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The effect of cigarette smoke extract on the activity of clarithromycin with anti-pseudomonal agents on growth and biofilm formation of *Pseudomonas aeruginosa*

By

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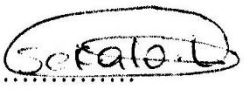
Declaration

I, **Sekalo Lebogang** declare that the content of this research project represents my own work. I strictly followed norms in academia, I acknowledged wherever I used other people's work and wherever I was assisted. I further declare that this work has not been submitted or presented before for any qualification at this institution or any other institution. This work is submitted in fulfilment of the requirements for a Master of Science degree at the University of Pretoria.

Signed..... 

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Executive Summary

The present study was undertaken with the initial objective of investigating the effects of six primary anti-pseudomonal antibiotics, namely amikacin, cefepime, ciprofloxacin, meropenem, piperacillin and tazobactam, on the planktonic growth of, and formation of biofilm by three different strains of the resilient respiratory pathogen, *Pseudomonas aeruginosa* [two drug-sensitive strains: the wild-type reference strain, PAO1(WT), and a clinical isolate (DS), as well as a multidrug resistant (MDR) clinically isolated variant of the pathogen]. These agents were investigated individually and in combination with the macrolide antibiotic, clarithromycin. Although all three test strains of *P. aeruginosa* are resistant to clarithromycin, this agent was included as an adjunct to the conventional anti-pseudomonal agents because of its inhibitory effects on various virulence factors of the pathogen. Following acquisition of the minimal inhibitory concentrations (MICs) of the individual anti-pseudomonal agents with respect to planktonic growth of, and biofilm formation by all three strains of *P. aeruginosa*, these experiments were repeated using the test anti-pseudomonal antibiotics in combination with clarithromycin. These results showed that amikacin, cefepime, ciprofloxacin and meropenem individually were potent inhibitors of the growth and formation of biofilm of the two susceptible strains of the *P. aeruginosa*, while, as expected, the MDR strain was highly resistant. When used in combination with clarithromycin, however, synergistic interactions with amikacin, cefepime and ciprofloxacin were observed, while additive activity was observed with the MDR strain.

This phase of the study was followed by investigating the effects of exposure of all three strains of *P. aeruginosa* to cigarette smoke condensate (CSC) on the anti-pseudomonal activities of amikacin, cefepime and ciprofloxacin individually and in combination with clarithromycin. Although brief exposure of all three strains of the pathogen to CSC had modest, albeit variable, augmentative effects on bacterial planktonic growth and biofilm formation, the antimicrobial activities of amikacin, cefepime and ciprofloxacin, both individually and in combination with clarithromycin, were unaffected by exposure to CSC.

In conclusion, although further research is necessary with respect to the effects of exposure of *P. aeruginosa* to CSC, the current study has highlighted two notable issues. Firstly, the inhibitory effects of several currently used anti-pseudomonal antibiotics, at therapeutically

relevant concentrations, on biofilm formation by *P. aeruginosa.* Secondly, the potential of clarithromycin to cause synergistic or additive interactions with the antibiotic-susceptible and –resistant strains, respectively.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

BHI	Brain heart infusion
CAP	Community-acquired pneumonia
CD	Cluster of differentiation
CDC	Centers for disease control
CF	Cystic fibrosis
CFU	Colony forming units
CLSI	Clinical and laboratory service
COPD	Chronic obstructive pulmonary disease
CRE	Carbapenem-resistant Enterobacterales
CS	Cigarette smoke
CSC	Cigarette smoke condensate
CYP3A4	Cytochrome P450 3A4
DC	Dendritic cells
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DS	Drug-sensitive
eDNA	Extracellular deoxyribonucleic acid
EF2	Elongation factor 2
EG	Ecthyma gangrenosum
EPS	Extracellular polymeric substance
ESBL	Extended-spectrum β-lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
Fe^{3+}	Iron
FIC	Fractional Inhibitory Concentration
FICI	Fractional Inhibitory Concentration Index
FLID	Flagellar cap protein
GNB	Gram-negative bacilli
HIV	Human immunodeficiency virus
ICU	Intensive care unit
Ig	Immunoglobulin
IL	Interleukin
LA	Luria agar

LB	Luria-Bertani broth
LPS	Lipopolysaccharides
Mbp	Megabase pairs
MDR	Multidrug-resistant
MIC	Minimum inhibitory concentration
NHLS	National Health Laboratory Service
NHSN	National Health Safety Network
NICD	National Institute of Communicable Diseases
NK	Natural killer
OD	Optical density
PAMPs	Pathogen-associated molecular patterns
PBPs	Penicillin-binding proteins
PBS	Phosphate-buffered saline
PRRs	Pattern recognition receptors
QS	Quorum sensing
RSA	Republic of South Africa
SDS	Sodium dodecyl sulphate
T3SS	Type III secretion system
TBS	Tryptic soy broth
TLRs	Toll-like receptors
URI	Upper respiratory infection
USA	United States of America
UTI	Urinary tract infection
VAP	Ventilator-associated pneumonia
WHO	World Health Organisation
WT	Wild type
XDR	Extensively drug-resistant

List of Units

µL	Microlitre
µm	Micrometer
µM	Micromolar
µg	Microgram
mL	Millilitre
mM	Millimolar
Min	Minutes
ng	Nanogram
U	Units
v/v	Volume per volume
w/v	Weight per volume
℃	Degrees Celsius

Chapter 1: Literature Review

1.1 Background of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa (*P. aeruginosa*) is a Gram-negative bacterium that can be found in a wide range of sites including soil, aquatic environments, plants, and indoor settings such as hospitals (Diggle and Whiteley, 2020; Hilliam et al., 2020). It can also be found in human faeces, and forms part of the normal flora of the skin, and nasopharyngeal mucosal surface (Iglewski., 1996; Diggle and Whiteley, 2020).

In 1882, French pharmacist Carle Gessard was the first to identify *P. aeruginosa* and named it *Bacillus pyocyaneus* based on the “blue/green” coloration of the phenazine compound pyocyanin (Gessard, 1984). Later the organism was renamed *P. aeruginosa*. The term *Pseudomonas aeruginosa* comes from three Greek words: pseudo’ meaning "false", monas meaning "single unit", and *aeruginosa* meaning "greenish blue" (Diggle and Whiteley, 2020). The genus *Pseudomonas* was first proposed in 1894 for all Gram-negative, rod-shaped aerobic bacilli with flagella (Harold and Rose, 1968; Wilson and Pandey, 2022). This genus is extremely broad, to the extent that there are currently over 144 species of *Pseudomonas* that have been discovered (Gomila et al., 2015).

1.2 Clinical conditions associated with *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is an opportunistic pathogen that causes invasive infections such as 1) chronic lung infection in cystic fibrosis (CF) patients; 2) acute ulcerative keratitis in patients that wear soft contact lenses extensively, and 3) bacteraemia in severe burn wounds, and of the respiratory tract, urinary tract, surgical sites, and bloodstream (Bodey et al., 1983; Hauser, 2009; Magret et al., 2011). It causes a local and systemic infection that mostly changes from benign to life-threatening (Tümmler and Klockgether, 2017). As mentioned above, *P. aeruginosa* infections can occur in many parts of the body and symptoms resemble those of other Gram-negative infections (Alhazmi, 2015; Paulsson et al., 2019).

Individuals presenting with *P. aeruginosa* respiratory tract infection appear to have more severe productive coughs that result in reduced appetite that, in turn, leads to weight loss (Stoltz

et al., 2015; Chai and Xu, 2020). In bone and joints, *P. aeruginosa* has been reported to cause osteochondritis with pain that persists for months in the neck and back region (Meher et al., 2016). Children with osteochondritis have pain and oedema for between three and four days (Cerioli et al., 2020). In skin and soft tissue, *P. aeruginosa* causes infections such as burn wound sepsis, psoriasis, ecthyma gangrenosum [EG], pyoderma, sepsis, hot tub folliculitis, septic shock, and chronic paronychia (Kujath and Kujath, 2010; Spernovasilis et al., 2021). Burn wound infection caused by *P. aeruginosa* occurs with systemic involvement presenting as fever, disorientation, hypotension, leukopenia, and hypothermia. Tenderness and moderate swelling are also the first signs of chronic paronychia (Hasannejad-Bibalan et al., 2021).

Otitis media, persistent suppurative otitis media, otitis externa, and aggressive external otitis are some of the diseases of the ear that are characterised by *P. aeruginosa* infection (Mittal et al., 2015). Symptoms of the ear include minor, local, and usually self-limited illness although it can progress to cancerous otitis. Pseudomonal eye infections include keratitis, endophthalmitis, conjunctivitis, ophthalmia neonatorum, scleral abscess and orbital cellulitis (Dhirachaikulpanich et al., 2021; Lin et al., 2022). *Pseudomonas aeruginosa* infections are frequently linked with permeable contact lenses, which can result in a necrotic, greyish stromal infiltration in epithelial injuries (Mittal et al., 2016). Pain, conjunctival hyperaemia, chemosis, lid oedema, reduced eyesight, hypopyon, or severe anterior uveitis with vitreous inclusion and pan ophthalmitis are all possible clinical features of infection with this microbial organism (Mittal et al., 2016; Treviño González et al., 2021).

1.3 Burden of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is the leading cause of opportunistic infections being responsible for chronic infections in both immunocompromised and immunocompetent patients (Paulsson et al., 2019). Up to 80% of immunocompromised individuals, including those with CF, organ transplant recipients, burn victims, those with cancer, diabetes and human immunodeficiency virus (HIV) infection, as well as those in intensive care units (ICUs), suffer from respiratory infections caused by *P. aeruginosa* (Juan et al., 2017; Paulsson et al., 2019).

Pseudomonas aeruginosa is a major cause of morbidity and mortality. It has been reported to be the second-leading infection among patients suffering from ventilator-associated pneumonia

(VAP) accounting for 16.6% of isolates as reported by the United States of America (USA) National Health Safety Network (NHSN) (Sievert et al., 2013). Globally, there is between an eight and 28% incidence rate of VAP with a mortality rate of between 24 and 76% (Bassetti et al., 2018). In the Republic of South Africa (RSA), *P. aeruginosa* is the primary source of morbidity and mortality in both immunocompromised and immunocompetent patients (Ohadian et al., 2020). The illnesses and deaths linked to *P. aeruginosa* infection in RSA account for 62% and 30%, respectively (Ramírez-Estrada et al., 2016; Ohadian et al., 2020). The RSA Department of Health reported that 37.4% of patients admitted to hospital with *P. aeruginosa* infection between 2010 and 2017 were found to be immunocompetent while 62.4% were immunocompromised. Notably, the death rate in immunocompetent patients was lower than that in immunocompromised patients (8% and 30%, respectively) (Bassetti et al., 2018; Parkins et al., 2018).

The burden of *P. aeruginosa* is increasing due to its inherent resistance to certain antibiotics. This ability to develop antibiotic resistance is a result of several mechanisms employed by these bacteria, as reviewed by Pang et al. (Pang et al., 2019). *Pseudomonas aeruginosa* are able to limit the ability of antibiotics to pass through the outer membrane of the bacteria (Lambert, 2002). In addition, they possess efflux systems that pump antibiotics out of the cell (Sun et al., 2014) and produce β-lactamases that are able to inactivate antibiotics (Berrazeg et al., 2015). Another way in which *P. aeruginosa* acquire antibiotic resistance is through mutational changes that lead to horizontal gene transfer (Breidenstein et al., 2011).

The development of resistance to currently available antibiotics leads to persistent infections with *P. aeruginosa* that has serious clinical implications (Lambert, 2002). For instance, an outbreak of multidrug resistant *P. aeruginosa* occurred in a tertiary academic hospital in Cape Town, South Africa in 2010 and 2011 leading to a fatality rate of 80% (Mudau et al., 2013). Furthermore, a study conducted by Adjei et al., at a private hospital in Durban, South Africa, investigated the resistance of 17 isolates of *P. aeruginosa* to certain drugs and reported that: 94% were resistant to piperacillin, 88% to impenem and ticarcillin, 82% to meropenem, 76% to ceftazidime and tazobactam, 82% to ciprofloxacin, and 29% to amikacin (Adjei et al., 2017).

1.3.1 Risk factors associated with *Pseudomonas aeruginosa* infection

As mentioned previously, *P. aeruginosa* infections are common in immunocompromised and immunocompetent individuals (Migiyama et al., 2021). In addition, it is common in children under the age of five years (Zhang et al., 2012; Gregson et al., 2021). In healthy infants, it causes community-acquired pseudomonal sepsis that often presents with diarrhoea. Infection with *Pseudomonas* is also common in the elderly with the older prevalence of *P. aeruginosa* infections increasing with age. *Pseudomonas aeruginosa* in adults has been associated with an increased risk of rare blood clotting incidents (Zhang et al., 2014). Individuals infected with HIV are also considered to be at risk of developing opportunistic *P. aeruginosa* infections, particularly when their cluster of differentiation (CD)4 T-lymphocyte count is lower than 200 cells per microlitre (cells/µL). *Pseudomonas* commonly causes pneumonia in these individuals contributing to high mortality in these cohorts (Akinjogunla et al., 2020; Nganou-Makamdop et al., 2021).

Pseudomonas infections in hospitals are usually associated with patients with VAP (Luyt et al., 2018). The high-risk of *P. aeruginosa* infection with VAP is due to endotracheal intubation of these individuals. The endotracheal tube damages the epithelial lining and serves as a surface for *P. aeruginosa* biofilm formation. This infection has been reported to account for 30% of the mortality rate of VAP patients (Ruffin and Brochiero, 2019). Antibiotic resistance also contributes to an increased risk of *P. aeruginosa* infections. The high incidence of multidrug resistant (MDR)/extensively drug resistant (XDR) or resistant *P. aeruginosa* rates is a global public health problem and is related to higher death and resource consumption (Raman et al., 2018; Horcajada et al., 2019). Prior antibiotic use and ICU hospitalisation are significant risk factors that correlate with MDR or XDR *P. aeruginosa* infections (Willmann et al., 2014; Raman et al., 2018). Importantly, the use of cigarettes plays a major role in developing lung emphysema and chronic obstructive pulmonary diseases (COPD); that skew the inflammatory mediator profile of macrophages and leads to increased disease susceptibility (Strzelak et al., 2018).

1.4 Characteristics of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a non-spore forming, motile Gram-negative bacillus that is approximately between 0.5 and 0.8 micrometres (µm) in size. The *P. aeruginosa* genome [5.5-7 megabase pairs (Mbp)] is composed of a large single circular chromosome and has a variety of plasmids (Kung et al., 2010; Cao et al., 2017). It is ubiquitous, found predominantly in soil, water, associated with the natural skin flora, as well as on other surfaces (Diggle and Whiteley, 2020; Wilson and Pandey, 2022). The bacterium can grow in aerobic and anaerobic conditions (Pallett et al., 2019) and is able to ferment glucose and lactose (Qin et al., 2019). It requires between 16 and 24 hours for *P. aeruginosa* to grow on solid media such as blood agar, MacConkey, Cetrimide, Luria and nutrient agar at temperatures ranging between 25 and 37 degrees Celsius (℃) (Troung et al., 2022).

On solid media, the bacterium produces large, opaque, convex colonies that have a rough edge and are light tan in colour (Kirisits et al., 2005; LaBauve and Wargo, 2015). *Pseudomonas aeruginosa* requires 24-hours to grow in media such as Luria-Bertani (LB) broth, nutrient broth and Brain Heart Infusion (BHI) broth forming a colourless to translucent yellow bacterial suspension or milky-like culture (Bouglé et al., 2017; Testa et al., 2019; Mellini et al., 2021). During growth, the bacteria produce different pigments, which include pyocyanin (blue-green), pyorubin (red-brown), pyoverdine (yellow-green) and fluorescent pigments when incubated on Cetrimide agar media for 24 hours at between 35 and 37 ℃ (Visca et al., 2007; Jayaseelan et al., 2014).

The cell wall of *P. aeruginosa* is composed of an inner and outer layer (Jones, 2017). The inner layer is composed mainly of phospholipids while the outer layer consists of an outer membrane composed of endotoxins or lipopolysaccharides (LPSs) (Jones, 2017; Huszczynski et al., 2020). Lipopolysaccharides are made up of three domains including lipid A, an oligosaccharide core, and the O antigen which is covalently bonded to the core (Alam et al., 2020). Lipopolysaccharides give *P. aeruginosa* a more negative charge and protects the membrane from harsh conditions. In addition, it adds to the structural integrity of the cell wall. Other molecules of the outer membrane include phospholipids, lipoprotein and surface proteins. The inner layer is connected to the outer layer by the peptidoglycan layer (Diggle and Whiteley, 2020; Huszczynski et al., 2020). The structure of the cell wall of *P. aeruginosa* is presented in Figure 1.1.

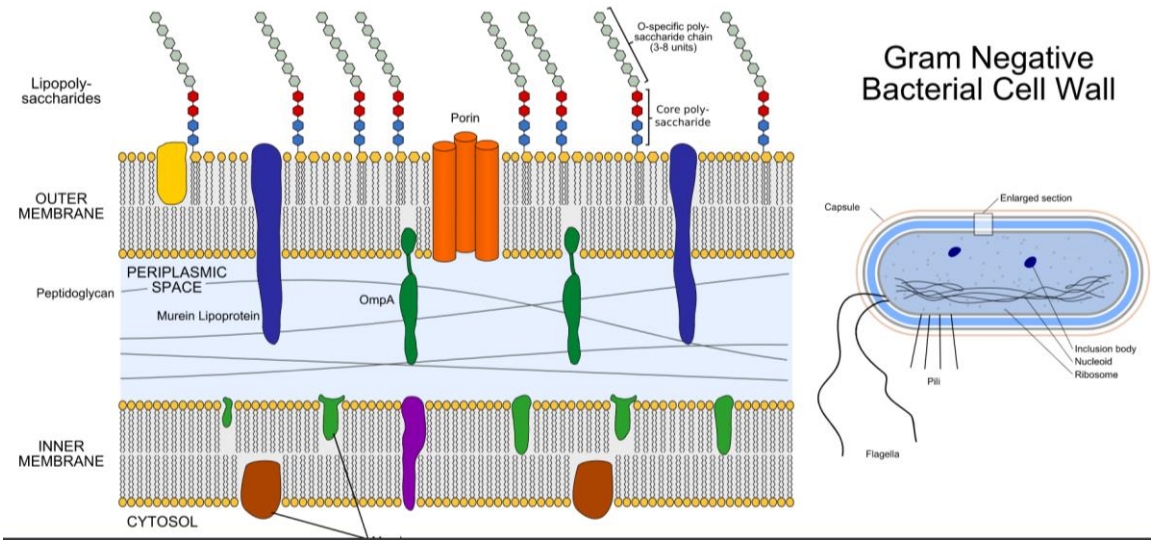


Figure 1.1: Gram-negative bacterial (*P. aeruginosa*) cell wall. (Permission granted: https://commons.wikimedia.org/wiki/File:Gram_negative_cell_wall.svg).

1.5 Virulence factors of *Pseudomonas aeruginosa*

Virulence factors are molecules that are produced by the bacterium to aid in evading the host immune system, thereby allowing the microbial agent to cause infection. *Pseudomonas aeruginosa* has several major virulence factors that are involved in the pathogenesis of the bacteria (Alonso et al., 2020; Qin et al., 2022). *Pseudomonas aeruginosa* have flagella, pili and receptors that act as adhesion components and, further secrete type III secretions, proteases and other toxins such as exotoxin A, lipases, phospholipases and pyocyanin (Sultana et al., 2016, Jurado-Martin et al., 2021). Lipases and phospholipases are enzymes that attack lipids in surfactant and host cell membranes (Curran et al., 2018). Pyocyanin, a blue-green pigment, can disrupt electron transport and redox cycling in host cells. Pyoverdine chelates free iron (Fe^{3+}), thereby giving the bacteria a competitive advantage as Fe^{3+} is needed by *P. aeruginosa* for growth (Lo et al., 2016). The lipid A and O-antigen of LPS, present in the outer membrane of *P. aeruginosa*, also assists in the pathogenesis of this microorganism by aiding the progression of an infection (Huszczynski et al., 2020). Furthermore, *P. aeruginosa* uses mechanisms such as the formation of biofilm and quorum-sensing (QS), that allow the bacteria to adapt to environmental changes (Zhao et al., 2020). Biofilm formation gives *P. aeruginosa* the ability to adapt to physiological changes when experiencing environmental stress such as being exposed to antibiotics, temperature changes or lack of nutrients (Higgins et al., 2021).

Factors that mediate motility and adhesion of bacteria are flagella and type IV pili that have the ability to bind to host epithelial gangliosides, asialoGM1 and asialoGM2 (Gellatly and Hancock, 2013; Haiko and Westerlund-Wikström, 2013). Along with LPS, these surface appendages are also highly inflammatory (Kutschera et al., 2021). The type III secretion system (T3SS) is activated once the pathogen is in contact with the host epithelial cells, and primarily introduces cytotoxins into the cytoplasm of the host cell (Tümmler and Klockgether, 2017). Proteases are also produced and released by *P. aeruginosa*. These proteases can harm response components, mucins, and destroy epithelial tight junctions of the host, allowing bacteria to spread (Hobden, 2002; Marsden et al., 2016). Figure 1.2 below summarises the virulence factors used by *P. aeruginosa* for increased pathogenesis.

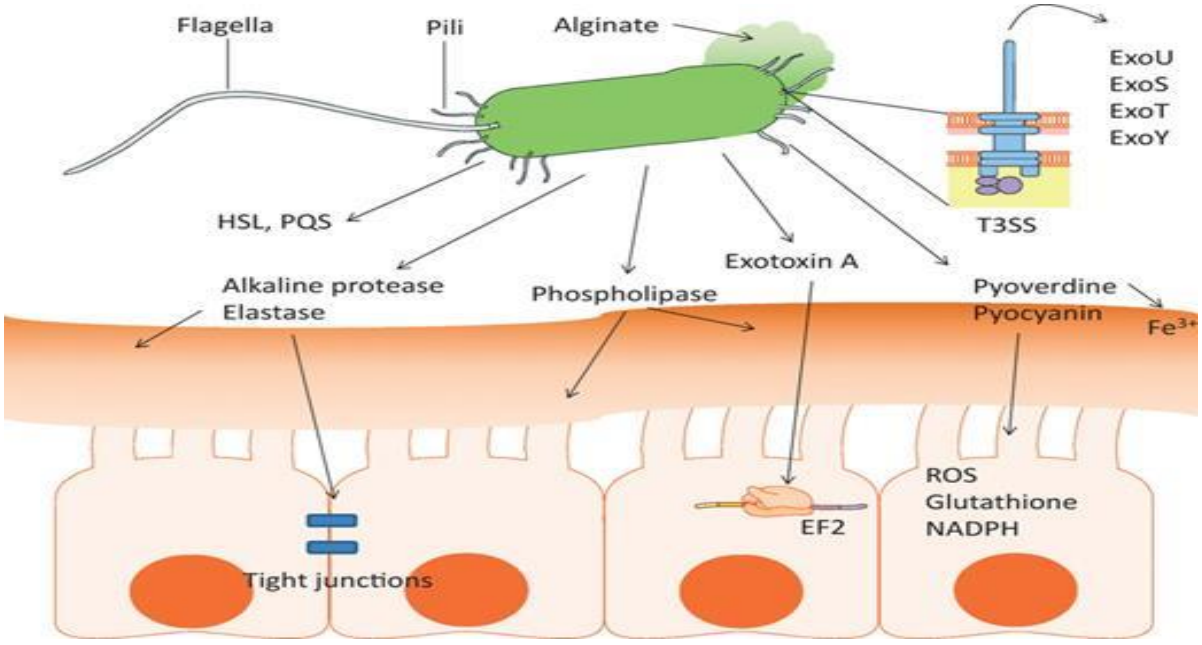


Figure 1.2: Summary of key virulence determinants of *P. aeruginosa* (Permission granted: Gellatly and Hancock, 2013).

1.6 Pathogenesis of *Pseudomonas aeruginosa*

As mentioned previously, *P. aeruginosa* is an opportunistic nosocomial pathogen that poses a high risk, particularly in immunocompromised patients having acute and chronic infections such as blood-stream infections in ICU, surgical site infections, hospital-acquired pneumonia, burn wounds, COPD, lung cancer, CF, leukaemia, chronic dermal wound infections, as well as

respiratory and urinary tract infections (Gellatly and Hancock, 2013; Jenny and Kingsbury, 2018). The transmission of this pathogen can be through hospitalisation or community-acquired. However, community-acquired infection with *P. aeruginosa* is uncommon and is almost always paired with an underlying defect in immunity (Huang et al., 2002; Killough et al., 2022).

Pseudomonas aeruginosa can cause acute or chronic infection of the airways. (Williams et al., 2010) Acute infection mostly occurs as a consequence of direct trauma such as epithelial damage due to smoke inhalation and intubation. Chronic infection occurs as a result of patients having an underlying medical condition that prevents an effective immune response being mounted against the secondary *P. aeruginosa* infection (Rada, 2017; Vilaplana and Marco, 2020). *Pseudomonas aeruginosa* pathogenesis is defined by numerous toxins, and virulence factors, as described above. Moreover, the pathogen is invasive and toxigenic (Jefferies et al., 2012; Golovkine et al., 2018). The pathogenesis of *P. aeruginosa* is characterised by invasion and dissemination; bacterial adherence and systemic or toxaemic diseases (Malhotra et al., 2019).

1.6.1 Bacterial adherence and colonisation

As mentioned above, increased risk of *P. aeruginosa* colonisation is usually associated with hospital admissions (Finch et al., 2015). During infection, the bacteria adhere to the host epithelial cells, resulting in cell invasion leading to tissue damage (Hickey et al., 2018). This is achieved by bacterial surface structures such as pili, flagella or surface-exposed protein (Veesenmeyer et al., 2009; Hickey et al., 2018). These factors are variable in their specificity and strength of binding. The stage where the bacteria bind to the host epithelial surface is called colonization (Hinsa and O'Toole, 2004; Paulsson et al., 2019).

1.6.2 Invasion and dissemination

During invasion, host tissues are damaged and the pathogen is disseminated into the bloodstream (Curran et al., 2018). Bacterial factors such as toxins, including exotoxin A, type I-IV proteins, lipase, phospholipase, and alkaline phosphate aid in the process of invasion (Curran et al., 2018; Paulsson et al., 2019). Exotoxin A inhibits host protein synthesis that leads to cell death, thereby inhibiting the host elongation factor 2 (EF2) (Schultz et al., 2000; Hickey et al., 2018). This inhibition leads to suppression of host immune response T3SS, which allows

P. aeruginosa to infiltrate the epithelial barriers directly by opposing wound healing during colonisation (via *ExoU*) and indirectly by promoting cell injury (recruiting and activating neutrophils) that leads to the symptoms of bacterial pneumonia (Hauser, 2009; Galle et al., 2012; Lampaki et al., 2019). Proteases work by degrading the immunoglobulins and fibrin and disrupting the tight junctions of host epithelial cells (Paulsson et al., 2019). It is also found to contribute to tissue damage in respiratory infections and decreases the host lung surfactant (Kipnis et al., 2006; Chakravarty et al., 2017).

1.6.3 Host immune response to *Pseudomonas* airway infection

An average human inhales approximately 10 000 litres (L) of air per day that contains potentially pathogenic microorganisms and other environmental particles (Ghimire and Neupane, 2020). Despite this, the healthy individual's lungs remain free from infections reflecting the efficiency of the innate immune response (Hartl et al., 2018; Zhou et al., 2020). The first line of defence against invading microorganisms is the physical barrier of the epithelium and the immunogenic defence is played by cell types such as neutrophils, macrophages and dendritic cells (DCs) of the innate immune system and T- and B-lymphocytes of the adaptive immune response (Rada, 2017). These cells work together to prevent colonisation and clear the *P. aeruginosa* pathogen from the host's airways (Lo, 2008; Mazor and Pastan, 2020).

1.6.3.1 Innate immune response against *Pseudomonas* aeruginosa infection

Innate immunity is the first line of host defence in fighting potentially pathogenic microorganisms. This nonspecific immune response is activated against pathogens without previous interaction with the invading organism (Huber-Lang et al., 2018). The innate immune system engages with several components to respond to *P. aeruginosa* infections such as DCs, macrophages, natural killer (NK) cells, neutrophils and the complement system (Lavoie et al., 2011). Pattern recognition receptors (PRRs) aid the innate immune response to identify the invading microorganism by recognising and binding to pathogen-associated molecular patterns (PAMPs), leading to host response stimulation (Li and Wu, 2021). Pattern recognition receptors are intra- and extra-cellular membrane-bound receptors and form part of the Toll-like receptor (TLR) family. Toll-like receptors are expressed by host cells to recognise microbial membrane components such as lipids, lipoproteins, nucleic acid and proteins (Hancock et al., 2012; Qin et al., 2022).

1.6.3.2 Epithelial cells

The nasal and tracheal passages are lined with the pseudostratified epithelial cells forming a physical barrier (Rao et al., 2021). The conducting passages have ciliated cell types (hair-like extensions: cilia) which work to brush out inhaled particles and transport the mucous and mucous-encased bacteria and other particles out of the lungs (Kouzaki et al., 2013; Aljohmani et al., 2022).

Airways are lined with secretory cells such as goblet cells which are responsible for the production, storage and secretion of mucin glycolipids, clara cells and type II epithelial cells which secrete bronchiolar surfactant (Galle et al., 2012). Mucins trap foreign particles which are bound to surfactant proteins and opsonize the microbial pathogen in preparation for phagocytosis by cells of the innate immune system (Whitsett and Alenghat, 2015). For lung defence, epithelia secrete many molecules such as complementary proteins which bind the inhaled pathogen and enhance phagocytosis (Chroneos et al., 2010; Huber-Lang et al., 2018).

Epithelial cells also secrete cytokines and chemokines while activating TLRs that allow for the recruitment and activation of an innate and adaptive immune response (Whitsett and Alenghat, 2015). During infection or inflammation of the lungs, degranulated phagocytic cells secrete cationic peptides such as LL-37, lysozyme, lactoferrin and β-defensins that neutralise the infection and resolve inflammation (Holt et al., 2008; Moradali et al., 2017; Yin et al., 2019).

1.6.3.3 Phagocytic cells

Neutrophils contain effective microbicidal molecules in their lysosomes such as reactive nitrogen species and reactive oxygen species, as well as lysozyme, elastase and defensin peptides which enable neutrophils to kill phagocytosed pathogens within the lungs (Hayes et al., 2011; Lavelle et al., 2016). Inflammatory cytokines and LPS stimulate neutrophils to become inflammatory and degranulate, that, in turn, leads to local tissue damage which is limited by non-inflammatory apoptosis (Gideon et al., 2019).

Alveolar macrophages, like neutrophils, play a crucial role in host defence by phagocytosing and killing internalised bacteria (Zahalka et al., 2022). In addition, macrophages secrete cytokines and chemokines (Raoust et al., 2009). The chemokines secreted by macrophages

recruit additional neutrophils to the site of infection. Macrophages in the lungs are also responsible for phagocytosing dying neutrophils and facilitating clearance of the infection and repairing damaged tissue (Lavoie et al., 2011).

1.6.3.4 Adaptive immune response against *Pseudomonas aeruginosa* airway infection

Unlike the innate immune response, the adaptive response is specific, acting when previous invading microorganisms are encountered (Moser et al., 2021). In case of a recurring infection, the secondary response is more rapid and effective than that of the primary exposure response leading to the clearance of the infection (Han et al., 2020). This response is facilitated by DCs that activate T-lymphocytes and macrophages (Murata and Kang, 2018). At the pathogen exposed region, such as peripheral tissue, mucosal surfaces and lymphoid tissue, there are many immature DCs that are efficient in taking-up pathogen (Ait-Oufella et al., 2014). Following the uptake of the pathogen and subsequent exposure to inflammatory cytokines, DCs mature leading to antigen processing and presenting to T-lymphocytes (Ait-Oufella et al., 2014; Ishak et al., 2020). As such, DCs link the innate immune response with that of the adaptive immune response. There is an initial release of pro-inflammatory cytokines, including interleukin (IL)-6 and IL-12, while specific T-lymphocyte proliferation has been reported to be downregulated by QS exposed DCs (Hoffman et al., 2016; Moser et al., 2021).

1.7. Planktonic growth of *Pseudomonas aeruginosa*

Planktonic bacteria are single-celled organisms that exist as floaters in their distinct habitats (Hernández-Jiménez et al., 2013). Planktonic cells can move passively through bodily fluids or actively along the surface using their flagella. This is considered to be a ‘transition phase’ in the growth of *P. aeruginosa* (Ciofu and Tolker-Nielson, 2019). The microbes then adhere by creating a reversible attachment to the surface, generating a monolayer of cells (Kreve and Reis, 2021; Chitlapilly Dass and Wang, 2022). This monolayer of cells is essential in the creation of bacterial biofilms, acting as surface attachment precursors for the microorganism (Dunne, 2002).

1.8. Biofilm formation by *Pseudomonas aeruginosa*

Pseudomonas aeruginosa, like many other bacteria, form biofilm for survival when exposed to unfavourable and potentially harmful environments such as exposure to antibiotics and the hosts immune system. This leads to chronic infections (Rasamiravaka et al., 2015). The biofilm of *P. aeruginosa* consists of bacterial aggregates that form on the surface of the abiotic or biotic solid medium producing extracellular polymeric substance (EPS) that enclose them (Heidari et al., 2022).

The EPS of *P. aeruginosa* biofilm is composed of extracellular deoxyribonucleic acid (eDNA), polypeptides and exopolysaccharides (Davey et al., 2003; Karatan and Watnick, 2009; Hemati et al., 2014). The *P. aeruginosa* biofilm structure comprises significant carbohydrate rich polymers and its extracellular matrix is composed mainly of alginate, *pel* and *psl* (Skariyachan et al., 2018). Alginate protects, maintains and stabilises the biofilm matrix and aids in retention of water and nutrients, while *pel* and *psl* are the main primary structures of the biofilm matrix. The main function of EPS is to provide structural support to the biofilm (López et al., 2010).

The development of biofilm involves three stages: (i) initial attachment of the bacteria to the surface, (ii) formation of microcolonies and biofilm maturation, and (iii) detachment of the biofilm (Lee and Yoon, 2017). Biofilm maturation is the developmental stage of *P. aeruginosa* and is of particular significance for the current study. The bacterium, *P. aeruginosa* produces lectin genes (*lecA* and *lecB*) that mediate the adhesion of *P. aeruginosa* pili with other cells during microcolony formation (Khan et al., 2020a; Uruén et al., 2021). The *pel* genes are mainly involved with the synthesis of polysaccharides and *psl* is needed for cell-to-cell interactions and maintaining the biofilm structure (Skariyachan et al., 2018).

The formation of biofilm is induced by QS, which facilitates intercellular communication between bacterial cells (Sana et al., 2019). The formation of biofilm is regulated by the *LasR* gene which binds to the *Psl* operon for regulation of expression of the *Psl*, *Rhl* and *Pel* systems, which are responsible for polysaccharide biosynthesis (Donlan, 2001; Gupta and Devi, 2020; Heidari et al., 2022). Figure 1.3 shows the developmental stages of *P. aeruginosa* biofilm. The process involves i) the planktonic stage, ii) attachment of bacteria to a surface, iii) production of the extracellular matrix, iv) maturation of biofilm structures, v) spatial differentiation, and vi) biofilm dispersal (Lee and Yoon et al., 2017).

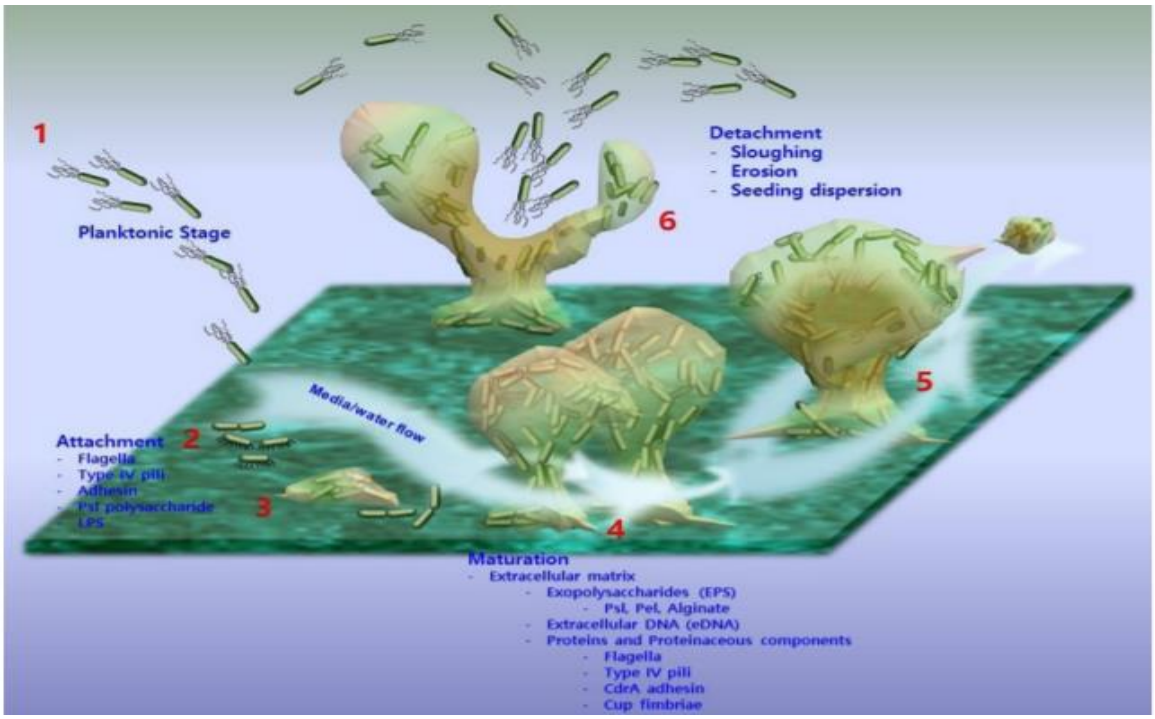


Figure 1.3: Diagrammatic presentation of the developmental stages of *P. aeruginosa* (Permission granted: Lee and Yoon et al., 2017).

1.9. Treatment of *Pseudomonas aeruginosa* infections

Treatment of infections caused by *P. aeruginosa* involves the administration of combinations of anti-pseudomonal drugs (Bassetti et al., 2018; Pang et al., 2019), which include flouroquinolones, beta (β)-lactams, carbapenems and aminoglycosides. Despite the availability of these antimicrobial programs, the development of a standard treatment regimen for *P. aeruginosa* has been challenging due to the ability of the bacteria to develop antimicrobial resistance (Kuti et al., 2016; Vanderwoude et al., 2020). Multiple studies have demonstrated the development of MDR (75%) and XDR (64%) strains with complete resistance to several anti-pseudomonal antibiotics occurring worldwide (Pang et al., 2019).

Multidrug-resistant *P. aeruginosa* is the acquired non-susceptibility to at least one agent in three or more antimicrobial categories (Gandra et al., 2019). Extensively drug-resistant *P. aeruginosa* is defined as non-susceptibility to more than one antibiotic in all of the antimicrobial categories such as aminoglycosides, carbapenems, cephalosporins, flouroquinolones, and penicillins (Sun et al., 2014, Gill et al., 2016). Multidrug-resistant *P.*

aeruginosa strains are treated with polymyxin and colistin but the use of these antibiotics is limited due to their high toxicity (Høyland-Kroghsbo et al., 2017).

1.9.1 Flouroquinolones

The flouroquinolones are oral antibiotics used to treat *P. aeruginosa* infections worldwide. The commonly used fluoroquinolones include ciprofloxacin, which has high activity against Gram-negative bacteria (Naber et al., 1994). Due to its efficacy in eliminating Gram-negative bacteria, ciprofloxacin is used to treat a number of conditions such as bronchitis, sinus infections, urinary tract infections, and chronic bacterial prostatitis (Fayyaz et al., 2015). Flouroquinolones are also recommended for treating children infected with *P. aeruginosa* especially those individuals with a history of therapeutic failure or resistance to multiple antibiotics (Naber, 1994; Riou et al., 2010).

1.9.2 Beta-lactams

The β-lactams are a class of antibiotics that contain a β-lactam ring in their molecular structure. These antibiotics act by inhibiting the cross-linking reaction in peptidoglycan synthesis (Paterson and Bonomo, 2005). This target used by β-lactams is called the penicillin-binding proteins (PBPs). The binding of β-lactams to the PBPs leads to termination of the transpeptidation process leading to loss of viability, lysis and autolytic processes that take place within the cell (Eckburg et al., 2019; Parkins et al., 2018). The β-lactam agents used for treating *P. aeruginosa* include cefepime, piperacillin and tazobactam, which are broad-spectrum antibiotics, being effective against Gram-positive and Gram-negative bacteria, such as *Staphylococcus aureus* and *P. aeruginosa,* respectively (Frank et al., 2003). The combination therapy of two anti-pseudomonal drugs e.g., a β-lactam antibiotic and aminoglycoside are recommended for initial treatment of *P. aeruginosa* infection (Dharmapalan et al., 2017). This regimen is recommended for patients with neutropenia, bacteraemia, sepsis, severe upper respiratory infections (URIs) or abscess formation (Liu et al., 2016; Dharmapalan et al., 2017; Pathak et al., 2020).

The β-lactam antibiotics are widely used in RSA and, indeed, globally (Frank et al., 2003). The combination of amoxicillin and clavulanic acid account for 54.9% of all prescribed β-lactams (Rodríguez-Baño et al., 2018). Amoxicillin is an active ingredient found in more than 95% of penicillins advocated for treating bacterial infections. Notably, penicillins are the most prescribed class of antibiotics in RSA (Dobias et al., 2017). The resistance of microbes to β-

lactams is a world-wide problem, including in RSA (Ekwanzala et al., 2018). *Pseudomonas aeruginosa* has developed several mechanisms of resistance against the β-lactams such as production of β-lactamase, alterations in drug permeability, activation of efflux pumps and changes in target molecules (Tshitshi et al., 2020). *Pseudomonas aeruginosa* infections are mostly treated with a combination of anti-pseudomonal β-lactams (penicillin or cephalosporin) and aminoglycosides (Rodríguez-Baño et al., 2018; Koulenti et al., 2019).

1.9.3 Aminoglycosides

Aminoglycosides are antibiotics that are either natural or semi-synthetic derived from the filamentous bacteria, actinomycetes (Krause et al., 2016). They have proven to be effective when used as first-line agents in the early days of antimicrobial chemotherapy (Paterson, 2000). Aminoglycosides are potent, broad-spectrum antibiotics, which act by inhibiting protein synthesis (Qin et al., 2019). Examples of the aminoglycoside antibiotics include amikacin and tobramycin, which are active against Gram-negative bacteria (Krause et al., 2016; Redpath et al., 2021). In a number of countries, high resistance levels of *P. aeruginosa* to amikacin have been reported, including; Turkey (49.7%), India (83.6%) and Egypt (26.7%). In RSA, amikacin resistant cases of *P. aeruginosa* have been reported in the Eastern Cape (35.8%), Western Cape (18.9%), Gauteng and Kwazulu-Natal (17%), North West (3.7%) and Limpopo (1.9%) (Ekwanzala et al., 2018; Kimera et al., 2020).

Tobramycin has shown to be clinically effective against *P. aeruginosa* (Olivares et al., 2017). Inhaled tobramycin was developed to reduce the aminoglycoside resistant *P. aeruginosa* strains and has been shown to succesfully eliminate the bacteria in the early stage of *P. aeruginosa* infection (Ratjen et al., 2009). The ability of *P. aeruginosa* to form biofilm, as well as the frequent mutation that *P. aeruginosa* strains undergo, are the main reasons behind these bacteria developing resistance against aminoglycosides (Khan et al., 2020a). There is increased resistance to most aminoglycosides, however, resistance for colistin and inhaled tobramycin remains low. The tobramycin resistant *P. aeruginosa* clones appear to be lower than the susceptible clones (Díez-Aguilar et al., 2018; Luan et al., 2020). The combination of tobramycin and ciprofloxacin has had significant effects in preventing the development of resistance and reducing bacterial load (Liu et al., 2016; Dobias et al., 2017).

1.9.4 Carbapenem

Carbapenems have the broadest spectrum of antibacterial activity with notable potency against both Gram-negative and Gram-positive bacteria (Doi, 2019). Carbopenems are regarded as 'last-line agents' or 'antibiotics of last resort' and are usually only used for seriously ill individuals (Paterson, 2000; Torres et al., 2007). The most commonly used carbapenem employed in the treatment of *P. aeruginosa* infection is meropenem, which is also a broad-spectrum agent (Doi, 2019). New carbapenems such a doripenem and biapem have also been found to have excellent activity against *P. aeruginosa*, however, these antibiotics have demonstrated little or no activity against strains that express resistance to the currently available antibiotics (Aguilera-Alonso et al., 2020).

On a positive note, the carbapenem resistance level against *P. aeruginosa* was reported to decrease in Africa from 41% to 23% between 2012 and 2017 (Tadesse et al., 2017). Meropenem has been shown to be the antibiotic with the highest resistance against *P. aeruginosa* (van Duin and Doi, 2017; Doi, 2019). In 2017, Adjei et al. conducted a study in Durban, RSA where resistance of several drugs were tested against *P. aeruginosa*. These researchers found that 88% of the isolates tested were resistant to imipenem and ticarrilin and 82% were resistant to meropenem (Adjei et al., 2017). In the global priority list of antibiotic resistant bacteria published by the World Health Organisation (WHO) in 2017, three pathogens appeared to be critical for research and new antibiotic development namely carbapenem-resistant Enterobacterales (CRE), carbapenem-resistant *P. aeruginosa* and carbapenem-resistant *Acinobacter baumanni* (van Duin and Doi, 2017; Castagnola et al., 2019).

1.9.5 Tazobactam

Tazobactam is an antibiotic that is highly active against the extended-spectrum β-lactamase (ESBL) producing Enterobacteriaceae and MDR *P. aeruginosa* (Ng et al., 2016). This antibiotic belongs to the class of β-lactamase inhibitors and is commonly combined with piperacillin or ceftolozane in the treatment of various bacterial infections (Harris et al., 2002). Piperacillin-tazobactam was approved for use in 1994 for treating VAP and hospital-acquired bacterial pneumonia (Harris et al., 2002; Kuti et al., 2016) and is considered as an alternative therapy for MDR *P. aeruginosa* (Mizrak et al., 2019). Tazobactam combinations have been shown to be 81% effective in treating hospitalised individuals infected with *P. aeruginosa*

compared to 61% of those who received polymyxin or aminoglycoside-based regimens. Therefore, tazobactam and β-lactams should be given preferentially, as opposed to polymyxin and aminoglycosides, to manage drug-resistant *P. aeruginosa* infections (Giacobbe et al., 2018). In a 2018 study, *Escherichia coli* and *P. aeruginosa* demonstrated resistance of 9.1% and 14.4%, respectively against the regimen comprising piperacillin and tazobactam (Schechter et al., 2018).

1.9.6 Clarithromycin

Clarithromycin was the first class of macrolide antibiotics, discovered in 1952 (Scheld et al., 1992) and is used as a second-line drug against respiratory tract infections such as chronic rhinosinusitis, community-acquired pneumonia (CAP), CF and COPD in immunocompromised patients (Rodvold, 1999; Kocsmár et al., 2021). Clarithromycin is soluble in water at 25 °C, well-absorbed, acid stable and can also be taken with food (Davidson, 2019). It has a molecular weight range of between 747 and 800 grams per mole (g/mol) and is metabolised by Cytochrome P450 3A4 (CYP3A4) resulting in numerous drug interactions (Alhajlan et al., 2013; Davidson, 2019). The side-effects of clarithromycin include gastrointestinal effects, abnormal taste, and elevated blood urea nitrogen (Takemori et al., 2020).

Clarithromycin has been administered at sub-minimum inhibitory concentration (MIC) ranges of between 200 and 250 mol/L in combination with other anti-pseudomonal agents such as, amikacin, for the treatment of drug-resistant *P. aeruginosa* isolates that cause acute polynephritis, CF and panbronchitis, with positive outcomes (Nomura et al., 2014). Including the macrolide antibiotics to anti-pseudomonal regimens has shown added benefit in inhibiting bacterial colonisation and production of virulence factors such as proteases and phospholipases (Imperi et al 2014). This treatment regimen also leads to inhibition of biofilm formation, resulting in a decrease in surface motility, micro-colony formation and QS (Tateda et al., 1991; Wargo et al., 2011). Improved treatment of *Pseudomonas* infections has also been reported globally with the inclusion of macrolides such as azithromycin, clarithromycin, erythromycin, and fidaxomicin to a regimen consisting of anti-pseudomonal agents (Okada et al., 2015; Kocsmár et al., 2021). The effectiveness of clarithromycin has been demonstrated by a 99% reduction in the number of colony forming units (CFU) of *P. aeruginosa* at two-times the MIC

after 72 hours of treatment (Alhajlan et al., 2013; Kocsmár et al., 2021). Figure 1.4 below shows the chemical structure of clarithromycin.

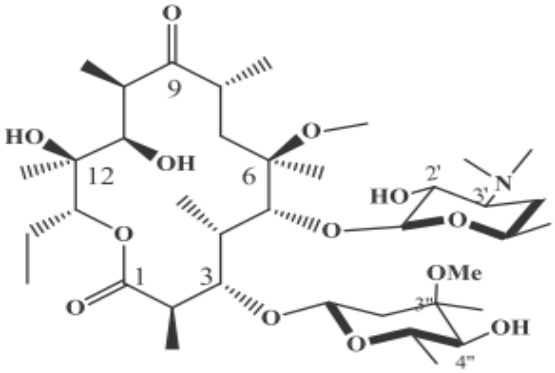


Figure 1.4: Chemical structure of clarithromycin, the first macrolide class of antibiotics discovered in 1952 (Permission requested: Li et al., 2019).

1.9.6.1 Resistance mechanisms of *Pseudomonas aeruginosa*

Due to the high antimicrobial resistance of Gram-negative bacilli (GNB) such as *P. aeruginosa, Accinobacter baumannii* and *Stenotrophomonas maltophilia*, treatment of patients infected with these microorganisms has become a daily challenge in hospitals (Paterson, 2006). These GNB have resistance mechanisms that are expressed by antibiotic-inactivating enzymes as well as non-enzymatic mechanisms (Paterson, 2006; Miller, 2016). The mechanisms employed by these bacteria include: i) mutation in the chromosomal gene that increases the intrinsic resistance mechanism such as efflux pumps and antibiotic-inactivating enzymes and, ii) horizontal transfer of genetic material that carries resistance genes such as plasmid-encoding β-lactamase, aminoglycosides-modifying enzymes or non-enzymatic molecules (Oliveira and Reygaert, 2022; Breijyeh et al., 2020).

Pseudomonas aeruginosa antibiotic resistance is multi-factorial, meaning, it occurs via innate, acquired and adaptive mechanisms (Han et al., 2020). The innate mechanisms of resistance of *P. aeruginosa* include restriction of outer membrane permeability, efflux systems that pump antibiotics out of the cell and β-lactamase production that inactivates the β-lactam antibiotics (Miller, 2016). Most of the antibiotics against *P. aeruginosa* need to penetrate the cell membrane, therefore restricted outer membrane permeability blocks antibiotics such as

clarithromycin, aminoglycosides, quinolones, β-lactams and polymyxins from entering the bacteria (Cox et al., 2018; Breijyeh et al., 2020). *Pseudomonas aeruginosa* produces antibiotic-inactivating enzymes to breakdown or modify the structure of β-lactam antibiotics rendering them ineffective. In addition, *P. aeruginosa* has twelve efflux pumps belonging to the Resistance-Nodulation-Division (RND) family that contribute to antibiotic resistance (Daury et al., 2016).

The acquired mechanism of resistance of *P. aeruginosa* includes mutational changes and horizontal gene transfer. The acquired mechanism leads to the development of multidrug resistant strains (Wilton et al., 2016). *Pseudomonas aeruginosa* can limit antibiotic uptake, block antibiotic targets and further increase the expression of efflux pumps by these mutational changes thereby enabling the bacteria to persist in the presence of antibiotics (Susilowati et al., 2017). The bacteria are also able to acquire resistance genes that include transformation, transduction and conjugation, via horizontal gene transfer (Sultana et al., 2016).

The adaptive mechanisms include transient alteration in gene and protein expression in response to environmental stimuli that leads to increased bacterial survival (Miller, 2016). This mechanism of developing resistance includes biofilm formation and the promotion of persister cells (Vanderwoude et al., 2020). Formation of biofilm makes the bacteria less sensitive to antimicrobial agents and this often results in chronic infections (Høyland-Kroghsbo et al., 2017). Persister cells that develop in biofilm are genetically not resistant to antibiotics, however, these bacteria can tolerate high concentrations of antibiotics. It has been observed *in vitro* and *in vivo* that *P. aeruginosa* persister cells and biofilm formation make the treatment of infections caused by these bacteria more difficult (Høyland-Kroghsbo et al., 2017; Khosravi et al., 2017, Soares et al., 2020).

1.10 Treatment of *Pseudomonas aeruginosa* in the planktonic phase of growth

The commonly used drugs employed to eradicate the planktonic bacteria are tested *in vitro*. These drugs includes: ampicillin, ciprofloxacin, clindamycin, erythromycin, gentamicin, and tetracycline (Švarcová et al., 2022). Planktonic cells are generally not resistant to antibiotic treatment as resistance mechanisms are largely at play in microbial biofilms. In a study conducted on *P. aeruginosa* in a planktonic growth phase, it was found that the vast majority

of cells were eliminated by fairly low, clinically achievable concentrations of fluoroquinolones, carbenicillin and ofloxacin (Patel et al., 2022).

1.11 Treatment of biofilm producing *Pseudomonas aeruginosa*

Biofilm formation by *P. aeruginosa* complicates the treatment of the infection, however, researchers have shown that certain drugs can inhibit the formation of biofilm by these bacteria (Cao et al., 2017). These drugs include: erythromycin, azithromycin, clarithromycin and roxithromycin. These antibiotics have been shown to inhibit the alginate protein that is needed for biofilm development (Wilton et al., 2016; Olivares et al., 2020). Among these antimicrobial agents, clarithromycin has shown potential in inhibiting biofilm formation even when used at sub-MICs (Kandemir et al., 2005; Dicicco et al., 2012). In addition, biofilm formation has been inhibited using combination therapy (Rodríguez-Baño et al., 2018). The drugs that are most commonly used in combination to treat biofilm-forming *P. aeruginosa* infections are, ceftazidime and ampicillin. These antibiotics have been shown to have improved efficacy in inhibiting the formation of biofilm in the presence of coumarin (Wilton et al., 2016). Moreover, the effectiveness of fosfomycin and tobramycin in preventing biofilm formation has been demonstrated *in vitro*. Aminoglycosides (amikacin and tobramycin) showed preventable action on the early stage (adhesion) of *P. aeruginosa* biofilm formation (Mataraci-Kara and Ozbek Celik, 2018). Meropenem, levofloxacin and colistin are some of the antibiotics that have shown effectiveness when combined with clarithromycin (Ozbek and Mataraci-Kara, 2016).

As mentioned previously, bacterial biofilm poses challenges to antibiotic therapies, often leading to treatment failure. For most classes of antibiotics, it requires a 10- to 1000-fold increase in the antibiotic concentration required to inhibit planktonic bacteria to treat biofilm microorganisms (Langton Hewer and Smyth, 2017). Treatment failure is a predisposing factor for the development of antibiotic resistance (Chen et al., 2021).

1.12. The effect of cigarette smoke on the host immune response

Use of tobacco products, and particularly smoking of cigarettes, causes many challenges to public health globally, contributing to exacerbation of most respiratory infections including those caused by *P. aeruginosa* (Al-Sawalha et al., 2017). The effect of smoking is not immediate but eventually causes long-lasting complications and damage (Basilico et al., 2016). Exposure to cigarette smoke (CS) increases the individuals susceptibility to respiratory tract

infections (Li et al., 2020), by compromising the normal function of the innate immune cells (neutrophils, monocytes, macrophages and DCs) (Xu et al., 2020) and enhancing mucus secretion (Chien et al., 2020). Cigarette smoke has also been shown to impair the oxidative burst which is required for intracellular killing following phagocytosis of microorganisms (Emma et al., 2022). Cigarette smoke exposure also affects the function of the adaptive immune components, inhibiting the proliferation and function of the T- and B-lymphocytes (Drannik et al., 2004) and immunoglobulin G (IgG) secretion (Chien., 2020). The number of DCs in the lungs of an individual who smokes cigarettes is lower and the lymph nodes are notably immature compared to those of nonsmoking individuals. This, in turn, leads to an absence of antigen presentation and costimulatory molecules (Al-Sawalha et al., 2017; Khabour et al., 2018; Taha et al., 2020).

Cigarette smoke has been shown to alter the phenotypic structure of *P. aeruginosa* and increase bacterial virulence (Li et al., 2020). Cigarette smoke affects bacterial proliferation and persistence and damages the nasopharyngeal microflora in the respiratory tract (Basilico et al., 2016). It has been shown to affect the pathogenesis, virulence factors, antibiotic resistance, biofilm formation, and treatment of microbial organisms (Voss et al., 2015; Basilico et al., 2016; Gilpin et al., 2019).

1.12.1 The effect of cigarette smoke on the pathogenesis of *Pseudomonas aeruginosa*

Cigarette smoke exposure affects the pathogenesis of *P. aeruginosa* by increasing the bacteria's oxidative stress (Voss et al., 2015). Elements present in CS are known to damage the airway epithelial cells leading to reduced clearance of the bacterial pathogen, increased bacterial adherence to the epithelium, mucus hypersecretion, reduced epithelial elastic properties in the lungs and impaired phagocytic activity by neutrophils and macrophage (Basilico et al., 2016). The increased secretion of mucus and depressed epithelial elastic properties result in chronic inflammation and colonisation which leads to a high rate of airway infections such as pneumonia and COPD (Gilpin et al., 2019; Xu et al., 2020).

1.12.2 The effect of cigarette smoke on planktonic growth of *Pseudomonas aeruginosa*

The effects of CS exposure on planktonic bacterial cultures have been described. For instance, exposure to cigarette smoke condensate (CSC) was found to inhibit the growth of *Bifidobacterium animalis* (Hu et al., 2015) while CSC exposure induced the formation of biofilms in other bacterial genera, such as *Staphylococcus aureus*, *P. aeruginosa*, *Streptococcal* species, as well as *Mycobacterium tuberculosis* without affecting the growth of these bacteria (Mutepe et al., 2013; Hutcherson et al., 2015, Cholo et al., 2020). However, Chien et al., found that exposure of planktonic microorganisms to cigarette smoke dampened pseudomonal growth in a dose-dependent manner (Chien et al 2020).

1.12.3 The effect of cigarette smoke on biofilm formation by *Pseudomonas aeruginosa*

Cigarette smoke has been found to promote the formation of biofilm by several human pathogens such as *S. aureus*, *Streptococcus mutans*, *Klebsiella pneumoniae*, *Porphyromonas gingivalis* and *P. aeruginosa* (Bravo-Gutiérrez et al., 2021). In the case of *P. aeruginosa*, CS has been found to promote the biofilm genes (*pilF* and *flgK*) and also suppress the QS gene (*rhlA*) (Antunes et al., 2011). Cigarette smoke induces changes in the *P. aeruginosa* phenotype to form biofilm and increases oxidative stress that, in turn, is associated with increased expression of *tpX*, which is the gene responsible for redox homeostasis (Maura et al., 2016; Bialous and Glantz, 2018; Bravo-Gutiérrez et al., 2021).

1.12.4 The effect of cigarette smoke on the treatment of *Pseudomonas aeruginosa* infection

The effect of CS exposure on the treatment of many respiratory infections has been reported and has been associated with poor treatment outcomes (Khan et al., 2020b). For instance, treatment of individuals who smoke cigarettes and are infected with *Helicobacter pylori*, a Gram-negative bacteria that can cause stomach cancer, require higher doses of the treatment regimen as well as extended treatment schedules when compared to patients not exposed to CS (Strzelak et al., 2018). Moreover, a recent study has shown that CS exposure can induce antibiotic resistance in *S. aureus* and has also been found to inhibit the efficacy of treatment of *Streptococcus. pneumoniae* (Avner et al., 2012; Xu et al., 2020; Behzadi et al., 2021).

1.12.5 The effect of cigarette smoke on drug resistance of *Pseudomonas aeruginosa*

Cigarette smoke has been reported to increase the incidence of resistance of *P. aeruginosa* to levofloxacin, gentamycin and cause upregulated expression of multidrug efflux pumps (Chien et al., 2020). Exposure to CS has also been reported to lead to upregulation of drug-resistance genes in *P. aeruginosa* (Kunz Coyne et al., 2022). The upregulated antibiotic-resistance genes that have been identified include the *oprD, mexEF-oprN* and *mvaT* genes, which encode for the membrane protein, efflux pumps and regulatory protein, respectively (Xu et al., 2020). In addition, Matapa et al. recently demonstrated that CS enhances the expression of the *mef*(A) and *erm*(B) genes, encoding for a drug efflux pump and ribosomal methylase, that reduces the affinity of macrolides for the ribosome, respectively in *in vitro* biofilm cultures of *S. pneumoniae.* Upregulation of these genes leads to increased resistance of this bacteria to macrolide antibiotics (Matapa, 2019).

Therefore, the main purpose of the current study was to determine the MIC of clarithromycin alone, and in combination with seven anti-pseudomonal drugs. Furthermore, the effect of cigarette smoke on clarithromycin alone and in combination with the anti-pseudomonal drugs on both planktonic and biofilm growth of *P. aeruginosa* was investigated.

Chapter 2: Aims and Objectives

2.1. Aims

To investigate: i) the effects of combining six different anti-pseudomonal agents individually with clarithromycin on their antimicrobial potencies against two susceptible strains and one multidrug resistant (MDR) strain of *Pseudomonas aeruginosa*, measured according to both planktonic growth and biofilm formation; and ii) the effects of exposure of the three test *P. aeruginosa* strains to cigarette smoke condensate (CSC) on susceptibility to the individual anti-pseudomonal agents in the absence and presence of clarithromycin.

2.2. Objectives

To determine:

- The antimicrobial activities of clarithromycin and six anti-pseudomonal agents individually against three strains of *P. aeruginosa* by determining the minimum inhibitory concentration (MIC) of each agent using optical density (OD) measurements for planktonic growth of bacteria and the crystal violet method for biofilm formation quantitation.
- The antimicrobial activities of clarithromycin in combination with the individual anti-pseudomonal agents against the three test strains of *P. aeruginosa* using the inhibitory interaction index (fractional inhibitory concentration index: FICI) according to the abbreviated checkerboard method.
- The effects of CSC *per se* on the planktonic growth of, and biofilm formation by the three strains of *P. aeruginosa* by determining the lowest concentration that affects bacterial growth and biofilm formation by these bacteria.
- The effects of CSC on the inhibitory activities of clarithromycin and the anti-pseudomonal agents, individually as well as in combination, using MIC and FICI determinations, respectively.

2.3. Study Design

This was an experimental, laboratory-based study that investigated the effect of CSC on the activities of clarithromycin and six anti-pseudomonal agents on the planktonic growth of, and biofilm formation by *P. aeruginosa* in *vitro*.

2.4. Study setting

The current study was a collaborative project between the Department of Immunology, Pathology Building, Faculty of Health Sciences, University of Pretoria (UP), Pretoria and the Department of Critical Care and Pulmonology, Faculty of Health Sciences, University of Witwatersrand, Johannesburg, both situated in Gauteng, South Africa. However, all the experimental procedures were conducted in the Department of Immunology, UP.

The study was approved by the Faculty of Health Sciences Research Ethics Committee, UP (Ethics approval number: 29/2021).

Chapter 3: Materials and Methods

3.1. Introduction

The planktonic growth phase of *Pseudomonas aeruginosa* is the free-living form of this bacteria (Rybtke et al., 2015; Lee and Yoon, 2017). The attachment of these free-floating planktonic components to a surface is the first stage in the development of biofilm (Gupta et al., 2016; Spicer et al., 2021). However, mature biofilms also produce planktonic bacteria at the end of the biofilm formation process (as shown in Chapter 1, Figure 1.3) (Rabin et al.,2015; Rybtke et al., 2015, Ciofu and Tolker-Nielsen, 2019). Biofilms allow bacteria to survive and spread in conditions that would otherwise not support the growth of planktonic organisms by providing an antimicrobial-resistant habitat, as well as by protecting the microorganisms from the host immune system (Gupta et al., 2016, Gupta and Devi, 2020, Donadu et al., 2022). Notably, the use of tobacco products and, in particular, the smoking of cigarettes has been shown to modify the bacterial surface and increase biofilm formation of *P. aeruginosa,* as well as other potentially pathogenic bacteria such as, *Staphylococcus aureus, Streptococcus mutans, Klebsiella pneumonia,* and *Porphyromonas gingivalis* (Hutcherson et al., 2015, Vargas Buonfiglio et al., 2018). The observed increase in biofilm formation by these microorganisms in the presence of cigarette smoke increases the propensity of individuals who smoke tobacco products to develop infectious diseases and to have a reduced response to treatment (Hutcherson et al., 2015).

The following methodology was performed in order to evaluate the effect of cigarette smoke extracts, in the form of cigarette smoke condensate (CSC), on the growth of, and biofilm formation by *P. aeruginosa* in the presence and absence of clarithromycin and six anti-pseudomonal agents.

3.2 Materials

3.2.1. Bacterial strains

The *P. aeruginosa* bacterial strains used in the present study include the reference strain, PAO1, which is sensitive to all anti-pseudomonal agents, and a drug-sensitive and a multidrug resistant (MDR) clinical isolate. These strains were provided by the National Institute of Communicable Diseases (NICD), of the National Health Laboratory Service (NHLS), Johannesburg, South Africa.

3.2.2 Antibiotics, chemicals, and reagents

The antibiotics used in the current study included the macrolide antimicrobial agent, clarithromycin, and six anti-pseudomonal agents, namely: ciprofloxacin (fluoroquinolone), cefepime (β-lactam cephalosporin), piperacillin (β-lactam agent), amikacin (aminoglycoside), meropenem (carbapenem), and tazobactam (β-lactamase inhibitor). The-antipseudomonal agents were selected on the basis of the recommendations by the American Thoracic Society and the Infectious Disease Society of America for the antimicrobial chemotherapy of *P. aeruginosa* pneumonia, namely: a β-lactam + an aminoglycoside, or a β-lactam + a fluoroquinolone, or an aminoglycoside + a fluoroquinolone. Clarithromycin does not inhibit the growth of *P. aeruginosa*, but was included to probe its adjunctive antimicrobial properties, which appear to be achieved via inhibitory effects on the synthesis of protein virulence by the pathogen. Clarithromycin and the anti-pseudomonal agents, with the exception of amikacin and ciprofloxacin, were dissolved in dimethyl sulphoxide (DMSO). Amikacin and ciprofloxacin were dissolved in deionised water (dH_2O) and 0.1 molar (M) hydrochloric acid (HCl), respectively. Clarithromycin was tested at a range from 0.25 to 250 micrograms per millilitre (µg/mL), while the other anti-pseudomonal agents were investigated at concentrations ranging between 0.06 and 64 µg/mL to determine the minimum inhibitory concentration (MIC) of each test agent.

Crystal violet (Sigma-Aldrich, St. Louis, MO, USA), used in the process of biofilm quantification, was prepared at a concentration of 1gram (g) per 100 mL in 95% ethanol.
Unless otherwise indicated, all other chemicals and reagents were obtained from Sigma-Aldrich.

3.2.3 Culture media

The bacteriological culture medium used for culturing planktonic *P. aeruginosa* was Luria-Bertani (LB) broth (Lasec, Johannesburg, RSA). *Pseudomonas aeruginosa* cultures were grown in 10% tryptic soy broth (TSB) for experiments investigating biofilm formation. Luria agar (LA) (Lasec) and blood agar (National Health Laboratory Service) plates were used for the detection and enumeration of bacterial colonies.

3.2.4 Cigarette Smoke Condensate

Cigarette smoke condensate (CSC) was purchased from Murty Pharmaceuticals (Lexington, KY, USA) as a 40 gram per litre (g/L) stock solution. A range of concentrations, from 0.4 to 400 µg/mL, was prepared using DMSO as solvent in a series of two-fold dilutions. The DMSO was used at a final concentration of 1% in all assays.

3.3 Methodology

3.3.1. Bacterial inoculum preparation

Vials of frozen stock cultures of the *P. aeruginosa* wild-type (PAO1) strain, the drug-sensitive clinical isolate, and the MDR isolate were thawed and inoculated (10 µL) onto blood agar plates and incubated for 24 hours at 37 °C in the presence of 5% carbon dioxide (CO_2) (ESCO, Horsham, PA, USA). Following incubation, one bacterial colony from each test strain was added to separate tubes containing 10 mL LB broth and incubated at 37 °C in a shaking incubator (Daihan Labtech Co., Ltd., Gyeonggi-do, KOR) at 180 revolutions per minutes (rpm) for 24 hours. Following the incubation period, the culture was diluted to obtain a bacterial suspension with an optical density (OD) of three at a wavelength of 600 nanometres (nm) using a PowerWaveX microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA). The culture was diluted with LB broth to yield a suspension containing approximately 10^7 colony-forming units (CFU)/mL.

3.3.2 Preparation of planktonic cultures

Planktonic cultures of *P. aeruginosa* were prepared as described previously (Van Duuren et al., 2017; Otani et al., 2018). Briefly, the planktonic culture inoculums, prepared as described

in Section 3.3.1, were diluted in LB broth to a concentration of 10^5 CFU/mL and a 200 µL volume of the diluted cultures was transferred into each well of a 96-well microplate. The cultures were mixed thoroughly using a Cooke AM69 microplate shaker (Dynatech AG, Zug, CH) for 20 seconds whereafter the plates were incubated at 37 °C for 24 hours with gentle shaking (Daihan Labtech Co., Ltd.). Following the incubation period, a ten-fold dilution of the culture was made and the OD measured using a PowerWaveX microplate spectrophotometer (BioTek Instruments, Inc.) set at a wavelength of 600 nm.

3.3.2.1 Quantification of planktonic growth of *Pseudomonas aeruginosa*

The planktonic cultures of the three *P. aeruginosa* test strains prepared as described in Section 3.3.2 were assessed for growth by determining the number of CFU/mL LB broth. A total of six serial 10-fold dilutions were made of the cultures by the addition of 50 µL of bacterial suspension to 450 µL of phosphate-buffered saline (PBS, pH 7.4) (Sigma-Aldrich). The diluted bacteria were mixed thoroughly between dilutions using a vortex mixer (Scientific Industries, Inc., Bohemia, NY, USA). A 10 µL aliquot of the various dilutions of the *P. aeruginosa* planktonic culture was plated, in triplicate, onto clearly marked LA plates. The plates were incubated for 24 hours at 37 °C in the presence of 5% CO_2 (ESCO), prior to enumeration of the CFUs on the LA plates.

3.3.2.2 Minimum inhibitory concentration determination of clarithromycin and anti-pseudomonal agents against planktonic growth of *Pseudomonas aeruginosa*

The antimicrobial activities of clarithromycin and the six test anti-pseudomonal antibiotics were evaluated by determining the minimum inhibitory concentration (MIC) of each agent against the planktonic growth of the reference strain PAO1, and the drug-sensitive and MDR clinical isolates of *P. aeruginosa*. A 96-well microplate was used for the experiments. The first well of each plate was designated as the control and the other wells were assigned for the various antibiotic treatments at different concentrations. The planktonic culture (200 µL), prepared as described above (Section 3.3.2), was added to each well of the 96-well microplate followed by the addition of 2 µL of appropriate solvent to the control wells and 2 µL of the various test agents at different concentrations (clarithromycin: 250 - 0.25 µg/mL and anti-pseudomonal drugs 64 - 0.06 µg/mL) to the wells designated to the various antibiotics. The microplate was mixed thoroughly using a Cooke AM69 microplate shaker (Dynatech AG) for 20 seconds, whereafter the plate was incubated at 37 °C in the presence of 5% CO_2 (ESCO) for 24 hours. Following the incubation period, the OD of each well was determined using a

PowerWaveX microplate spectrophotometer (BioTek Instruments, Inc.) set at a wavelength of 600 nm. The MIC of each antibiotic was regarded as the lowest concentration of the antibiotic that inhibited the growth of *P. aeruginosa* compared to that of the untreated controls.

3.3.2.3 Effect of clarithromycin in combination with the test anti-pseudomonal agents on planktonic growth of *Pseudomonas aeruginosa*

The inhibitory activity of clarithromycin, in combination with the test anti-pseudomonal agents, was assessed using the same procedure as described above (Section 3.3.2.2). The clarithromycin was combined with each anti-pseudomonal drug at their respective MICs followed by a double dilution, using the abbreviated checkerboard method (Martinez-Irujo et al., 1996). A 2 µL volume of each clarithromycin combination was added to the designated wells of a 96-well microplate containing cultures (200 µL) of the different test strains of *P. aeruginosa*. As described above, the plates were mixed thoroughly before being incubated at 37 °C in the presence of 5% CO_2 (ESCO) for 24 hours. Following the incubation period, the OD was measured at 600 nm using a PowerWaveX microplate spectrophotometer (BioTek Instruments, Inc.). The lowest concentration of the combined antibiotics that showed inhibition in the formation of biofilm was used to calculate the fractional inhibitory concentration index (FICI). This was calculated by determining the fraction inhibitory concentration (FIC) of each agent in that combination. The FICI was determined as the sum of the FICs of the individual antibiotics. The FICI was calculated as follows:

- The FIC of each antibiotic = MIC of the antibiotic in a combination / MIC of the antibiotic alone.

- FICI = FIC of drug clarithromycin + FIC of drug B (test anti-pseudomonal agent)

The inhibitory interaction of the antibiotics was interpreted as being synergistic, additive, indifferent or antagonistic when the FICI values were ≤ 0.5, between 0.5 and 1.0, between 1.0 and 4.0 or >4.0, respectively (Naghmouchi et al., 2011; Andréjak et al., 2012; Kitazaki and Myhre, 2017).

3.3.2.4 The effect of cigarette smoke condensate on the planktonic growth of *Pseudomonas aeruginosa*

A planktonic culture of each of the three *P. aeruginosa* strains was prepared as described in Section 3.3.2. The effect of CSC on the planktonic growth of *P. aeruginosa* was determined following the same method as described above (Section 3.3.2.2). Briefly, a 200 µL volume of the prepared culture was added to each well of a 96-well microplate. This was followed by the addition of 2 µL of varying concentrations of CSC (0.4 - 400 µg/mL) to each well, while 2 µL of DMSO (used as the solvent) was added to the control well. As described above, the plate was mixed thoroughly before being incubated at 37 °C in the presence of 5% CO_2 (ESCO) for 24 hours.

3.3.2.5 The effect of cigarette smoke condensate on the antimicrobial activity of clarithromycin and the test anti-pseudomonal agents individually, as well as in combination

In order to investigate the effect of CSC on the activity of clarithromycin and the six anti-pseudomonal agents on the reference strain, PAO1, and the drug-sensitive and MDR clinical isolates of *P. aeruginosa*, the concentration of CSC (as determined in Section 3.3.2.4) that led to an increase in planktonic bacterial growth of all three strains of *P. aeruginosa* was selected. Planktonic culture (200 µL), prepared as described in Section 3.3.2, was added to each well of two 96-well microplates. Two microlitres of the concentration of CSC that was found to cause increased bacterial growth was added to the bacterial suspension and the plates mixed thoroughly using a Cooke AM69 microplate shaker (Dynatech AG). The various test antibiotics, individually and in combination with clarithromycin, were added to the appropriately designated wells and the microplates were mixed thoroughly again before being incubated at 37 °C in the presence of 5% CO_2 (ESCO) for 24 hours.

3.3.3 Preparation of biofilm cultures

Cultures to investigate biofilm formation by *P. aeruginosa* were prepared as described for planktonic growth above (Section 3.3.2). Similar to the preparation of the planktonic cultures, 10^5 CFU/mL of each of the three test strains of *P. aeruginosa* were inoculated into 10% TSB growth medium. The diluted bacteria (200 µL) were added to each well of a 96-well

microplate, mixed thoroughly, and incubated at 37 °C in the presence of 5% CO_2 (ESCO) for 48 hours without shaking to allow for the development of biofilm.

3.3.3.1 Quantification of biofilm formation by *Pseudomonas aeruginosa*

Biofilm formation was quantitated using a modified crystal violet staining procedure (Otani et al., 2018). Following the 48-hour incubation period to allow for the development of biofilm, the TSB growth medium from each well was gently aspirated using a Pasteur pipette. Each well was washed with 200 µL of dH_2O to remove the remaining medium and unattached cells and the plate was left to air-dry for 10 minutes. Crystal violet (1%) staining solution (200 µL) was added to each well of the 96-well microplate and incubated for 30 minutes at room temperature (24 °C). Following this incubation step, the excess crystal violet was removed from the wells by aspiration using a Pasteur pipette and each well was washed three times with 200 µL of dH_2O to remove any unbound crystal violet. The plate was then left to air-dry for 10 minutes and the biofilm-associated crystal violet was extracted by adding 200 µL of 95% ethanol to each well. The contents of each well were transferred to a clean microplate and the OD measured at 600 nm using a PowerWaveX microplate spectrophotometer (Bio-Tek instrument, Inc.). It is important to note that the purpose of these experiments was to investigate the effects of the test anti-pseudomonal agents alone and in combination with clarithromycin, as well as those of CSC alone and in combination with the individual antibiotics with and without clarithromycin, on biofilm formation, as opposed to determining their antimicrobial activities against the various strains of *P. aeruginosa* encased in pre-formed biofilm. In this experimental setting, the test agents and the bacterial strains were added together to the bacteriological culture media and not retrospectively to pre-formed biofilm cultures of the pathogen.

3.3.3.2 Minimum inhibitory concentration determination of clarithromycin and anti-pseudomonal agents against biofilm formation by *Pseudomonas aeruginosa*

The antimicrobial activities of clarithromycin and the six test anti-pseudomonal antibiotics were evaluated by determining the MIC of each agent against the formation of formation by the reference strain PAO1, and the drug-sensitive and MDR clinical isolates of *P. aeruginosa*.

The methodology was followed as described for planktonic growth (Section 3.3.2.2) for determining the MICs of the test antibiotics on biofilm formation by the three *P. aeruginosa*

test strains. Following the addition of clarithromycin (250 - 0.25 μg/mL), the six anti-pseudomonal drugs (64 - 0.06 μg/mL) and appropriate solvent controls, the plate was mixed thoroughly and incubated at 37 °C in the presence of 5% CO_2 (ESCO) for 48 hours. The effects of clarithromycin and the anti-pseudomonal agents on biofilm formation, compared to the solvent-treated control bacteria, were quantitated using the crystal violet procedure described above (Section 3.3.3.1). The MIC of each test agent was taken as the lowest concentration of that antibiotic that inhibited biofilm formation when compared to the solvent-treated control bacteria.

3.3.3.3 Effect of clarithromycin in combination with the test anti-pseudomonal agents on biofilm formation by *Pseudomonas aeruginosa*

The activity of clarithromycin, in combination with the test anti-pseudomonal agents, on biofilm formation was assessed using the same procedure as described above (Section 3.3.3.2). The clarithromycin was combined with each anti-pseudomonal drug at their respective MICs followed by a double dilution, using the abbreviated checkerboard method (Martinez-Irujo et al., 1996). A 2 μL volume of each clarithromycin combination was added to the designated wells of a 96-well microplate containing cultures (200 μL) of the different test strains of *P. aeruginosa*. As described above, the plates were mixed thoroughly before being incubated at 37 °C in the presence of 5% CO_2 (ESCO) for 48 hours to allow for biofilm formation. Following the incubation period, the crystal violet method was used for quantitating biofilm formation. The MICs of the combination of clarithromycin and the respective test agents were determined as the lowest concentration of the various combinations of agents that inhibited the formation of biofilm by *P. aeruginosa* compared to that of the solvent-treated controls.

3.3.3.4 The effect of cigarette smoke condensate on biofilm formation by *Pseudomonas aeruginosa*

A planktonic culture of each of the three *P. aeruginosa* strains was prepared as described in Section 3.3.2. The effect of CSC on biofilm formation by *P. aeruginosa* was determined following the same method as described above (Section 3.3.3.2). Following incubation at 37 °C in the presence of 5% CO_2 (ESCO) for 48 hours, biofilm was quantitated using the crystal violet staining procedure as described above (Section 3.3.3.1). Biofilm formation was taken as a measure of the intensity of the crystal violet colour compared to that of the solvent-treated control wells.

3.3.3.5 The effect of cigarette smoke condensate on the antimicrobial activity of clarithromycin and the test anti-pseudomonal agents individually, as well as in combination

In order to investigate the effect of CSC on the activity of clarithromycin and the six anti-pseudomonal agents on the reference strain, PAO1, and the drug-sensitive and MDR clinical isolates of *P. aeruginosa*, the concentration of CSC that led to an increase in planktonic bacterial growth of all three strains of *P. aeruginosa* was selected. Planktonic culture (200 µL), prepared as described in section 3.3.2, was added to each well of two 96-well microplates. Two microlitres of the concentration of CSC that was found to cause increased bacterial growth was added to the bacterial suspension and the plates mixed thoroughly using a Cooke AM69 microplate shaker (Dynatech AG). The various test antibiotics, individually and in combination with clarithromycin, were added to the appropriately designated wells and the microplates were mixed thoroughly again before being incubated at 37 °C in the presence of 5% CO_2 (ESCO) for 48 hours, as described above. The crystal violet method was used to quantitate the biofilm formation by the bacteria in order to determine the MICs of the individual antibiotics in the presence of CSC. In the case of the antibiotic combinations in the presence of CSC, the fractional inhibitory interaction index was determined as described above, Section 3.3.2.3.

Chapter 4: Results

4.1. Introduction

Pseudomonas aeruginosa planktonic growth is a free-living form of bacterial growth (Rybtke et al., 2015; Lee and Yoon, 2017, Muhammad et al., 2020). The attachment of these free-floating planktonic components to a surface is the first stage in the development of biofilm (Gupta et al., 2016; Spicer et al., 2021). However, mature biofilms also produce planktonic bacteria at the end of the biofilm formation process (as shown in Chapter 1, Figure 1.3) (Rabin et al., 2015; Rybtke et al., 2015, Ciofu and Tolker-Nielsen, 2019). Biofilms allow bacteria to survive and spread in conditions that would otherwise not support the growth of planktonic organisms by providing an antimicrobial-resistant habitat, as well as by protecting the microorganisms from the host immune system (Gupta et al., 2016, Gupta and Devi, 2020, Donadu et al., 2022). Notably, the use of tobacco products and, in particular, the smoking of cigarettes has been shown to modify the bacterial surface and increase biofilm formation (Hutcherson et al., 2015, Vargas Buonfiglio et al., 2018).

It has been found that most *P. aeruginosa* strains showed moderate or low metabolic activity when grown in biofilm, which confers intrinsic antibiotic resistance, particularly for antibiotics that target cellular processes associated with metabolic activity and active growth, making *P. aeruginosa* infection difficult to treat (Lee and Yoon, 2017, Muhammad et al., 2020).

The present chapter presents the results of minimum inhibitory concentration (MIC) determinations of clarithromycin and the test anti-pseudomonas agents alone and in combination on both planktonic growth of, and biofilm formation by three strains of *P. aeruginosa*. Furthermore, the results of the effect of exposure to cigarette smoke condensate (CSC) on these MIC values are presented.

4.2. Minimum inhibitory concentrations of clarithromycin and the anti-pseudomonal agents on *pseudomonas aeruginosa* planktonic growth and biofilm formation.

A representative 96-well microplate indicating the minimum inhibitory concentrations (MICs) for the vaious test agents against planktonic growth of *P. aeruginosa* PAO1 following 24 hours of incubation is shown in Figure 4.1.

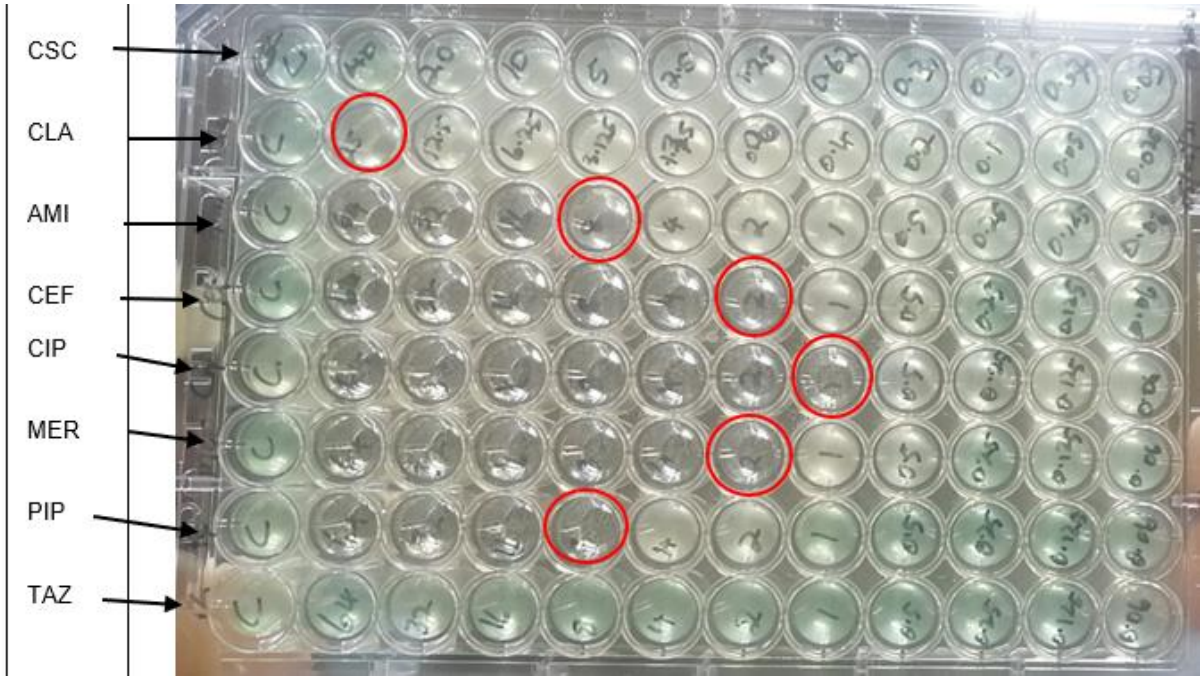


Figure 4.1: A representative 96-well microplate showing the minimum inhibitory concentrations of clarithromycin and test anti-pseudomonal agents, as well as the effects of CSC (in the absence of antibiotics) on *P. aeruginosa* PAO1. The clear wells indicate no bacterial growth and the wells that are cloudy represent growth of *P. aeruginosa*. The concentrations of clarithromycin tested ranged from 250 to 0.25 µg/mL and the concentrations of the anti-pseudomonal drugs were tested at a range from 64 to 0.06 µg/mL. Cigarette smoke condensate was tested at concentrations ranging from 40 to 0.4 µg/mL. The lowest concentration at which bacterial growth was inhibited (as circled in red) was considered to be the MIC for each antibiotic.

Abbreviations: AMI, amikacin; CEF, cefepime, CIP, ciprofloxacin; CLA, clarithromycin; CSC, cigarette smoke condensate; MER, meropenem; PIP, piperacillin; TAZ, tazobactam

A representative 96-well microplate indicating the MICs for the various test agents against biofilm formation by *P. aeruginosa* PAO1 following 48 hours of incubation is shown in Figure 4.2.

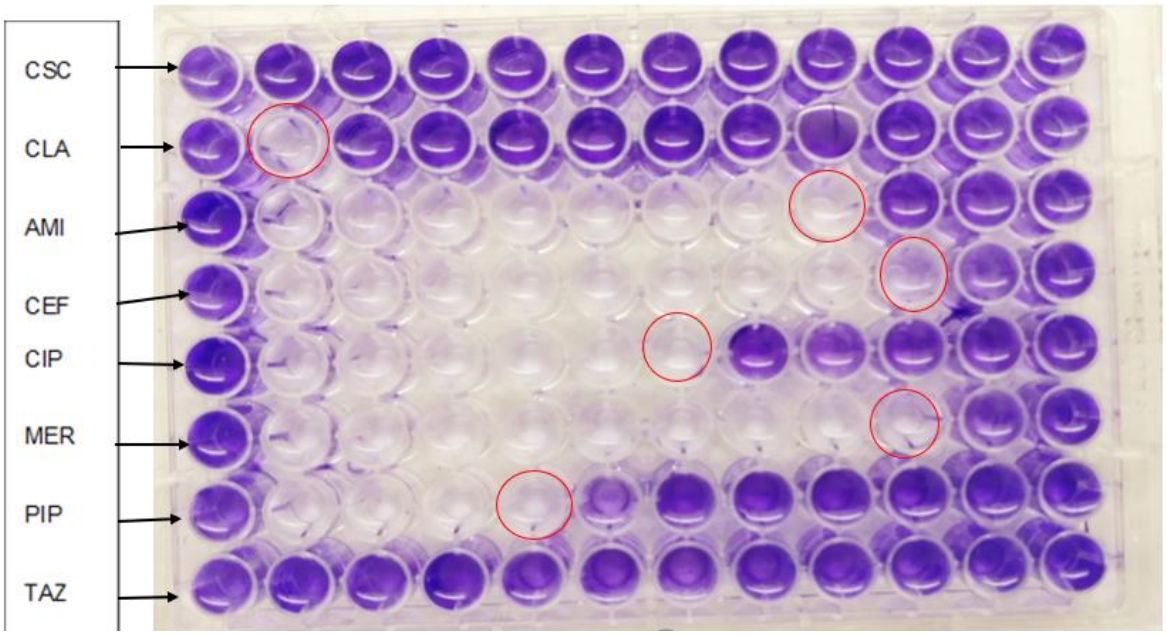


Figure 4.2: A representative 96-well microplate showing the MICs of clarithromycin and other test anti-pseudomonal agents, as well as the effects of CSC, against biofilm formation by *P. aeruginosa* PAO1. The clear wells indicate no biofilm formation and the wells that are coloured purple represent biofilm formation by *P. aeruginosa* with the higher the intensity of the colour indicating the more biofilm formed. The concentrations of clarithromycin tested ranged from 250 to 0.025 µg/mL and the concentrations of the anti-pseudomonal drugs were tested at a range from 64 to 0.06 µg/mL. Cigarette smoke condensate was tested at concentrations ranging from 40 to 0.4 µg/mL. The lowest concentrations at which biofilm formation was inhibited (as circled in red) were considered to be the MIC values for each antibiotic.

Abbreviations: AMI, amikacin; CEF, cefepime, CIP, ciprofloxacin; CLA, clarithromycin; CSC, cigarette smoke condensate; MER, meropenem; PIP, piperacillin; TAZ, tazobactam

The MICs of clarithromycin and the six anti-pseudomonal agents on the planktonic growth of, and biofilm formation by the three different strains of *P. aeruginosa* [*viz*. reference strain PAO1, which is sensitive to all anti-pseudomonal agents (WT), and a drug-sensitive (DS) and a multidrug resistant (MDR) clinical isolates] are shown in Tables 4.1 and 4.2, respectively. The results presented are representative of three sets of experiments performed in triplicate.

Table 4.1: Minimum inhibitory concentrations of clarithromycin and six anti-pseudomonal agents on planktonic growth of *Pseudomonas aeruginosa.*

Agent (µg/mL)	**Strains**		
	WT	**DS**	**MDR**
Clarithromycin	125	125	250
Amikacin	4	2	64
Cefepime	2	4	>64
Ciprofloxacin	0.5	0.5	32
Meropenem	4	8	>64
Piperacillin	16	16	>64
Tazobactam	>64	>64	>64

Data derived from 3 separate experiments with 3 replicates for each test system in each experiment.

The MIC of clarithromycin was found to be the same for the planktonic growth of the WT and DS strains of *P. aeruginosa* (125 µg/mL); however, the MIC for this antibiotic against the MDR strain was two-fold higher (250 µg/mL). The MICs of ciprofloxacin and piperacillin were comparable between the WT and the DS *P. aeruginosa* strains in planktonic culture. However, in the case of amikacin, the MIC for the DS strain was two-fold lower than that of the WT, while the MIC for meropenem was two-fold higher for the DS strain compared to that of the *P. aeruginosa* WT. Notably, tazobactam was found to have no effect on the planktonic growth of any of the three stains of *P. aeruginosa* at the highest concentration tested (64 µg/mL). The MDR strain also displayed far greater resistance to the other antibiotics tested, with the highest concentrations of clarithromycin and amikacin used being required to inhibit the growth of planktonic *P. aeruginosa.* As was the case with tazobactam, cefepime,

meropenem and piperacillin did not inhibit the growth of the MDR bacterial strain at the highest concentration of the antibiotics tested.

Table 4.2: Minimum inhibitory concentrations of clarithromycin and six anti-pseudomonal agents on biofilm formation by *Pseudomonas aeruginosa.*

Agent (µg/mL)	**Strains**		
	WT	**DS**	**MDR**
Clarithromycin	125	125	125
Amikacin	0.25	0.25	8
Cefepime	1	0.5	64
Ciprofloxacin	1	0.5	32
Meropenem	0.5	0.5	>64
Piperacillin	16	8	>64
Tazobactam	>64	>64	>64

Data derived from 3 separate experiments with 3 replicates for each test system in each experiment.

In contrast to what was found with the planktonic growth of *P. aeruginosa*, the MIC of clarithromycin against the formation of biofilm by all three test strains was the same (125 µg/mL). Unexpectedly, biofilm formation by the WT and DS strains of *P. aeruginosa* was inhibited at lower concentrations of amikacin, cefepime, ciprofloxacin, and meropenem than were found to inhibit planktonic growth. In the case of piperacillin, biofilm formation by the WT strain was inhibited at the same concentration as planktonic growth (16 µg/mL), while biofilm formation by the DS strain was inhibited at a two-fold lower concentration than planktonic growth. Interestingly, biofilm formation by the MDR strain was inhibited by amikacin at a concentration of 8 µg/mL when compared to the MIC of this antibiotic of 64 µg/mL against planktonic growth. Similar MICs were obtained for the other test antibiotics against biofilm formation by the MDR strain.

4.3. Fractional inhibitory concentration indices (FICI) for clarithromycin in combination with three anti-pseudomonal agents

Due to lack of inhibition of both planktonic growth and biofilm formation by all three strains of *P. aeruginosa* at the highest concentration of tazobactam tested, this antibiotic was not tested in combination with clarithromycin. In addition, due to unforeseen delays in starting the experimental work due to restricted access to the laboratory during COVID, two other agents with relatively high MICs, specifically meropenem and piperacillin, were excluded from the experiments investigating the effect of clarithromycin in combination with these anti-pseudomonas agents. The results for amikacin, cefepime and ciprofloxacin, in combination with clarithromycin are shown in Tables 4.3 and 4.4, respectively.

Table 4.3: FICI values for combinations of clarithromycin with anti-pseudomonal agents against planktonic growth of *Pseudomonas aeruginosa* strains.

Agent (μg/mL)	**Strains**		
	WT	**DS**	**MDR**
Clarithromycin + Amikacin	0.5	0.5	0.25
Clarithromycin + Cefepime	0.25	0.25	0.25
Clarithromycin + Ciprofloxacin	0.25	0.25	0.25

Table 4.4: FICI values for combinations of clarithromycin and anti-pseudmonal agents against biofilm formation by *Pseudomonas aeruginosa* strains.

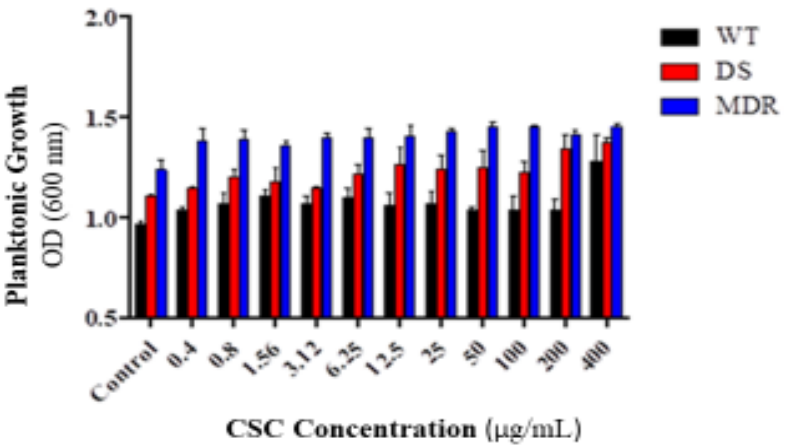
Agent (μg/mL)	**Strains**		
	WT	**DS**	**MDR**
Clarithromycin + Amikacin	0.25	0.5	1
Clarithromycin + Cefepime	0.125	0.25	≥1
Clarithromycin + Ciprofloxacin	0.125	0.125	1

The combination of clarithromycin with the three selected anti-pseudomonal agents was found to result in a synergistic inhibition of planktonic growth of the WT, DS and MDR strains of *P. aeruginosa*. Notably, with respect to the MDR strain of the pathogen, additive interactions were evident when clarithromycin was combined with the various anti-pseudomonal agents.

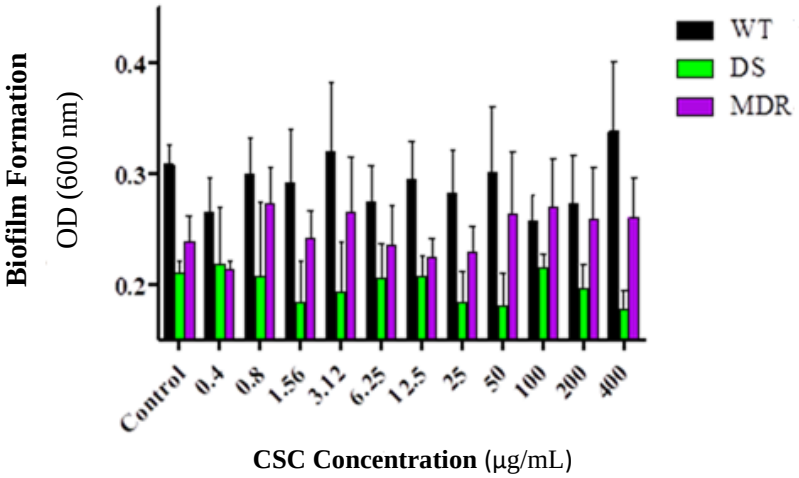
Essentially similar findings were noted in the case of biofilm formation, notable synergy observed with the WT and DS strains on exposure to the clarithromycin/cefepime and clarithromycin/ciprofloxacin combinations. Once again, however, additive interactions were observed on exposure of the MDR strain to the combinations of amikacin, cefepime or ciprofloxacin with the MDR strain of *P. aeruginosa.*

4.4. The effect of cigarette smoke condensate on planktonic growth of, and biofilm formation by *Pseudomonas aeruginosa.*

The results for the effects of CSC on the planktonic growth of, and biofilm formation by the three strains of *P. aeruginosa* are presented in Figure 4.6 A and B, respectively.



A.



B.

Figure 4.6. Effect of cigarette smoke condensate (CSC) on planktonic growth of (A) and biofilm formation by (B) the WT, DS and MDR strains of *Pseudomonas aeruginosa.* The results are expressed as the means ± SD of two different experiments, with triplicate repeats, compared to the solvent control.

Treatment of the three strains with CSC was found to result in increased, albeit modest, planktonic growth of *P. aeruginosa,* In the case of the WT strain, planktonic growth increased with exposure to CSC reaching a plateau at 0.8 µg/mL. Growth was found to increase again at a concentration of 400 µg/mL CSC. A slight dose-response increase in planktonic growth of the DS strain of *P. aeruginosa* exposed to CSC was also observed. The planktonic growth of the MDR strain was found to increase on exposure to the lowest concentration of CSC used (0.4 µg/mL) whereafter the growth of the bacteria reached a plateau.

The effects of exposure of all three strains of the pathogen were, however, variable, with no clear dose-related responses evident.

4.5 The effect of cigarette smoke condensate on the minimum inhibitory concentrations of clarithromycin and the anti-pseudomonal agents.

The MICs of clarithromycin and the three selected anti-pseudomonal drugs, in the presence of CSC, on the planktonic growth of, and biofilm formation by the WT, DS and MDR strains of *P. aeruginosa* are shown in Tables 4.5 and 4.6, respectively.

Table 4.5: Minimum inhibitory concentrations of clarithromycin and anti-pseudomonal agents against planktonic cultures of *Pseudomonas aeruginosa* exposed to cigarette smoke condensate.

Agent (µg/mL)	**Strains**					
	WT		**DS**		**MDR**	
CSC	**Without**	**With**	**Without**	**With**	**Without**	**With**
Clarithromycin	125	250	125	125	250	250
Amikacin	4	2	2	1	64	NA
Cefepime	2	0.5	4	1	>64	32
Ciprofloxacin	0.5	0.25	0.5	0.25	32	16

Table 4.6: Minimum inhibitory concentrations of clarithromycin and anti-pseudomonal agents against biofilm formation by *Pseudomonas aeruginosa* exposed to cigarette smoke condensate.

Agent (μg/mL)	**Strains**					
	WT		**DS**		**MDR**	
CSC	**Without**	**With**	**Without**	**With**	**Without**	**With**
Clarithromycin	125	125	125	125	125	125
Amikacin	0.25	0.5	0.25	0.5	8	8
Cefepime	1	1	0.5	0.5	64	64
Ciprofloxacin	1	0.5	0.5	0.25	32	16

The results demonstrate that CSC has a seemingly, albeit modest, negative effect on the MICs of each antibiotic against planktonic growth and biofilm formation, However, the differences observed are mostly small, generally of the order of one dilution**,** possibly the result of minor variations in procedure.

4.6. Fractional inhibitory concentration indices for the effect of cigarette smoke condensate on the activity of clarithromycin in combination with the anti-pseudomonal agents.

The effects of CSC on the inhibitory activities of the combinations of clarithromycin with amikacin, cefepime or ciprofloxacin on both planktonic and biofilm cultures of the three strains of *P. aeruginosa* are shown in Tables 4.7 and 4.8, respectively.

Table 4.7: FICI values for clarithromycin in combination with the anti-pseudomonal agents against planktonic growth of *Pseudomonas aeruginosa* exposed to cigarette smoke condensate.

Agent (μg/mL)	**Strains**					
	WT		**DS**		**MDR**	
CSC	**Without**	**With**	**Without**	**With**	**Without**	**With**
Clarithromycin + Amikacin	0.5	0.5	0.5	1	0.25	≥1
Clarithromycin + Cefepime	0.25	0.5	0.25	0.5	0.25	1
Clarithromycin + Ciprofloxacin	0.25	0.5	0.25	0.5	0.25	1

Table 4.8: FICI values for clarithromycin in combination with the anti-pseudomonal agents against biofilm formation by *Pseudomonas aeruginosa* exposed to cigarette smoke condensate.

Agent (μg/mL)	**Strains**					
	WT		**DS**		**MDR**	
CSC	**Without**	**With**	**Without**	**With**	**Without**	**With**
Clarithromycin + Amikacin	0.25	0.25	0.5	1	1	≥1
Clarithromycin + Cefepime	0.125	0.125	0.25	0.25	≥1	1
Clarithromycin + Ciprofloxacin	0.125	0.125	0.125	0.125	1	≥1

With respect to planktonic growth, following exposure to CSC, the FICI values of the anti-pseudomonal agents in combination with clarithromycin against all three of the *P. aeruginosa* strains were either mostly unaffected or slightly attenuated and largely reflected the results shown for the antibiotic combinations in Table 4.3 in the absence of CSC. Similarly, in the case of biofilm formation, the FICI values of the anti-pseudomonal agents in combination with clarithromycin against the WT and DS strains of the pathogen were either unaffected or

minimally altered following exposure to CSC, largely reflecting the results shown in Table 4.4 in the absence of CSC.

Chapter 5: General Discussion and Conclusion

The key findings of the experimental work presented in this dissertation are summarised as follows:

- With respect to planktonic growth, all three strains of *Pseudomonas aeruginosa* were resistant to clarithromycin with minimum inhibitory concentration (MIC) values of ≥125 μg/mL. In the case of the recognised anti-pseudomonal agents, the order of potency based on the MIC values for the reference strain, PAO1 (WT) of the pathogen was ciprofloxacin > cefepime > amikacin = meropenem > piperacillin > tazobactam. The corresponding order of potency for the clinical isolate of the pathogen was ciprofloxacin > amikacin > cefepime > meropenem > piperacillin > tazobactam. As expected, the multidrug resistant (MDR) strain of the pathogen demonstrated resistance to all six anti-pseudomonal agents.
- In the case of biofilm formation by the WT and drug-sensitive (DS) strains of the pathogen, the MIC values for clarithromycin and the six anti-pseudomonal agents were, mostly lower, indicative of increased antimicrobial activity, than those associated with planktonic growth of the pathogen, while those of the MDR strain were essentially equivalent. In this context, it is important to note that these experiments were designed to investigate the effects of the test antibiotics added to the various strains of the pathogen at the outset of the experiment, as opposed to retrospective addition to *P. aeruginosa* encased in pre-formed biofilm, a setting in which the opposite would be expected *i.e.* decreased antimicrobial activity (Lutz *et al.*,2021). The results are therefore indicative of the ability of the test antimicrobial agents, when added directly to susceptible strains of *P. aeruginosa,* to inhibit biofilm formation, a potentially important protective activity of these agents.
- The most notable finding of the current study was the observed synergy of the drug-susceptible strains of *P. aeruginosa* to amikacin, cefepime and ciprofloxacin when cultured in the presence of clarithromycin, independently of growth in either standard bacteriological or biofilm-promoting growth medium. These effects of clarithromycin were less evident with the MDR strain of the pathogen, being additive as opposed to synergistic due, most likely, to the pre-existing sensitivity of these agents to amikacin, cefepime and ciprofloxacin.
- Exposure of all three strains of *P. aeruginosa* to cigarette smoke condensate (CSC) alone (in the absence of the antibiotics) resulted in modest increases in planktonic growth,

which varied according to the strain of the pathogen. Similar effects were evident in the setting of exposure all three strains of the pathogen to CSC in biofilm-promoting growth media. With respect to the effects of CSC on antibiotic activity, exposure of all three strains of *P. aeruginosa* to the individual anti-pseudomonal agents or clarithromycin in the presence of CSC did not result in detectable attenuation of the anti-pseudomonal activities of the test antimicrobial agents. Likewise, addition of CSC to the combination of clarithromycin with amikacin, cefepime or ciprofloxacin during the growth of all three strains of the pathogen under both planktonic and biofilm-promoting culture conditions, donly minimally affected the synergistic antimicrobial interactions of the test antibiotics, while the additive effects previously noted with the MDR strain were minimally affected. The failure of CSC to affect anti-pseudomonal antibiotic activity may reflect aspects of experimental design, specifically the short duration of exposure of all strains of the pathogen to CSC. In this context, it is noteworthy that Xu et al. recently reported that prolonged exposure (10 – 15 passages) of *P. aeruginosa* to cigarette smoke extract is necessary to achieve induction of the *nfxc* drug-resistant phenotype. Future studies should therefore focus on extended exposure of the pathogen to CSC, initially in a basal medium (to eliminate possible neutralisation of constituents of CSC by components of complex growth media) followed by transfer to bacteriological culture media.

Limitations of the current study include: i) a somewhat over-ambitious study design, encompassing three strains of *P. aeruginosa*, seven antibiotics used individually, as well as in combination, with and without CSC, and two types of assay of bacterial growth; ii) lack of a comparison of the effects of the various test agents individually and in combination on *P. aeruginosa* encased in pre-formed biofilm relative to those of direct exposure of the pathogen to the test antibiotics as performed in the current study; iii) as already mentioned, the probable necessity for extended exposure of the pathogen to CSC prior to culture in the absence and presence of antibiotics; and iv) although beyond the scope of this dissertation, measurement of the effects of other types of macrolide, both 14- and 15-membered, on the reversal of *P. aeruginosa* MDR. These issues can be resolved in future studies.

In conclusion, irrespective of apparent limitations, the current study has highlighted two notable issues. Firstly, the inhibitory effects of currently used anti-pseudomonal antibiotics on biofilm formation. Secondly, the potential of clarithromycin to attenuate multidrug

resistance of *P. aeruginosa* cultured under both planktonic and biofilm-forming growth conditions.

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