) (Cellier et al., 2007). In response to this nutrient capture, microorganisms have evolved mechanisms to retain these nutrients and produce siderophores that remove Fe^{2+} from host proteins, e.g., *P. aeruginosa* produces a yellowish-green fluorescence extracellular compound called pyoverdin, which functions as a siderophore (Cox and Adams, 1985).

SOS Response

The SOS response of bacteria controls DNA repair and damage. The SOS response is an inducible DNA repair pathway (Podlesek and Žgur, 2020) and one of the essential transcriptional responses to environmental stress in bacteria. During the battle between amoebae and bacteria, several exogenous and endogenous factors trigger the SOS response. Exogenous factors include UV irradiation, oxidative compounds, acids, organic mutagens, antibiotics, and physical stressors. Simultaneously, the replicative helicase continues untwisting DNA, and the replisome (an enzyme complex that replicates DNA) bounces over template lesions on the leading and lagging strands (Indiani, 2013). The bacterial response to DNA damage (SOS response) involves two proteins: LexA (a repressor) and RecA (an inducer). The response of LexA is switched off while the cells are healthy; RecA is active when any DNA damage occurs. In a healthy bacterial cell, the LexA protein binds to the "SOS box" which is coded for by more than 50 genes. Meanwhile, RecA floats around the cell, looking for damaged DNA. If any damaged DNA is found, it binds to it and stimulates the breaking of the LexA protein from the SOS box (Figure 5). In order to repair damaged DNA, the SOS box

also turns on proteins that promote hibernation or dormancy of the cell to form biofilms, and in some bacteria, it turns on the production of antibiotic resistance genes (Žgur-Bertok, 2013; Van der Veen & Abee, 2010).

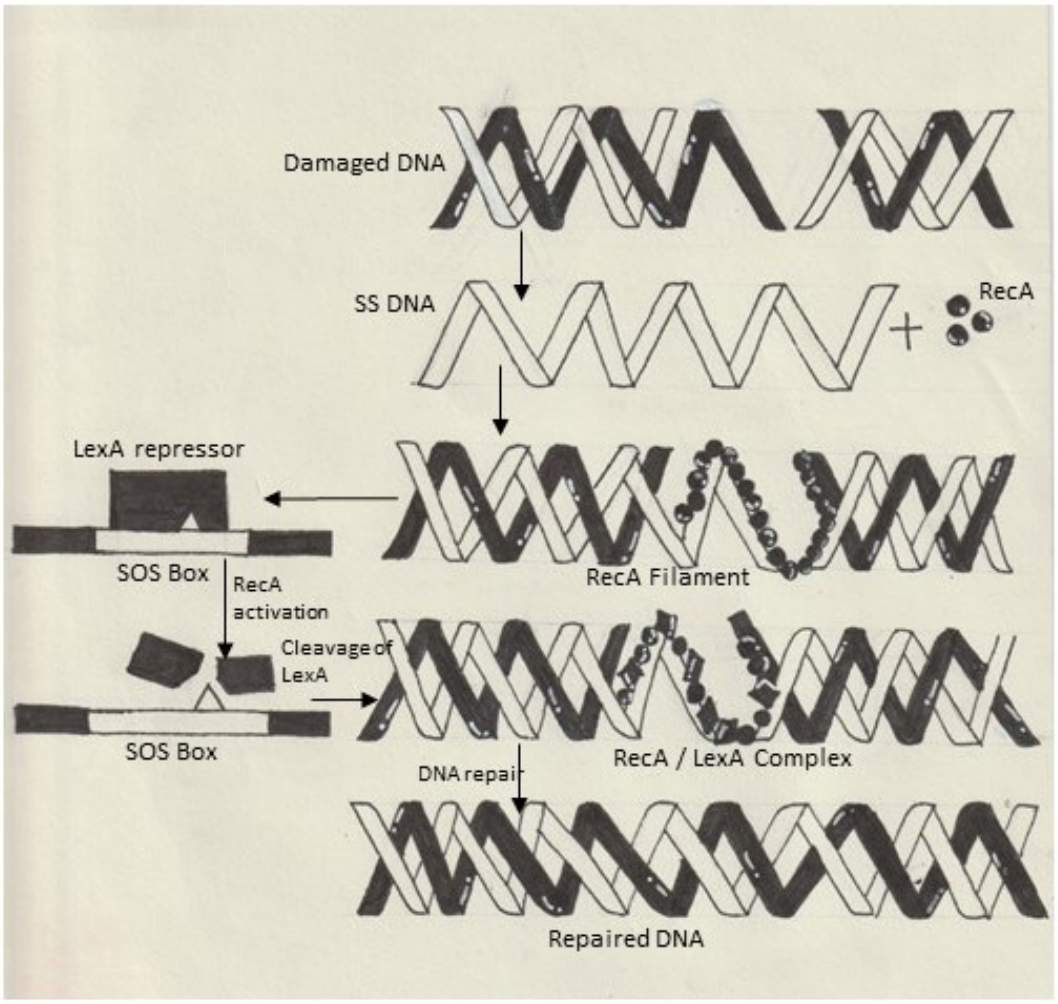

Figure 5: The SOS system has enhanced DNA-repair capacity. LexA binds to the SOS box, blocking transcription of SOS genes. When activated by DNA damage, the co-protease RecA causes LexA to self-cleave and vacate the SOS box, allowing expression of SOS genes (adapted from Memar et al., 2020).

Free-living amoebae promote extracellular DNA uptake by P. aeruginosa

Many naturally competent bacteria are capable of actively transporting extracellular DNA fragments through their cell envelope and cytoplasm via horizontal gene transfer and access to the nutrients in extracellular DNA (Mell & Redfield, 2014). Naturally competent bacteria actively pull DNA fragments from their environments into their cells. In Gram-negative

bacteria, DNA uptake happens in two stages-DNA uptakes across the outer membrane and DNA translocation across the inner membrane. The double-strand extracellular DNA is bound at the cell surface, then DNA is pulled through the type II secretion pore by retraction of cell surface fibres of the T4P family. One strand of the DNA is translocated intact into the cytoplasm by the Rec2/ComEC protein, and the other is degraded. The new strand recombines with a homologous sequence in the chromosome, displacing the resident strand (Mell & Redfield, 2014).

The requirements of DNA uptake depends on the nutritional needs of the recipient cell, the occurrence of DNA damage, the capability of incoming DNA to recombine with chromosomal DNA, and the impact of this recombination on fitness (Mell & Redfield, 2014). The battle between bacteria (e.g., *P. aeruginosa* in our case) and amoebal phagosomes may damage bacterial DNA. During phagolysosome fusion, RNS (reactive nitrogen species) produces peroxynitrite ($ONOO^-$) which can damage DNA. The need to repair damaged DNA promotes DNA uptake through transformation from the environment, and it is possible that the presence of amoebae (e.g., *A. polyphaga* and *W. magna*) in association with *P. aeruginosa* might promote DNA uptake from the environment.

1.2 Water quality of hot tubs and whirlpools and public health risks

Hot tubs and whirlpools act as reservoirs of *P. aeruginosa* and numerous nosocomial and community outbreaks associated with use of these facilities have been reported (Zichichi et al., 2000; Berrouane et al., 2000). The dissemination of pseudomonad infections in hot tubs and whirlpools water is related to many factors including mode of contamination, water quality maintenance, plumbing and drainage systems, complex and hard to clean piping, high temperature, aeration and agitation (Lutz and Lee, 2011).

Water quality maintenance

Hot tub and whirlpool water temperature ranges are from 35°C-45°C, and the in-use temperature limit is ≤40°C (Health Canada, 2015), temperatures that are ideal for *P.*

aeruginosa growth. Disinfection procedure may eliminate other microbes susceptible to disinfectants, leaving a niche opportunity for *P. aeruginosa* (Bédard et al., 2016). Whenever disinfectant levels fall below recommended levels in hot tubs, endogenous *P. aeruginosa* can replicate in the water (Bodey et al., 1983). The higher temperature, combined with the added agitation and aeration of hot tubs water, and biofilm growth may cause rapid degradation of chlorine levels, resulting in chlorine-based disinfectants being ineffective for control of *P. aeruginosa* (Muraca et al., 1987; Price and Ahearn, 1988).

Plumbing and drainage systems

The piping systems of swimming pools and hot tubs have intermittent recirculation and are more complicated than standard sinks and showers. Multifaceted piping makes cleaning more problematic. Higher *P. aeruginosa* positivity are typically found in swab samples taken from side-wall tiles, gutter drains, jets, and strainer baskets, rather than in water samples themselves, indicating that higher concentrations can exist in the biofilm (Lutz and Lee, 2011). The drain close beneath the level of hot tubs and whirlpools may be an unrecognized source of *P. aeruginosa* infections (Yasmina et al., 2000). The area of the drain attached to the tub stays moist, providing a suitable environment in which *P. aeruginosa* form microcolonies under a protective slime layer. When water is added during the use of hot tubs, *P. aeruginosa* colonies in the drain may become dislodged (Yasmina et al., 2000).

Public health risks in hot tubs for P. aeruginosa infections

P. aeruginosa causes folliculitis and acute otitis externa among bathers in hot tubs and pools, and outbreaks are largely caused by serotype O11 strains (Roser et al., 2014); albeit, other serotypes can also cause infections (Maniatis et al., 1995; Highsmith et al., 1985). *P. aeruginosa* associated infection among hot tubs users include respiratory infections, Pontiac fever, gastrointestinal disease, urinary tract infections, ear infections, and eye and skin infections (Darla et al., 2007). Forty-seven outbreaks associated with treated recreational water (hot tubs/spas and water playgrounds), were associated with *Pseudomonas* spp. infection between 2000-2014 in the USA and in Puerto Rico ((Hlavsa et al., 2018). In an outbreak in Tennessee among 60 members who regularly used a health spa, 37 developed otitis externa and mastitis due to infection from *P. aeruginosa* serotype O11associated with a swimming

pool that had not been chlorinated for two days due to equipment malfunction (Gustafson et al., 1983). Fifteen out of twenty-five people were infected with *Pseudomonas* after using a hotel pool or hot tub in Colorado, USA, and all were positive for *P. aeruginosa* or other *Pseudomonas* species (CDC, 2000). In another study, 108 (50 water and 58 swab) samples were obtained from eight indoor swimming pools and three hot tubs from central Ohio, USA. Twenty-three samples were positive for *P. aeruginosa*, of which 16 were from hot tubs. *P. aeruginosa*-positive samples were much more likely to come from swabs than from water samples. The swab sample's location included side-wall tiles, gutter drains, jets and strainer baskets indicated biofilm-rich location (Lutz & Lee, 2011). In a separate study, of 40 bathers, 25 developed *Pseudomonas* folliculitis from *P. aeruginosa* contamination after 2-4 days of exposure from swimming pools or whirlpools in Israel, which had not been chlorinated and monitored routinely (Cohen-Dar et al., 2012). An outbreak of folliculitis caused by *P. aeruginosa serotype* O7 occurred among 26 out of 36 people who used a hotel whirlpool in Newfoundland, Canada (Ratnam et al., 1986).

1.3 Scope of the Study

The primary aim of my research was to generate novel data on antimicrobial-resistant *P. aeruginosa* from water in hot tubs and whirlpools in Alberta. Secondly, the thesis examined the persistence and transformability of extracellular DNA in *P. aeruginosa* from water in hot tubs and whirlpools, a phenomenon that has not yet been well studied (Lutz and Lee, 2011). Antimicrobial-resistant extracellular DNA, present in water from hot tubs and whirlpools, could help transform *P. aeruginosa*, leading to amplification and dissemination of antibiotic resistant phenotypes in environmental systems. Therefore, this study also examined the natural competency of extracellular DNA uptake in *P. aeruginosa* collected from hot tubs and whirlpools from plasmids carrying antibiotic resistance genes, and their ability to stably transform. Thirdly, antibiotic-resistant *P. aeruginosa* interactions with free-living amoeba are unknown (Greub and Raoult, 2004), and this research attempted to examine whether free-living amoebae present in hot tubs and whirlpools water could enhance early extracellular DNA uptake by *P. aeruginosa*.

The goals of this thesis were to:

- Characterize antimicrobial-resistants *P. aeruginosa* from water in hot tubs and whirlpools in central southern Alberta, and assess carriage of antibiotic-resistant genes present among those *P. aeruginosa* isolates.

- Determine whether extracellular DNA carrying antimicrobial resistance genes can transform *P. aeruginosa* isolates into resistant phenotypes within biofilms.

- Determine whether extracellular DNA carrying antimicrobial resistance genes can transform *P. aeruginosa* in the presence of amoebae (e.g., *A. polyphaga* and *W. magna*) in a biofilm environment, and how amoebae might impact transfer of extracellular DNA to *P. aeruginosa* isolates.

This study will help improve our understanding of the health risks associated with water in hot tubs and whirlpools systems due to the growth and activity of *P. aeruginosa* by addressing antibiotic resistance, horizontal transfer of AMRs genes and co-existence with free-living protozoa.

2. Materials & Methods

2.1 Antimicrobial resistance profiling of *P. aeruginosa*

2.1.1 Sample collection, preparation, and storage condition

Individual isolates of *P. aeruginosa* were obtained from the Alberta Provincial Laboratory for Public Health (ProvLab), in Edmonton, Alberta. Three hundred fifty-three *P. aeruginosa* isolates came from water in hot tubs and whirlpools in southern Alberta, where the ambient water temperature was >30°C and were collected between April 2014 to May 2015. Individual water samples were processed by membrane filtration and grown on m-PAC agar (Thermo Fisher Scientific, USA) for the selective recovery and enumeration of *P. aeruginosa*. Out of 353 *P. aeruginosa* isolates, 84 were selected randomly for purity check and were sub-cultured on tryptic soy agar (TSA [Difco™, France]). Pure cultures were confirmed as being *P. aeruginosa* by comprehensive biochemical testing using a Vitek-2 Automated Bacterial Identification System (bioMerieux, France).

From the selective 84 isolates, 80 isolates were confirmed as *P. aeruginosa*, from where, 45 isolated were randomly chosen for antimicrobial susceptibility tests using Kirby-Bauer's disk diffusion method on Mueller-Hinton agar (Difco™, France). The method, guidelines and zone diameter breakpoints limits ("A breakpoint is a chosen concentration (mg/L) of an antibiotic which defines whether a species of bacteria is susceptible or resistant to the antibiotic") were followed according to CLSI (CLSI, 2018). If the MIC is less than or equal to the susceptibility breakpoint the bacteria is considered susceptible to the antibiotic. If the MIC is greater than this value the bacteria is considered intermediate or resistant to the antibiotic (BSAC, 2021), the designations of resistance/susceptible were based on the Clinical and Laboratory Standards Institute (CLSI, 2018). Antimicrobial susceptibility results were validated by testing the reference strain *P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922, *E. coli* ATCC 35218 and *S. aureus* ATCC 25923 against 36 selected antimicrobial agents. The 45 *P. aeruginosa* hot tub isolates and the reference strains were stored at -80°C until used.

2.1.2 AMR profiling using Kirby-Bauer's disk diffusion method

Select *P. aeruginosa* isolates were enriched on TSA medium at 37°C for 18-24 h. Three to five well-isolated colonies were selected from the TSA plates and transferred to 5 mL phosphate-buffered saline (PBS) medium by sterile loop and mixed well. The suspension density was adjusted to achieve a 0.5 McFarland standard using a DensiCHEK Plus Standards Kit (Biomérieux, New Zealand). Within 15 min after adjusting the inoculum suspension's turbidity, a sterile cotton swab was dipped, rotated several times, and pressed firmly on the inside wall of the tube above the fluid level to remove excess inoculum from the swab. The swab was streaked over the whole surface of the 150 mm Mueller-Hinton agar (MHA) plate, and the lid was kept partially open for 3 to 5 min to absorb the moisture. Antimicrobial discs (purchased from Oxoid™, Thermo Scientific™) were dispensed using an antimicrobial susceptibility disk dispenser (Thermo Scientific™ Remel™, 150 mm, 12-place). After confirming that the discs were evenly distributed at least 24 mm apart, a maximum of 12 discs were placed on one 150 mm MHA plate and the plates were incubated at 37°C within 15 min after the discs were applied. The plates were examined after 16-18 h of incubation. The complete inhibition zone diameter and the disc's diameter was measured to the nearest whole

millimeter using a ruler. The inhibition zones' sizes were interpreted by referring to Tables 2A-2I of Clinical and Laboratory Standards Institute (CLSI, 2018). Thirty-six antibiotics belonging to ten different classes were tested. Each of the 45 *P. aeruginosa* isolates was tested in triplicate against 36 antibiotics, and then the whole experiment was conducted three times. For each run *P. aeruginosa* ATCC 27853 was used as positive control, *E. coli* ATCC 25922, *E. coli* ATCC 35218, and *Staphylococcus aureus* ATCC 25923 were used as negative controls.

Table 1: Antibiotics and their associated concentrations used for the susceptibility testing of *P. aeruginosa* isolates (Thermo Scientific, Oxoid, USA).

Antibiotic Classes	Antibiotics	Concentration (µg/ml)
Cell wall inhibitors	Ampicillin	10
	Cloxacillin	5
	Mecillinam	10
	Vancomycin	5
	Polymyxin B	30
	Colistin Sulphate	25
Protein synthesis inhibitors	Streptomycin	10
	Chloramphenicol	30
	Tetracycline	30
	Erythromycin	15
Carbapenems	Ertapenem	10
	Doripenem	10
	Imipenem	10
	Meropenem	10
Fluoroquinolone	Ciprofloxacin	5
	Enrofloxacin	5
	Levofloxacin	5
	Ofloxacin	5
	Lomefloxacin	10
	Norfloxacin	10
Cephalosporins	Cefotaxime	30
	Cephalothin	30
	Cefepime	30
	Ceftazidime	30
Folic acid synthesis inhibitors	Sulfamethoxazole/Trimethoprim	25
	Trimethoprim	5
Nitrofurantoin	Nitrofurantoin	100
Monobactams	Aztreonam	30
Penicillin	Piperacillin	100
	Ticarcillin	75
	Piperacillin /Tazobactam	110
Aminoglycosides	Amikacin	30
	Tobramycin	10
	Gentamicin	10
	Fosfomycin	50
	Netilmicin	30

2.1.3 PCR analysis of P. aeruginosa isolates to detect antimicrobial resistance genes

The 45 isolates of *P. aeruginosa* were analyzed for antimicrobial resistance genes by PCR analysis. *P. aeruginosa* ATCC 27853 was used as a positive control for targeted 33 antimicrobials resistant genes and *E. coli* ATCC 25922, *E. coli* ATCC 35218, and *S. aureus*

ATCC 25923 as negative controls. No template DNA was used as a blank sample for PCR reactions.

A loopful of *P. aeruginosa* inoculum was taken from the LB agar plate and added to 100µL nuclease-free water in a 96 well plate. The plate containing all the *P. aeruginosa* isolates, positive and negative controls, was boiled at 95°C for 10 min. The lysates were transferred to Eppendorf tubes and centrifuged at 5000 rpm for 5 min and the supernatant removed. The cell lysates were kept at -20°C until further use.

PCR amplification was conducted to identify the genes responsible for the resistance of *P. aeruginosa*. Each reaction was performed using a 25µL mixture (final concentration) consisting of 12.5µL of GoTaq G2 Hot Start colourless master mix (Promega, USA), 1µL forward and 1µL reverse primer, 8.5 µL nuclease-free water and 2µL template DNA. Denaturation occurred for 2 min at 95°C followed by 35 cycles of 1 min at 95°C, 30 s at 63°C and 1 min at 72°C with a final extension step of 10 min at 72°C. All thermal cycling conditions were executed using a three-step cycling protocol. The PCR assay was run on 3% agarose gel electrophoresis with SYBR®Safe (Invitrogen, UK) DNA Gel staining. The same PCR conditions were maintained for all the 33 genes.

Gel electrophoresis

The agarose gel was prepared from 0.75g of GelPilot® LE Agarose (Qiagen, Toronto, ON, Canada) and added 50 mL of 50X TAE buffer (1L 50X TAE stock, 242g Tris base, 57.1mL acetic acid, 100 mL 0.5M EDTA). The mixture was microwaved and intermittently swirled in the solution until all particles were dissolved. The agarose solution was poured into a casting stand, and one µl of SYBR Safe DNA stain (Invitrogen, ThermoFisher Scientific, USA) per 10 mL of agarose gel was added to the solution before cooling. The gel was set at room temperature (22°C-25°C) for 30 min, and wells were loaded with 10µL of the sample with 2µL of GelPilot® Loading Dye (Qiagen, Toronto, ON, Canada) and 3 µL of GelPilot® 1kb Plus Ladder (Qiagen, Toronto, ON, Canada). The agarose gel was run at 110V for 50 min and imaged using the Bio-Rad ChemiDoc™ MP imaging system (Bio-Rad, Mississauga, ON, Canada) to look for the presence of targeted genes).

Table 2: Nucleotide sequences of primers used for PCR amplification of AMR *P. aeruginosa* gene screening.

No.	Gene Type	Gene	Primer	Sequence (5` - 3`)	Annealing Temp	Reference	Sample type & source
1	Porin	oprL	oprL-F oprL-R	ATGGAAATGCTGAAATTCGGC CTTCTTCAGCTCGACGCGACG	57	De Vos et al. (1997)	*P. aeruginosa*, Clinical
2.	Porin	oprD	oprD-F oprD-R	CGTCGCTTCGGAACCTCAACTA GCCGTGACCTCGAACCTGA	63	Schiavano et al. (2017)	*P. aeruginosa*, Swimming Pool.
3.	QS	gyrB	gyrB-F gyrB-R	CCTGACCATCCGTCGCCACAAC CGCAGCAGGATGCCGACGCC	57	Aghamollaei et al. (2015)	*P. aeruginosa*, Clinical
4.	QS	lasI	lasI-F lasI-R	ATGATCGTACAAATTGGTCGG GTCATGAAACCGCCAGTC	57	Aghamollaei et al. (2015)	*P. aeruginosa*, Clinical
5.	QS	lasR	LasR-F LasR-R	ATGGCCTTGGTTGACGGT GCAAGATCAGAGAGTAATAAGA CCC	57	Aghamollaei et al. (2015)	*P. aeruginosa*, Clinical
6.	Efflux Pump	ampC	ampC-F ampC-R	CGGCTCGGTGAGCAAGACCTTC AGTCGCGGATCTGTGCCTGGTC	57	Ozer et al. (2012)	*P. aeruginosa*, Clinical
7.	Efflux Pump	mexC1,2	mexC1,2-F mexC1,2-R	ATCCGGCACCGCTGAAGGCTGCG CGGATCGAGCTGCTGGATGCGCG	57	Ozer et al. (2012)	*P. aeruginosa*, Clinical
8.	Efflux Pump	mexC3,4	mexC3,4-F mexC3,4-R	GTACCGGCGTCATGCAGGGTCC TTACTGTTGCGGCGCAGGTGACT	57	Ozer et al. (2012)	*P. aeruginosa*, Clinical
9.	Biofilm	ndvB	ndvB-F ndvB-R	GGCCTGAACATCTTCTTCACC GATCTTGCCGACCTTGAAGAC	56	Saffari et al. (2017)	*P. aeruginosa*, Clinical
10.	T6SS	tssC1	tssC1-F tssC1-R	CTCCAACGACGCGATCAAGT TCGGTGTTGTTGACCAGGTA	56	Saffari et al. (2017)	*P. aeruginosa*, Clinical

(Continued)

Table 2: (Continued)

No.	Gene Type/Target/Activity	Gene	Primer	Sequence	Annealing Temp	Reference	Sample Type & Source
11.	Effector Protein/Toxin	exoT	exoT-F exoT-R	AATCGCCGTCCAACTGCATGCG TGTTCGCCGAGGTACTGCTC	58	Kaszab et al. (2011)	*P. aeruginosa*, Soil
12.	Effector Protein/Toxin	exoY	exoY-F exoY-R	CGGATTCTATGGCAGGGAGG GCCCTTGATGCACTCGACCA	58	Kaszab et al. (2011)	*P. aeruginosa*, Soil
13.	Effector Protein/Toxin	exoU	exoU-F exoU-R	CCGTTGTGGTGCCGTTGAAG CCAGATGTTCACCGACTCGC	58	Kaszab et al. (2011)	*P. aeruginosa*, Soil
14.	Effector Protein/Toxin	exoS	exoS-F exoS-R	GCGAGGTCAGCAGAGTATCG TTCGGCGTCACTGTGGATGC	58	Kaszab et al. (2011)	*P. aeruginosa*, Soil
15.	T3SS	popB	popB-F popB-R	TTTGAATTCTCAGATCGCTGCCGGTCG TTTGAATTCTCAGATCGCTGCCGGTCG	55	Feltman et al. (2001)	*P. aeruginosa*, Clinical
16.	β-lactam, Biofilm	NDM	NDM-F NDM-R	ATGGAATTGCCCAATATTATGC CGAAAGTCAGGCTGTGTTG	57	Ismail & Mahmoud (2018)	*P. aeruginosa*, Clinical
17.	β-lactam-Carbapenemase	blaNDM-2	NDM-2-F NDM-2-R	GGTTTGGCGATCTGGTTTTC CGGAATGGCTCATCACGATC	52	Poirel et al. (2010)	*P. aeruginosa*, Clinical
18	β-lactam-Carbapenemase	blaNDM-1	NDM-1-F NDM-1-R	GGCGGAATGGCTCATCACGA CGCAACACAGCCTGACTTTC		Yong et al. (2009)	*K. pneumoniae*, Clinical
19.	β-lactam-Carbapenemase	blaIMP	IMP-F IMP-R	GGAATAGAGTGGCTTAAYTCTC GGTTTAAYAAAACAACCACC	52	Poirel et al. (2010)	*K. pneumoniae*, Clinical
20.	Metallo-β-lactamases	blaIMP-1	IMP-1-F IMP-1-R	ACCGCAGCAGAGTCTTTGCC ACAACCAGTTTTGCCTTACC	55	Shibata et al. (2003)	*P. aeruginosa*

(Continued)

Table 2: (Continued)

No.	Gene Type	Gene	Primer	Sequence	Annealing Temp	Reference	Sample Type & Source
21.	Metallo-β-lactamases	blaIMP-2	IMP-2-F IMP-2-R	GTTTTATGTGTATGCTTCC AGCCTGTTCCCATGTAC	55	Shibata et al. (2003)	*P. aeruginosa*
22.	β-lactam-Carbapenemase	blaSPM	SPM-F SPM-R	AAAATCTGGGTACGCAAACG ACATTATCCGCTGGAACAGG	52	Poirel et al. (2010)	*P. aeruginosa*, Clinical
23.	Metallo-β-lactamases	blaSPM-1	SPM-1-F SPM-1-R	GCGTTTTGTTTGTTGCTC TTGGGGATGGAGACTAC	55	Shibata et al. (2003)	*P. aeruginosa*
24.	β-lactam-Carbapenemase	blaAIM	AIM-F AIM-R	CTGAAGGTGTACGGAAACAC GTTCGGCCACCTCGAATTG	52	Poirel et al. (2010)	*P. aeruginosa*, Clinical
25.	β-lactam-Carbapenemase	blaVIM	VIM-F VIM-R	GATGGTGTTTGGTCGCATA CGAATGCGCAGCACCAG	52	Poirel et al. (2010)	*P. aeruginosa*, Clinical
26.	β-lactam-Carbapenemase	blaVIM-1	VIM-1-F VIM-1-R	AGTGGTGAGTATCCGACAG ATGAAAGTGCGTGGAGAC	55	Tsakris et al. (2000)	*P. aeruginosa*, Clinical. Imipenem, Meropenem resistant.
27.	Metallo-β-lactamases	blaVIM-2	VIM-2-F VIM-2-R	ATGTTCAAACTTTTGAGTAAG CTACTCAACGACTGAGCG	55	Poirel et al., 2000	*P. aeruginosa*, Clinical
28	β-lactam-Carbapenemase	blaOXA-48	OXA-F OXA-R	GCGTGGTTAAGGATGAACAC CATCAAGTTCAACCCAACCG	52	Poirel et al. (2010)	*Enterobacter cloacae*, Clinical
29.	Metallo-β-lactamases	blaGIM	GIM-F GIM-R	TCGACACACCTTGGTCTGAA AACTTCCAACTTTGCCATGC	52	Ellington et al., (2007)	*P. aeruginosa*, Reference strain

(Continued)

Table 2: (Continued)

No.	Gene Type	Gene	Primer	Sequence	Annealing Temp	Reference	Sample Type & Source
30.	Metallo-β-lactamases	blaBIC	BIC-F BIC-R	TATGCAGCTCCTTTAAGGGC TCATTGGCGGTGCCGTACAC	52	Ellington et al., (2007)	*P. aeruginosa*, Reference strain
31.	β-lactam-Carbapenemase	blaSIM	SIM-F SIM-R	TACAAGGGATTCGGCATCG TAATGGCCTGTTCCCATGTG	52	Poirel et al. (2010)	*A. baumannii*, Clinical
32.	β-lactam-Carbapenemase	blaDIM	DIM-F DIM-R	GCTTGTCTTCGCTTGCTAACG CGTTCGGCTGGATTGATTTG	52	Poirel et al. (2010)	*A. baumannii*, Clinical
33.	β-lactam-Carbapenemase	blaKPC	KPC-Fm KPC-Rm	CGTCTAGTTCTGCTGTCTTG CTTGTCATCCTTGTTAGGCG	52	Poirel et al. (2010)	*K. pneumoniae*, Clinical

2.2 Extracellular DNA transformation of *P. aeruginosa*

This portion of the study was conducted in static broth culture conditions at room temperature (22°C-25°C). A *P. fluorescens* strain carrying a green fluorescent protein (GFP) plasmid containing ampicillin and tetracycline-resistance was used as the original plasmid donor. An environmental *P. aeruginosa* isolate obtained from ProvLab (originally isolated from a contaminated hot tubs/whirlpools) was used as the plasmid recipient to assess competence of the strain in taking up plasmid DNA. *P. aeruginosa* ATCC 27853 was used as the positive control. The GFP *P. fluorescens* ATCC 13525 and *P. aeruginosa* ATCC 27853 were obtained from the American Type Culture Collections (ATCC).

2.2.1 Growth media, strains, and growth conditions

The *P. fluorescens* and *P. aeruginosa* strains were grown overnight at 37°C in LB broth containing, per litre, 10 g of Bacto Tryptone, 5 g of yeast extract, and 5 g of NaCl (Difco™, France) supplemented with the appropriate antibiotic concentrations (ampicillin 100 µg/ml).

Table 3: Bacterial strains and plasmids used in this study.

Strain or Plasmid	Resistance Phenotype	Use
P. fluorescens ATCC 13525 (GFP)	tet, amp	Donor cell
P. aeruginosa ATCC 27853	tet, amp	Recipient cell
P. aeruginosa isolate Strain 09 (hot tub isolate)	amp, erythromycin, cloxacillin, mecillinam, cephalothin	Recipient cell

2.2.2 Recipient inoculum preparation

From the stock culture collection (-80^{0}C) a loopful of the inocula was streaked on LB agar plate containing 100 µg/ml ampicillin and incubated overnight at 37^{0}C. After overnight enrichment, a colony was selected from the LB agar plate and diluted with 100 ml LB broth and incubated overnight at 37^{0}C in a shaking incubator with agitation at 200 rpm. A one ml overnight culture was diluted with 19 ml fresh LB broth, incubated for an additional 4h to achieve the bacterial growth log phase and then incubated for an additional two hours to an optical density of 0.6. These cultures were centrifuged for 10 min at 6,500 x g before resuspension in the appropriate sterile water volume (200 µl) of water was added in order to get the 100-fold increased concentration of bacteria.

2.2.3 Donor strain and plasmid extraction

The plasmid donor strain, a GFP strain of *P. fluorescens*, was grown aerobically at 37^{0}C overnight in LB broth supplemented with 100 µg/ml ampicillin with agitation at 200 rpm using a shaking incubator. The bacterial cells were centrifuged at 14000g for 10 min, and the cell pellets were washed twice with 0.9% sterile saline solution and resuspended in the same saline solution. The concentration of resulting donor inoculants was measured by colony-forming units (CFU) by counting on LB agar plates. The plasmid was extracted from *P. fluorescens* ATCC 13525 (GFP) containing *amp* and *tet* genes using the QIAprep Spin Miniprep Kit (Qiagen, Germany), according to the manufacturer's protocols. The concentration of the extracted plasmid DNA was measured by NanoDrop™ 2000/2000c spectrophotometer (Thermo Fisher Scientific, USA). Sterility of all plasmid DNA samples was verified before each use by plating on LB agar.

2.2.4 Transformation by electroporation

In preliminary experiments assessing electrocompetence of wildtype *P. aeruginosa* isolates from hot tubs, a single isolate was found to be particularly competent to uptake plasmids and with little effect on viability (known as Strain 09). This *P. aeruginosa* isolate was used as the plasmid recipient, and the strain was grown at 37°C in LB medium to a concentration of ~10^9-10^{10} viable bacteria/ml of medium and equally distributed into four microcentrifuge tubes. The cells were harvested by centrifugation at 4°C for three min at 14000xg. The cell pellet was washed twice with 10% glycerol. The washed pellet was resuspended on 500 µl of 10% glycerol and 500 ng purified plasmid DNA of *P. fluorescens* was mixed with 100 µl of electrocompetent cells of *P. aeruginosa* isolate and transferred to a 2 mm gap width electroporation cuvette. The pulse (setting: 25 µF, 200 ohms, 2.5 kV) was applied using a Bio-Rad Gene PulserXcell (Bio-Rad). After electroporation, one ml of LB broth was added, and cells were transferred to a small glass tube (13X100 mm) and shaken for 0, 1 and 2 h at 37°C. After incubation, the cells were harvested in a one ml microcentrifuge tube and 900 µl of the supernatant was discarded, and the cell pellet was resuspended in one ml LB broth. The mixture was then plated on LB agar plates containing 100 µg/ml ampicillin. The plates were incubated at 37°C for 24-48 h until colonies were visible. Cells electroporated without added DNA were used as a control.

2.2.5 Natural transformation protocols

P. aeruginosa Strain 09 cells were grown on LB agar plates from the stock culture and the plates were incubated overnight at 37°C. A prominent colony growing from an LB agar plate containing 100 µg/ml ampicillin was selected from the plate, added to 20 ml LB broth, and it incubated overnight at 37°C in a shaking incubator. From the enriched broth, one ml was taken and added to 200 ml of LB broth and incubated overnight at 37°C in a shaking incubator to achieve the exponential phase of *P. aeruginosa* growth.

The transformation broth was prepared by adding 25 ml of de-chlorinated sterilized Edmonton tap water to 25 ml of LB broth from the exponential *P. aeruginosa* phase and adding 10 µL of plasmid DNA at a concentration of 156 $\mu g.mL^{-1}$ (measured by NanoDrop™ 2000/2000c spectrophotometer, Thermo Fisher Scientific, USA), which was extracted from the GFP *P. fluorescens* ATCC13525. The assay was left at room temperature (22°C-25°C) for the natural occurrence of transformation. As a positive control for the wildtype transformation assay, the electroporated plasmid DNA containing *P. aeruginosa* isolates was used.

2.2.6 Monitoring the transformation

The natural transformation of GFP plasmid of *P. fluorescens* ATCC 13525 into the *P. aeruginosa* cells was checked every 24 h, and 25 µL of the broth from the assay was inoculated on LB agar plates containing 100, 200 and 500 µg/ml ampicillin and incubated overnight at room temperature (22°C-25°C). Colonies were checked under an epifluorescence microscope (EVOS® Imaging System, Thermo Fisher Scientific, USA). The visibility of GFP *P. aeruginosa* under a fluorescence microscope provided the verification of the transformation of plasmid DNA containing the *tet* and *amp* gene. As a positive control, the electroporated GFP *P. aeruginosa* was enriched in the same medium at room temperature (22°C-25°C). After 24 h 25 µL enriched broth from the assay was inoculated on LB agar plates containing 100, 200 and 500 μgml^{-1} ampicillin and incubated overnight at room temperature (22°C-25°C). The overnight enriched colonies were checked every 24 h for the first few weeks and every other day for 118 days under the epifluorescence microscope to observe how long the GFP plasmid was stable inside the recipient *P. aeruginosa* cell. The nutrient medium (20% LB broth) was added every week to keep the organism live.

2.2.7 PCR confirmation of P. aeruginosa

PCR amplification of DNA from the transformed *P. aeruginosa* isolates was conducted to confirm the transformation of the antimicrobial-resistant GFP plasmid gene to the *P. aeruginosa* isolate. The real-time PCR assay targeted the *gyrB* gene because *gyrB* has been designed and evaluated as a highly sensitive and specific assay for *P. aeruginosa* strains and closely related pseudomonads. The assay consisted of a highly sensitive and specific novel primer sets (gyrB_722F: GGCGTGGGTGTGGAAGTC, 400 nM and gyrB_788R: TGGTGAAGCAGAGCAGGTTCT, 400 nM) and a hybridization probe (gyrB_746MGB_P: FAM-TGCAGTGGAACGACA-NFQMGB, 250 nM) as described by Lee et al. (2011). The GFP transformed recipient *P. aeruginosa* isolate was cultured on LB agar containing ampicillin (100 µg/ml) and incubated overnight at 37^0C. The colonies from the LB agar plates were observed under an epifluorescence microscope to first verify fluorescence visibility, and the colonies showing fluorescence were selected for PCR confirmation. Individual colonies of the *P. aeruginosa* cultures were collected from the LB agar plate and were suspended in 500 µL of molecular grade water and boiled at 95°C on a hot plate for 10 min to lyse the cell and release the DNA. Then, the lysates were transferred to Eppendorf tubes and centrifuged at 5000 rpm for 5 min. The cell lysates were kept at -20°C until further use. 20 µL qPCR reactions were performed on the ABI 7500 Fast Real-Time thermocycler containing 15 µL Mastermix (final concentrations of 1X PrimeTime Gene Expression Master Mix (IDT), 200 µg/mL Bovine Serum Albumin (Sigma), 400 nM forward and reverse primers, 250 nM probe (IDT), nuclease-free water (Life Technologies, Thermo Fisher Scientific, USA) plus 5 µL of the template using MicroAmp Optical 96-well Reaction Plates (Life Technologies, Thermo Fisher Scientific, USA). The cycling conditions were 95°C for 3 min (holding) then 40 cycles of the following: 95°C for 5 s and 60°C for 30 s). The assay included *P. aeruginosa* (ATCC 27853) as a positive control and no template as a negative control for each PCR reaction to ensure that the reaction was contamination-free.

2.3 Extracellular DNA transformation to *P. aeruginosa* in the presence of free-living amoebae

2.3.1 Preparing P. aeruginosa for amoeba co-culture experiment

From the frozen state (-80°C) *P. aeruginosa* Strain 09 isolates were grown for 18 h at 37°C on LB agar, which contained, per litre, 10 g of Bacto Tryptone, 5 g of yeast extract, and 5 g of NaCl (Difco™, France) before co-culture experiments. A single colony was picked from the agar plate, and a suspension of *P. aeruginosa* in 5 ml dechlorinated filtered sterile Edmonton tap water was prepared to obtain 1 OD at 600 nm (10^9 CFU/ml).

2.3.2 Amoebae culture

Free-living amoeba *A. polyphaga* (human corneal scraping isolate, ATCC®30461) and *Willaertia magna* (ATCC®50035) were obtained from the American Type Culture Collection. The amoeba's vegetative trophozoites were obtained from the axenic culture in serum casein glucose yeast extract medium (SCGYEM) at 25°C for 96 h in 25 cm^2 cell-culture flasks before the experiments, as previously described (Nowakowska & Oliver, 2013). To obtain vegetative trophozoites, the SCGYEM was refreshed every 24 h before co-culture experiments. The amoeba trophozoites were resuspended in 15 ml conical tubes by scraping off the bottom of the cell-culture flasks. The trophozoites were centrifuged at 4000 g for 5 min, and the supernatants were removed, and the pellet washed three times with dechlorinated filtered sterile tap water. Pellets were then re-suspended in sterile tap water to a concentration of approximately $1x10^5 mL^{-1}$ trophozoites using a hemocytometer (Fisher Scientific™, Hampton).

2.3.3 Co-culture of A. polyphaga and W. magna with P. aeruginosa

A. polyphaga or *W. magna* trophozoites and *P. aeruginosa* cells were co-infected in a 500 µL capacity chamber slide (Thermo Scientific™ Nunc™ Lab-Tek™ II) to a multiplicity of infection (MOI) of 100 bacteria to 1 trophozoite in filtered sterile tap water containing 20% LB broth. Three milliliters of the mixed suspension were dispensed in 15 ml conical tubes and centrifuged at 500x g for 5 min to initiate physical interaction between *A. polyphaga* or *W. magna* trophozoites and *P. aeruginosa* cells and transferred to the chamber slides. The mixed

cultured chamber slides were incubated in triplicate at room temperature. The experiments were performed three times. The chamber slides provided direct observation of *P. aeruginosa* and amoeba interactions within real-time and within an *in vitro* system. The co-culture assay was run for 118 days. The nutrient medium (20% LB broth) was added every week to keep the organism live. From 0 days (2h) to 118 days the amoebae-*Pseudomonas* interaction was observed every day for the first few weeks and then every other day. Co-cultures were examined using the Invitrogen EVOS® FL Auto Imaging system (Thermo Fisher Scientific, USA).

2.3.4 Transformation of extracellular GFP plasmid DNA to P. aeruginosa isolates in the presence of FLA

Two amoebae, *A. polyphaga* and *W. magna*, were grown in chamber slides on 400 µL 20% SCGYEM mixed with dechlorinated tap water. The co-culture was performed with the *P. aeruginosa* isolate with each of the two amoebae. After 48 h of growth of the amoeba-*Pseudomonas* co-culture, the plasmid DNA of *P. fluorescens* (the plasmid DNA extraction procedure is described in section 2.2.3) was added (2 µL of concentration 156 µg/mL); no DNA was used as a negative control and fluorescent *P. aeruginosa* (electroporated GFP *P. fluorescens* plasmid DNA to *P. aeruginosa* isolate) was used as a positive control.

2.3.5 Fluorescent microscopy and image processing

The chamber slide containing biofilms with amoeba and *P. aeruginosa* was observed periodically by microscopy to check the amoeba-bacteria interactions, their physical location (in planktonic, in biofilm and in food vacuoles of the trophozoites) and the transfer of GFP plasmid extracellular DNA to *P. aeruginosa*. Random brightfield and fluorescent images and time-lapse videos of the flow-cell were taken during the study period.

3. Characterization of antimicrobial resistant *P. aeruginosa* contaminating hot tubs in Alberta

3.1 Introduction

Waterborne pathogens remain a universal health hazard. *P. aeruginosa* is an opportunistic water-based environmental pathogen (Ashbolt 2015b; Garvey et al., 2018) that causes a wide range of diseases including pneumonia and folliculitis, as well as eye and ear infections. It is recognized as a pathogen of major concern with respect to antibiotic resistance making infections difficult to treat. Susceptible individuals may be infected through aerosols, contact with contaminated water, and fomites (Rogues et al., 2007), and hot tubs and whirlpools are a known source of infection from *P. aeruginosa*.

There are several reasons why *P. aeruginosa* is prolific in hot tubs and whirlpools (refer to section 1.1.3.1). Hot tub piping systems are complex and inaccessible for cleaning, and this complexity may enhance biofilm formation (Berrouane et al., 2000). The elevated temperatures in these recreational water sources also permits human-adapted strains to potentially flourish in these systems. The presence of scale and corrosion in the inaccessible area of the piping systems, as well as water stagnation and low flow in the dead ends allow the formation of micro-niches that are protected from circulating disinfectants, thereby supporting biofilm growth.

To the author's knowledge, this is the first extensive study of hot tubs and whirlpools water from the central-southern Alberta, Canada for the presence of antimicrobial resistant *P. aeruginosa*, and there are limited studies overall on the prevalence of antibiotic-resistant *P. aeruginosa* in these recreational venues. Lutz & Lee (2011) conducted a surveillance study to determine the background prevalence and antimicrobial resistance profile of *P. aeruginosa* in swimming pools and hot tubs and found that 96% of the isolates were multidrug resistant. Therefore, the present research study could provide important information for understanding antibiotic prevalence in *P. aeruginosa* in hot tubs in Alberta, and help support a clinical approach to treating infection associated with these recreational venues.

3.2 Results and Discussion

A total of 45 *P. aeruginosa* isolates were collected from hot tubs and whirlpools in southern Alberta, Canada. Antibiotic resistance tests were performed according to CLSI (CLSI, 2018) standards to establish the susceptibility and resistance profiles of these 45 *P. aeruginosa* isolates against 36 antibiotics belonging to ten different classes. Among those 36 antibiotics, 21 were clinically relevant antibiotics represented by seven classes based on CLSI (2018). According to CLSI (2018), the everyday use of antibiotic groups for *P. aeruginosa* related to clinical therapy include cell wall inhibitors (polymyxin B), penicillins (piperacillin, ticarcillin), β-lactam combination agents (piperacillin-tazobactam), cephalosporins (cefepime, ceftazidime), monobactams (aztreonam), carbapenems (doripenem, imipenem, and meropenem), aminoglycosides (amikacin, tobramycin, gentamicin and netilmicin) and fluoroquinolones (ciprofloxacin, levofloxacin, ofloxacin, lomefloxacin and norfloxacin) (Bassetti et al., 2018). As such this portion of the study focused on characterizing phenotypic resistance to antibiotics, and genes involved in mediating resistance, across the 45 *P. aeruginosa* isolates collected from contaminated water in hot tubs and whirlpools in Alberta, Canada.

Table 4: Clinically relevant antibiotics with breakpoints diameter values (CLSI, 2018; Lutz & Lee, 2011).

Sl No	Name of the clinically important antibiotics for *Pseudomonas* treatment	Disk Content (µg/ml)	Interpretive Categories and Zone Diameter Breakpoints, nearest whole mm			Resistance observed in *P. aeruginosa* isolates from hot tubs (yes/no)
			S	I	R	
1	**Penicillins**	100	≥21	15-20	≤14	
	Piperacillin					ND*
2	**B-Lactam combined agents**					
	Piperacillin-tazobactam	100/10	≥21	15-20	≤14	No
	Ceftazidime-avibactam	30/20	≥21	-	≤20	No
	Ceftolozane-tazobactam	30/10	≥21	17-20	≤16	ND*
	Ticarcillin-clavulanate	75/10	≥24	16-23	≤15	ND*
3	**Cephems**					
	Ceftazidime	30	≥18	15-17	≤14	ND*
	Cefepime	30	≥18	15-17	≤14	ND*
4	**Monobactams**					
	Aztreonam	30	≥22	16-21	≤15	No
5	**Carbapenems**					
	Doripenem	10	≥19	16-18	≤15	Yes
	Imipenem	10	≥19	16-18	≤15	No
	Meropenem	10	≥19	16-18	≤15	No
6	**Aminoglycosides**					
	Gentamicin	10	≥15	13-14	≤12	No
	Tobramycin	10	≥15	13-14	≤12	No
	Amikacin	30	≥17	15-16	≤14	No
	Netilmicin	30	≥15	13-14	≤12	No
7	**Fluoroquinolones**					
	Ciprofloxacin	5	≥21	16-20	≤15	No
	Levofloxacin	5	≥17	14-16	≤13	No
	Norfloxacin	10	≥17	13-16	≤12	No
	Lomefloxacin	10	≥22	19-21	≤18	No
	Ofloxacin	5	≥16	13-15	≤12	No
	Gatifloxacin	5	≥18	15-17	≤14	ND*

**ND- Not determined; S-Susceptible, I-Intermediate, R-Resistant*

Among all 45 *P. aeruginosa* isolates collected from hot tubs in Alberta, resistance was observed to 15 of the 36 antibiotics tested in the laboratory (Figure 6). Of the 21 antibiotics listed as being clinically important for treating *P. aeruginosa* infections (Table 4), fifteen of these antibiotics were tested by disc diffusion growth inhibition assays for resistance among the isolates. Of the 15 clinically important antibiotics surveyed in this study and listed in Table 4, resistance was only observed against doripenem (carbapenems) and in only one isolate [2% frequency] (Table 4, Figure 6). This doripenem-resistant isolate was also resistant to the carbapenem known as ertapenem (Figure 6), but susceptible to other carbapenem class drugs (imipenem, meropenem). This same isolate was considered multidrug resistant to eight other antibiotics, including antibiotics in drug classes associated with cell wall inhibitors (ampicillin,

cloxacillin, mecillinam, vancomycin), cephalosporins (cephalothin), folic acid inhibitors (trimethoprim, sulfamethoxazole/trimethoprim), and nitrofurans (nitrofurantoin) [10 antibiotics in total]. All 45 *P. aeruginosa* isolates from hot tubs were susceptible to the recommended drugs within the fluoroquinolone (Table 8), penicillin, aminoglycoside (Table 12), β-lactam combination agents (Table 11) and monobactam classes (Table 4) (Table 10). In contrast, Lutz & Lee (2011) found that *P. aeruginosa* from hot tubs in central Ohio area in the United States were resistant against several treatment-recommended antimicrobials in their study, including aminoglycosides (amikacin 9%, gentamicin 9%, tobramycin 9%), carbapenems (imipenem 26%, meropenem 4%), monobactams (aztreonam 22%), and penicillin (ticarcillin/ clavulanic acid 4%). The data suggests that antibiotics recommended for treatment of *P. aeruginosa* infections in Alberta, except for doripenem, may be sufficient for treating infections associated with contaminated recreational water venues.

Interestingly, all of the 45 *P. aeruginosa* isolates analyzed from hot tubs in Alberta shared a common 'backbone' of resistance to four antimicrobials belonging to three different cell wall inhibitor drugs (ampicillin, cloxacillin and mecillinam) and a cephalosporin (cephalothin) indicating a common antibiotic resistance pattern in all isolates contaminating the recreational water source (Figure 6). In addition to this common and shared backbone of resistance, all 45 strains were resistant to at least two or more antibiotics [i.e., 6 in total] (Figure 7); the combinations of these varied among the 45 isolates, but these results designate all isolates as being multidrug resistant. Outside of the resistance backbone, resistance to vancomycin and trimethoprim were the next most commonly observed resistance patterns (43/45 isolates for each antibiotic [96%]). This was followed by nitrofurantoin (41/45 isolates [91%]), erythromycin (31/45 isolates [69%]) and a combination agent - sulfamethoxazole/trimethoprim (26/45 isolates [58%]).

Forty-three isolates were multidrug resistant to seven or more antibiotics and 13 strains were resistant to up to 10 different antibiotics (Figure 7), albeit the resistance patterns varied among all the isolates (Figure 6). Thirty-seven multidrug isolates were inclusive with eight or more antibiotics and twenty-one isolates were resistant to nine or more antibiotics of several different classes (Figure 6 and 7).

Of those antibiotic classes for which resistance was observed in *P. aeruginosa*, the carbapenems had the lowest level of resistance (Figure 6, Table 7), with only 1/45 isolates (2%) demonstrating resistance to doripenem, and 4/45 [9%] isolates resistant to ertapenem. The highest levels of resistance were observed against various cell wall inhibitors (Table 5), nitrofurans (Table 10), first generation cephalosporins (Table 9), some protein synthesis inhibitors (Table 6) and folic acid inhibitors (Table 10). All *P. aeruginosa* strains were susceptible to antibiotics in the fluoroquinolone class (Table 8).

Pseudomonas aeruginosa Isolate Number	Antibiotic	ampicillin	cloxacillin	mecillinam	cephalothin	vancomycin	trimethoprim	nitrofurantoin	erythromycin	sulfamethoxazole/trimethoprim	chloramphenicol	streptomycin	ertapenem	Polymyxin B	cefotaxime	doripenem	Number of Antibiotics Isolate Was Resistant To
	13	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	10
	15	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	10
	20	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	10
	21	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	10
	28	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	10
	345	1	1	1	1	1	1	1	1	1	0	0	1	0	0	0	10
	309	1	1	1	1	1	1	1	0	1	0	0	1	0	0	1	10
	2	1	1	1	1	1	1	1	1	1	0	1	0	0	0	0	10
	9	1	1	1	1	1	1	1	1	1	0	1	0	0	0	0	10
	26	1	1	1	1	1	1	1	1	1	0	1	0	0	0	0	10
	47	1	1	1	1	1	1	1	1	1	0	1	0	0	0	0	10
	7	1	1	1	1	1	1	1	1	1	0	0	0	0	1	0	10
	3	1	1	1	1	1	1	1	1	0	0	1	0	1	0	0	10
	14	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	9
	16	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	9
	23	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	9
	25	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	9
	353	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	9
	65	1	1	1	1	1	1	1	1	0	0	0	1	0	0	0	9
	56	1	1	1	1	1	1	1	1	0	0	0	0	1	0	0	9
	8	1	1	1	1	1	1	1	1	0	0	0	0	0	1	0	9
	29	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	8
	30	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	8
	50	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	8
	17	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	8
	77	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	8
	254	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	8
	5	1	1	1	1	1	1	0	1	1	0	0	0	0	0	0	8
	10	1	1	1	1	1	1	1	0	1	0	0	0	0	0	0	8
	11	1	1	1	1	1	1	1	0	1	0	0	0	0	0	0	8
	18	1	1	1	1	0	1	1	0	1	1	0	0	0	0	0	8
	27	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	8
	62	1	1	1	1	1	1	1	0	0	0	0	1	0	0	0	8
	72	1	1	1	1	1	1	1	0	1	0	0	0	0	0	0	8
	270	1	1	1	1	1	1	1	0	1	0	0	0	0	0	0	8
	279	1	1	1	1	1	1	1	0	1	0	0	0	0	0	0	8
	337	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	8
	4	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	7
	297	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	7
	316	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	7
	22	1	1	1	1	0	1	1	1	0	0	0	0	0	0	0	7
	6	1	1	1	1	1	0	0	1	1	0	0	0	0	0	0	7
	1	1	1	1	1	1	1	0	0	1	0	0	0	0	0	0	7
	12	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	6
	287	1	1	1	1	1	0	1	0	0	0	0	0	0	0	0	6
	P. aeruginosa ATCC 27853	1	1	1	1	1	1	1	0	1	0	0	0	0	0	0	8
	Number of Isolates Having Resistance to the Listed Antibiotic	45	45	45	45	43	43	41	31	26	6	5	4	2	2	1	

Figure 6: Heatmap antibiogram of *P. aeruginosa* isolates collected from water in hot tubs and whirlpools in Alberta, Orange cells indicate a backbone of resistance common to all recreational water strains. Red cells indicate resistance to the listed antibiotic but for which not all strains were resistant, white cells reflect susceptibility. The cell value "1" represent resistant and "0" represent susceptible. Although 36 antibiotics were used in the screen, only those for which resistance was observed are presented in the heatmap.

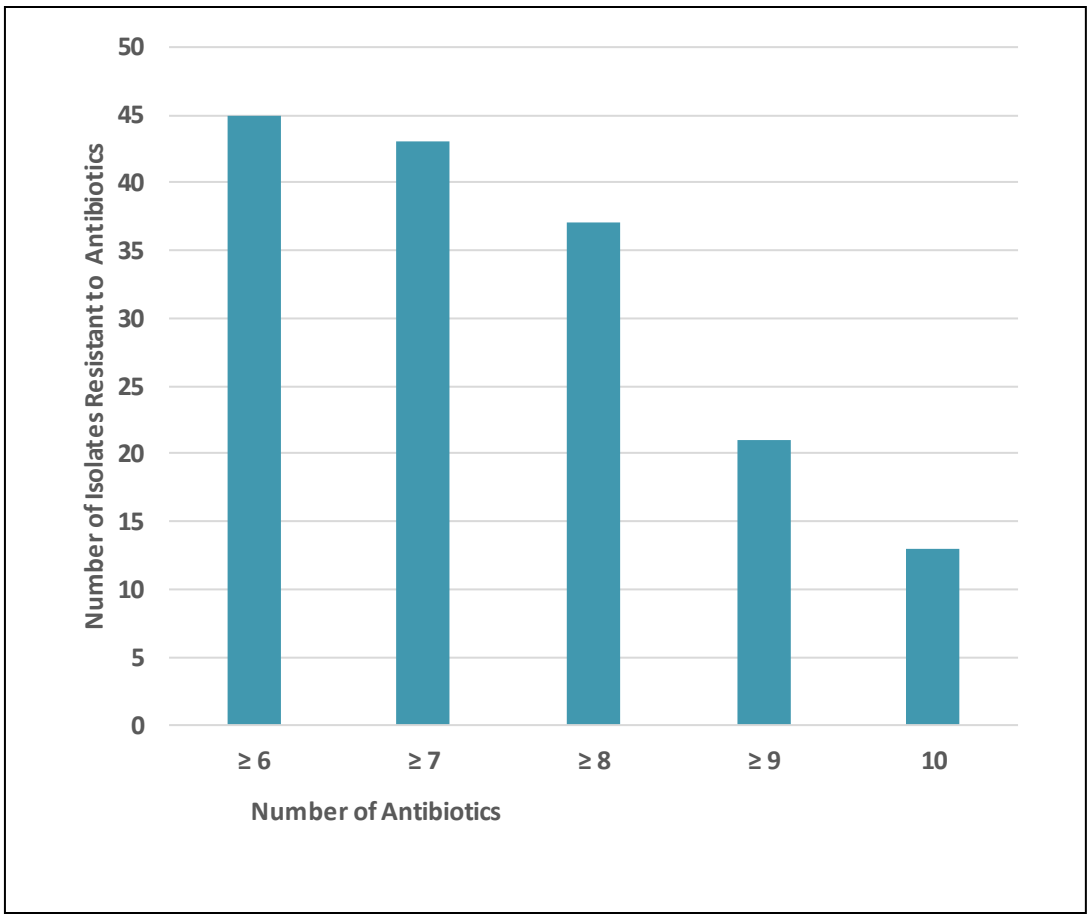

Figure 7: *P. aeruginosa* isolates correspond to resistant antibiotics. All 45 *P. aeruginosa* isolates were resistant to at least six or more antibiotics, 43 isolates were resistant to seven or more antibiotics, 37 isolates were resistant to eight or more antibiotics, 21 isolates were resistant to nine or more antibiotics and 13 isolates were resistant to 10 antibiotics.

Resistance to antibiotics were observed in six different classes of antibiotics (cell wall inhibitor, cephalosporin, folic acid synthesis inhibitor, nitrofuran, protein synthesis inhibitor and carbapenem), indicating that all *P. aeruginosa* from hot tubs in Alberta can be considered as multidrug resistant strains (i.e., defined as resistance to one or more drugs in three different classes). Although multiclass resistance was observed, none of the strains were completely resistant to all drugs represented in the class. For example, in terms of cell wall inhibitors, all 45 *P. aeruginosa* isolates were resistant to ampicillin, cloxacillin, mecillinam, but 96% were resistant to vancomycin, and only two isolates were resistant to polymyxin B. All the isolates were susceptible to the cell wall inhibitor known as colistin sulphate (Table 5). Among the four tested protein synthesis inhibitors, 69% of *P. aeruginosa* isolates were found to be resistant to

erythromycin, whereas only 11% and 13% were resistant to the protein synthesis inhibitors streptomycin and chloramphenicol, respectively. None of the *P. aeruginosa* isolates were resistant to the protein synthesis inhibitor, tetracycline (Table 6). Among four carbapenems, all isolates were susceptible to imipenem and meropenem, whereas four strains were resistant to ertapenem and only one strain was resistant to doripenem (Table 7). In the case of cephalosporins (Table 9), all strains were susceptible to cefepime and ceftazidime, but 2 strains were resistant to cefotaxime. Interestingly, all *P. aeruginosa* isolates were resistant to the 1st generation cephalosporin known as to cephalothin (Table 9). Among two folic acid synthesis inhibitors 96% of strains were resistant to trimethoprim and 58% were resistant to the dual combination of sulfamethoxazole/trimethoprim (Table 10). The nitrofuran class was represented by only one drug, nitrofurantoin, for which 91% of isolates were resistant (Table 10). All strains were susceptible to the single representative monobactam (aztreonam), as well as all representative penicillins (piperacillin, ticarcillin, piperacillin/tazobactam) [Table 11] and all representative aminoglycosides (amikacin, tobramycin, gentamicin, fosfomycin, and netilmicin) [Table 12]. A summary of antibiotic class resistance is provided in Figure 8.

Table 5: Results of susceptibility testing of antibiotics (cell wall inhibitors) use for *P. aeruginosa* isolates (n=45) collected from hot tubs/whirlpools from central-southern Alberta, Canada, and percentage of resistance and susceptible values (referred from CLSI, 2018) of *P. aeruginosa*).

Antibiotics	**Disk concentration (µg/ml)**	**Ref. Zone diameter breakpoint for *P. aeruginosa* (mm)**		**Inhibition zone (average±SD) for reference strains**				***P. aeruginosa* isolates**			
				***P. aeruginosa* ATCC 27853**	***E. coli* 25922**	***E. coli* 35218**	***S. aureus* 25923**	**Susceptible**		**Resistance**	
		S≥	**R<**					**N. (%)**	**Inhibition zone (average±SD)**	**N. (%)**	**Inhibition zone (average±SD)**
Ampicillin	10	-	-	0±0	26.00±1.00	0±0	43.00±1.00	-	-	45 (100)	0±0
Cloxacillin	5	-	-	0±0	0±0	7.33±0.58	34.67±1.53	-	-	45 (100)	0±0
Mecillinam	10	-	-	0±0	28.67±0.58	21.67±1.53	16.00±0.00	-	-	45 (100)	0±0
Vancomycin	5	-	-	0±0	0±0	0±0	17.67±0.58	2 (4.44)	8.00±0.58	43 (95.55)	0±0
Polymyxin B	30	14	14	17.00±1.73	14.33±2.08	15.00±2.00	12.00±2.16	43 (95.55)	16.68±1.55	2 (4.44)	12.83±0.71
Colistin Sulphate	25	-	-	17.00±1.00	17.66±0.58	15.33±0.58	0±0	45 (100)	18.03±1.67	-	-

Table 6: Results of susceptibility testing of antibiotics (protein synthesis inhibitors) use for *P. aeruginosa* isolates (n=45) collected from hot tubs/whirlpools from central-southern Alberta, Canada, and percentage of resistance and susceptible values.

Antibiotics	**Disk concentration (µg/ml)**	**Ref. Zone diameter breakpoint for *P. aeruginosa* (mm)**		**Inhibition zone (average±SD) for reference strains**				***P. aeruginosa* isolates**			
				***P. aeruginosa* ATCC 27853**	***E. coli* 25922**	***E. coli* 35218**	***S. aureus* 25923**	**Susceptible**		**Resistance**	
		S≥	**R<**					**N. (%)**	**Inhibition zone (average±SD)**	**N. (%)**	**Inhibition zone (average±SD)**
Streptomycin	10	-	-	13.33±0.58	18.00±1.00	0±0	19.33±0.58	40(93.3)	12.75±4.43	5(11.11)	0±0
Chloramphenicol	30	-	-	12.33±1.53	33.33±0.58	10.33±0.58	24.33±1.53	39 (86.66)	18.37±5.80	6 (13.33)	0±0
Tetracycline	30	-	-	18.00±1.00	27.33±0.58	27.67±1.53	33.33±0.58	45 (100)	16.92±3.32	-	-
Erythromycin	15	-	-	10.67±1.15	12.33±0.58	16.67±0.58	29.67	14 (31.11)	11.04±4.38	31 (68.88)	0±0

Table 7: Results of susceptibility testing of antibiotics (carbapenems) use for *P. aeruginosa* isolates (n=45) collected from hot tubs/whirlpools from central-southern Alberta, Canada, and percentage of resistance and susceptible values (referred from CL, 2018) of *P. aeruginosa*.

Antibiotics	**Disk content (µg/ml)**	**Ref. Zone diameter breakpoint for *P. aeruginosa* (mm)**		**Inhibition zone (average±SD) for reference strains**				***P. aeruginosa* isolates**			
								Susceptible		**Resistance**	
		S≥	**R<**	***P. aeruginosa* ATCC 27853**	***E. coli* 25922**	***E. coli* 35218**	***S. aureus* 25923**	**N. (%)**	**Inhibition zone (average±SD)**	**N. (%)**	**Inhibition zone (average±SD)**
Ertapenem	10	-	-	27.33±1.15	33.67±6.11	38.00±1.00	24.00±1.00	41(91.11)	20.10±7.44	4 (8.88)	0±0
Doripenem	10	15	15	36±1.00	31.67±2.89	32.67±2.89	35.50±3.10	44(97.77)	33.02±4.82	1 (2.22)	9.89±8.58
Imipenem	10	15	15	28.33±0.58	35.33±1.15	34.33±0.58	55.250.96	45 (100)	25.98±5.65	-	-
Meropenem	10	15	15	32.67±5.77	32.00±4.36	31.00±3.47	28.00±1.82	45(100)	31.83±7.25	-	-

Table 8: Results of susceptibility testing of antibiotics (fluoroquinolones) use for *P. aeruginosa* isolates (n=45) collected from hot tubs/whirlpools from central-southern Alberta, Canada, and percentage of resistance and susceptible values (referred from CLSI, 2018) of *P. aeruginosa*.

Antibiotics	**Disk content (µg/ml)**	**Ref. Zone diameter breakpoint for *P. aeruginosa* (mm)**		**Inhibition zone (average±SD) for reference strains**				***P. aeruginosa* isolates**			
								Susceptible		**Resistance**	
		S≥	**R<**	***P. aeruginosa* ATCC 27853**	***E. coli* 25922**	***E. coli* 35218**	***S. aureus* 25923**	**N. (%)**	**Inhibition zone (average±SD)**	**N. (%)**	**Inhibition zone (average±SD)**
Ciprofloxacin	5	15	15	37.67±2.08	40.67±0.58	38.67±1.53	35±1.15	45 (100)	38.81±3.18	-	-
Enrofloxacin	5	-	-	25.33±0.58	39.33±0.58	35.67±0.58	32.67±0.58	45 (100)	26.38±3.92	-	-
Levofloxacin	5	13	13	27.67±1.53	39.67±3.21	38.33±0.58	33.00±1.83	45 (100)	32.17±3.75	-	-
Ofloxacin	5	12	12	22.33±0.58	34.33±0.58	33.67±0.58	31.33±1.15	45 (100)	27.72±3.81	-	-
Lomefloxacin	10	18	18	30.00±1.73	35.00±1.00	26.00±1.00	28.33±1.53	45 (100)	32.64±3.34	-	-
Norfloxacin	10	12	12	35.00±0.00	39.67±0.58	39.67±0.58	29.00±0.00	45 (100)	37.87±2.41	-	-

Table 9: Results of susceptibility testing of antibiotics (cephalosporins) use for *P. aeruginosa* isolates (n=45) collected from hot tubs/whirlpools from central-southern Alberta, Canada, and percentage of resistance and susceptible values (referred from CLSI, 2018) of P. *aeruginosa*.

Antibiotics	**Disk content (µg/ml)**	**Ref. Zone diameter breakpoint for *P. aeruginosa* (mm)**		**Inhibition zone (average±SD) for reference strains**				***P. aeruginosa* isolates**			
								Susceptible		**Resistance**	
		S≥	**R<**	***P. aeruginosa* ATCC 27853**	***E. coli* 25922**	***E. coli* 35218**	***S. aureus* 25923**	**N. (%)**	**Inhibition zone (average±SD)**	**N. (%)**	**Inhibition zone (average±SD)**
Cefotaxime	30	-	-	27.00±1.00	37.67±0.58	38.00±1.00	30.00±0.00	43 (95.55)	18.34±3.84	2 (4.44)	0±0
Cephalothin	30	-	-	0±0	17.67±0.58	20.33±0.58	44.00±1.00	-	-	45 (100)	0±0
Cefepime	30	14	14	30.33±0.58	34.33±0.58	33.33±0.58	29.67±0.58	45 (100)	30.62±3.77	-	-
Ceftazidime	30	14	14	31.00±1.00	33.67±1.15	33.33±2.08	21.00±0.82	45 (100)	32.46±2.24	-	-

Table 10: Results of susceptibility testing of antibiotics (folic acid synthesis inhibitors, nitrofuran, monobactams) use for *P. aeruginosa* isolates (n=45) collected from hot tubs/whirlpools from central- southern Alberta, Canada, and percentage of resistance and susceptible values (referred from CLSI, 2018) of *P. aeruginosa*.

Antibiotics	**Disk content (µg/ml)**	**Ref. Zone diameter breakpoint for *P. aeruginosa* (mm)**		**Inhibition zone (average±SD) for reference strains**				***P. aeruginosa* isolates**			
				***P. aeruginosa* ATCC 27853**	***E. coli* 25922**	***E. coli* 35218**	***S. aureus* 25923**	**Susceptible**		**Resistance**	
		S≥	**R<**					**N. (%)**	**Inhibition zone (average±SD)**	**N. (%)**	**Inhibition zone (average±SD)**
Folic acid synthesis inhibitors											
Sulfamethoxazole /Trimethoprim	25	-	-	0±0	33.00±1.00	25.67±0.58	38.67±0.58	19 (42.22)	12.89±4.79	26 (57.77)	0±0
Trimethoprim	5	-	-	0±0	32.67±1.53	29.00±1.00	28.00±0.00	2 (4.44)	12.67±0	43 (95.55)	0±0
Nitrofuran											
Nitrofurantoin	100	-	-	0±0	24.67±0.58	24.67±1.15	24.00±0.00	4 (8.88)	10.92±3.14	41 (91.11)	0±0
Monobactams											
Aztreonam	30	15	15	29.00±1.00	36.00±1.00	34.67±0.58	15.25±2.36	45 (100)	29.17±2.64	-	-

Table 11: Results of susceptibility testing of antibiotics (penicillin) use for *P. aeruginosa* isolates (n=45) collected from hot tubs/whirlpools from central- southern Alberta, Canada, and percentage of resistance and susceptible values (referred from CLSI, 2018) of *P. aeruginosa*.

Antibiotics	**Disk content (µg/ml)**	**Ref. Zone diameter breakpoint for *P. aeruginosa* (mm)**		**Inhibition zone (average±SD) for reference strains**				***P. aeruginosa* isolates**			
								Susceptible		**Resistance**	
		S≥	**R<**	***P. aeruginosa* ATCC 27853**	***E. coli* 25922**	***E. coli* 35218**	***S. aureus* 25923**	**N. (%)**	**Inhibition zone (average±SD)**	**N. (%)**	**Inhibition zone (average±SD)**
Piperacillin	100	14	14	32.33±1.53	30.33±1.53	17.67±2.08	45.67±0.58	45 (100)	31.72±2.52	-	-
Ticarcillin	75	15	15	28.00±1.00	30.33±0.58	6.33±5.69	48.25±3.20	45 (100)	28.25±2.78	-	-
Piperacillin/ Tazobactam	110	14	14	33.33±2.08	31.67±0.58	32.00±1.00	46.67±1.53	45 (100)	34.17±2.40	-	-

Table 12: Results of susceptibility testing of antibiotics (aminoglycosides) use for *P. aeruginosa* isolates (n=45) collected from hot tubs/whirlpools from central-southern Alberta, Canada, and percentage of resistance and susceptible values (referred from CLSI, 2018) of *P. aeruginosa*.

Antibiotics	**Disk content (µg/ml)**	**Ref. Zone diameter breakpoint for *P. aeruginosa* (mm)**		**Inhibition zone (average±SD) for reference strains**				***P. aeruginosa* isolates**			
				***P. aeruginosa* ATCC 27853**	***E. coli* 25922**	***E. coli* 35218**	***S. aureus* 25923**	**Susceptible**		**Resistance**	
		S≥	**R<**					**N. (%)**	**Inhibition zone (average±SD)**	**N. (%)**	**Inhibition zone (average±SD)**
Amikacin	30	14	14	26.00±1.00	27.33±0.58	21.67±0.58	26.33±0.58	45 (100)	25.73±2.73	-	-
Tobramycin	10	12	12	26.67±1.53	25.00±0.00	20.33±1.53	27.50±0.58	45 (100)	26.09±2.48	-	-
Gentamicin	10	12	12	24.00±1.00	25.33±1.53	20.67±0.58	28.00±0.81	45 (100)	22.26±2.80	-	-
Fosfomycin	50	-	-	34.33±0.58	32.67±0.58	32.33±0.58	32.67±0.58	45 (100)	22.06±4.85	-	-
Netilmicin	30	12	12	21±0.00	27.67±0.58	25.67±0.58	28.33±0.58	45 (100)	23.03±2.76	-	-

Among the tested antibiotic classes, *P. aeruginosa* isolates were susceptible to fluoroquinolones, monobactam, aminoglycosides, protein synthesis inhibitors, and carbapenem, yet resistant to cell wall inhibitors, cephalosporins, folic acid synthesis inhibitors and nitrofuran (Figure 8). Almost all isolates including the positive control *P. aeruginosa* (ATCC 27853) were resistant to four cell wall inhibitors and a cephalosporin -cephalothin. All the tested *P. aeruginosa* isolates were susceptible to penicillin (piperacillin, ticarcillin) and β-lactam combinations (piperacillin/tazobactam). Carbapenems were the least resistant group of antibiotics against *P. aeruginosa* isolates. All 45 isolates showed susceptibility to aminoglycosides and fluoroquinolones. All 45 isolates were susceptible to one of the protein synthesis inhibitors - tetracycline. Among 45 isolates, all the isolates showed resistance to a folic acid synthesis inhibitor, trimethoprim. Almost all isolates (91%) showed resistance to nitrofuran (nitrofurantoin) and no monobactam has been found resistant against any of the tested *P. aeruginosa* isolates. The disk diffusion antimicrobial susceptibility testing showed that among the antibiotics tested, fluoroquinolones, aminoglycosides, β-lactam combined agents (piperacillin/tazobactam), penicillin (piperacillin, ticarcillin), and monobactam (aztreonam) were the most active compounds. All the *P. aeruginosa* isolates tested were susceptible to these active antibiotics.

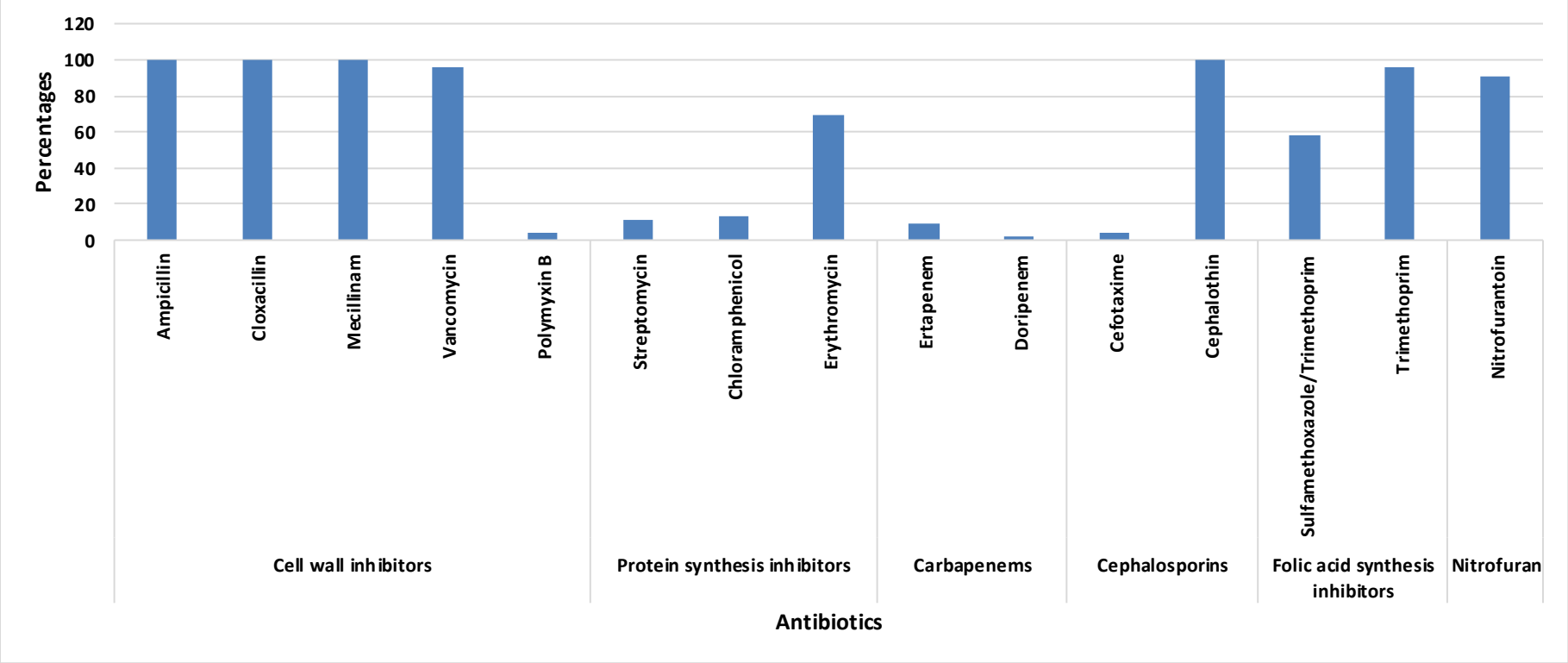

Figure 8: Antibiotic resistant patterns of 45 *P. aeruginosa* isolates from water in hot tubs and whirlpools. Almost all isolates were found resistant to cell wall inhibitors. Among the protein synthesis inhibitors, 69% of isolates were resistant to erythromycin. Carbapenems were the least resistant group of antibiotics against *P. aeruginosa* isolates. All 45 isolates shown resistant to one cephalosporin (cephalothin) and only two isolates (4.44%) found resistant to another cephalosporin (cefotaxime). A folic acid synthesis inhibitor- trimethoprim found resistant to all 43 isolates (96%) while trimethoprim combined with another folic acid synthesis inhibitor- sulfamethoxazole, the combination (sulfamethoxazole/ trimethoprim) drug found effective (resistant to 58% isolates). Nitrofuran (Nitrofurantoin) was resistant to 42 isolates (91%).

The presence of selected antimicrobial resistance genes in each of the isolates were tested by PCR (Table 2 [Chapter 2]). The PCR results indicated that most of the listed antibiotic resistance genes were found in the *P. aeruginosa* isolates, except for those associated with β-lactams and metallo- β-lactamase genes (Table 13, Figure 9). The most common types of genes associated with antimicrobial resistance included porins and efflux pumps, which are known to be dominant mechanisms for resistance in *P. aeruginosa* and were present in all the strains. The quorum sensing genes *LasI*, *Las R* and *gyrB* were also found in all hot tub strains. Some variability in occurrence of effector proteins/toxins genes was observed, especially exoU and exoS (Table 13, Appendix-3), and it is common to observe variability in the distribution of genes for effector proteins among strains (Sánchez-Vallet, et al., 2018).

The acquisition of most resistance phenotype requires genotype changes. Genetic changes could be either mutations (e.g., point mutations, deletions and insertions) or the acquisition by horizontal transfer of antibiotic resistance genes (Corona & Martinez, 2013). Exposure to antibiotics such as ciprofloxacin can promote genetic mutation in *Mex* efflux in *P. aeruginosa* (Ahmed et al., 2018). Resistant to β-lactams and quinolones is related to the presence of porins, and resistance to polymyxins and aminoglycosides comes from changing Lipid A structures in LPS. Although no β-lactam resistance genes were observed in the isolates, phenotypic resistance was observed against some β-lactam antibiotics (carbapenems, cephalosporins). There may be two possible reasons to explain this observed phenotypic antibiotic resistance in the absence of specific genes linked to resistance. This could include - limiting the uptake of antibiotics due to decreased outer membrane permeability as a result of porin mutation or through the action of efflux transporters. *P. aeruginosa* is intrinsically resistant against a wide range of antibiotics because of the presence of porins and several efflux pumps (Xiaoyan et al., 2015). At least 90% of the uptake of cephaloridine occurs through porin channels (Zimmermann, 1977).

In the tested *P. aeruginosa* isolates, no β-lactam genes were found but porin genes (*oprL*, *oprD*) were found which indicates those porin genes which are responsible for

resistance to cell wall inhibitors (ampicillin, cloxacillin, mecillinam, vancomycin and polymyxin B) and two β-lactams (cephalosporins (cefotaxime, cephalothin) and carbapenems (ertapenem, doripenem)). Efflux pumps can pump-out β-lactams, quinolones and aminoglycosides. No resistance was observed to quinolones and aminoglycosides among *P. aeruginosa* isolates from hot tubs and whirlpools, and could be related to the presence of efflux pump genes (*ampC*, *mexC1,2*, *mexC3,4*). The efflux pump MexXY-OprM is known to expel aminoglycosides (Pang et al., 2019), and it could be possible that this pump may not present in *P. aeruginosa* isolates resulting in susceptibility to aminoglycosides. Similarly, efflux pumps MexAB-OprM and MexEF-OprN are able to pump out quinolones, and the potential absence of those pumps among *P. aeruginosa* isolates could result in susceptibility against quinolones. Unfortunately, the presence of these pumps were not examined in this thesis.

Quorum sensing associated genes (*lasI, lasR, gyrB*) and biofilm genes (*ndvB*) were present in the *P. aeruginosa* isolates from hot tubs and whirlpools. The presence of these genes indicates virulence characteristics and biofilm production capabilities of *P. aeruginosa* isolates. The T3SS gene *popB* was found in all the *P. aeruginosa* isolates which is responsible for resistance to aminoglycosides and polymyxins. T3SS interacts on LPS of cell membrane and injects cytotoxins (*ExoS, ExoY, ExoT* and *ExoU*) directly into the host cell. No *P. aeruginosa* isolates were found resistant to aminoglycosides, but few isolates (two out of 45) were shown to be resistant to polymyxin B. The T6SS effector transporter gene *tssC1* was also found in all our *P. aeruginosa* isolates. Effector protein genes (*ExoS, ExoY, ExoT* and *ExoU*) were also found in our *P. aeruginosa* isolates. Effector proteins also alter host cells to suppress host defense mechanisms and facilitate infections.

Table 13: Classes of antibiotic resistance genes present in the *P. aeruginosa* isolates, compared with *P. aeruginosa* ATCC 25853, *S. aureus* ATCC 25923, *E. coli* ATCC 35218 and *E. coli* ATCC 25922.

Gene Types	Name of Genes	No. of *P. aeruginosa* isolates (% prevalence)	*P. aeruginosa* ATCC 25853	*S. aureus* ATCC 25923	*E. coli* ATCC 35218	*E. coli* ATCC 25922
Porin	*oprL*	45 (100)	**Present**	**Absent**	**Absent**	**Absent**
	oprD	45 (100)	**Present**	**Absent**	**Absent**	**Absent**
Efflux Pump	*ampC*	45 (100)	**Present**	**Absent**	**Absent**	**Absent**
	mexC1,2	45 (100)	**Present**	**Absent**	**Absent**	**Absent**
	mexC3,4	45 (100)	**Present**	**Absent**	**Absent**	**Absent**
QS	*lasI*	45 (100)	**Present**	**Absent**	**Absent**	**Absent**
	lasR	45 (100)	**Present**	**Absent**	**Absent**	**Absent**
	gyrB	45 (100)	**Present**	**Absent**	**Absent**	**Absent**
Biofilm	*ndvB*	45 (100)	**Present**	**Absent**	**Absent**	**Absent**
T3SS	*popB*	45 (100)	**Present**	**Absent**	**Absent**	**Absent**
T6SS	*tssC1*	45 (100)	**Present**	**Absent**	**Absent**	**Absent**
Effector Protein	*exoY*	40 (89)	**Present**	**Absent**	**Absent**	**Absent**
	exoU	19 (42)	**Absent**	**Absent**	**Absent**	**Absent**
	exoS	25 (56)	**Absent**	**Absent**	**Absent**	**Absent**
	exoT	45 (100)	**Present**	**Absent**	**Absent**	**Absent**

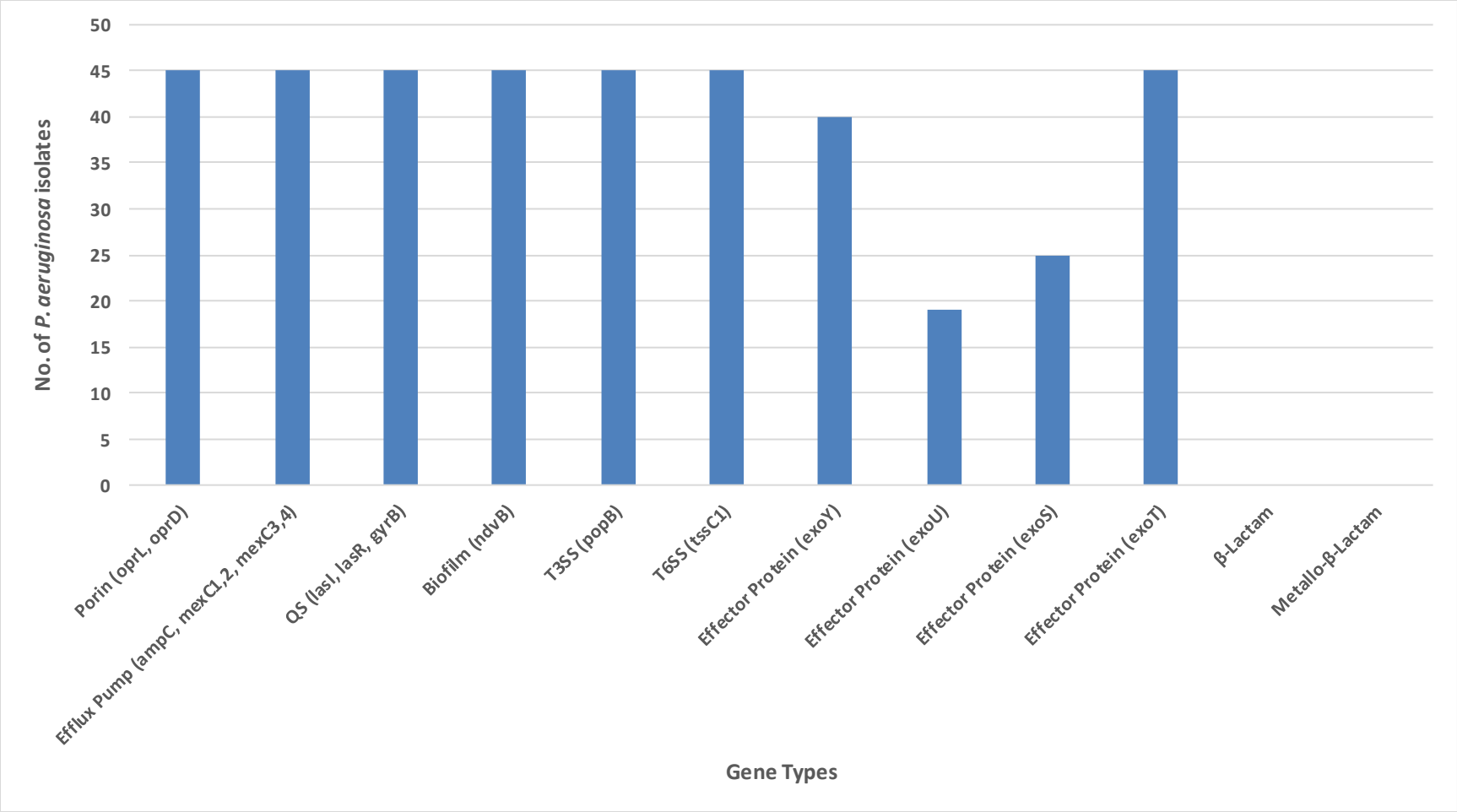

Figure 9: Classes of antibiotic resistance genes present in the *P. aeruginosa* isolates collected from hot tubs in Alberta. No AMR and β-Lactam biofilm (*NDM*) genes were found in the tested *P. aeruginosa*. Meanwhile, the presence of porin and efflux pump genes represent the transformation mechanism of AMR genes to *P. aeruginosa*.

4. Transformation of extracellular DNA to *P. aeruginosa* in the biofilm environment - Role of amoeba in facilitating gene transformation.

4.1 Introduction

Natural transformation is a process that allows competent bacteria to obtain exogenous naked DNA from the environment. It acts as a mechanism to enable the acquisition and dissemination of antibiotic resistance determinants throughout bacterial populations. Similarly, plasmid transformation is one of the leading mechanisms of horizontal gene transfer, wherein extracellular circular DNA is taken up by competent bacterial cells, playing a prominent role in the rapid spread of antibiotic resistance genes (Nolan et al., 2020). Although uptake of DNA from the environment is important for bacterial transformation, some bacteria such as a *P. aeruginosa* also produce a large amount of extracellular DNA [up to 18 µg/ml] (Dong et al., 2008), a feature important for biofilm formation in *P. aeruginosa* (Nolan et al., 2020). The uptake and production of extracellular DNA by bacteria are controlled by quorum sensing (Ibanez de Aldecoa et al., 2017; Nolan et al., 2020).

Extracellular DNA is an essential element of the extracellular matrix of multicellular communities, e.g., biofilm formed by bacteria, archaea, and fungi (Chimileski et al., 2014; Okshevsky & Meyer, 2015) and extracellular DNA production has been detected in laboratory conditions among *Pseudomonas* spp., including *P. stutzeri* and *P. aeruginosa* (Stewart et al., 1983). In some experiments, the natural transformation was also shared by closely related species, including *P. alcaligenes, P. mendocina* and *P. pseudoalcaligenes* (Carlson et al., 1983). Many naturally competent bacteria can actively transport extracellular DNA fragments throughout their cell envelope and into their cytoplasm (Mell & Redfield, 2014). Natural competence and transformation have not been reported in many bacteria species, including *P. aeruginosa*. Natural competence also allows bacteria to use extracellular DNA for nutritional purposes (Redfield, 1993; Redfield et al., 1997; Solomon & Grossman, 1996). The natural competency of recipient bacteria (*E. coli*) increases under lower nutrient levels as well as lower oxygen levels (Finkel & Kolter, 2001). To mitigate nutrient deficiency, extracellular DNA can serve as the source of carbon and energy to support bacterial growth. Mechanisms of DNA

uptake go beyond nutrient acquisition alone and include the ability to evolutionarily adapt to stressful situations.

The source of extracellular DNA can be homospecific or heterospecific. Homospecific environmental DNA uptake was observed in *Haemophilus influenzae* and *Neisseria gonorrhoeae* (Smith et al., 1995). After degrading one strand, another strand may be uptake, a process determined by the "uptake of signal sequences" similarly observed in *Haemophilus influenzae* (Smith et al., 1995). Finally, after the recombination process, new DNA is integrated into the chromosome. Bacteria commonly use transformation as a mechanism of antimicrobial resistance by exchanging ARGs (antimicrobial resistance genes) (von Wintersdorff et al., 2016). Antimicrobials can induce competence in many bacterial species by stimulating ARGs' transformation (Prudhomme et al., 2006; Charpentier et al., 2011).

Pseudomonas spp. have been reported to be capable of natural transformation (Petra et al., 2002; Jerónimo et al., 2012). According to Lutz and Lee (2011), *P. aeruginosa*'s extracellular DNA persistency and transformability in hot tubs and whirlpools water have not been well studied. Since *P. aeruginosa* is capable of natural transformation in biofilms (Nolan et al., 2020), and hot tubs and whirlpools represent substrates suitable for biofilm formation (Lutz and Lee, 2011), this study attempted to examine whether extracellular DNA encoding ARGs could transform *P. aeruginosa* in a biofilm setting. Using a plasmid (pAB1) having a broad host range for *Pseudomonas* species, and carrying a green fluorescent protein (GFP) tag as well as ampicillin and tetracycline resistance (appendix 4) the work presented in this Chapter examines the natural competency of wildtype strains in a biofilm environment.

In addition, since natural biofilms often represent mixed communities of microbes, the potential for transformation may also be affected by other community members. Free-living amoebae (FLA) may co-exist with bacteria in biofilm communities, and are predators of bacteria and grazers of biofilms. *P. aeruginosa* is one of several amoebae-resistant bacteria (Matz et al., 2008; Thomas et al., 2006). *P. aeruginosa*`s capacity to secrete toxins and form biofilms support the avoidance of amoebae phagocytosis. *P. aeruginosa* within a biofilm can kill associated amoebae by quorum sensing and the type III secretion system, and *P.*

aeruginosa has developed anti-digestion mechanisms that supports survival inside the amoebae (Matz et al., 2008). In addition, various anti-phagocytic strategies enable *P. aeruginosa* to escape from amoebal phagocytes (Lovewell et al., 2014). Moreover, the SOS response supports *P. aeruginosa* to repair damaged DNA and increases transformation of DNA from the environment (Podlesek and Žgur, 2020). Given that stress is a potent trigger of bacterial transformation and that a complex predator/prey interaction exists between *P. aeruginosa* and many FLAs, it was hypothesized that the presence of FLA in a biofilm may facilitate plasmid DNA transformation in *P. aeruginosa*. Using fluorescent microscopy, the current study examined *in vitro* uptake of extracellular ARGs by *P. aeruginosa* in the presence of the free-living amoeba *Acanthamoebae polyphaga* and *Willaertia magna,* two water-related FLA species.

4.2 Results and Discussion

The occurrence of natural transformation enables bacteria to overcome environmental challenges during harsh and extreme conditions and maintain population numbers. During such situations, some bacteria instinctively release DNA from the cells into the environment, free to be taken up by competent recipient bacterial cells. Not all bacteria take up extracellular DNA from their environment, and therefore the focus of this research was to determine whether wildtype strains of *P. aeruginosa* isolated from hot tubs were capable of stable transformation of plasmids encoding ARGs.

Artificial competency of P. aeruginosa`s transformability

To assess the ability of strains to become stably transfected with ARG-containing plasmids, electroporation was used to artificially introduce foreign DNA into *P. aeruginosa* isolates collected from hot tubs in Alberta. Wildtype hot tub strains of *P. aeruginosa* were shown to stably transform a *P. fluorescens*-derived plasmid containing GFP and ARGs through electroporation. One *P. aeruginosa* isolate in particular, strain 9, appeared particularly adept at stably incorporating plasmid DNA, as determined by a strong GFP fluorescent phenotype even after 49 days (Figure 10). These electroporation results demonstrate that some wildtype *P. aeruginosa* hot tub strains can be stably transfected with plasmids and are therefore competent at incorporating foreign DNA into the cell.

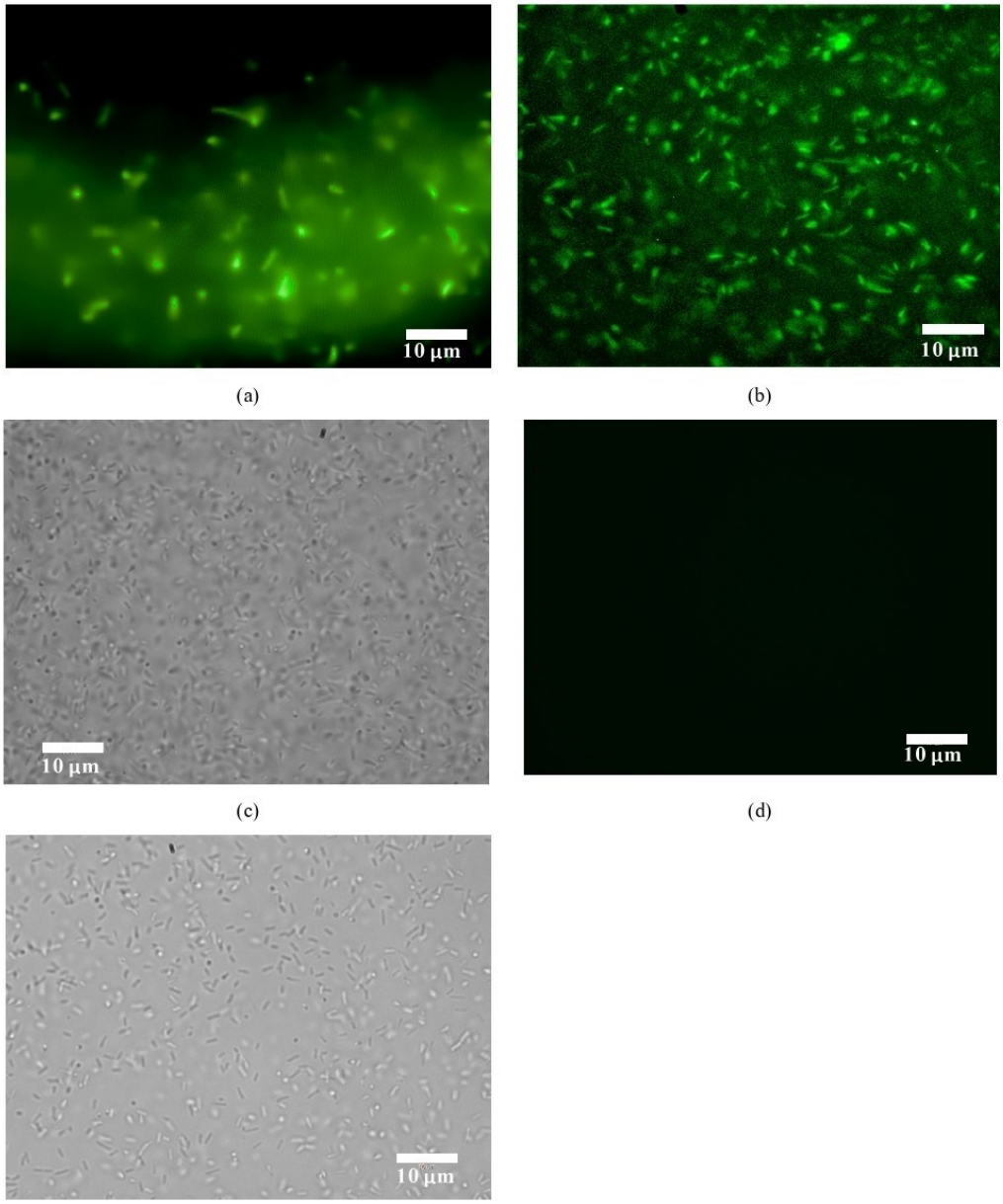

Figure 10: Artificial transformation of *P. aeruginosa* by electroporation and assessed after 49 days in culture. Photomicrographs are as follows: a) GFP *P. fluorescens* (ATCC13525) before DNA extraction under epifluorescent microscopy with 400X magnification under oil immersion. b) Transfected *P. aeruginosa* isolate no. 9 by electroporation (extracellular DNA from *P. fluorescens*) under epifluorescent microscopy. C) Bright field view of transfected *P. aeruginosa* isolate no. 9 by electroporation. d) Non-transfected *P. aeruginosa* isolate no. 9 under epifluorescent microscopy with 400X magnification under oil immersion. e) Bright field view of non-transfected *P. aeruginosa* isolate no. 9 with the same magnification under oil immersion.

Natural competency of P. aeruginosa`s transformability

Since wildtype strains of *P. aeruginosa* were competent at incorporating foreign DNA through artificial means (i.e., electroporation), the next logical step was to examine the natural transformation capabilities of *P. aeruginosa* in a monoculture biofilm environment. Experiments were conducted by inoculating recipient bacteria and donor plasmid DNA into a single tube and incubating the culture over time. The transformation assay was monitored every 24 h for the first few weeks and every other day until 118 days; observations were made under epifluorescence microscopy (EVOS® Imaging system for fluorescence, Thermo Fisher Scientific, USA). Not surprisingly, the natural transformation took much longer to occur in culture, with fluorescent *P. aeruginosa* cells observable after 49 days (Figure 11). However, as was observed with electroporation studies, naturally transformed cells continued to express a stable GFP phenotype after 118 days of culture (Figure 11). After transformation, isolates were confirmed as *P. aeruginosa,* based on positive results from the species-specific *gyrB* gene qPCR assay (Appendix-2). Fluorescence intensity was variable among the naturally transformed sample isolates and the positive electroporated control transformants, with higher intensity noted in electroporated cells (Figure 10), suggesting that although these plasmids were naturally taken up by *P. aeruginosa*, the efficiency was lower and have resulted in a lower plasmid copy numbers in the naturally-transformed cells. The data demonstrate that wildtype hot tub strains of *P. aeruginosa* are naturally competent at taking up foreign plasmid DNA and that this transformation is stable over time and through many generations, suggesting that plasmids may be an important source of dissemination of antibiotic resistance genes in a hot tub environment.

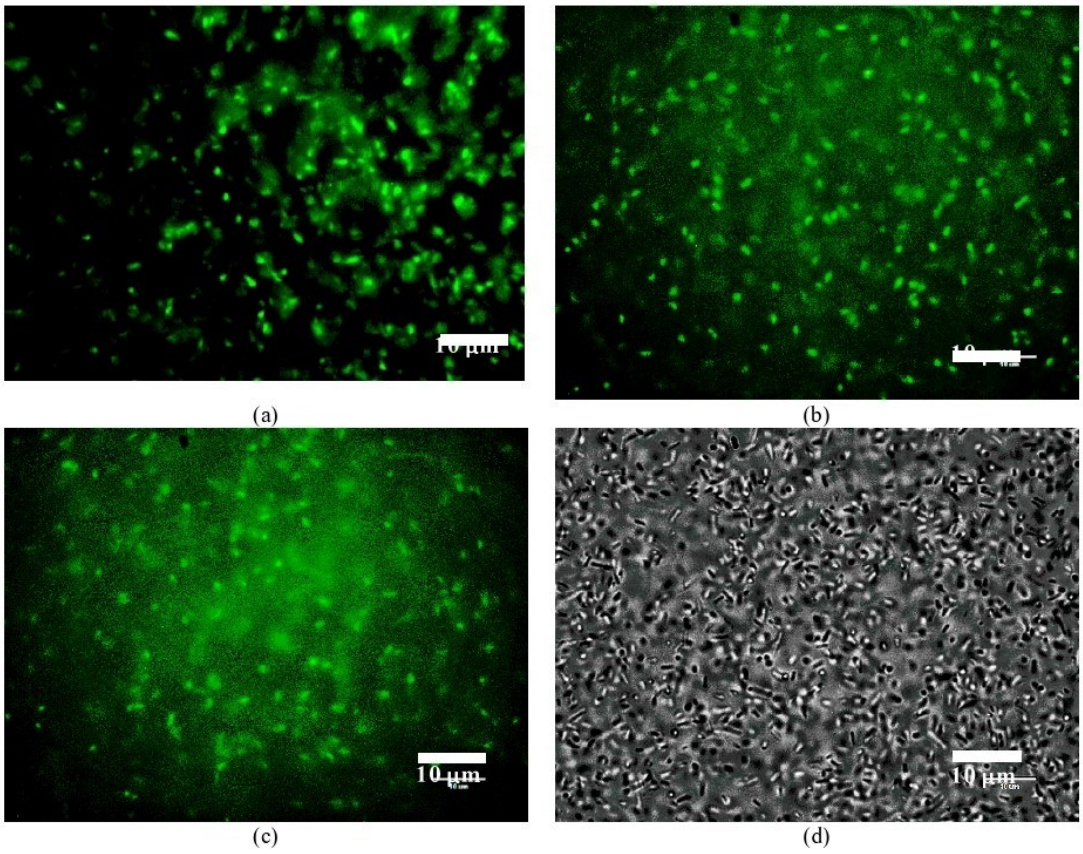

Figure 11: Natural transformation of extracellular DNA under the epifluorescent microscope with 400X magnification under oil immersion. The GFP visibility of plasmid DNA transformed to *P. aeruginosa* isolate no 9 (a), (b) and (c), after 49, 105 and 118 days, respectively. (d) The eDNA transformed to *P aeruginosa* isolate no. 9 (after 49 days, bright field view).

Natural competency of P. aeruginosa`s transformability in the presence of FLA

To further examine the natural competency of *P. aeruginosa* isolates for taking up exogenous DNA in a biofilm, a mixed culture experiment with *P. aeruginosa* and free-living amoebae (*A. polyphaga* and *W. magna*) was carried out. It was hypothesized that the presence of FLA could promote the early uptake of extracellular plasmids and enhance natural competency of *P. aeruginosa* due to stress induction associated with predator/prey interactions and better mimic the ecological conditions in hot tubs and whirlpools.

To assess whether natural transformation can occur in the presence of a mixed community of microbes, the naturally-competent wildtype *P. aeruginosa* strain 9 was cultured in the presence of two different FLA species – *A. polyphaga* and *W. magna* trophozoites. The *P. aeruginosa*

isolate and two species of FLA trophozoites were inoculated into culture tubes, and extracellular plasmid DNA [GFP *P. fluorescens* (ATCC 13525)] added after 48 h of co-incubation. Interestingly, the GFP plasmid DNA was transformed in the *P. aeruginosa* isolate after only nine days in the presence of *A. polyphaga* and after 14 days in the presence of *W. magna* (Figure 12, Figure 13). PCR amplification targeting the species-specific (*P. aeruginosa*) *gyrB* gene confirmed successful transformation of the GFP plasmid DNA to the recipient *P. aeruginosa* isolate in the presence of amoebae (Appendix-2).

Importantly, however, was the observation that both *A. polyphaga* and *W. magna* began forming cysts in mixed cultures as early as 18 hours after co-incubation with *P. aeruginosa*, even before exogenous plasmid DNA was added to the culture. Since plasmid DNA was added 48 hours after co-inoculation in the transformation experiment, the effect of FLA on inducing plasmid uptake and transformation in *P. aeruginosa* is uncertain. It could be that although the 'prey' (i.e., *P. aeruginosa*) was in abundance, the conditions for the predators (i.e., FLAs) was less than ideal, causing them to rapidly encyst during co-culture. Since *P. aeruginosa* are considered to be amoeba-resistant bacteria (José Maschio et al., 2015; Greub & Raoult, 2004), it is possible that these culture conditions triggered encystation of amoeba as a means of protecting themselves from these bacteria. The study found no internalization of *P. aeruginosa* into *A. polyphaga* and *W. magna* over time. Likewise, it is possible that these culture conditions also led to stress-related signaling in the bacterial population (i.e., through quorum sensing), activating systems such as the SOS response – a mechanism known to increase environmental DNA uptake in bacteria (Baharoglu et al., 2010; Yakimov et al., 2021), potentially explaining why transformation occurred quicker in *P. aeruginosa* in the presence of FLA. Unfortunately, these experiments were not repeated in the current study, and neither were the experiments run simultaneously to control for extraneous experimental effects that might contribute to the enhanced uptake of DNA, and so the significance of this enhanced transformation effect due to FLA is uncertain. Nevertheless, the work lays a foundation for understanding the complex interactions between predators and prey in a biofilm environment, and what effect it may have on HGT antibiotic-resistance.

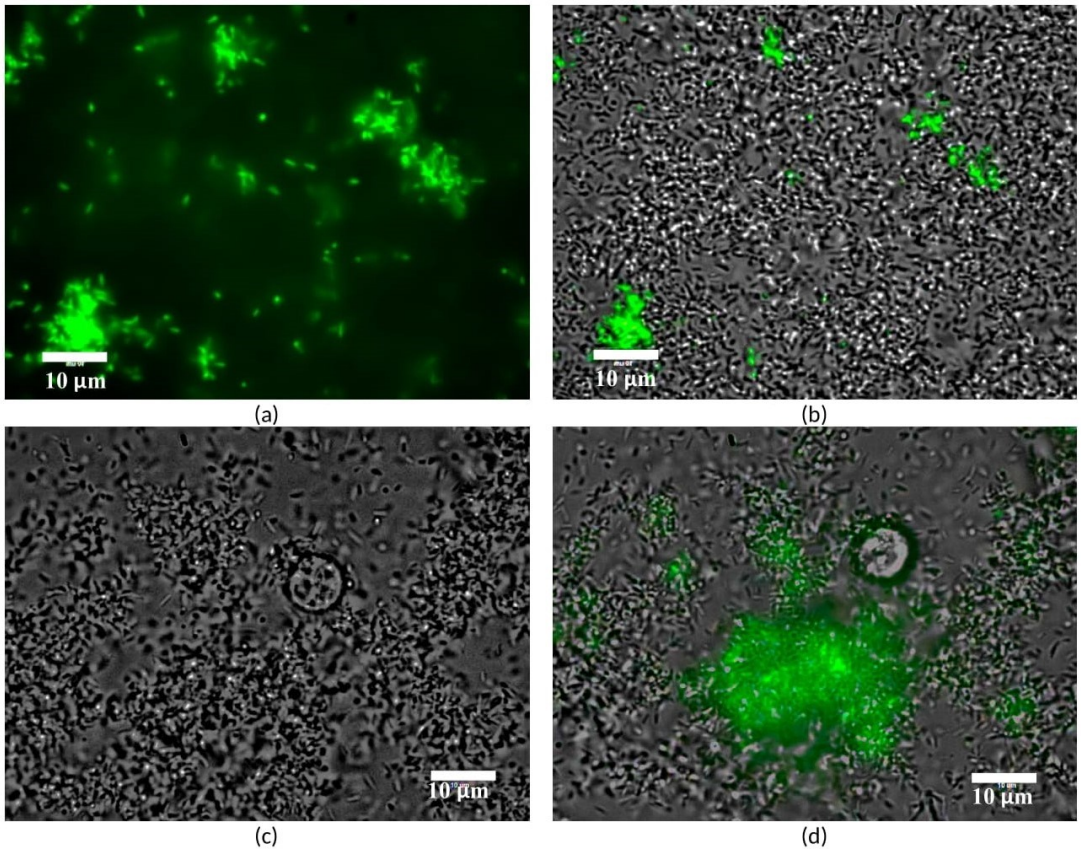

Figure 12: GFP plasmid extracellular DNA transformed to *P. aeruginosa* (isolate no. 9) in the presence of *A. polyphaga* at room temperature (25°C): (a) green fluorescent image after nine days co-incubation; (b) combined green fluorescent and bright field image in same frame after nine days incubation, with the green fluorescence of *active P. aeruginosa* among biofilms representing the transformation; (c) after 11 days incubation, cyst formation of *A. polyphaga* observed in bright field image along with *P. aeruginosa* biofilm; (d) combined green fluorescent and bright field image in same frame showing the transformation of GFP plasmid extracellular DNA with green fluorescent colour.

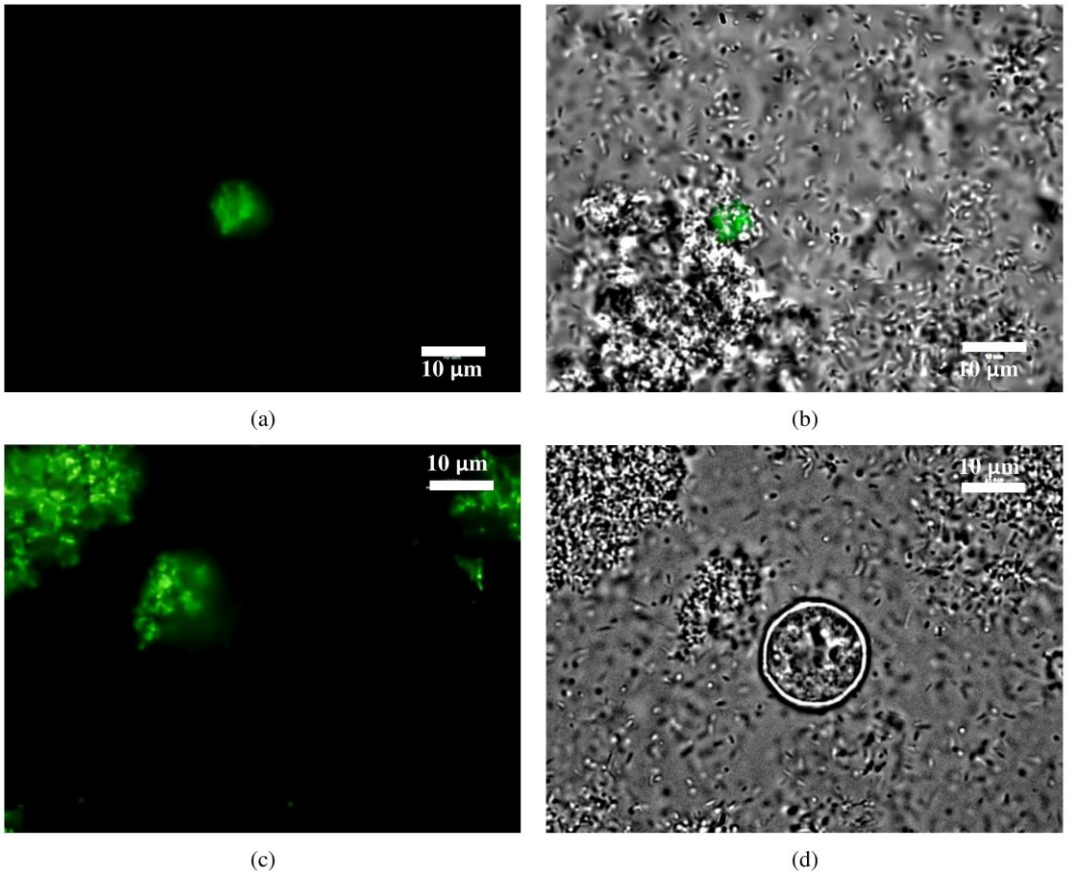

Figure 13: Extracellular DNA transformation to the recipient *P. aeruginosa* in the presence of *W. magna* at room temperature (25°C): (a) green, fluorescent view after 14 days; (b) combined green fluorescent and bright-field view after 14 days; (c, d) fluorescence micrograph after 21 days of cyst formation and corresponding bright field micrograph, and demonstrating a higher number of GFP-positive *P. aeruginosa* after extracellular DNA transformation.

Indeed, some studies have demonstrated that biofilm bacteria have several survival mechanisms at their disposal while living in an ecosystem including predatory FLA, but in most cases the exact mechanisms of survival are unknown. Dey (2019) demonstrated that *P. aeruginosa* can enter into a viable but nonculturable (VBNC) state in a biofilm environment as a response to various environmental conditions (including pipe materials and biocides) and association of VBNC *P. aeruginosa* with *A. polyphaga* results in resuscitation of *P. aeruginosa* to an active form within 2h. Acanthamoebae are also able to reactivate VBNC *Legionella pneumophila* into an active pathogenic state (*Steinert et al., 1997*). It has also been demonstrated that *E. coli* can survive within FLA including *Acanthamoeba castellanii, A.*

astronyxis and *Naegleria fowleri* (Jung, 2011). The mechanisms of *E. coli*`s intracellular survival in FLAs remain unknown but relates to the ability of the bacteria to inhibit the fusion of lysosomes with phagosomes as a critical step in the intracellular survival of this bacterium (Bozue and Johanson,1996). The survival of internalized *Legionella spp.* in FLA is still poorly understood, and limited information is available on how pathogenic *Legionella* interacts with FLA in its natural biofilm environment *(*Shaheen and Ashbolt, 2021). FLA species supported intracellular growth of *L. pneumophila,* but FLA often exhibit a feeding preference, avoiding amoeba-resistant bacteria in favor of non-pathogenic bacteria *(*Shaheen et al., 2019*).* In a study by Shaheen et al. (2019), phagocytosis of *L. pneumophila by A. polyphaga* and *V. vermiformis,* but not by *W. magna,* in the presence of *E. coli* indicated that the recognition systems for food were different among FLA species. Furthermore, the prolonged presence of undigested *E. coli* in trophozoites containing *L. pneumophila* indicated that *L. pneumophila* may actively interfere with the amoeboid digestion process *(*Shaheen et al., 2019*).* Consequently, it is hypothesized that the *P. aeruginosa* strains isolated from hot tubs may be resistant to *A. polyphaga* and *W. magna,* triggering encystation in these amoebas and possibly activating anti-amoeboid mechanism in the bacteria (such as SOS, quorum sensing, etc.), and which may enhance the natural competency of the bacterial population leading to HGT of antibiotic resistance

P. aeruginosa has three dominant quorum sensing systems: las, rhl and pqs (Wilder et al., 2011). *P. aeruginosa* isolates from hot tubs and whirlpool possessed *las* genes (both *lasI* and *lasR*) indicating quorum sensing may play a key role in the transformation process in these isolates (Chapter 3, Figure 9). Biofilms act as a nutrition source during DNA repair and transformation (Ibanez de Aldecoa et al., 2017). About 50% to 90% of the total organic carbon of biofilms is extracellular polymeric matrix (Flemming et al., 2000), and biofilms provide an ideal niche for the exchange of plasmids (Donlan, 2002). The nature of the static broth culture condition allowed for substrate-mediated formation of biofilms and likely the production of extracellular DNA. Extracellular DNA stimulates the production of type IV pili, which are promoters of natural transformation (Ellison et al., 2018; Salzer et al., 2014). These experimental results suggest that biofilm production could be an important factor in the transfer and spread of plasmids carrying ARGs in nature, informing potential surveillance and mitigation strategies to reduce further environmental and clinical dissemination.

5. Discussion & Conclusion

The goal of the research carried out in this thesis was to evaluate antimicrobial resistance patterns of naturally occurring *P. aeruginosa* from water in hot tubs and whirlpools in Alberta, and to determine whether these naturalized strains could acquire antibiotic resistance genes from within the biofilm communities they are typically found in. The most significant findings of this thesis research were as follows:

- There was a significant level of multiclass and multidrug antibiotic resistance observed in *P. aeruginosa* isolates collected from water in hot tubs and whirlpools in Alberta, with some isolates resistant to up to 10 different antibiotics, but with all strains possessing a core resistance profile to select cell wall inhibitors and cephalosporin. Worryingly, some isolates displayed resistance to carbapenems.

- Wildtype *P. aeruginosa* isolates collected from hot tubs in Alberta appear to be naturally competent for acquiring antimicrobial-resistant extracellular DNA from biofilms, and for which they become stably transformed.

- In the presence of free-living amoeba (*A. polyphaga* and *W. magna*) and in a biofilm environment, bacteria maintain competency, and very preliminary data suggest that bacterial competency may be enhanced.

Each of these significant findings, and their relative importance are discussed in detail below–

Antibiotic Resistance in P. aeruginosa isolated from Hot tubs

The findings of this study indicate that hot tub isolates of *P. aeruginosa* possess a high level of multidrug resistance to a range of antibiotic classes and generations. All isolates were resistant to the cell wall inhibitors ampicillin, cloxacillin and mecillinam, as well as the cephalosporin known as cephalothin. Other dominant resistance patterns included those having resistance to vancomycin (96%), trimethoprim (96%), and nitrofurantoin (91%). Although significant multidrug resistance was observed, the overall patterns of resistance observed among all isolates suggested that the antibiotics recommended as a first line of therapy according to CLSI 2018, are likely to be effective against hot tub infections caused by *P.*

aeruginosa, with one exception - doripenem. This finding alone is extremely important given the growing concern regarding the rapid emergence of carbapenem-resistance in bacteria around the globe (Corbella et al., 2000; Meletis et al., 2016; Potter et al., 2016). The World Health Organization has ranked carbapenem-resistant *P. aeruginosa* as a *critical* priority pathogen within their global priority listing, placing it in the highest-risk category for public health, and for which research and development for new antibiotic treatments is desperately needed. Although only one isolate displayed doripenem resistance, several hot tub strains also showed resistance to ertapenem (4/45 [9%]), but all isolates were susceptible to meropenem and imipenem. In total, 11% (n=5/45) of all *P. aeruginosa* hot tub isolates displayed some form of carbapenem resistance. Although the present study did not find *P. aeruginosa* isolates resistant to imipenem or meropenem, Lutz & Lee (2011) observed in their recreational water study that 26% of *P. aeruginosa* isolates were resistant to imipenem and 4% were resistant to meropenem. Collectively, the data demonstrates a worrying trend in the widespread occurrence of global carbapenem resistance in *P. aeruginosa* strains found in hot tubs, suggesting that recreational water systems might facilitate the dissemination of drug resistant phenotypes in opportunistic and possible pathogenic strains of the bacteria.

Carbapenem drugs are considered the most effective drug against *P. aeruginosa* (Codjoe & Donkor, 2017). There are three major mechanisms by which bacteria become resistant to carbapenems: carbapenemase enzyme production, efflux pumps and porin mutations (Suay-García & Pérez-Gracia, 2019). Carbapenemase genes were not found in any of the tested *P. aeruginosa* isolates, but rather the study found that *P. aeruginosa* strains from hot tubs contained pump-oriented genes (ampC, mexC1,2 and mexC3,4) and porin-mediated genes (oprL and oprD) (Table 13, Figure 9, Appendix-3). Elamin et al. (2017) found limited or decreased uptake of antibiotics by *P. aeruginosa* due to cell membrane permeability, and active efflux. Doripenem, like other β-lactam antibiotics, react with penicillin-binding proteins (PBSs) to form stable acyl enzymes. This inactivates the PBPs, leading to a weakened cell wall, which eventually ruptures because of osmotic pressure forces (Stratton, 2005). Doripenem has high affinity for PBP2 and PBP3 in *P. aeruginosa* (Davies et al., 2008).

Of the primary antibiotics clinically relevant for treating *P. aeruginosa* infections (Table 4), Lutz & Lee, (2011) found *P. aeruginosa* isolates from their recreational water study that were resistant to amikacin (9%), aztreonam (22%), ceftriaxone (4%), gentamicin (9%), imipenem (26%), meropenem (4%), ticarcillin/clavulanic acid (4%), and tobramycin (9%), whereas in the current study in Alberta, Canada, demonstrated that all hot tub isolates were susceptible to the above mentioned antibiotics. Important to note however, was the relatively low prevalence of resistance to most of these clinically-relevant antibiotics in the Lutz & Lee (2011) study. However, their data also suggests an emergence of resistance in hot tub strains against CLSI's (2018) list of clinically-relevant antibiotics for treatment of *P. aeruginosa* infections. It is possible that the differences in these resistance patterns among the two studies may relate to variations in the use of different antibiotics for treatment of *P. aeruginosa* clinical infections geographically, and therefore the populations of *P. aeruginosa* strains that are shed by bathers using hot tubs. It is believed that one of the primary sources of *P. aeruginosa* colonization in hot tubs comes from the skin of bathers themselves (Chandrasekar et al., 1984), in addition to naturalized environmental saprozoic strains that might also be present in the biofilms.

In terms of other trends in resistance, several studies reinforce the findings of widespread resistance of *P. aeruginosa* clinical strains to cell wall inhibitors, in particularly early generation drugs. Like the present study, in which 100% of *P. aeruginosa* isolate were resistant to ampicillin and 96% resistant to nitrofurantoin, Lutz & Lee (2011) also found high rates if resistance to these antibiotics in their hot tub strains (74% and 96%, respectively). Similar patterns were found in clinical isolates of *P. aeruginosa* from respiratory tract infections, where 100% of isolates were resistant to ampicillin (Gad et al., 2008), and from urinary tract infections in pregnant women, where 50% of *P. aeruginosa* isolates were resistant to nitrofurantoin (Okonko et al., 2009).

The observation that environmental isolates of *P. aeruginosa* possess high level multidrug resistance is of significant concern, particularly in light of the general biology of this organism. *P. aeruginosa* is an opportunistic and saprozoic environmental bacterium. As such, it has the capacity to exponentially grow in an environmental reservoir such as a poorly maintained hot tub, and this environmental *replication* can inherently lead to widespread dissemination and

amplification of antibiotic resistance genes, quite possibly among pathogenic strains. Furthermore, since some hot tub strains appear to be naturally competent at transforming environmental DNA within biofilms, this provides a possible mechanism for how the environment may be key to expanding the reservoir of resistance and in disseminating transmission of multidrug resistant strains.

Antibiotic resistant P. aeruginosa isolated from hot tubs are naturally competent to acquire extracellular DNA

The presence of multiclass and multidrug resistant *P. aeruginosa* from water in hot tubs and whirlpools in Alberta, Canada, raises the possibility that the water could serve as a potential route in which new resistance genes of *P. aeruginosa* enter and disseminate within the biofilm population. Antibiotic resistance is the most commonly recognized manifestation of horizontal gene transfer. Although substantial research has provided insight into the molecular mechanisms of horizontal gene transfer and antibiotic resistance, little is known about the functions of these systems in natural environments. Horizontal gene transfer is one of the key mechanisms of microbial adaptation and can lead to; a) different phenotypic traits, including metabolic capabilities, b) spread of virulence factors, c) development of heavy metal resistance and antibiotic resistance, and which could be transferred along with mobile genetic elements (Hannan, 2010). The results of the transformation study provide evidence that extracellular DNA may be taken up by wildtype strains of *P. aeruginosa* typically found in hot tubs and whirlpools. The successful transformation of extracellular DNA in bacterial monocultures was observed after 49 days, suggesting that *P. aeruginosa* appears to be naturally competent to uptake extracellular DNA.

Natural genetic transformation enables the recipient bacteria to acquire novel genes, thus promoting the emergence of antibiotic resistance (Cvitkovitch, 2001). Hence, natural transformation can be an important mechanism to adapt to changing environments, which is common among different bacteria species. A total of 82 bacteria species, including *Bacillus subtilis Streptococcus pneumoniae*, and *Vibrio cholerae*, are now known to be naturally transformable (Johnston et al., 2014). High transformation ability was observed in *Bacillus subtilis* (Ashikaga et al., 2000). Zhang et al. (2018) reported that stresses, such as starvation

and exposure to antibiotics enhanced the transformation efficiency between *B. subtilis* strains. Stress fosters the exchange of genetic material to promote the survival of the community (Zhang et al., 2018). Plasmids may come from dead bacterial cells (from the environment) by HGT from the biofilm source (Hasegawa et al., 2018). Aligning with above studies, this research has demonstrated that *P. aeruginosa* is capable of natural transformation of extracellular plasmid DNA in biofilms. More attention is required in antibiotic resistant bacteria targeting the natural environment (Kimihiro et al., 2020). This research lays for the foundation for further investigations to find specific mechanisms behind the plasmid DNA uptake by *P. aeruginosa* isolates in their natural ecosystem, particularly in water of hot tubs and whirlpools.

P. aeruginosa`s transformation competency may be enhanced in presence of free-living amoeba in a biofilm

The capabilities of *P. aeruginosa* to acquire extracellular plasmid DNA in a biofilm monoculture environment presupposes its competency in mixed-culture biofilms. The research presented in this thesis, demonstrate the natural competency of *P. aeruginosa* for extracellular plasmid DNA when co-cultured with FLAs (*A. polyphaga* and *W. magna*), and raises the intriguing possibility that FLA may actually help facilitate competency. Transformation occurred by 9 or 14 days in FLA co-culture experiments (*A. polyphaga* and *W. magna*, respectively) compared to 49 days under monoculture conditions. However, the fact that only a single experimental replicate was performed precludes the study from making any firm conclusions. Nevertheless, this work provides a foundation for which to address this possibility and the underlying mechanisms that could be at play. This study found that both species of amoebae (*A. polyphaga* and *W. magna*) did not ingest *P. aeruginosa* but quickly encysted when co-cultured with the bacteria, suggesting that extracellular signals for growth of amoeba were not ideal. It is possible that stress associated through predator/prey relationship, such as the SOS response may stimulate the early uptake of extracellular DNA by *P. aeruginosa*. Alternatively, there may be other factors involved in this early transformation, which need to be explored based on this initial finding. Nevertheless, the observation that *P. aeruginosa* strains from hot tubs and whirlpools in Alberta are multidrug/multic lass resistant, and that these strains are naturally competent and stably acquire AMR genes from mobile genetic

elements within a biofilm environment, raises the worrying prospect that hot tubs and whirlpools may be focal reservoirs of emerging antibiotic resistant *P. aeruginosa*.

The prevalence of FLA in swimming pools and recreational waters is high (Mavridou et al., 2014). The recommended concentration of free chlorine (1-3 ppm) in public swimming pools and hot tubs may fail to prevent the growth of FLA (Nastaran et al., 2020). FLAs are resistant to other disinfectants in the dosage used for swimming pools and artificial recreational waters (Kiss et al., 2014). In addition to biocide resistance, the presence of *P. aeruginosa* may prompt early encystment of amoeba. Hot tubs and whirlpools are suitable ecological niches for biofilm formation, the presumptive experimental stress situations (e.g., QS and SOS response) may better represent the ecological conditions in hot tubs and whirlpools.

The transformation experiments were accomplished with several limitations. The experiments were done in ambient temperature (22-25°C), which may not reflect the temperature conditions experienced in hot tubs (37- 40°C). Artificial culture conditions where nutrients are in abundance may also not reflect the limiting nutrients environments found in hot tub water. Limited nutrients induce the stress response in bacteria and could possibly increase transformation. The number of *P. aeruginosa* and FLA were controlled to keep MOI 100:1 respectively, which may differ in actual water systems in hot tubs and whirlpools. Moreover, transformation experiments were undertaken in monoculture assay, no other organisms were present in the assay other than *P. aeruginosa* and amoebae which limits the real time situations in hot tub and whirlpool water. Keeping all the above-mentioned limitations apart, the transformation experiment results still provide an experimental basis to address these questions. Although the transformation experiments were only performed once, and the results are not conclusive, they are intriguing and provide basis for better understanding transformation in natural premise plumbing ecosystems. Therefore, a comprehensive study is required with a reasonable number of experimental replicates to assess the statistical significance of the results.

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Appendices

APPENDIX 1: PCR analysis results of P. aeruginosa isolates:

No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
Name of the Sample/Tested genes	oprL	oprD	gyrB	lasI	lasR	ampC	mexC1,2	mexC3,4	ndvB	tssC1	exoT	exoY	exoU	exoS	popB	NDM	NDM-2	NDM-1	IMP	IMP-1	IMP-2	SPM	SPM-1	AIM	VIM	VIM-1	VIM-2	OXA	GIM	BIC	SIM	DIM	KPC-Fm
S1	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S2	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S3	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S4	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S5	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S6	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S7	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S8	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S10	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S12	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

S13	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S14	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S15	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S16	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S17	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S18	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S19	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S20	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S21	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S22	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S23	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S24	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S25	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S26	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S27	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S28	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S29	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S30	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S47	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S50	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S56	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S62	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

S65	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S72	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S77	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S254	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S270	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S279	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S287	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S297	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S309	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S316	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S337	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S345	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S353	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. aeruginosa (GFP)	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. aeruginosa (RFP)	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. aeruginosa (F1)	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

P. aeruginosa (F2)	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S. aureus (ATCC 25923)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E. coli (ATCC 35218)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E. coli (ATCC 25922)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. aeruginosa (ATCC 25853)	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

APPENDIX 2: qPCR analysis results of P. aeruginosa isolates using gyrB gene

qPCR amplification results of species specific (*P. aeruginosa*) gyrB gene, *P. aeruginosa* ATCC 27853 as positive control, samples named E, S and T were three transformed *P. aeruginosa* isolates:

Experiment Results Report

2020_October_9_gyrB

Experiment Summary

Experiment Name	:2020_October_9_gyrB
Experiment Type	:Quantitation - Standard Curve
File Name	:2020_October_9_gyrB.eds
Run Started	:2020 Oct 09 11:00:07 AM
Run Finished	:2020 Oct 09 11:44:00 AM
Run Duration	:43 minutes 52 seconds
Date Modified	:2020 Oct 09 12:06:52 PM
User	:
Number of wells used	:5
Number of wells with results	:5
Instrument Name	:
Instrument Type	:Applied Biosystems 7500 Fast Instrument
Comments	:samples for Shaheen/Murad

Results Summary

Sample	Target	Quantity (Mean)	Quantity (Std Dev)	CT (Mean)	CT (Std Dev)
E	gyrB			13.46	
POSITIVE	gyrB			13.95	
S	gyrB			12.69	
T	gyrB			14.09	

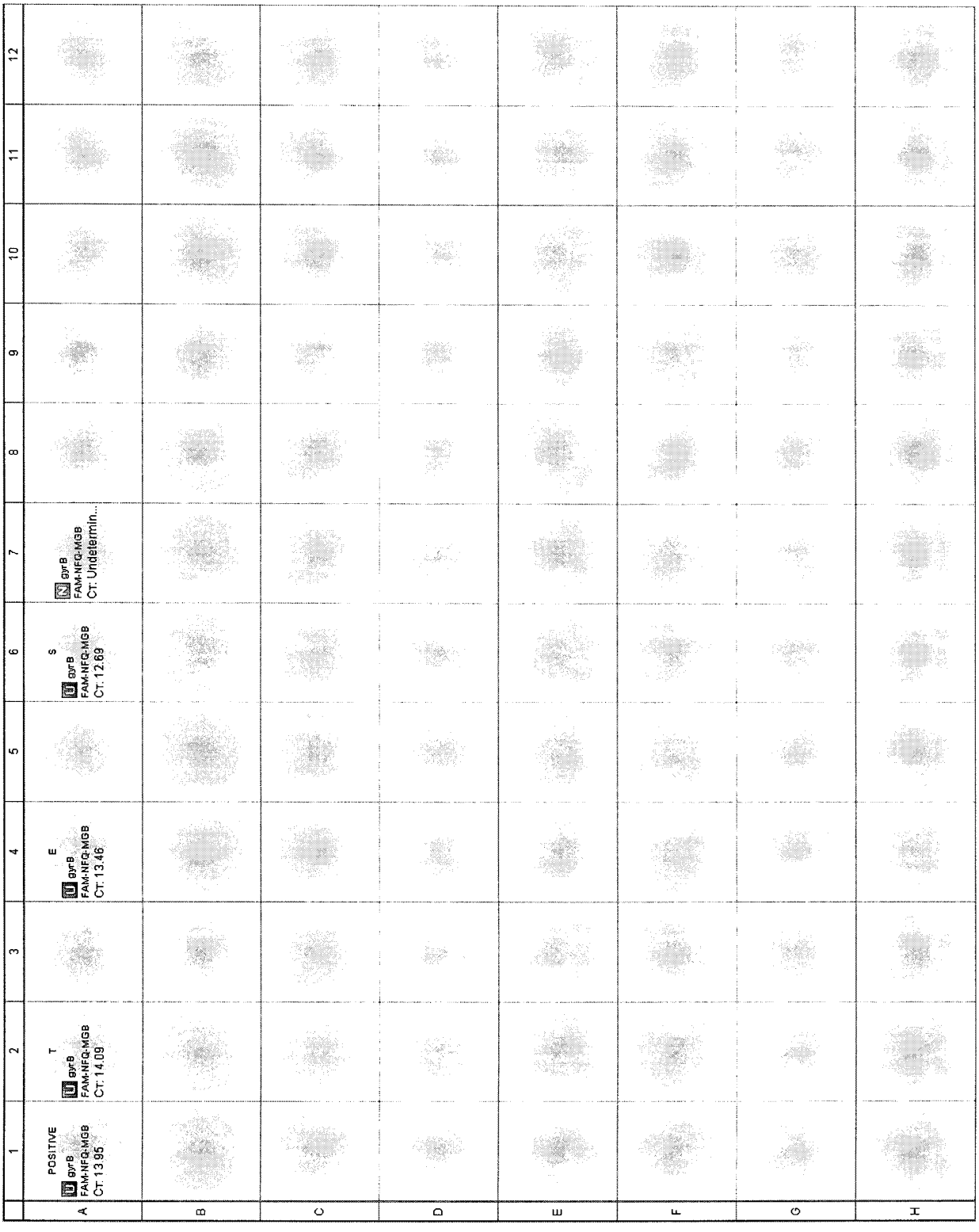
Plate Layout
1
2
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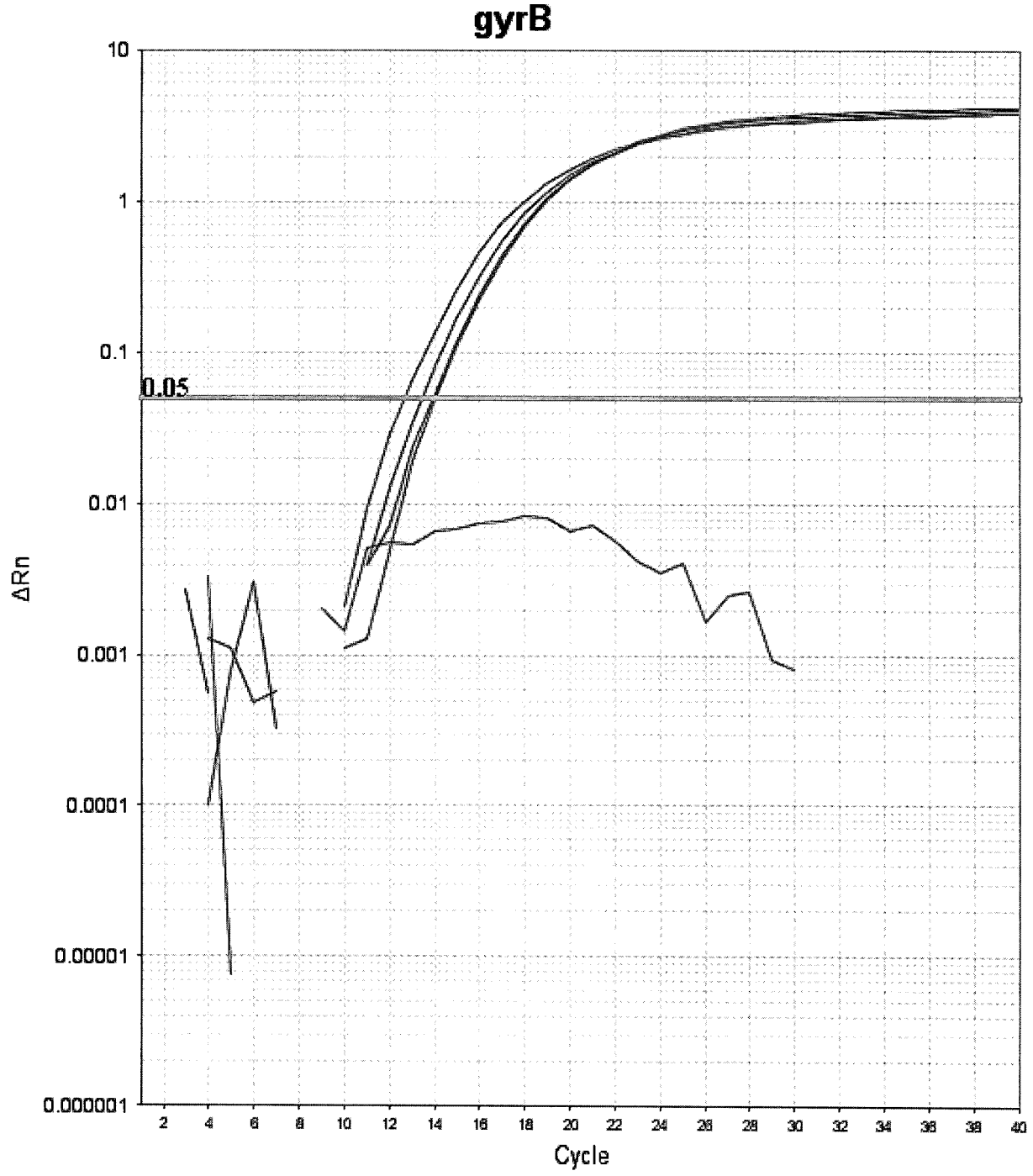
12
A
B
C
D
E
F
G
H
POSITIVE
U gyrB
FAM-NFQ-MGB
Ct: 13.95
T
U gyrB
FAM-NFQ-MGB
Ct: 14.09
E
U gyrB
FAM-NFQ-MGB
Ct: 13.46
S
U gyrB
FAM-NFQ-MGB
Ct: 12.69
N gyrB
FAM-NFQ-MGB
Ct: Undetermin...

Amplification Plot (ΔRn vs. Cycle)



Results Table

Well	Sample	Target	Task	Quantity	CT
A1	POSITIVE	gyrB	UNKNOWN		13.9546
A2	T	gyrB	UNKNOWN		14.0938
A4	E	gyrB	UNKNOWN		13.463
A6	S	gyrB	UNKNOWN		12.6935
A7		gyrB	NTC		Undetermined

QC Summary

Total Wells	96	Processed Wells	5	Targets Used	1
Well Setup	5	Flagged Wells	0	Samples Used	4

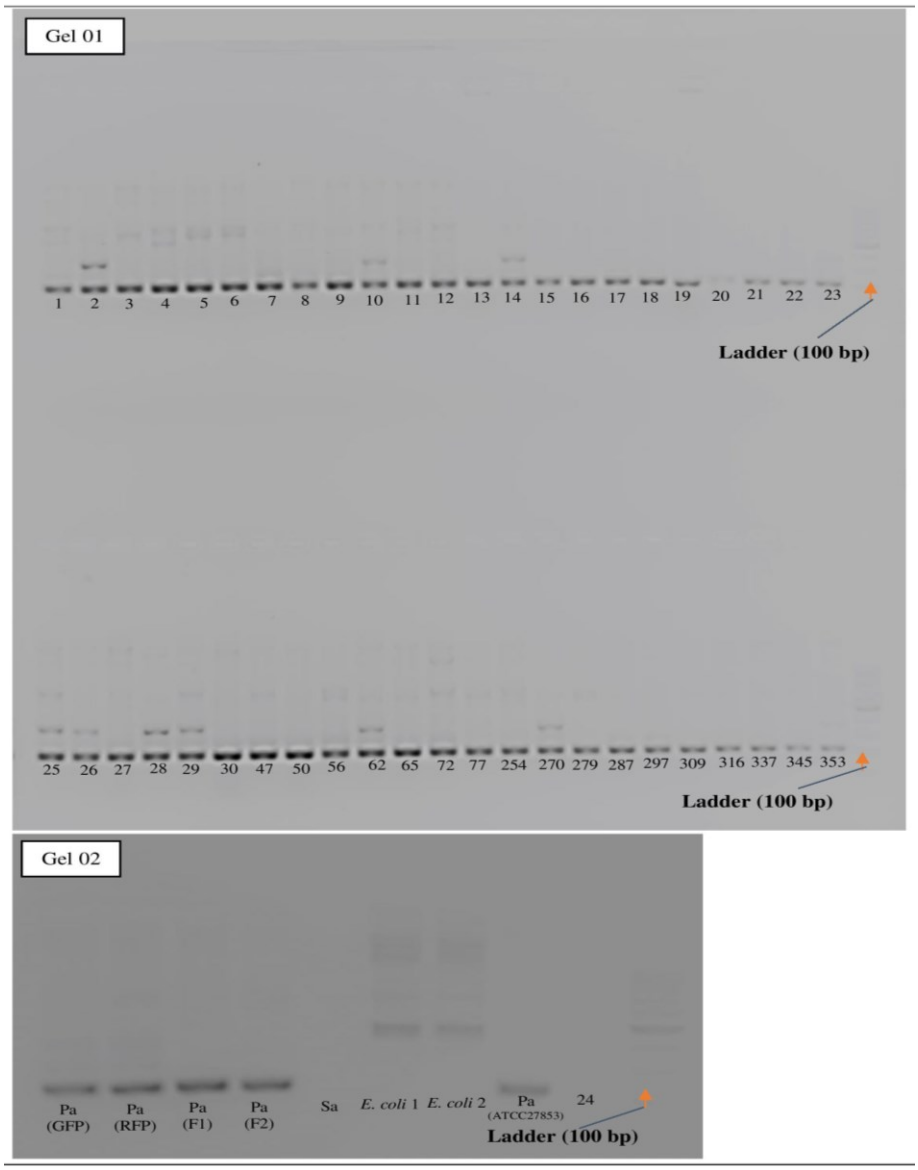
Flag	Name	Frequency	Locations
AMPNC	Amplification in negative control	0	
BADROX	Bad passive reference signal	0	
BLFAIL	Baseline algorithm failed	0	
CTFAIL	C_T algorithm failed	0	
EXPFAIL	Exponential algorithm failed	0	
HIGHSD	High standard deviation in replicate group	0	
NOAMP	No amplification	0	
NOISE	Noise higher than others in plate	0	
NOSIGNAL	No signal in well	0	
OFFSCALE	Fluorescence is offscale	0	
OUTLIERRG	Outlier in replicate group	0	
SPIKE	Noise spikes	0	
THOLDFAIL	Thresholding algorithm failed	0	
ZPR	Zero Passive Reference	0	

APPENDIX 3: PCR Amplification results of antimicrobial resistant genes present in P. aeruginosa isolates:

Name of the Gene: gyrB [222 bp]

Gel 01: Numeric value of each lane represent *P. aeruginosa* isolates number and far right is the ladder (100 bp)

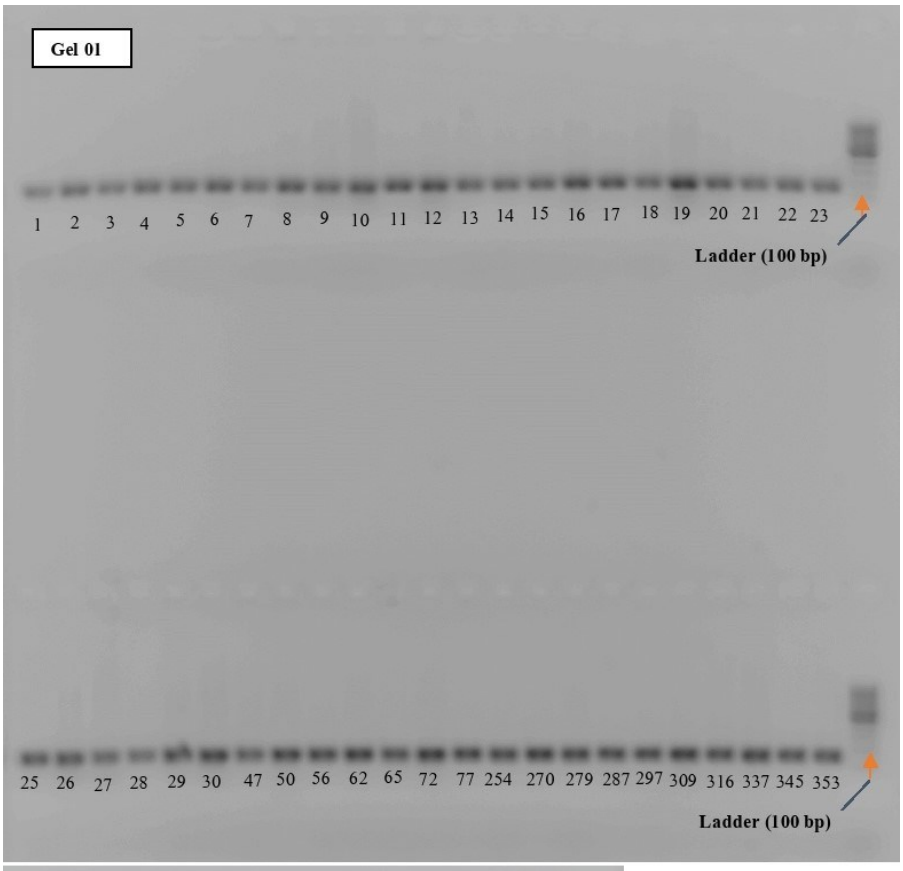
Gel 02: Lane 01: Pa GFP (*P. aeruginosa*), Lane 02: Pa RFP (*P. aeruginosa*), Lane 03: Pa F1 (*P. aeruginosa*), Lane 04: Pa F2 (*P. aeruginosa*), Lane 05: Sa (*Staphylococcus aureus* ATCC 25923), Lane 06: *E. coli* 1 (*Escherichia coli* ATCC 35218) (Non-specific band), *E. coli* 2 (*Escherichia coli* 25922) (Non-specific band), Lane 07: *P. aeruginosa* ATCC 27853, Lane 08: *P. aeruginosa* ATCC 27853, Lane 09: *P. aeruginosa* isolate no 24. Lane 10: ladder (100 bp).

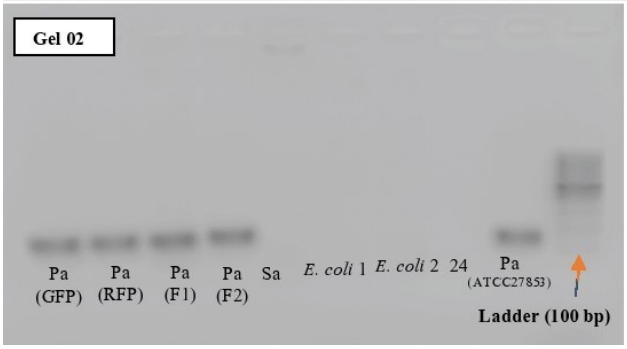


Name of the Gene: exoT [152 bp]

Gel 01: Numeric value of each lane represent *P. aeruginosa* isolates number and far right is the ladder (100 bp)

Gel 02: Lane 01: Pa GFP (*P. aeruginosa*), Lane 02: Pa RFP (*P. aeruginosa*), Lane 03: Pa F1 (*P. aeruginosa*), Lane 04: Pa F2 (*P. aeruginosa*), Lane 05: Sa (*Staphylococcus aureus* ATCC 25923), Lane 06: *E. coli* 1 (*Escherichia coli* ATCC 35218), *E. coli* 2 (*Escherichia coli* 25922), Lane 07: *P. aeruginosa* ATCC 27853, Lane 08: *P. aeruginosa* ATCC 27853, Lane 09: *P. aeruginosa* isolate no 24. Lane 10: ladder (100 bp).

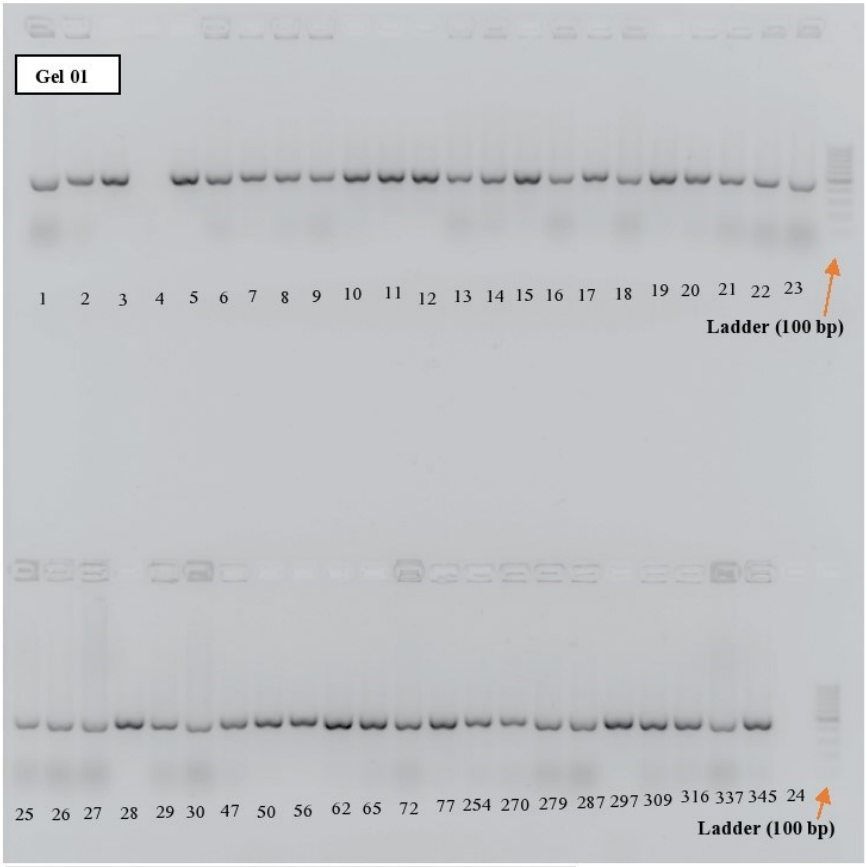


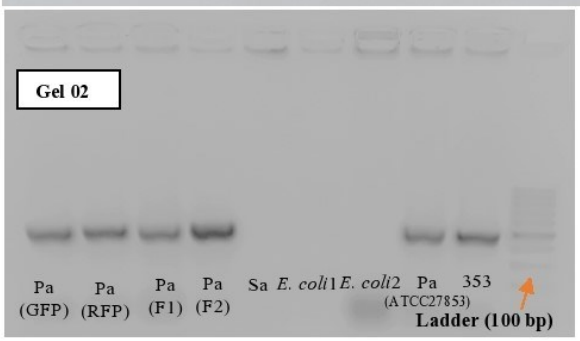


Name of the Gene: oprL [504 bp]

Gel 01: Numeric value of each lane represent *P. aeruginosa* isolates number and far right is the ladder (100 bp)

Gel 02: Lane 01: Pa GFP (*P. aeruginosa*), Lane 02: Pa RFP (*P. aeruginosa*), Lane 03: Pa F1 (*P. aeruginosa*), Lane 04: Pa F2 (*P. aeruginosa*), Lane 05: Sa (*Staphylococcus aureus* ATCC 25923), Lane 06: *E. coli* 1 (*Escherichia coli* ATCC 35218), *E. coli* 2 (*Escherichia coli* 25922), Lane 07: *P. aeruginosa* ATCC 27853, Lane 08: *P. aeruginosa* ATCC 27853, Lane 09: *P. aeruginosa* isolate no 353. Lane 10: ladder (100 bp).

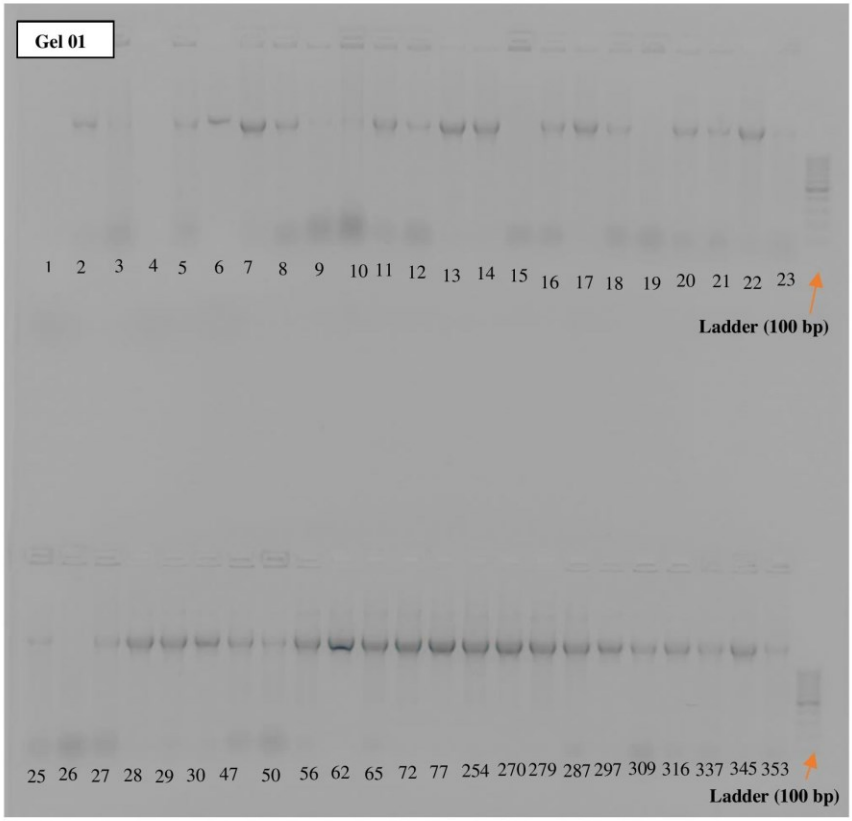


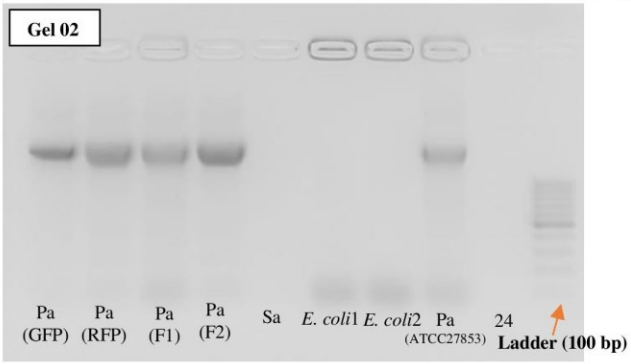


Name of the Gene: oprD [~1.5kb]

Gel 01: Numeric value of each lane represent *P. aeruginosa* isolates number and far right is the ladder (100 bp)

Gel 02: Lane 01: Pa GFP (*P. aeruginosa*), Lane 02: Pa RFP (*P. aeruginosa*), Lane 03: Pa F1 (*P. aeruginosa*), Lane 04: Pa F2 (*P. aeruginosa*), Lane 05: Sa (*Staphylococcus aureus* ATCC 25923), Lane 06: *E. coli* 1 (*Escherichia coli* ATCC 35218), *E. coli* 2 (*Escherichia coli* 25922), Lane 07: *P. aeruginosa* ATCC 27853, Lane 08: *P. aeruginosa* ATCC 27853, Lane 09: *P. aeruginosa* isolate no 24. Lane 10: ladder (100 bp).

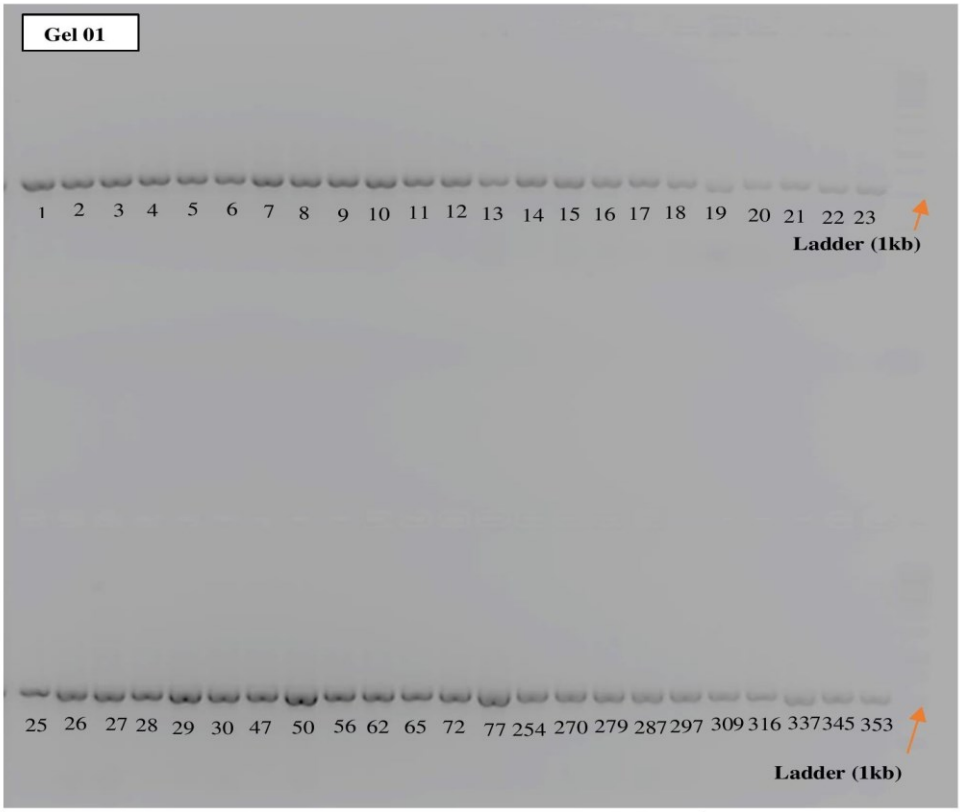


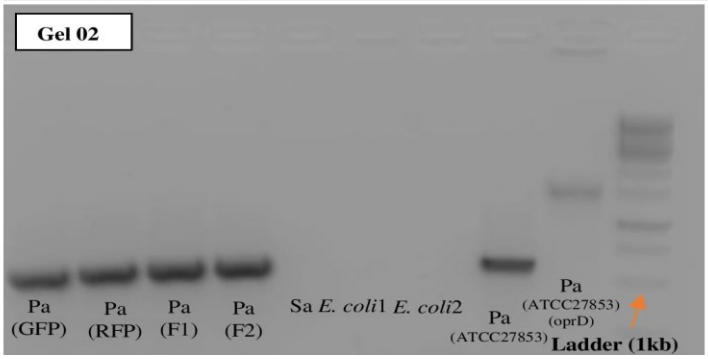


Name of the Gene: lasI [600 bp]

Gel 01: Numeric value of each lane represent *P. aeruginosa* isolates number and far right is the ladder (1kb)

Gel 02: Lane 01: Pa GFP (*P. aeruginosa*), Lane 02: Pa RFP (*P. aeruginosa*), Lane 03: Pa F1 (*P. aeruginosa*), Lane 04: Pa F2 (*P. aeruginosa*), Lane 05: Sa (*Staphylococcus aureus* ATCC 25923), Lane 06: *E. coli* 1 (*Escherichia coli* ATCC 35218), *E. coli* 2 (*Escherichia coli* 25922), Lane 07: *P. aeruginosa* ATCC 27853, Lane 08: *P. aeruginosa* ATCC 27853, Lane 09: *P. aeruginosa* ATCC 27853 (oprD). Lane 10: ladder (1kb).

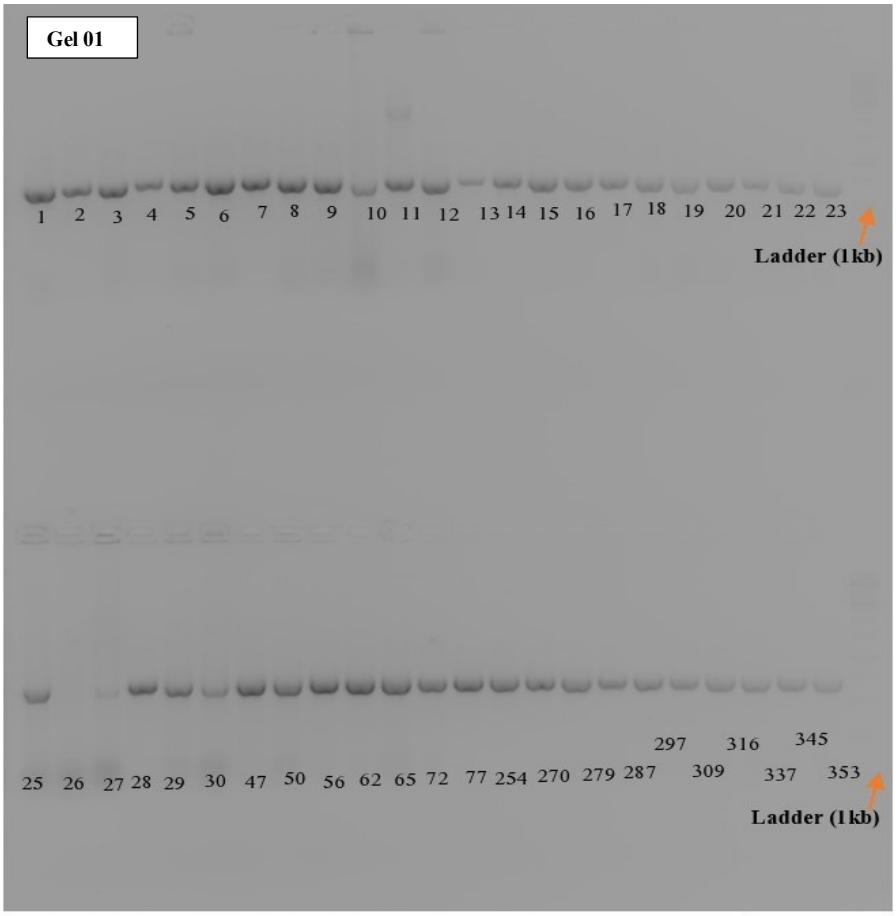


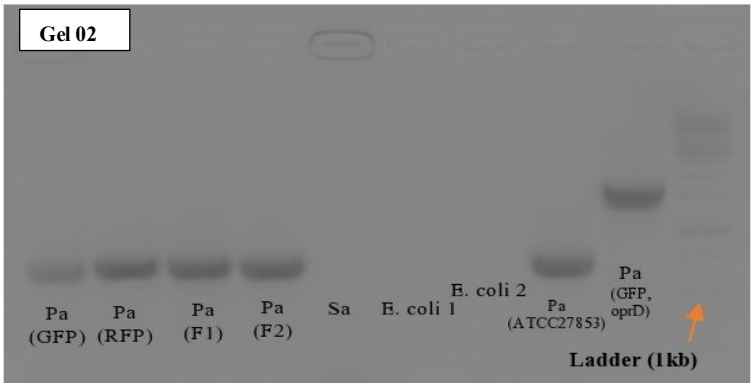


Name of the Gene: lasR [700 bp] (An addition gene oprD ran along with LasR (Gel 02, Lane 09))

Gel 01: Numeric value of each lane represent *P. aeruginosa* isolates number and far right is the ladder (1kb).

Gel 02: Lane 01: Pa GFP (*P. aeruginosa*), Lane 02: Pa RFP (*P. aeruginosa*), Lane 03: Pa F1 (*P. aeruginosa*), Lane 04: Pa F2 (*P. aeruginosa*), Lane 05: Sa (*Staphylococcus aureus* ATCC 25923), Lane 06: *E. coli* 1 (*Escherichia coli* ATCC 35218), *E. coli* 2 (*Escherichia coli* 25922), Lane 07: *P. aeruginosa* ATCC 27853, Lane 08: *P. aeruginosa* ATCC 27853, Lane 09: *P. aeruginosa* ATCC 27853 (oprD). Lane 10: ladder(1kb),

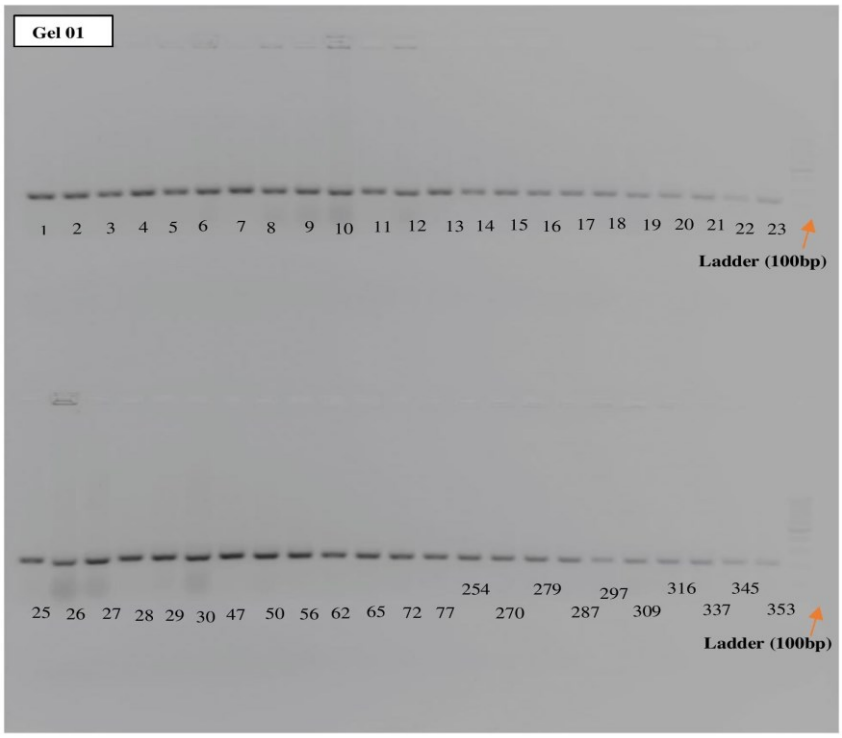


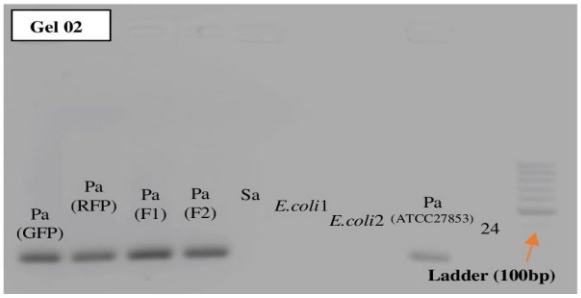


Name of the Gene: ampC [218 bp]

Gel 01: Numeric value of each lane represent *P. aeruginosa* isolates number and far right is the ladder (100bp)

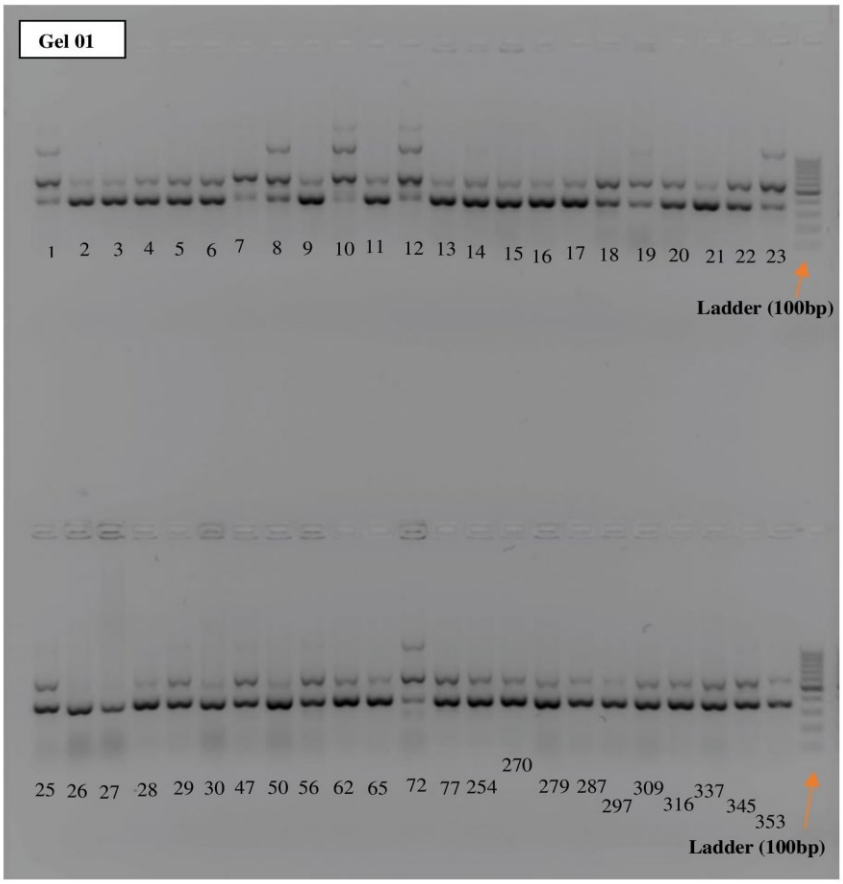
Gel 02: Lane 01: Pa GFP (*P. aeruginosa*), Lane 02: Pa RFP (*P. aeruginosa*), Lane 03: Pa F1 (*P. aeruginosa*), Lane 04: Pa F2 (*P. aeruginosa*), Lane 05: Sa (*Staphylococcus aureus* ATCC 25923), Lane 06: *E. coli* 1 (*Escherichia coli* ATCC 35218), *E. coli* 2 (*Escherichia coli* 25922), Lane 07: *P. aeruginosa* ATCC 27853, Lane 08: *P. aeruginosa* ATCC 27853, Lane 09: *P. aeruginosa* isolate no. 24. Lane 10: ladder (100bp).

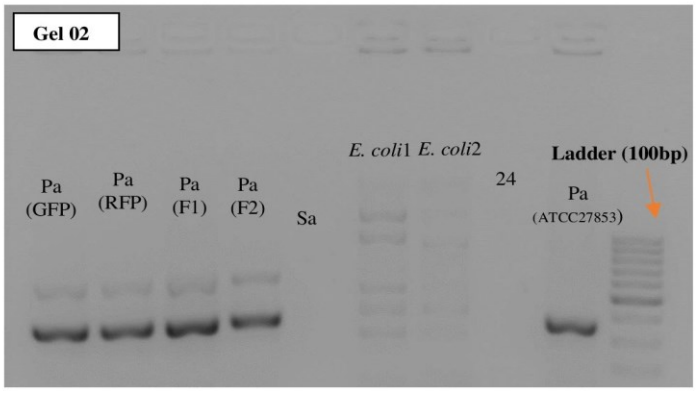




Name of the gene: mexC1,2 [344bp]

Multiplex amplification products showing expression of the mexC1 and mexC2 genes:
Gel 01: Numeric value of each lane represent *P. aeruginosa* isolates number and far right is the ladder (100bp).
Gel 02: Lane 01: Pa GFP (*P. aeruginosa*), Lane 02: Pa RFP (*P. aeruginosa*), Lane 03: Pa F1 (*P. aeruginosa*), Lane 04: Pa F2 (*P. aeruginosa*), Lane 05: Sa (*Staphylococcus aureus* ATCC 25923), Lane 06: *E. coli* 1 (*Escherichia coli* ATCC 35218), *E. coli* 2 (*Escherichia coli* 25922), Lane 07: *P. aeruginosa* ATCC 27853, Lane 08: *P. aeruginosa* isolate no.24, Lane 09: *P. aeruginosa* ATCC 27853, 10: ladder(100bp).



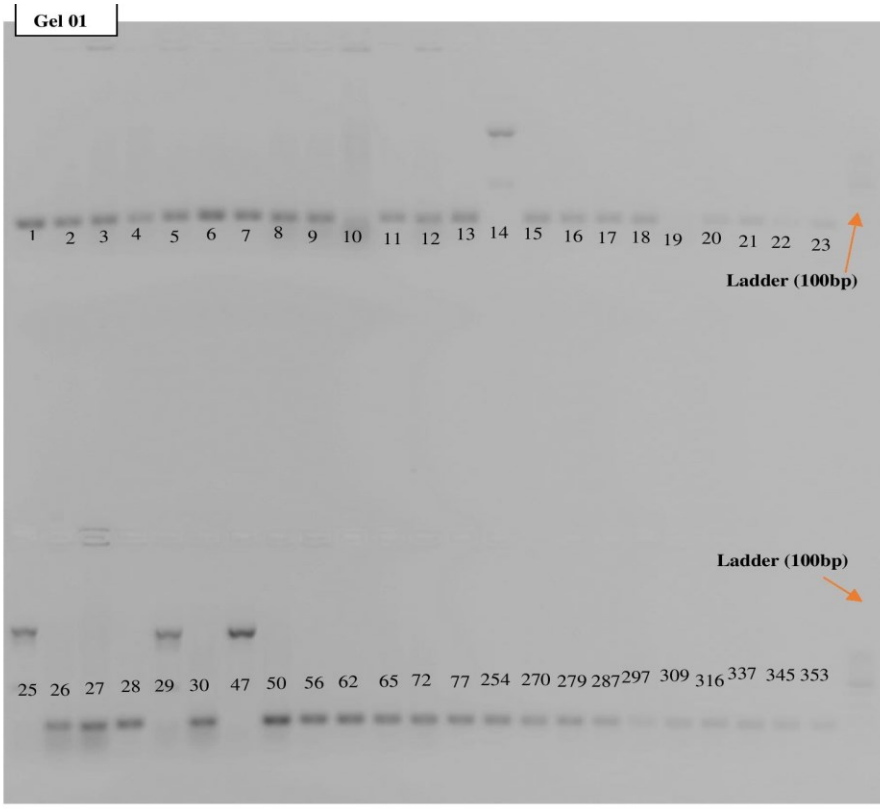


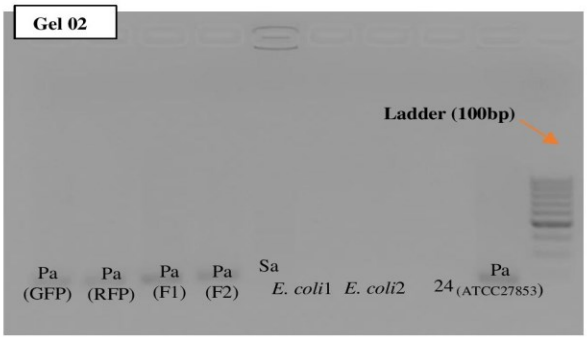
Name of the gene: mexC3,4 [164bp]

Multiplex amplification products showing expression of the mexC3 and mexC4 genes:

Gel 01: Numeric value of each lane represent *P. aeruginosa* isolates number and far right is the ladder (100bp).

Gel 02: Lane 01: Pa GFP (*P. aeruginosa*), Lane 02: Pa RFP (*P. aeruginosa*), Lane 03: Pa F1 (*P. aeruginosa*), Lane 04: Pa F2 (*P. aeruginosa*), Lane 05: Sa (*Staphylococcus aureus* ATCC 25923), Lane 06: *E. coli* 1 (*Escherichia coli* ATCC 35218), *E. coli* 2 (*Escherichia coli* 25922), Lane 07: *P. aeruginosa* ATCC 27853, Lane 08: *P. aeruginosa* isolate no.24, Lane 09: *P. aeruginosa* ATCC 27853, 10: ladder(100bp).

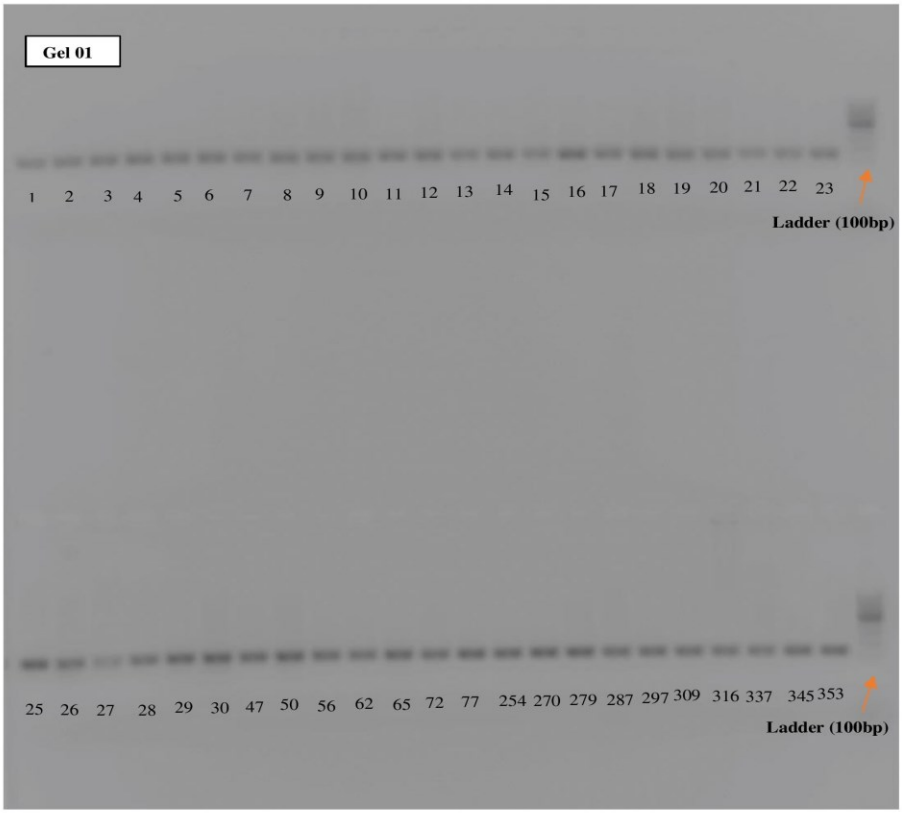


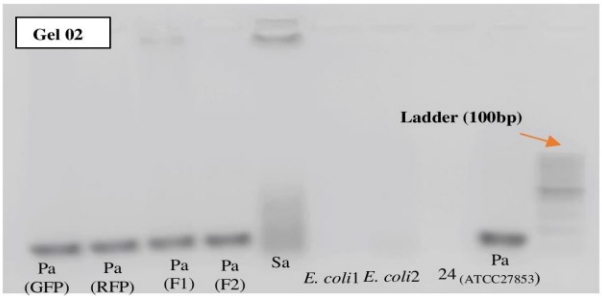


Name of the gene: ndvB [157 bp]

Gel 01: Numeric value of each lane represent *P. aeruginosa* isolates number and far right is the ladder (100bp).

Gel 02: Lane 01: Pa GFP (*P. aeruginosa*), Lane 02: Pa RFP (*P. aeruginosa*), Lane 03: Pa F1 (*P. aeruginosa*), Lane 04: Pa F2 (*P. aeruginosa*), Lane 05: Sa (*Staphylococcus aureus* ATCC 25923), Lane 06: *E. coli* 1 (*Escherichia coli* ATCC 35218), *E. coli* 2 (*Escherichia coli* 25922), Lane 07: *P. aeruginosa* ATCC 27853, Lane 08: *P. aeruginosa* isolate no.24, Lane 09: *P. aeruginosa* ATCC 27853, 10: ladder(100bp).

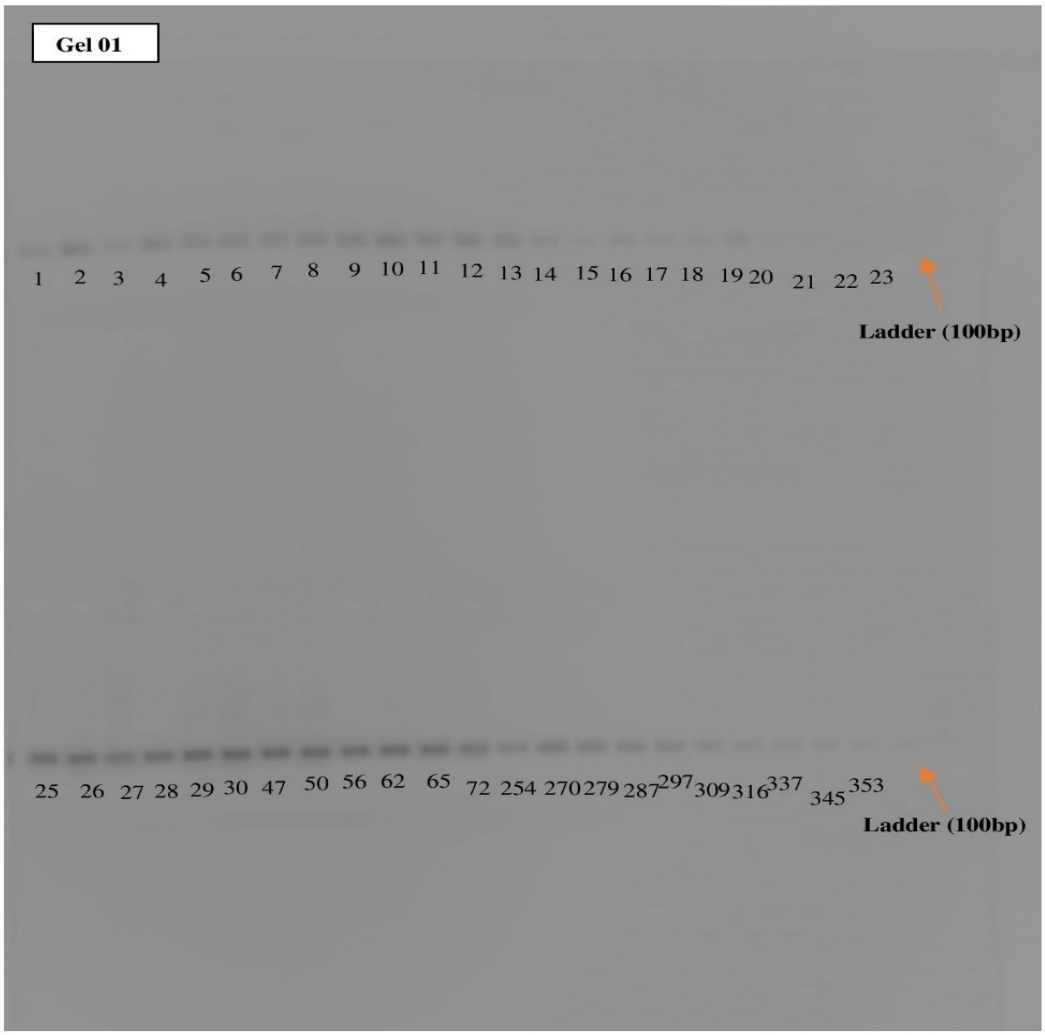


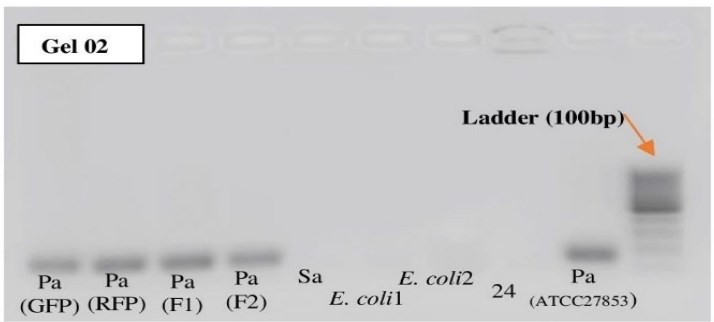


Name of the gene: tssC1 [150 bp]

Gel 01: Numeric value of each lane represent *P. aeruginosa* isolates number and far right is the ladder (100bp).

Gel 02: Lane 01: Pa GFP (*P. aeruginosa*), Lane 02: Pa RFP (*P. aeruginosa*), Lane 03: Pa F1 (*P. aeruginosa*), Lane 04: Pa F2 (*P. aeruginosa*), Lane 05: Sa (*Staphylococcus aureus* ATCC 25923), Lane 06: *E. coli* 1 (*Escherichia coli* ATCC 35218), *E. coli* 2 (*Escherichia coli* 25922), Lane 07: *P. aeruginosa* ATCC 27853, Lane 08: *P. aeruginosa* isolate no.24, Lane 09: *P. aeruginosa* ATCC 27853, 10: ladder(100bp).

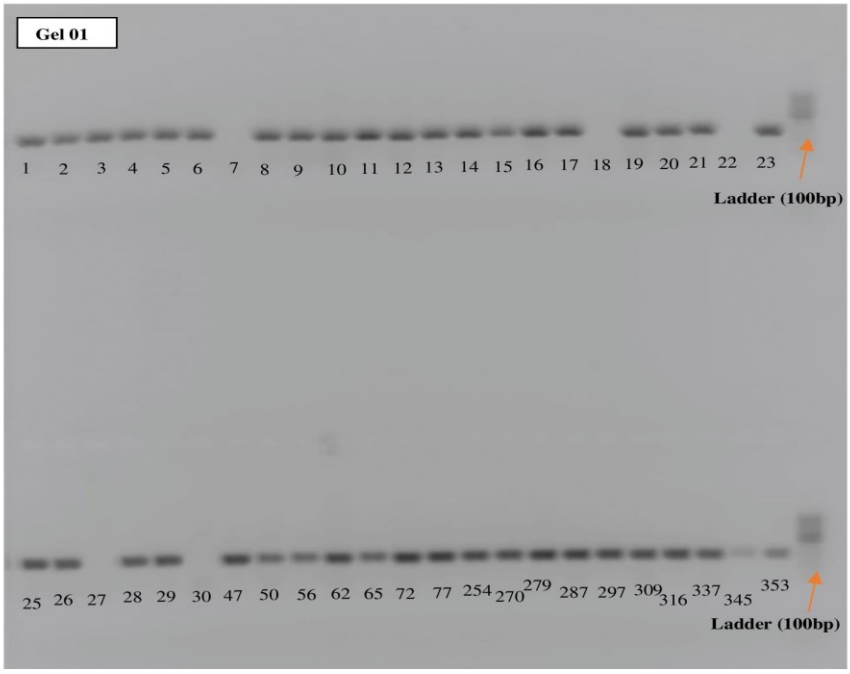


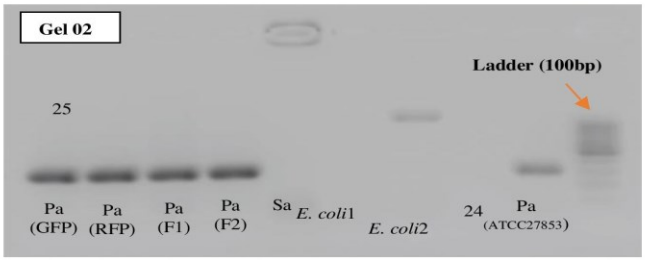


Name of the gene: exoY [289 bp]

Gel 01: Numeric value of each lane represent *P. aeruginosa* isolates number and far right is the ladder (100bp).

Gel 02: Lane 01: Pa GFP (*P. aeruginosa*), Lane 02: Pa RFP (*P. aeruginosa*), Lane 03: Pa F1 (*P. aeruginosa*), Lane 04: Pa F2 (*P. aeruginosa*), Lane 05: Sa (*Staphylococcus aureus* ATCC 25923), Lane 06: *E. coli* 1 (*Escherichia coli* ATCC 35218), *E. coli* 2 (*Escherichia coli* 25922), Lane 07: *P. aeruginosa* ATCC 27853, Lane 08: *P. aeruginosa* isolate no.24, Lane 09: *P. aeruginosa* ATCC 27853, 10: ladder(100bp).

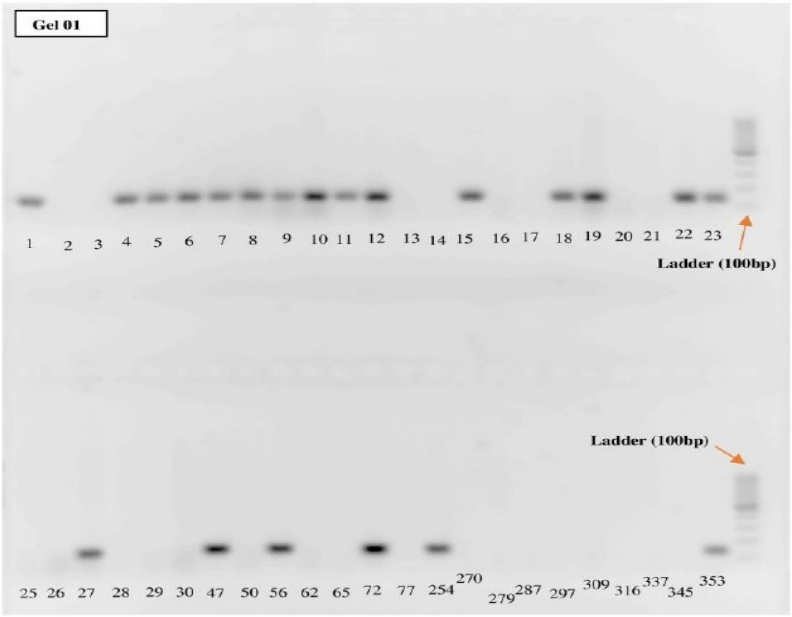


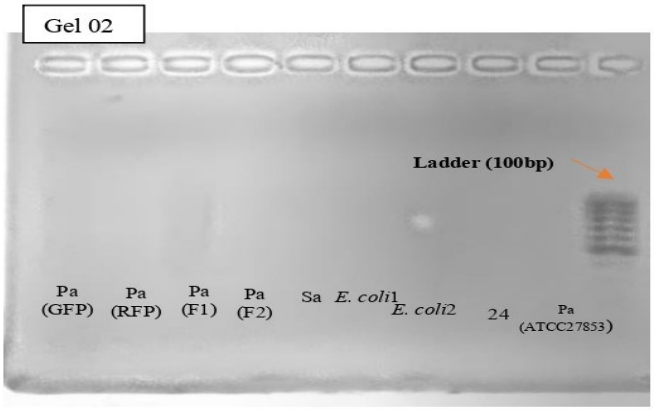


Name of the gene: exoU [134 bp]

Gel 01: Numeric value of each lane represent *P. aeruginosa* isolates number and far right is the ladder (100bp).

Gel 02: Lane 01: Pa GFP (*P. aeruginosa*), Lane 02: Pa RFP (*P. aeruginosa*), Lane 03: Pa F1 (*P. aeruginosa*), Lane 04: Pa F2 (*P. aeruginosa*), Lane 05: Sa (*Staphylococcus aureus* ATCC 25923), Lane 06: *E. coli* 1 (*Escherichia coli* ATCC 35218), *E. coli* 2 (*Escherichia coli* 25922),



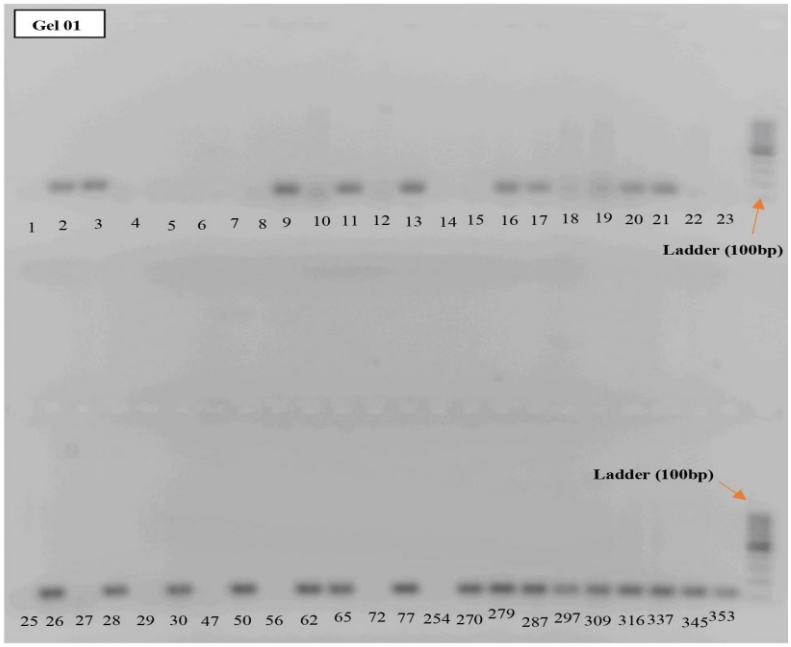


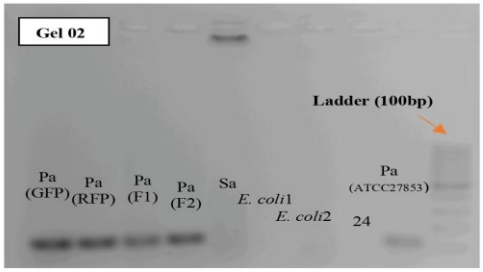
Lane 07: *P. aeruginosa* ATCC 27853, Lane 08: *P. aeruginosa* isolate no.24, Lane 09: *P. aeruginosa* ATCC 27853, 10: ladder(100bp).

Name of the gene: exoS [118 bp]

Gel 01: Numeric value of each lane represent *P. aeruginosa* isolates number and far right is the ladder (100bp).

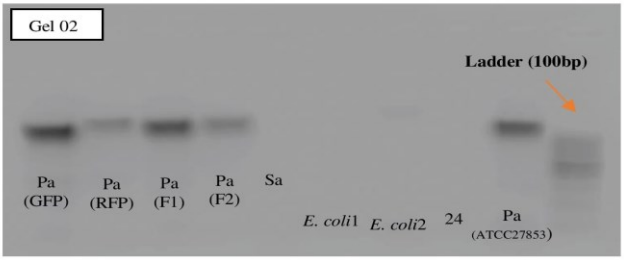
Gel 02: Lane 01: Pa GFP (*P. aeruginosa*), Lane 02: Pa RFP (*P. aeruginosa*), Lane 03: Pa F1 (*P. aeruginosa*), Lane 04: Pa F2 (*P. aeruginosa*), Lane 05: Sa (*Staphylococcus aureus* ATCC 25923), Lane 06: *E. coli* 1 (*Escherichia coli* ATCC 35218), *E. coli* 2 (*Escherichia coli* 25922), Lane 07: *P. aeruginosa* ATCC 27853, Lane 08: *P. aeruginosa* isolate no.24, Lane 09: *P. aeruginosa* ATCC 27853, 10: ladder(100bp).





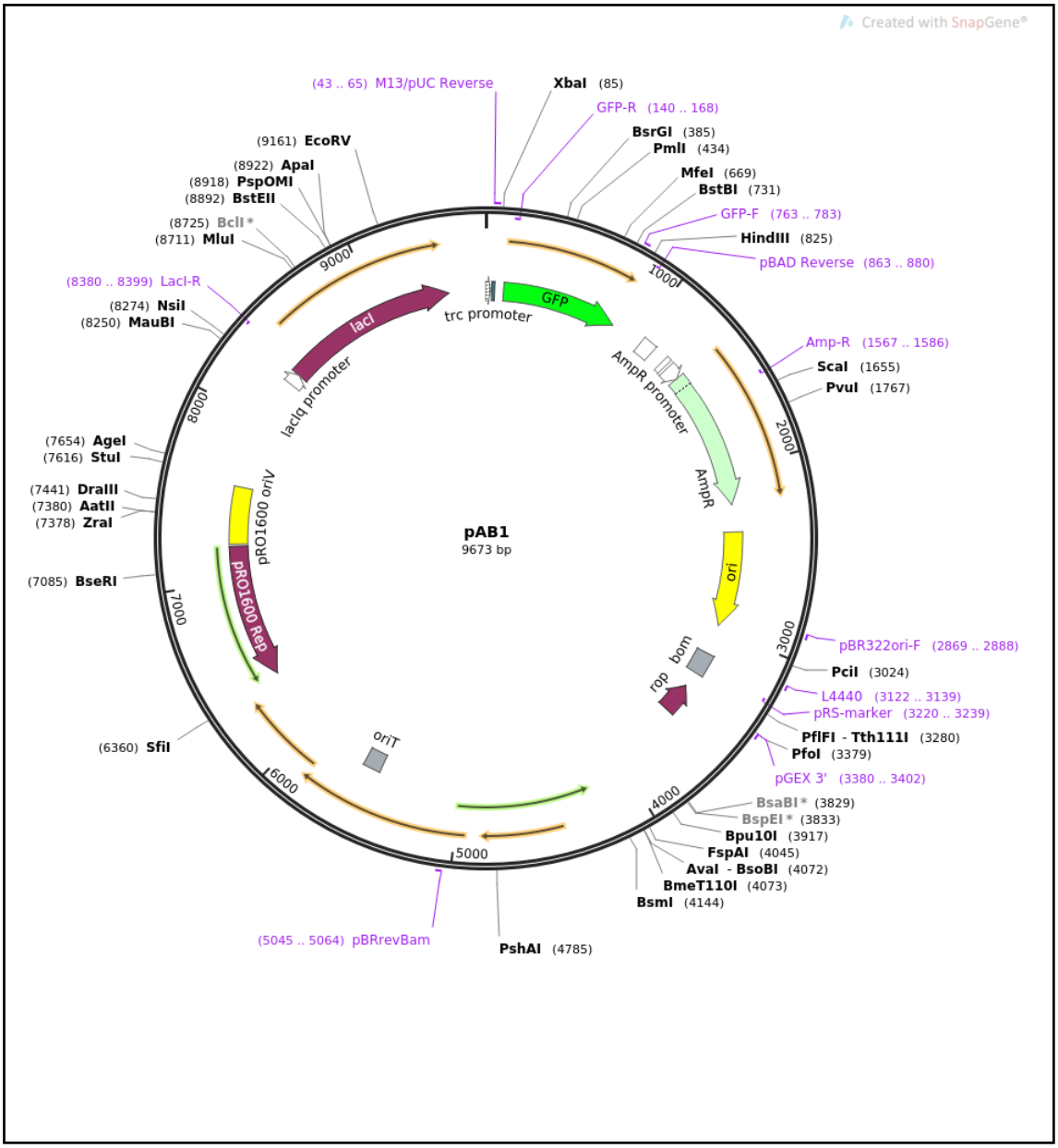
Name of the gene: popB [~1.5 K]

Gel 01: Numeric value of each lane represent *P. aeruginosa* isolates number and far right is the ladder (100bp). Gel 02: Lane 01: Pa GFP (*P. aeruginosa*), Lane 02: Pa RFP (*P. aeruginosa*), Lane 03: Pa F1 (*P. aeruginosa*), Lane 04: Pa F2 (*P. aeruginosa*), Lane 05: Sa (*Staphylococcus aureus* ATCC 25923), Lane 06: *E. coli* 1 (*Escherichia coli* ATCC 35218), *E. coli* 2 (*Escherichia coli* 25922), Lane 07: *P. aeruginosa* ATCC 27853, Lane 08: *P. aeruginosa* isolate no.24, Lane 09: *P. aeruginosa* ATCC 27853, 10: ladder(100bp).



APPENDIX 4: P. aeruginosa plasmid pAB1 contains GFP genes and ampicillin (AMP) resistant genes:

Broad host range of plasmid (pAB1) for *Pseudomonas*, containing GFP genes and ampicillin resistant genes (Addgene plasmid # 62547; http://n2t.net/addgene:62547; RRID:Addgene_62547) (Walters et al., 2003).



(The End)