Phenotypic and genotypic resistance characteristics of *Pseudomonas aeruginosa* isolates

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Thesis Title

Phenotypic and genotypic resistance characteristics of *Pseudomonas aeruginosa* isolates

Thesis Abstract

Pseudomonas aeruginosa causes both contact lens and non-contact lens-related keratitis (corneal infection). This opportunistic bacterium naturally has the ability to resist the mechanism of action of many antibiotics which are used for treatment. *P. aeruginosa* resistance patterns and the mechanism of resistance in isolates from keratitis are not well understood. This thesis described the phenotypic and genotypic patterns of antimicrobial resistance and compared these between ocular isolates of *P. aeruginosa* from Australia (contact lens) and India (non-contact lens). Changes in the antimicrobial susceptibility between isolates over time were also analysed.

Susceptibility to antibiotics, multipurpose disinfecting solutions and disinfectants was analysed for twenty-seven Australian isolates from contact lens-related keratitis and f orty non-contact lens-related isolated from India. The whole genomes of fourteen Australian (historical and recent) and twelve Indian isolates were sequenced using Illumi na® MiSeq®. Computational analysis of the genomes was performed to analyse their core and pan genomes and these were examined for the presence of acquired resist ance genes, virulence genes, gene mutations, and these compared to their phenotypic resistance to antibiotics.

Indian isolates possessed large pan genomes with more acquired resistance (30) genes and larger numbers of genetic variations. The Indian isolates contained clones of t hree sequence types ST308, ST316 and ST491, whereas Australian isolates contained only one sequence type ST233. Isolates with larger gene variations had mutations in the DNA mismatch repair system. Most multi-drug resistant Indian (non-contact lens) isolates were *exoU* +.

Indian isolates had large accessory genes compared to Australian isolates and this increased the pan genome size of the Indian isolates. The number of core genome mut ations were larger in the Indian isolates a median of 50006 (IQR=26967-50600) compared to Australian isolates a median of 26317 (IQR=25681-33780).

There were differences between isolates from Australia and India with respect to their antibiotic resistance and associated genes. Indian strains had more genetic diversity and were multi-drug resistant. However, there was no evidence of substantial genetic or phenotypic changes within isolates from their respective countries.

Thesis submission for the degree of Doctor of Philosophy

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Publication Details #1

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I confirm that where I have used a publication in lieu of a chapter, the listed publication(s) above meet(s) the requirements to be included in the thesis. I also declare that I have complied with the Thesis Examination Procedure.

In the name of Allah (God), the most beneficent, the most merciful

Dedication

To my Father Mohammad Saleem Khan,

Mother Saeeda Bibi

&

Grand-mother Maryum Bibi

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Abstract

Pseudomonas aeruginosa causes both contact lens and non-contact lens-related keratitis (corneal infection). This opportunistic bacterium naturally has the ability to resist the mechanism of action of many antibiotics which are used for treatment. *P. aeruginosa* resistance patterns and the mechanism of resistance in isolates from keratitis are not well understood. This thesis described the phenotypic and genotypic patterns of antimicrobial resistance and compared these between ocular isolates of *P. aeruginosa* from Australia (contact lens) and India (non-contact lens). Changes in the antimicrobial susceptibility between isolates over time were also analysed.

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Abbreviations

Abbreviations	Description
aGM1	Asialo-ganglio side M1
AHL	acyl homoserine lactones
AMR	Antimicrobial resistance
CDS	Coding sequences
CL	Contact lens
FIC	Fractional inhibitory concentration
FQ	Fluoroquinolones
LPS	Lipopolysaccharides
MBC	Minimum bactericidal concentration
MDR	Multi-drug resistance
MFP	Membrane fusion protein
MGE	Mobile genetic elements
MIC	Minimum inhibitory concentration
RND	Resistance nodulation-division
MK	Microbial keratitis
MLST	Multi-locus sequence typing
MNP	Multi-nucleotide polymorphism
MPDS	Multi-purpose disinfecting solutions
PASP	*Pseudomonas aeruginosa* Small Protease
QRDR	Quinolone-resistance determining region
QS	Quorum sensing
RND	Resistance nodulation division
SNP	Single nucleotide polymorphism
ST	Sequence type
TIIISS	Type three secretion system
tRNA	Transfer RNA

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Thesis structure

This thesis is presented in a series of seven chapters including three publications and one manuscript under review. There is a possibility of repetition in the methods of these chapters however, each chapter has been written as a stand-alone separate publication. The results are presented in this thesis from chapter 2-6. Chapter 2 is a combination of a published article with additional unpublished data. Chapter 3 contains unpublished data but chapter 4, 5, 6 are all published or under review.

CHAPTER 1 summarises the introduction and literature background consisting of resistance mechanisms of *P. aeruginosa* and its reported resistance in keratitis from different regions. It also explains the genomic diversity of this bacterium in terms of resistance and pathogenesis in the eye infections particularly.

CHAPTER 2 presents the comparative susceptibility of ocular *P. aeruginosa* isolates from contact lens related infection from Australia. The first half of the chapter is comprised of a published article which included the antibiotic susceptibilities of historical isolates collected in 2001-2006. The second half included the recent contact lens related isolates from Australia collected in 2018-2019 which were compared for the antibiotic susceptibilities to the historical isolates to analyse the differences over time.

CHAPTER 3 compared the antibiotic susceptibilities of isolates from Australia (contact lens related isolates) India (non-contact lens related isolates). The comparison was conducted to analyse the phenotypic differences in the antibiotic susceptibilities in two distinct regions with different antibiotic prescribing regulations.

CHAPTER 4 investigated the genomic differences in the ocular clones of sequence type 308 of *P. aeruginosa* over time. The ST308 isolates of *P. aeruginosa* of this study were compared

to the clones of previously published study. The isolates were compared because they shared sequence type 308 isolated from same place in India but twenty years later.

CHAPTER 5 presents the acquired resistance of fluoroquinolones. Fluoroquinolones are considered as first drug of choice for keratitis treatment and usually the reported resistance is due to mutations in the chromosomal genes. However, novel fluoroquinolone resistance genes were found in ocular *P. aeruginosa* isolates.

CHAPTER 6 presents the comparison of genomic resistance characteristics of ocular *P. aeruginosa* isolates from Australia and India. The whole genomes of the isolates were evaluated from both regions. Isolates were compared for acquired resistance genes, single nucleotide polymorphism, mutations in the DNA mismatch repair system genes, core genome and pangenome.

CHAPTER 7 discussed and summarized the findings from Chapters 2-6 and suggested future directions.

1 Introduction and literature review

1.1 Infections caused by *Pseudomonas aeruginosa*

P. aeruginosa is a cause of both community-acquired and hospital-acquired (nosocomial) infections. Corneal infections (or microbial keratitis, mostly due to contact lens use), otitis externa (commonly found in immunocompromise such as diabetes mellitus) and infections of skin and soft tissues (for example patients with diabetic foot infections) are examples of community-acquired infections. Hospitalized patients may be colonized with *P. aeruginosa* more easily because these bacteria are isolated from many places within hospitals (Bodey et al., 1983, Jones et al., 2002, Jackson et al., 1951, Bennett, 1974). According to a Centre for Disease Control and Prevention, *P. aeruginosa* is liable for 8% of nosocomial infections and usually these are caused by carbapenem resistant multidrug resistant isolates (2%) (Sievert et al., 2013). However, in intensive care units the percentage of *P. aeruginosa* infections varies between 13.2 and 22.6% (Erbay et al., 2003, Lizioli et al., 2003). *P. aeruginosa* infections commonly acquired during a hospital stay include surgical site infection, bloodstream infection, pneumonias, urinary tract infection, and skin infection in burn wounds. Chronic sinopulmonary colonization and repeated lung infections from *P. aeruginosa* are seen in patients with cystic fibrosis (Rello et al., 2002, Kollef et al., 2005). Patients with immunodeficiencies, for example infection with human immune deficiency virus and cases of organ transplants (heart, lung, bone-marrow etc.), are more prone to develop *P. aeruginosa* infections resulting in septicaemia and bacteraemia (Lee et al., 2006, Vidal et al., 1999).

1.2 Microbial keratitis

The ocular surface (cornea, conjunctiva and tear film) protects the internal parts of the eye from entry and invasion by microorganisms. Infection of the cornea, called microbial keratitis, can be caused by microorganisms including bacteria, fungi, viruses and parasites. Bacteria are considered the major cause of microbial keratitis worldwide (Bharathi et al., 2009, Pens et

al., 2008, Verani et al., 2009, Green et al., 2008) accounting for 60-70% of infections. Bacterial keratitis is associated with predisposing factors (Musch et al., 1983) such as prior surgery, corneal scars, epithelial defects, corneal oedema, dry eye, glaucoma, lagophthalmos, blepharitis and contact lens wear (Gopinathan et al., 2009, Dart et al., 1991) and can result in loss of vision, even blindness (McLeod et al., 1995, Keay et al., 2006b). Keratitis leads to corneal transplantation in 20% of patients (Hoddenbach et al., 2014).

Contact lenses have been used for decades for refractive, cosmetic and therapeutic purposes. However, there are complications associated with contact lens use. Corneal infection is rare but is the most severe complication of contact lens wear, occurring in around 4 per 10000 wearers per year (Cheng et al., 1999, Poggio et al., 1989, Stapleton et al., 2008a) and can cause visual loss in 10-15% cases (Cheng et al., 1999, Stapleton et al., 2008b, Choy et al., 2008). The estimated annual incidence of MK per 10,000 CL wearers, ranges between 1.2, for daily wear rigid gas permeable (RGP) lenses and 25.4 for extended wear of silicon hydrogel lenses (Stapleton et al., 2008a). Contact lens wear is a major cause of keratitis in developed countries (Fleiszig, 2006, Liesegang, 1997, Stapleton, 2020). According to recently published statistics by Centre for Disease Control and Prevention, 45 million people in the USA are contact lens wearers. As one, now fairly old, estimate suggests there are 140 million contact lens wearers in the world, (Nichols et al., 2013), which results in significant numbers of wearers with disease and vision loss (Stapleton et al., 2008b). While the incidence is unchanged, the number cases of contact lens-related keratitis has increased due to the elevated use of cosmetic lenses (Hedayati et al., 2015).

1.2.1 Signs and symptoms of microbial keratitis

The clinical diagnosis of microbial keratitis depends on the patient history, risk factors and the morphological features of the corneal lesion (Saini et al., 2003). Initial symptoms include discomfort and foreign body sensation and progressive pain is considered a hallmark of disease (Keay et al., 2009). In contact lens related microbial keratitis, the pain is progressive

following lens removal (Keay et al., 2009). During acute disease, photophobia and lid swelling are common (Keay et al., 2009).

Generally corneal infection presents with a corneal infiltrate and overlying epithelial defect. Eye care practitioners use clinical clues to recognize ocular surface infections (Rietveld et al., 2004), but some differential signs may help to predict whether the infection is bacteria, fungal, viral or amoebic (Theodore et al., 1985, Thomas et al., 2005). For example, keratitis caused by *Pseudomonas* sp. and *Acanthamoeba* sp. can appear to be similar upon initial presentation, which limits diagnosis (Dahlgren et al., 2007).

More severe microbial keratitis is associated with vision loss, prolonged disease duration and high cost to treat (Dart et al., 2008, Stapleton et al., 2008b, Keay et al., 2008). Somewhat counterintuitively, one study has shown a longer disease duration in culture negative cases compared to culture positive cases, however surgical intervention is more common in culture positive cases (Bhadange et al., 2015).

Laboratory diagnosis for microbial keratitis is recommended (Jones, 1981) but this does increase the time and cost to diagnosis (McDonnell, 1996). As it is critical to start antimicrobial treatment as soon as possible (Keay et al., 2006a), initial therapy is almost always empirical (Norina et al., 2008, Dart et al., 2008, Stapleton et al., 2008a). Viable culture may be used to confirm the causative organism however, 50% of cases are culture negative (Bhadange et al., 2015). Misdiagnosis can result in longer use of topical medications (Bhadange et al., 2015).

1.2.2 Risk factors for contact lens related microbial keratitis

Often the failure to follow hygiene and compliance instructions has been hypothesised as underlying contact lens complications (Abdelkader, 2014). Risk factors for keratitis include overnight wear of soft contact lenses (Stapleton et al., 2008a, Cheng et al., 1999, Dart et al., 2008), improper contact lens handling including hygiene practices and replacement frequency of storage cases (Lim et al., 2016, Keay et al., 2007, Stapleton et al., 2012). Other risk factors include poor compliance with contact lens disinfection and cleaning of accessories (Panthi et

al., 2014, Stapleton et al., 2008b), smoking, higher socioeconomic class and purchase of the contact lenses through the internet (Stapleton et al., 2008a). Use of disinfecting solution for longer times then recommended by the manufacturers, cosmetic contact lens wear, and the use of certain contact lens multipurpose disinfection solutions are also important risk factors (Sauer et al., 2016, Sharma et al., 2003, Lim et al., 2016).

The risk factor responsible for non-CL related microbial keratitis in a working age population is mostly ocular trauma (Sethi et al., 2010, Keay et al., 2006a). Ocular trauma includes ocular injury (when eye is scratched), surgery or trauma from vegetation or associated with contaminated water (Chidambaram et al., 2018). Non-CL related microbial keratitis with contaminated water is more common in developing countries (Srinivasan et al., 1997, Ung et al., 2019).

1.2.3 *Pseudomonas aeruginosa* keratitis

Pseudomonas aeruginosa is the most commonly isolated bacterium from microbial keratitis associated with contact lens wear, (Sharma et al., 2013, Dart et al., 1991, Galentine et al., 1984, Schein et al., 1989, Green et al., 2008, Fong et al., 2007) accounting for 60–70% of culture-proven cases (Cheng et al., 1999, Dart et al., 1991, Stapleton et al., 2007). This may be because it adheres well to contact lenses (Dutta et al., 2012) and lens cases, and can develop a biofilm,(Szczotka-Flynn et al., 2009, McLaughlin et al., 1998) which makes it is less susceptible to most disinfecting regimens (Vijay et al., 2015, McLaughlin et al., 1998, Szczotka-Flynn et al., 2009). Contamination of contact lens accessories and disinfecting solutions has been implicated in *Pseudomonas aeruginosa* keratitis (Boost and Cho, 2005, Mayo et al., 1987). *P. aeruginosa* also causes non-contact lens-related microbial keratitis, but at a lower rate of 2-7% of cases per year (Hooi and Hooi, 2005, Green et al., 2008).

1.2.4 Prevention of Microbial keratitis

Approaches to help reduce the incidence of microbial keratitis include limiting contact lens contamination and ocular trauma in CL-related and non-CL related keratitis respectively.

Different multipurpose disinfecting solutions (MPDS) are used to disinfect daily wear contact lenses. Multipurpose disinfecting solutions are formulated to contain combinations of various disinfectants to prevent growth of bacteria, including *P. aeruginosa*, and fungi. The International Organization for Standardization (ISO) has established requirements and methods for testing the efficacy of lens care products (ISO, 2014) (Rosenthal et al., 2002). However, the efficacy of MPDS against *P. aeruginosa* can vary based on the formulation of MPDS (Laxmi Narayana et al., 2018). The efficacy of MPDS can be reduced due to structural or defensive mechanisms in bacteria such as formation of biofilms (Szczotka-Flynn et al., 2009). There are reports that bacteria can become resistant to the disinfectants used in MPDS (Russell, 1999, Abjani et al., 2017).

1.2.5 Management of *P. aeruginosa* keratitis

1.2.5.1 Treatment and diagnostics

Corneal infection is secondary only to cataract as a leading cause of ocular morbidity and blindness worldwide (WHO, 1973) https://www.who.int/blindness/causes/priority/en/index8.html. Effective therapy, using appropriate antimicrobials, and timely identification of the causative agent is necessary for the proper treatment of keratitis (McLeod et al., 1995).

Intensive topical antibiotic therapy is commenced immediately after diagnosis. The frequency of drops is reduced with the improvement in clinical response (Upadhyay et al., 2015, Gokhale, 2008) but usually does not reduce to below four times per day. Ciprofloxacin monotherapy is often the first choice of treatment (Gokhale, 2008) for mild keratitis (McAllum et al., 2003, Green et al., 2019, Daniell, 2003). For severe keratitis cases, ciprofloxacin or ofloxacin monotherapy may not be effective, and other options may be prescribed including later generation fluoroquinolones or combination fortified therapy (McAllum et al., 2003, Amer Awan et al., 2010), such as 5% cefazolin and 1.4% tobramycin or gentamicin (Gokhale, 2008).

However, there are increasing reports on the emergence of fluoroquinolone resistance from various regions in the world (Alexandrakis et al., 2000, Garg et al., 1999, Subedi et al., 2018b). The use of inappropriate antibiotics, variations in clinical practice and availability of antibiotics in different countries are some of the possible reasons behind elevated resistance to antibiotic (Ventola, 2015). The variation in the antibiotic susceptibility *P. aeruginosa* keratitis isolates in different countries (Zhang et al., 2008, Oldenburg et al., 2013, Garg et al., 1999, Kowalski et al., 2003, Green et al., 2019, Kowalski et al., 2001) might be due to the possession and acquisition of multiple resistance mechanisms by this bacterium (Poole, 2001, Livermore, 2002). The development of multi-drug resistant *P. aeruginosa* in the eye can be devastating, as this may result in more cases of vision loss due to scaring or perforation (Egrilmez and Yildirim-Theveny, 2020).

1.3 **Antimicrobial resistance**

Antimicrobial resistance (AMR) is now the biggest barrier to successful treatment of many infections. The main causes behind AMR are the excessive use of antibiotics in animals and humans, sale of antibiotics over the counter, poor hygiene and release of nonmetabolized antibiotics or their residues through waste/manure (Aslam et al., 2018). A rising concern is the predicted increased usage of antibiotics in food animals up to 67% by 2030 (Van Boeckel et al., 2015). Antibiotic resistance has been described as a global health crisis (Ventola, 2015). According to the Centre for Disease Control and Prevention in the United States, at least 2.8 million people develop an antibiotic resistant infection and more than 35,000 people die each year. It is estimated that by 2050 about 444 million people will be infected with AMR bacteria (Aslam et al., 2018). The World Health Organisation has listed resistance development by several microbes as a major global health problem (WHO, 2017). Microbes of concern include the ESKAPE pathogens, which consist of *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterococcus species* (Rice, 2008).

1.3.1 Resistance in ocular *P. aeruginosa* strains

P. aeruginosa keratitis caused by antibiotic resistant isolates has been reported from different countries (Table 1-1). Overall, *P. aeruginosa* susceptibility to either ciprofloxacin or moxifloxacin is around 80% from included studies (Kaliamurthy et al., 2013, Oldenburg et al., 2013, Lichtinger et al., 2012). However, resistance in South Asian countries (Vazirani et al., 2015, Fernandes et al., 2016a, Subedi et al., 2018b) is rising. Resistance to ciprofloxacin (100% of strains in one study) was highest in India (Garg et al., 1999). A recent study from India found that some ocular *P. aeruginosa* isolates were resistant to all classes of antibiotics including, fluoroquinolones, aminoglycosides, beta-lactams and polymyxin B (Subedi et al., 2018b).

Table 1-1 Resistance mechanisms of ocular *P. aeruginosa* strains.

Resistance phenotype (frequency)	Region	Resistance genotype	Reference
Chloramphenicol (100%)	Singapore	Not provided	(Tan et al., 1995)
Ciprofloxacin (100%) Cefazolin (100%) Chloramphenicol (90%) Norfloxacin (72%) Gentamicin (63%) Amikacin (10 %)	India	Not provided	(Garg et al., 1999)
Ciprofloxacin (15%)	India	Not provided	(Kunimoto et al., 1999)
Ciprofloxacin & Ofloxacin (2 %) Gentamicin & Tobramycin (0.6%)	South Florida	Not provided	(Alexandrakis et al., 2000)
Ciprofloxacin (3.3%)	India, United Kingdom	*GyrA, par C*	(Lomholt and Kilian, 2003)
Ciprofloxacin (23%) Amikacin (6%) Ofloxacin (16%) Norfloxacin (15%) Neomycin (23%) Gentamicin (7%) Tobramycin (5%) Chloramphenicol (53%)	South America	Not provided	(Chalita et al., 2004)
Gatifloxacin (13.2%) Ciprofloxacin (10.5%) Ofloxacin (13.2%) Fusidic acid (100%) Gentamicin (10.5%) Chloramphenicol (100%)	Europe	Not provided	(Morrissey et al., 2004)
Ciprofloxacin (9%) Ofloxacin (36%) Amikacin (24%) Gentamicin (30%) Tobramycin (25%)	India	Not provided	(Smitha et al., 2005)
Ceftazidime (4%) Gentamicin (2 %) Ciprofloxacin (4%)	USA	Not provided	(Yeh et al., 2006)
Fluoroquinolones (11%)	Australia, India	Not provided	(Choy et al., 2008)
Ciprofloxacin (7%) Ofloxacin (7%) Levofloxacin (3%) Tobramycin (10%)	China	Not provided	(Zhang et al., 2008)

Resistance phenotype (frequency)	Region	Resistance genotype	Reference
Chloramphenicol (>50%)	United Kingdom	Not provided	(Shalchi et al., 2011)
Moxifloxacin (35%)	South India	Not provided	(Oldenburg et al., 2013)
Ciprofloxacin (29%) Gatifloxacin (7%) Ofloxacin (9%) Cefotaxime (12%) Ceftazidime (8%) Amikacin (2%) Gentamicin (9%) Tobramycin (5%)	India	Not provided	(Borkar et al., 2014)
Neomycin (44%) Sulfasoxazole (94%) Tobramycin (6%) Gentamicin (6%) Moxifloxacin (3%) Ciprofloxacin (2 %) Ceftazidime (2 %) Polymyxin (2 %)	San Francisco	Not provided	(Peng et al., 2018)
Ciprofloxacin (61%) moxifloxacin (53%) Levofloxacin (46%) Gentamicin (53%) Imipenem (69%) Polymyxin (30%) Cefepime (38%) Ceftazidime (76%) Ticarcillin (92%) Aztreonam (23%)	India	Multiple acquired genes reported including *blaOXA-50, blaPAO, aph(3')-IIb, aph(3")-IIb, aph(6)-Id*	(Subedi et al., 2018b)
Chloramphenicol (>50%) Moxifloxacin (>80%)	United Kingdom	Not provided	(Lee et al., 2019)

1.3.2 Resistance mechanisms in *Pseudomonas aeruginosa*

After the discovery of the first antibiotic, it was assumed that the mortality and morbidity associated with infections would have resolved. However, it quickly became apparent that some microbes were resistant to antibiotics. Resistance mechanisms appeared to be inherently present in *Pseudomonas aeruginosa* which is naturally resistant to beta-lactams (including cephalothin and ampicillin) due to the possession of an inducible AmpC beta-lactamase (Livermore, 2002).

P. aeruginosa, along with *S. maltophilia* and *Burkholderia spp.* are becoming more clinically important because of their high-level multi-drug resistance (Poole, 2001). *P. aeruginosa* has become notoriously difficult to treat with antibiotics and disinfectants because of its innate resistance mechanisms and its ability to acquire new resistance mechanisms (Hancock, 1998). Its intrinsic resistance mechanisms include low cell wall permeability and active efflux pumps and production of antibiotic inactivating enzymes (Lambert, 2002). *P. aeruginosa* can also acquire resistance via several mechanisms such as gene mutation and horizontal gene transfer (Breidenstein et al., 2011). This bacterium uses different mechanisms to resist each antibiotic. However, it confers resistance to an individual antibiotic via multiple mechanisms. For example, penetration of the cell by fluoroquinolones can be blocked. Additionally, targets can also be modified to avoid the action of fluoroquinolones.

Traditional resistance mechanisms of *P. aeruginosa,* intrinsic, acquired and adaptive resistance are shown in Figure 1-1. Other than these more traditional resistance mechanisms of *P. aeruginosa,* this bacterium can develop phenotypic resistance by forming biofilms. The lungs of cystic fibrosis patients become rapidly colonised by *P. aeruginosa* which form large biofilms (Moreau-Marquis et al., 2008). These biofilms can make even sensitive strains of *P. aeruginosa* 1000 times resistant to antibiotics (Rasmussen and Givskov, 2006). Also, bacterial cells in biofilms are able to transfer resistant genes, so exacerbating the transmission of resistance (Rasmussen and Givskov, 2006, Stalder and Top, 2016). Resistance mechanisms for different classes of antibiotics in *P .aeruginosa* are discussed in the sections below.

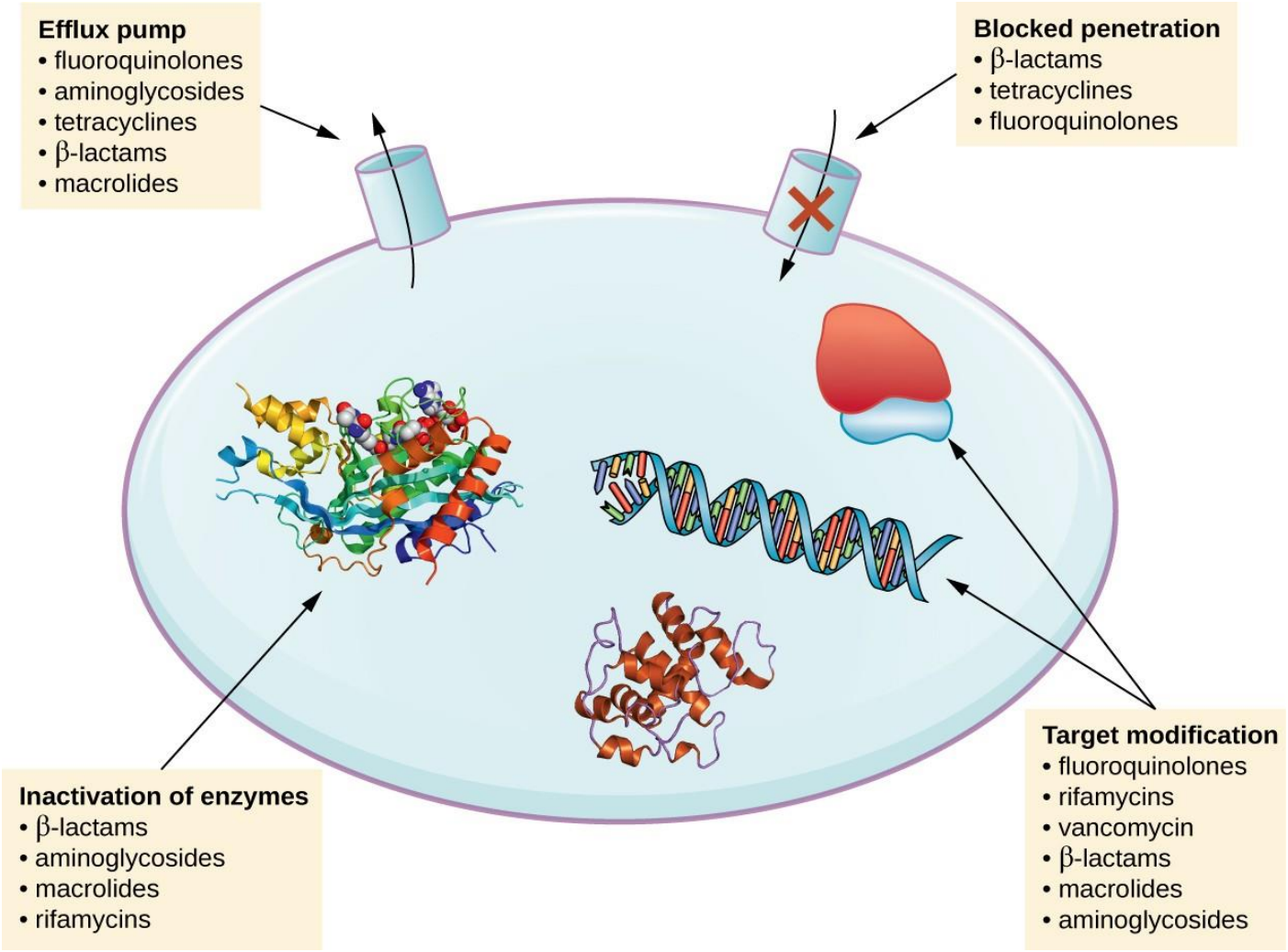

Figure 1-1 Antibiotic resistance mechanisms of *Pseudomonas aeruginosa*.

The figure is reproduced from https://courses.lumenlearning.com/microbiology/chapter/drug-resistance/

1.3.3 Intrinsic resistance

Intrinsic resistance refers to mechanisms which are present in the average strain of a given species irrespective of prior antibiotic exposure or the growth conditions. The outer membrane of gram-negative bacteria acts as a semipermeable barrier to various antibiotics and other molecules as it allows the entry into cells of small hydrophilic molecules and excludes larger molecules (Nikaido and Hancock, 1986). For example, quinolones which are hydrophilic molecules have restricted entry into cells through water-filled porins in the outer-membrane. Intrinsic resistance is often due to outer membrane permeability or possession of efflux pumps (Hancock, 1998), and these can work together to increase resistance. Indeed, the major mechanism for intrinsic resistance is believed to be a reduction in outer membrane permeability which results in slow penetration of antibiotic into cells (Hancock, 1998). In cases where efflux is not involved in resistance, for example with beta-lactams, production of antibiotic-modifying enzymes, such as beta-lactamases, can also confer intrinsic resistance (Hancock and Bell, 1988).

1.3.3.1 Outer membrane permeability

Membrane barriers that regulate permeability are involved in intrinsic resistance (Livermore, 1984). Almost all gram-negative bacteria possess these cell membrane resistance mechanisms, but *P. aeruginosa* appears to have a very high level of intrinsic resistance as it has ~12–100-fold less cell membrane permeability than *Escherichia coli* (Nikaido and Hancock, 1986). The possession of outer membrane proteins in *P. aeruginosa* called porins is a major resistance mechanism (Fernández and Hancock, 2012). *P. aeruginosa* produces several different types of porins with OprF being the major porin present in all strains (Brinkman et al., 2000). Different types of *P. aeruginosa* porins are listed in Table 1-2. Porins can combine with other membrane proteins to form efflux pumps.

Intrinsic antimicrobial resistance owes much to the presence of efflux systems in *P. aeruginosa* (Poole, 2001). Efflux pumps can mediate resistance to multiple antibiotics (Nikaido, 1998). Often, intrinsic resistance in *P. aeruginosa* involves antimicrobial efflux systems that use the

three proteins, MexA, MexB and OprM (Poole et al., 1993). Mutations in these proteins can lead to increased susceptibility to quinolones, beta-lactams (excluding imipenem), tetracyclines and chloramphenicol (Poole et al., 1996a, Poole et al., 1996b, Bianco et al., 1997). These efflux pumps are regulated and expressed by specific genes (Poole, 2001) and are therapeutic targets for *Pseudomonas* infections (Poole, 2001).

1.3.3.1.1 MexAB-OprM

The possession of these porin proteins results in intrinsic resistance to fluoroquinolones, beta-lactams, tetracycline, macrolides, chloramphenicol, novobiocin, trimethoprim and sulphonamides (Poole, 2001). Beta-lactams are poor substrates for MexAB-oprM and its expression is usually only related to resistance to meropenems (Li et al., 1994). The *oprM* gene is highly conserved in *P. aeruginosa* and contributes to antimicrobial resistance in most strains (Poole et al., 1996b, Bianco et al., 1997). Possession of MexAB-OprM may also be important for the resistance of biofilm-grown *P. aeruginosa* to certain antimicrobials (Brooun et al., 2000). A repressor protein MexR negatively regulates the expression of m*exAB-oprM*. Additionally, MexR also negatively autoregulates the *mexR* (*nalC*) regulatory gene (Srikumar et al., 2000) which in turn controls the expression of *nalB*. Mutations in the *nalB* result in resistance to fluoroquinolones, tetracycline and chloramphenicol (Jalal et al., 1999, Jalal and Wretlind, 1998, Alonso et al., 1999).

1.3.3.1.2 MexCD-OprJ

MexCD-OprJ was initially detected as a determinant of fluroquinolone resistance (Poole et al., 1996a, Hirai et al., 1987). However, it also mediates resistance to other antimicrobial agents including macrolides, chloramphenicol, novobiocin, tetracycline, trimethoprim and some beta-lactams (Srikumar et al., 1997, Köhler et al., 1996). Initially, MexCD-OprJ was believed to be involved in resistance to only 4th generation beta-lactams such as cefpirome and cefepime but later its ability to accommodate other beta-lactams such as cefoperazone and ceftazidime has

been proven (Srikumar et al., 1997). MexCD-OprJ works in a similar may to MexAB-OprM to expel antibiotics, but unlike MexAB-oprM it can be used to export beta-lactams such as carbenicillin and aztreonam (Srikumar et al., 1997). The expression of this efflux system is regulated by mutations in *nfxB* (Masuda et al., 1995).

1.3.3.1.3 MexEF-OprN

MexEF-OprN is usually dormant in wild type strains of *P. aeruginosa* (Köhler et al., 1997). However, after mutations in *nfxC,* it becomes active (Masuda et al., 1995, Köhler et al., 1997). Generally, in *nfxC* mutants where MexEF-OprN is expressed, strains are resistant to fluoroquinolones, chloramphenicol, and trimethoprim. *OprN* can be repressed by various substances such as salicylate which results in resistance to imipenem (Ochs et al., 1999).

1.3.3.1.4 MexXY-OprM

MexXY lacks a specifically linked outer membrane protein but utilizes OprM (Mine et al., 1999), especially in strains lacking MexAB. In *P. aeruginosa,* expression of MexXY-OprM produces resistance to aminoglycosides, tetracyclines, erythromycin (Aires et al., 1999, Li and Poole, 2001) and fluoroquinolones (Mine et al., 1999, Aires et al., 1999). Elimination of the regulatory gene *mexY* leads to susceptibility of MexXY-OprM isolates to aminoglycosides and fluoroquinolones (Masuda et al., 2000). An additional gene *mexZ* (also known as a*mrR*) represses the expression of *mexXY* and deletion of this gene enhances *mexXY* transcription in *P. aeruginosa* strains (Aires et al., 1999).

1.3.3.1.5 OprD

Resistance to imipenem is often due to a decrease of the outer membrane protein OprD. OprD is a specialized porin which has a specific role in the uptake of positively charged amino acids such as lysine. Loss of OprD is frequently associated with resistance to imipenem, which requires this porin to cross the outer membrane (Quale et al., 2006). Interestingly, unlike for

imipenem, resistance to meropenem is not affected by loss of *oprD*, indicating that these carbapenems cross the outer membrane by different channels (Lynch et al., 1987). *MexT* decreases the transcription of *oprD* (Köhler et al., 1999).

Table 1-2 Efflux pumps in *Pseudomonas aeruginosa* involved in antibiotics resistance.

Efflux components			**Regulatory genes**	**Target antibiotics**	**References**
MFP	**RND**	**Porin (OEP)**			
MexA	MexB	OprM	*mexR*	Fluoroquinolones, beta-lactams, chloramphenicol, tetracycline, novobiocin, trimethoprim, sulphonamides, macrolides	(Poole et al., 1993, Li et al., 1995)
MexC	MexD	OprJ	*nfxB*	Fluoroquinolones, beta-lactams, chloramphenicol, tetracycline, novobiocin, trimethoprim, macrolides,	(Poole et al., 1996a)
MexE	MexF	OprN	*mexT*	Fluoroquinolones, trimethoprim, chloramphenicol	(Köhler et al., 1997)
MexX	MexY	OprM	*mexZ*	Fluoroquinolones, aminoglycoside, tetracyclines, erythromycin	(Mine et al., 1999, Aires et al., 1999)

MFP= membrane fusion protein, RND= resistance nodulation-division, OEP=outer efflux protein.

1.3.4 Acquired antibiotic resistance

The mechanisms of acquired resistance to various antibiotics are listed in Table 1-3. In addition to intrinsic resistance, *P. aeruginosa* can also become resistant to antibiotics through acquired resistance. Acquired resistance is comprised of two types: mutations of genes and horizontal gene transfer.

1.3.4.1 Mutational resistance

Spontaneous mutations in genes can lead to resistance to antibiotics. The mutation frequency varies between different genes that mediate resistance to different antibiotics (Breidenstein et al., 2011). Various conditions can enhance mutations, such as growth in the presence of a DNA-damaging agents (Tanimoto et al., 2008) or even growth as biofilms (Driffield et al., 2008). Mutational frequencies differ between strains of *P. aeruginosa* with some becoming hypermutators that have the ability to mutate large number of genes and so acquire resistance to multiple antibiotics (Maciá et al., 2005). Similarly, hypermutations in the regulatory genes of various efflux pumps in *P. aeruginosa* also leads to resistance to several antibiotics. 36% of 128 *P. aeruginosa* strains isolated from the lungs of 30 cystic fibrosis patient were hypermutated because of mutations in *mutL* and *mutS* regulating MutS and MutL respectively in the DNA mismatch repair system (Oliver et al., 2000).

An important resistance mechanism is mutations in genes encoding *mexR* and *nfxB,* the regulators of the efflux pumps MexAB–OprM and MexCD–OprJ respectively (Stickland et al., 2010). These mutations often confer resistance to multiple antibiotics. Mutations in OprD can reduce the uptake of imipenem and therefore lead to resistance (Ochs et al., 1999). Similarly, mutations in the targets of antibiotics can also lead to resistance. For example, mutations in *gyrA* and *gyrB* (DNA gyrases) as well as *parC* and *parE* (topoisomerase IV) can reduce fluoroquinolone binding affinity, which leads to resistance (Dunham et al., 2010). Mutations to penicillin binding proteins are associated with *P. aeruginosa* resistance to beta-lactams (Srikumar et al., 1999). Beta-lactamases, enzymes that can destroy beta-lactam antibiotics, are regulated by chromosomal genes inherent in *P. aeruginosa.* When these chromosomal

genes are mutated or over-expressed, the enzymes are over-expressed which leads to rapid modification of the antibiotics (Giwercman et al., 1990).

Table 1-3. Mechanisms of action and resistance to antibiotics commonly used for treatment of *P. aeruginosa* infections.

Class	Antibiotics	Mechanism of action	Resistance mechanisms
Penicillin	Ticarcillin	Cell wall synthesis inhibition	De-repression of chromosomal beta-lactamase. Overexpression of the MexAB-OprM multi-drug efflux pump due to a *NalB* mutation. Specific plasmid-mediated beta-lactamases.
	Carbenicillin		
	Tazobactam		
	Piperacillin	Cell wall synthesis inhibition	De-repression of chromosomal beta-lactamase. Overexpression of the MexAB-OprM multi-drug efflux pump due to a *NalB* mutation. For the fourth generation cephalosporins cefepime and cefpirome, overexpression of the MexCD-OprJ multi-drug efflux pump due to an *NfxB* mutation.
Cephalosporin	Ceftazidime		
	Cefoperazone		
	Cefepime		
	Cefpirome		
Aminoglycosides	Gentamicin	Protein synthesis inhibition	Overexpression of the MexXY efflux pump in impermeability type resistance due to a mutation in the regulatory gene *MexZ*. Plasmid-mediated production of modifying enzymes.
	Tobramycin		
	Amikacin		
Fluoroquinolone	Ciprofloxacin	DNA synthesis inhibition	Target site mutations in the GyrA (or sometimes the *GyrB*) topoisomerase subunit; Overexpression of multi-drug efflux pumps due to *NalB*, *NfxB* or *NfxC* mutations.
Polymyxin	Polymyxin B	Cell membrane synthesis inhibition	Outer membrane LPS changes due to PhoP/PhoQ regulatory mutations.
Carbapenem	Imipenem	Cell wall synthesis inhibition	Loss of specific outer membrane porin channel, OprD; Reduction in levels of OprD due to a *NfxC* mutation that also upregulates multi-drug resistance due to MexEF-OprN; For meropenem overexpression of the MexAB-OprM multi-drug efflux pump due to a *NalB* mutation.
	Meropenem		

DNA= deoxyribose nucleic acid, LPS= lipopolysaccharide

1.3.4.2 Horizontally acquired resistance

Horizontally acquired resistance occurs by the transfer of genes across strains and species and is a key source of genetic diversity in bacterial populations, resulting in adaptation and evolution. This transfer is commonly mediated by mobile genetic elements (MGEs) which include plasmids, transposons, integrons, prophages and resistance islands. These MGEs can harbour resistance genes and can be acquired and transferred via conjugation, transformation or transduction (Breidenstein et al., 2011). **Conjugation** is a process that transfers DNA through direct physical contact between donor and recipient cells (Arber, 2014). **Transduction** is the transfer of DNA from one bacterium to another that is mediated by infection with bacteriophages. During **transformation**, bacteria take up free fragments of DNA and incorporate it into their own genome (Pang et al., 2019, Arber, 2014).

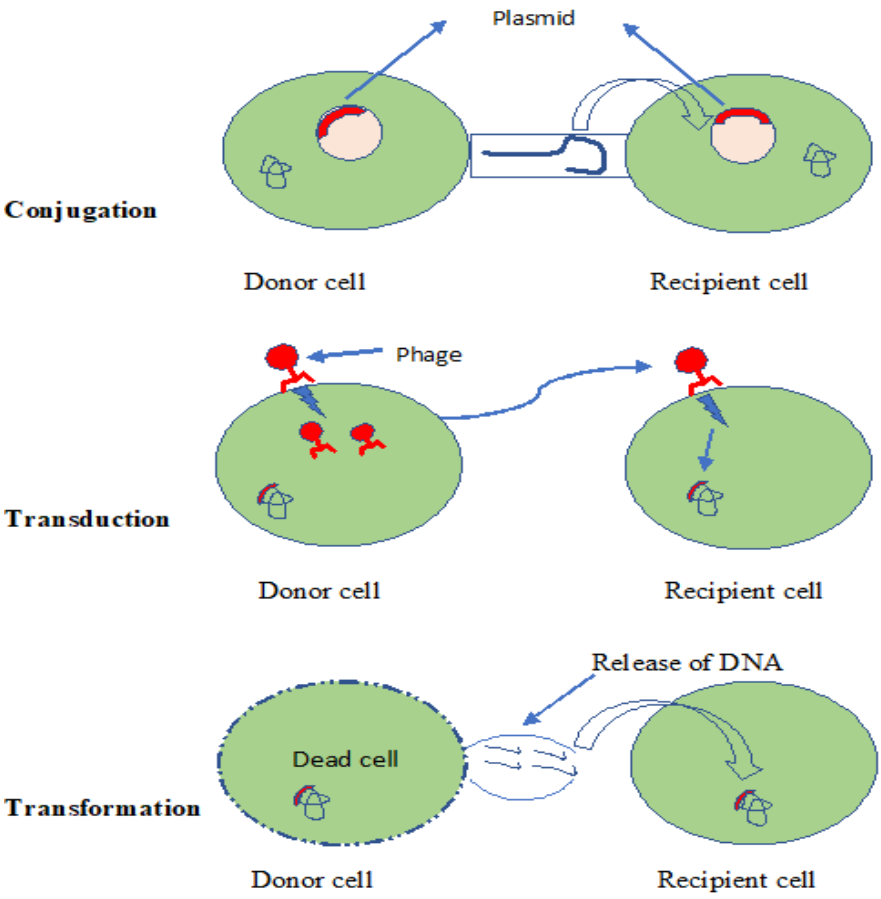

Figure 1-2 Mechanisms of horizontal gene transfer in *P. aeruginosa.*

(Recreated from Pang Z, Raudonis R, Glick BR, Lin TJ, Cheng Z. Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and alternative therapeutic strategies. *Biotechnol Adv*. 2019;37(1):177-192. doi:10.1016/j.biotechadv.2018.11.013).

The transfer of genes on plasmids can increase antibiotic resistance and confer resistance to multiple antibiotics, as plasmids often harbour multiple gene cassettes, some of which contain resistance genes (Rozwandowicz et al., 2018). Multiple plasmids can be acquired by bacteria and plasmids can interact with other mobile genetic elements, causing persistence in the host (Dionisio et al., 2019). Horizontal transfer of resistance genes via integrons occurs by insertion of mobile gene cassettes into specific genetic sites via site-specific recombination. Integrons play a critical role in dissemination of antibiotic resistance among *P. aeruginosa* strains (Rowe-Magnus and Mazel, 2002). Different types of acquired resistance genes of *P. aeruginosa* are listed in Table 1-4.

1.3.5 Mobile genetic elements

Ten percent of the *P. aeruginosa* genome is defined as a flexible genome which can be composed of genomic islands (GIs). Integrons, plasmids, transposons, insertion sequences and bacteriophages are also a part of the flexible genome in *P. aeruginosa* and are referred to as mobile genetic elements (MGEs) (Hacker and Carniel, 2001), (Partridge et al., 2018). MGEs promote intracellular DNA mobility which support the acquisition and dissemination of resistance genes (Kung et al., 2010a). Transposons and insertion sequences are detached DNA segments which can move freely to different locations of a cell's DNA (Al-Nayyef et al., 2015). Integrons carry genes to specific sites, plasmids are involved in exchange genes by conjugation, and bacteriophages can add additional gene by transduction. Additionally, extracellular DNA segments can also be up taken by the bacterial genomes directly. All these interactions of MGEs underpin the process of evolution and aiding the development of multi-drug resistant strains (Partridge et al., 2018).

In *P. aeruginosa,* one or more MGEs can be involved in the resistance of *P. aeruginosa* isolated from different infections (Labuschagne et al., 2008, Boonkerd et al., 2009, Subedi et

al., 2018c). Integrons in *P. aeruginosa* obtain gene cassettes and convert them into functional genes by site specific recombination. Usually, integrons carry multiple resistance genes (Belotti et al., 2015, Subedi et al., 2018c). There are three main classes of integrons class 1,2, and 3 (Mazel et al., 1998, Mazel, 2004). Class 1 and 2 have been found in *P .aeruginosa* carrying resistance genes from different infections, including keratitis (Subedi et al., 2018c). Involvement of transposons in the dissemination of resistance gene has also been reported in *P. aeruginosa* keratitis isolates (Subedi et al., 2018c) and from other infections (Abril et al., 2019, Chopra et al., 2003). Insertion sequences have been associated with antibiotic resistance in *P. aeruginosa* (Evans and Segal, 2007, Fowler and Hanson, 2014). Similarly, plasmids carrying multiple antibiotic resistance genes either alone or in association with transposons and integrons have been reported (Botelho et al., 2017). Plasmids contribute towards bacterial fitness by encoding functional genes for virulence, resistance, metabolism and other functions (Frost et al., 2005).

1.3.5.1 Acquired genes responsible for resistance to specific antibiotics:

1.3.5.1.1 Beta-lactam resistance genes

Other than inducible beta-lactamases located on its chromosome, some *P. aeruginosa* strains acquire integron related genes which can hydrolyse beta-lactams (Poirel et al., 2001a) and plasmids encoding new beta-lactamases, that confer resistance to penicillins and cephalosporins (Livermore, 1995a). The extended spectrum beta-lactamases (confer resistance to most beta-lactam antibiotics) carried on plasmids are of great concern, along with metallo beta-lactamases which inactivate cephalosporins (Bush, 2010). The PER-1 beta-lactamase was the first extended spectrum beta-lactamase identified in *P. aeruginosa* from a Turkish patient hospitalised in Paris, France in 1991 (Vahaboglu et al., 1997). Six types of *P. aeruginosa* metallo beta-lactamases (MBLs), which belong to class B beta-lactamases that hydrolyze most beta-lactam-based antibiotics, have been described. These include imipenemase (IMP), Verona integron-encoded metallo beta-lactamase (VIM), Sao Paulo

metallo beta-lactamase (SPM), Germany imipenemase (GIM), New Delhi metallo beta-lactamase (NDM) and Florence imipenemase (FIM) (Hong et al., 2015). These genes, which can be carried by genetic elements such as integrons and plasmids (Yan et al., 2006, Pang et al., 2019), and have been identified in *P. aeruginosa* from various infections and countries (Nordmann and Naas, 1994, Yu et al., 2006).

1.3.5.1.2 Aminoglycoside resistance genes

The enzymatic modification of aminoglycosides by aminoglycoside-acetyltransferases (AAC), aminoglycoside-adenyl transferases (AAD) and aminoglycoside-phosphotransferases (APH) can confer resistance to aminoglycosides in *P. aeruginosa.* These enzymes can be coded on mobile genetic elements such as integrons, transposons, plasmids, genomic islands, and other genetic structures (Ramirez et al., 2013, Teixeira et al., 2016). The associated modifications to the aminoglycosides can lead to reduced affinity for the 30S bacterial ribosomal subunit, the main target of aminoglycosides (Vakulenko and Mobashery, 2003). Two aminoglycoside resistance genes in *P. aeruginosa* clinical isolates, *aacA29a* and *aacA29b*, can be located at the 5′ and 3′ end of the carbapenem-hydrolyzing beta-lactamase VIM-2 gene cassette in a class I integron (Poirel et al., 2001a). Other acquired aminoglycoside resistance genes encode methylases, and the first 16SrRNA methylase was discovered in a Japanese *P. aeruginosa* clinical isolate carrying *rmtA* in 2003 (Yokoyama et al., 2003) and another was identified in South Korea in 2009 (Jin et al., 2009). The other variants of *rmt, rmtD* (Tada et al., 2018) and *rmtB* (Ge et al., 2011), also mediate resistance to aminoglycosides.

1.3.5.1.3 Fluoroquinolone resistance genes

In *P. aeruginosa,* resistance to fluoroquinolones has been commonly associated with chromosomal mutations in genes encoding topoisomerase IV or gyrase (Nouri et al., 2016) or mutations in the regulation of efflux pumps (Nikaido, 1998, Srikumar et al., 1999). However, during the past decade, acquired resistance genes for fluoroquinolones such as *crpP* (Chávez-

Jacobo et al., 2018, Chávez-Jacobo et al., 2019) and *qnrVC1* (Kocsis et al., 2019) genes have been identified in *P. aeruginosa*. *CrpP* can be carried on the plasmid pUC-*crpP* and confers resistance to ciprofloxacin in *Escherichia coli* (Chávez-Jacobo et al., 2019). In *P. aeruginosa*, *crpP* is associated with resistant to ciprofloxacin and low level resistance to norfloxacin. In *P. aeruginosa*, *crpP* can be carried on the pUM505 plasmid (Chávez-Jacobo et al., 2018). Another acquired fluoroquinolone resistance gene of *P. aeruginosa* is *qnrVC1,* which can be carried on an integron (Kocsis et al., 2019). However, none of these fluoroquinolone resistance determinants have been reported in ocular *P. aeruginosa* isolates.

1.3.5.1.4 Polymyxin resistance genes

Resistance to polymyxins through the chromosomal mutations was believed to be the only mechanism in almost all the bacteria prior to 2016. Since then, there has been the emergence of plasmid-mediated resistance via the *mcr-1* gene in the *E. coli* strain SHP45 (Liu et al., 2016). Later, another plasmid-mediated gene *mcr-2* was found in *E.coli,* and the prevalence of *mcr-2* has been reported to be higher than *mcr-1* (Xavier et al., 2016). Mcr-2-mediated resistance to polymyxins has also been reported internationally (Skov and Monnet, 2016, Perreten et al., 2016, Bernasconi et al., 2016, Fernandes et al., 2016b). The major epidemiological concern is the potential for *mcr* genes to spread into health care-associated extensive drug-resistant (XDR) pathogens, including *P. aeruginosa*, which would lead to truly untreatable infections. Recently, *mcr-1* has been reported in *P. aeruginosa* from Pakistan (Hameed et al., 2019).

Table 1-4. Acquired genes found in *P. aeruginosa.*

Gene	Variant	Source and infection type	Year	Country	Reference
Class A extended spectrum beta-lactam	PER-1	Urinary tract infection	1993	France	(Nordmann et al., 1993)
		Intensive care units	2005	Turkey	(Kolayli et al., 2005)
		Intensive care units	2000	Belgium	(Claeys et al., 2000)
		Intensive care units	2001	Italy	(Luzzaro et al., 2001)
		Multiple hospital infections	2007	Poland	(Empel et al., 2007)
		Hospital	2008	Hungry	(Libisch et al., 2008a)
		Hospital	2008	Serbia	(Libisch et al., 2008a)
		Hospital	2011	Tunisia	(Ktari et al., 2011)

Gene	Variant	Source and infection type	Year	Country	Reference
Class A extended spectrum beta-lactam	PER-1	Hospital	2006	Japan	(Yamano et al., 2006)
		Hospital	2014	China	(Qing et al., 2014)
		Not specified	2012	Greece	(Ranellou et al., 2011)
		Burn patients	2010	Iran	(Mirsalehian et al., 2010)
	PER-2	Hospital	2006	Bolinvia	(Celenza et al., 2006)
	VEB-3	Clinics	2006	China	(Jiang et al., 2006)
	GES-1	Wound infection	2004	Brazil	(Castanheira et al., 2004)
		Respiratory tract infection	2002	France	(Chopra et al., 2003)
		Hospital	2005	Argentina	(Pasteran et al., 2005)
	GES-8 (IBC-2)	Urinary tract infection	2001	Greece	(Mavroidi et al., 2001)
	GES-9	Rectal swab	2009	France	(Poirel et al., 2005a)

Gene	Variant	Source and infection type	Year	Country	Reference
Class A extended spectrum beta-lactam	GES-13	Respiratory tract infection	2010	Greece	(Kotsakis et al., 2010)
	SHV-2a	Respiratory tract infection	1999	France	(Naas et al., 1999)
		Hospital	2009	Tunisia	(Mansour et al., 2009a)
	SHV-5	Hospital	2004	Greece	(Poirel et al., 2004)
	SHV-12	Hospital	2001	Thailand	(Chanawong et al., 2001)
		Burn patient	2010	Japan	(Uemura et al., 2010)
	TEM-4	Hospital	1999	France	(Poirel et al., 1999)
	TEM-21	Hospital	2002	France	(Chopra et al., 2003)

Gene	Variant	Source and infection type	Year	Country	Reference
Class A extended spectrum beta-lactam	TEM-24	Hospital	2000	France	(Marchandin et al., 2000)
	TEM-42	Hospital	1996	France	(Mugnier et al., 1996)
	CTX-M-1	Cystic fibrosis	2006	Netherlands	(al Naiemi et al., 2006)
	CTX-M-2	Pneumonia	2009	Brazil	(Picão et al., 2009b)
		Hospital	2006	Bolivia	(Celenza et al., 2006)
	CTX-M-3	Hospital	2014	China	(Qing et al., 2014)
	CTX-M-14	Hospital	2014	China	(Qing et al., 2014)
	CTX-M-15	Hospital	2014	China	(Qing et al., 2014)
	CTX-M-43	Hospital	2006	Bolivia	(Celenza et al., 2006)
	BEL-1	Hospitals	2005	Belgium	(Poirel et al., 2005b)
	BEL-2	Urinary tract infection	2010	Belgium	(Poirel et al., 2010a)

Gene	Variant	Source and infection type	Year	Country	Reference
Class A extended spectrum beta-lactam	BEL-3	Hospital	2010	Spain	(Juan et al., 2010)
	PME-1	Hospital	2011	USA	(Tian et al., 2011)
Class B metallo beta-lactamases	IMP-1	Hospital	2009	Japan	(Zhao et al., 2009)
	IMP-1	Hospital	2003	South Korea	(Lee et al., 2003)
		Hospital	2007	Brazil	(Martins et al., 2007)
		Hospital	2009	China	(Qu et al., 2009)
		Hospital	2007	Turkey	(Ozgumus et al., 2007)
		Hospital	2004	Singapore	(Koh et al., 2004)
		Hospital	2009	Thailand	(Boonkerd et al., 2009)
		Hospital	2007	Japan	(Ohara et al., 2007)
	IMP-2	Hospital	2007	Japan	(Ohara et al., 2007)
	IMP-4	Hospital	2011	Malaysia	(Khosravi et al., 2011)

Gene	Variant	Source and infection type	Year	Country	Reference
Class B metallo beta-lactamases	IMP-4	Hospital	2005	Australia	(Peleg et al., 2005)
	IMP-5	Hospital	2006	Portugal	(Brízio et al., 2006)
	IMP-6	Hospital	2012	South Korea	(Yoo et al., 2012)
		Hospital	2014	China	(Chen et al., 2014)
	IMP-7	Hospital	2002	Canada	(Gibb et al., 2002)
		Peritonitis	2002	Malaysia	(Ho et al., 2002)
		Hospital	2007	Slovakia	(Ohlasova et al., 2007)
		Pneumonia	2007	Japan	(Kouda et al., 2007)
		Hospital	2004	Singapore	(Koh et al., 2004)
		Sepsis	2009	Czech Republic	(Hrabák et al., 2009)
		Hospital	2015	Denmark	(Hammerum et al., 2015)

Gene	Variant	Source and infection type	Year	Country	Reference
Class B metallo beta-lactamases	IMP-9	Hospital	2006	China	(Xiong et al., 2006)
	IMP-10	Urine and stool	2002	Japan	(Iyobe et al., 2002)
	IMP-11	Not available	Not available	Japan	Accession no. **AB074437**
	IMP-13	Hospital	2005	Italy	(Pagani et al., 2005)
		Hospital	2009	Austria	(Duljasz et al., 2009)
		Hospital	2012	France	(Fournier et al., 2012)
		Hospital	2011	Belgium	(Naas et al., 2011)
	IMP-14	Hospital	2012	Thailand	(Piyakul et al., 2012)
	IMP-15	Respiratory infection	2010	Mexico	(Quinones-Falconi et al., 2010)
		Hospital	2013	Spain	(Gilarranz et al., 2013)
		Hospital	2014	Germany	(Castanheira et al., 2014)
	IMP-16	Hospital	2004	Brazil	(Mendes et al., 2004)

Gene	Variant	Source and infection type	Year	Country	Reference
Class B metallo beta-lactamases	IMP-18	Tracheal aspirant of accident victim	2006	USA	(Hanson et al., 2006)
		Hospital	2008	Mexico	(Garza-Ramos et al., 2008)
		Hospital	2009	Puerto Rico	(Wolter et al., 2009a)
	IMP-19	Not available	Not available	Japan	Accession no. **AB184876**
		Urinary tract infection	2013	Italy	(Pollini et al., 2013)
	IMP-20	Not available	Not available	Japan	Accession no. **AB196988**
	IMP-21	Not available	Not available	Japan	Accession no. **AB204557**
	IMP-22	Hospital	2009	Austria	(Duljasz et al., 2009)
		Hospital	2009	Italy	(Pellegrini et al., 2009)
	IMP-25	Not available	Not available	China	Accession no. **EU352796**

Gene	Variant	Source and infection type	Year	Country	Reference
Class B metallo beta-lactamases	IMP-26	Hospital	2013	Malaysia	(Kim et al., 2013)
			2010	Singapore	(Koh et al., 2010)
	IMP-29	Intensive care unit patient	2012	France	(Jeannot et al., 2012)
	IMP-30	Hospital	2013	Russia	(Pegg et al., 2013)
	IMP-31	Not available	Not available	Germany	Accession no. **KF148593**
	IMP-33	Hospital	2013	Italy	(Deshpande et al., 2013)
	IMP-35	Hospital	2013	Germany	(Pournaras et al., 2013)
	IMP-37	Not available	Not available	France	Accession no. **JX131372**
	IMP-40	Not available	Not available	Japan	Accession no. **AB753457**
	IMP-41	Not available	Not available	Japan	Accession no. **AB753458**
	IMP-43	Hospital	2013	Japan	(Tada et al., 2013)
	IMP-44	Hospital	2013	Japan	(Tada et al., 2013)

Gene	Variant	Source and infection type	Year	Country	Reference
Class B metallo beta-lactamases	IMP-45	Canine origin	2014	China	(Wang et al., 2014)
	IMP-48	Not available	Not available	USA	Accession no. **KM087857**
		Hospital	1999	Italy	(Lauretti et al., 1999)
		Hospital	2006	France	(Corvec et al., 2006)
		Hospita	2000	Greece	(Tsakris et al., 2000)
		Hospital	2014	Germany	(Castanheira et al., 2014)
		Hospital	2013	Italy	(Pollini et al., 2013)
	VIM-2	Hospital	2009	Tunisia	(Mansour et al., 2009b)
		Hospital	2012	Thailand	(Piyakul et al., 2012)
		Hospital	2009	Austria	(Duljasz et al., 2009)
		Respiratory infection	2010	Mexico	(Quinones-Falconi et al., 2010)

Gene	Variant	Source and infection type	Year	Country	Reference
Class B metallo beta-lactamases	VIM-2	Hospital	2012	Thailand	(Piyakul et al., 2012)
		Hospital	2009	India	(Castanheira et al., 2009)
		Hospital	2008	Kenya	(Pitout et al., 2008)
		Hospital	2008	Hungry	(Libisch et al., 2008b)
		Hospital	2010	Malaysia	(Khosravi et al., 2010)
		Hospital	2005	South Korea	(Walsh et al., 2005)
		Hospital	2005	Japan	(Walsh et al., 2005)
		Hospital	2005	France	(Walsh et al., 2005)
		Hospital	2005	Greece	(Walsh et al., 2005)
		Hospital	2005	Italy	(Walsh et al., 2005)

Gene	Variant	Source and infection type	Year	Country	Reference
Class B metallo beta-lactamases	VIM-2	Hospital	2005	Portugal	(Walsh et al., 2005)
		Hospital	2005	Spain	(Walsh et al., 2005)
		Hospital	2005	Croatia	(Walsh et al., 2005)
		Hospital	2005	Poland	(Walsh et al., 2005)
		Hospital	2005	Chile	(Walsh et al., 2005)
		Hospital	2005	Venezuela	(Walsh et al., 2005)
		Hospital	2005	Argentina	(Walsh et al., 2005)
		Hospital	2005	USA	(Walsh et al., 2005)
		Hospital	2005	Belgium	(Walsh et al., 2005)
		Hospital	2005	Germany	(Walsh et al., 2005)

Gene	Variant	Source and infection type	Year	Country	Reference
Class B metallo beta-lactamases	VIM-2	Hospital	2005	Turkey	(Walsh et al., 2005)
	VIM-3	Hospital	2001	Taiwan	(Yan et al., 2001)
	VIM-4	Hospital	2005	Greece	(Walsh et al., 2005)
		Hospital	2005	Sweden	(Walsh et al., 2005)
		Hospital	2005	Poland	(Walsh et al., 2005)
		Hospital	2004, 2008	Hungary	(Libisch et al., 2008b (Libisch, 2004 #1188))
		Hospital	2014	France	(Castanheira et al., 2014)
Class D beta-lactamases	OXA-2	Hospital	1997	Turkey	(Danel et al., 1997)
	OXA-10	Burn infection	1993	Turkey	(Hall et al., 1993)
	OXA-1 or OXA-31	Hospital	2001	France	(Aubert et al., 2001)
	OXA-56 or OXA-128	Hospital	2010	China	(Liu et al., 2010)

Gene	Variant	Source and infection type	Year	Country	Reference
Class D beta-lactamases	OXA-18	Hospital	1997	France	(Philippon et al., 1997)
	OXA-45	Hospital	2003	North America	(Toleman et al., 2003)
	OXA-40	Hospital	2009	Spain	(Sevillano et al., 2009)
	OXA-198	Ventilator associated pneumonia	2011	Belgium	(El Garch et al., 2011)
Carbapenemases	GES-2	Nosocomial pneumonia	2001	South Africa	(Poirel et al., 2001b)
	GES-5	Burn and wound infection	2006	China	(Wang et al., 2006)
		Hospitals	2008	South Africa	(Labuschagne et al., 2008)
		Blood stream infection	2009	Brazil	(Picão et al., 2009a)
		Hospital	2014	Turkey	(Iraz et al., 2014)
	GES-18	Respiratory tract infection	2013	Belgium	(Bebrone et al., 2013)

Gene	Variant	Source and infection type	Year	Country	Reference
Carbapenemases	KPC-2	Hospital	2011	Columbia	(Cuzon et al., 2011)
		Hospital	2009	Puerto Rico	(Wolter et al., 2009a)
		Hospital	2009	Trinidad and Tobago	(Akpaka et al., 2009)
		Hospital	2010	USA	(Poirel et al., 2010b)
		Intensive care unit	2011	China	(Ge et al., 2011)
		Hospital	2013	Argentina	(Ramírez et al., 2013)
		Intensive care unit	2012	Brazil	(Jácome et al., 2012)
	KPC-5	Hospital	2009	Puerto Rico	(Wolter et al., 2009b)

1.3.6 Adaptive resistance

Adaptive resistance is a type of resistance that bacteria develop to survive in stressed environments, such as in the presence of antibiotics, which results in alterations in gene or protein expression. This alteration is usually temporary and can be reversed upon removal of the stimulus (Sandoval-Motta and Aldana, 2016). *P. aeruginosa* may alter its normal growth and function to limit the expression of targets for antibiotics. Once the antibiotic concentration is lowered the organisms will resume its normal growth patterns (Lewis, 2010). In *P. aeruginosa,* the major adaptive resistance mechanisms are biofilm formation (Costerton et al., 1999) or production of drug persister cells (or drug tolerant cells) (Balaban et al., 2013).

In *P. aeruginosa*, adaptive resistance to polycationic antibiotics occurs after pre-exposure. The polycationic antibiotics pass through the outer membrane via self-promoted uptake (Hancock and Bell, 1988) and displace divalent cations from LPS (Hancock, 1998). This results in disruption of the membrane and cell death. Upon pre-exposure to the antibiotics, the operon *arnBCADTEF* is activated and this mediates the addition of 4-aminoarabinose to the Lipid A of LPS (Olaitan et al., 2014). This blocks self-promoted uptake leading to resistance (Hancock, 1998). Induction of beta-lactamases is another adaptive resistance mechanism in *P. aeruginosa* that results in the inactivation of beta-lactam antibiotics (Livermore, 1995a). Overexpression of genes encoding efflux pumps can occur during exposure to sub-inhibitory concentrations of antibiotics. For example, exposure to aminoglycosides can induce the MexXY efflux pump (Hocquet et al., 2003, Breidenstein et al., 2011), which results in the antibiotic being rapidly removed from cells.

Environmental or physiological stimuli can also be responsible for the development of antibiotic tolerant cells. In biofilms, quorum sensing occurs in cells, which modifies production of many genes including those involved in upregulation of efflux pumps, enzymes, and gene regulators (Rutherford and Bassler, 2012). Biofilms slow the penetration of antibiotics into and slow access to nutrients. This can lead to different metabolic activity, whereby cells in the outer biofilm layer are metabolically active and cells in the inner part of the biofilm grow more slowly

(Breidenstein et al., 2011). These slow growing cells can be relatively resistant to many antibiotics (Lewis, 2010). Like biofilms, swarming cells of *P. aeruginosa* exhibit adaptive resistance to polymyxin B, gentamicin and ciprofloxacin (Lai et al., 2009, Overhage et al., 2008). Swarming is a form of motility on viscous or semi-solid media which also results in the dysregulation of many genes and greater resistance to antibiotics (Overhage et al., 2008).

1.3.7 Type III secretion system and resistance of *P. aeruginosa* isolates

P. aeruginosa uses a broad range of virulence determinants to initiate an infection. One of those virulence determinants is the type III secretion system (T3SS) which helps to escape the phagocytic activity in the host (Ghosh, 2004). Four effector proteins are secreted by the *P. aeruginosa* type III secretion system; ExoS, ExoT, ExoU and ExoY (Frank, 1997). Not all strains of *P. aeruginosa* possess all the effector proteins (Feltman et al., 2001, Fleiszig et al., 1997). Usually *exoS* (which contributes to the invasive phenotype of strains) and *exoU* (which contributes to the cytotoxic phenotype of strains) are mutually exclusive (Hauser, 2009). ExoS and ExoT inhibit phagocytosis by disrupting actin cytoskeletal rearrangement, focal adhesins and signal transduction cascades important for phagocytic function (Barbieri and Sun, 2004). ExoU and ExoY are cytotoxins with phospholipase and adenylate cyclase activities respectively (Yahr et al., 1995). Presence of *exoU* has been associated with antibiotic resistance (Subedi et al., 2018a), often with MDR phenotype (Horna et al., 2019, Yousefi-Avarvand et al., 2015) and worst clinical outcomes in the keratitis *P. aeruginosa* isolates (Borkar et al., 2013) and other infections (Sullivan et al., 2014). Previously, presence of *exoU* was corelated with an increased resistance to fluoroquinolones (Sullivan et al., 2014, Wong-Beringer et al., 2008, Shen et al., 2015) but recently resistance to beta-lactams was also reported in the *exoU P. aeruginosa* isolates (Subedi et al., 2018a). The frequency of resistance in the *exoU* isolates is more compared to *exoS* isolates (Garey et al., 2008, Cho et al., 2014) due to an un-defined mechanism .

1.3.8 Rationale for research

While there are various epidemiological studies reporting resistance of *P. aeruginosa* keratitis isolates. The genetic basis of this resistance has not often been investigated.

1.4 Thesis aims and objectives

The overall aim of this thesis was to study the resistance mechanisms in ocular isolates of *P. aeruginosa* which was divided into the following aims:

I. To compare antibiotic susceptibilities of Australian and Indian P*. aeruginosa* keratitis isolates.

II. To explore multipurpose disinfecting solution sensitivity in the Australian (CL related) isolates and compare this to their antibiotic sensitivity.

III. To analyse the genomic differences among *P. aeruginosa* isolates from Australian and Indian keratitis and examine changes over time.

IV. To investigate the genomic differences in clonal isolates of *P. aeruginosa* recovered over time.

2 Susceptibility of Contact Lens-Related *Pseudomonas aeruginosa* Keratitis Isolates from Australia to Multipurpose Disinfecting Solutions, Disinfectants, and Antibiotics

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I certify that all co-authors of this work have agreed to submission of this work as a part of this thesis.

tvst

Article

Susceptibility of Contact Lens-Related *Pseudomonas aeruginosa* Keratitis Isolates to Multipurpose Disinfecting Solutions, Disinfectants, and Antibiotics

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Purpose: This study analyzed the susceptibilities of 17 contact lens (CL)-related keratitis isolates of *Pseudomonas aeruginosa* from Australia to antibiotics, multipurpose contact lens disinfecting solutions (MPDS), and disinfectants through minimum inhibitory (MIC) and minimum bactericidal concentrations.

Methods: Antibiotics included ciprofloxacin, levofloxacin, gentamicin, tobramycin, piperacillin, imipenem, ceftazidime, and polymyxin B. The MPDS OPTI-FREE PureMoist, Complete RevitaLens OcuTec, Biotrue, and Renu Advanced Formula and the constituent disinfectants; alexidine dihydrochloride, polyquaternium-1, polyaminopropyl biguanide, and myristamidopropyl dimethylamine (Aldox) were analyzed. The combined susceptibility of disinfectants based on the MPDS formulation was assessed through fractional inhibitory concentration.

Results: All isolates were susceptible to levofloxacin and gentamicin, 2/17 were resistant to ciprofloxacin; 1/17 was resistant to tobramycin, piperacillin, and polymyxin; and 3/17 were resistant to ceftazidime whereas 12/17 were resistant to imipenem. Of the four MPDSs, for Renu Advanced Formula 8/17 strains have an MIC $\leq$ 11.36 for OPTI-FREE PureMoist 14/17 strains have an MIC $\leq$ 11.36% for Complete RevitaLens 9/17 strains have an MIC $\leq$ 11.36, and for Biotrue 5/17 strains have MIC = 11.36. All strains were killed by 100% MPDS. At the concentrations used in the MPDSs, individual disinfectants were not active. From three tested isolates, no synergy was found in dual combinations of disinfectants. However, synergy was found for triple combination of disinfectants for three tested strains.

Conclusions: Australian CL-related isolates of *P aeruginosa* were susceptible to most antibiotics. There was variability in susceptibility to different MPDS. Individual disinfectant excipients had limited activity. The combination of the disinfectants showed synergy, antagonism, and no interaction.

Translational Relevance: This study will help to choose better preventive and treatment measures for microbial keratitis.

Introduction

Contact lenses have been used for decades for refractive, cosmetic, and therapeutic purposes. Although contact lenses have optical and vocational benefits, they are associated with certain complications. Corneal infection is rare but is the most severe complication of contact lens wear, occurring in around 4 per 10,000 wearers per year,[1] and can cause visual loss in 10% to 15% cases.[1] *Pseudomonas aeruginosa* is the most commonly isolated bacterium from contact lens-related microbial keratitis.[2] This may be due to its strong adhesion to contact lenses and contact lens cases compared with other microorganisms.[3] *P aeruginosa* can also develop biofilms on these surfaces,[4] which facilitates persistence of the organism.[5]

Contact lens multipurpose disinfecting solutions (MPDS) are used to minimize the numbers of bacteria on lenses for the safe use of daily wear contact

2.1 Introduction

Contact lenses have been used for decades for refractive, cosmetic and therapeutic purposes. Whilst contact lenses have optical and vocational benefits, they are associated with certain complications. Corneal infection is rare but is the most severe complication of contact lens wear, occurring in around 4 per 10000 wearers per year (Stapleton et al., 2008a) and it can cause visual loss in 10-15% cases (Stapleton et al., 2008a). *Pseudomonas aeruginosa* is the most commonly isolated bacterium from contact lens-related microbial keratitis (Cheng et al., 1999, Green et al., 2008, Galentine et al., 1984, Schein et al., 1989). This may be due to its ability to adhere to contact lenses and contact lens cases compared to other microorganisms (Henriques et al., 2005). *P. aeruginosa* can also develop biofilms on these surfaces (Szczotka-Flynn et al., 2009) which facilitates persistence of the organism (Vijay et al., 2015).

Contact lens multipurpose disinfecting solutions (MPDS) are used to minimize the numbers of bacteria on lenses for the safe use of daily wear contact lenses. Daily wear is the most common wear schedule for contact lens wearers in many countries (Efron et al., 2013). However, there are reports that bacteria can become resistant to these disinfectants (Russell, 1999) which raises concerns about the effectiveness of these solutions. Resistance to disinfecting solutions may be due to inherent resistance associated with the cytotoxic phenotype of *P. aeruginosa* (Lakkis and Fleiszig, 2001), the surface charge of the bacterial cell (Bruinsma et al., 2006), or expression of outer membrane proteins such as OprR (Tabata et al., 2003). Harbouring *qac* genes (Subedi et al., 2018d) may confer resistance to disinfectants, and although this has been shown with ocular isolates of *Staphylococcus aureus* (Shi et al., 2016), this has not been seen in the limited number of *P. aeruginosa* strains evaluated . *Qac* genes can occur on class 1 integrons along with genes for antibiotic resistance, and this raises concern of cotransfer of these genes amongst bacterial populations. Resistance may not only be associated with the possession of *qac* genes, but also with genes conferring virulence traits such as *exoU* and *exoS* (Borkar et al., 2014).

Various antibiotics are used for the treatment of microbial keratitis, but emerging resistance to the antibiotics (Livermore, 2002) from the possession of inherent and acquired resistance mechanisms is increasing (Moshirfar et al., 2006). Emerging resistance of ocular isolates of *P aeruginosa* has been reported internationally (Subedi et al., 2018d) with variation in their resistance profile to antimicrobials (Kowalski et al., 2001). Inherent resistance mechanisms include low membrane permeability, expression of efflux pumps, production of antibiotic-inactivating enzymes, and mutation of resistance genes (Hancock and Speert, 2000). Acquired genes confer resistance while inserted into mobile genetic elements such as integron and transposons (Subedi et al., 2018c), which can then migrate around bacterial populations. The severity of infections caused by *P aeruginosa* and its ability to acquire resistance and virulence genes, which give it the potential to resist almost all antibiotic classes, increases the concern about *P aeruginosa* infections (Hancock and Speert, 2000). In the management of corneal infection, despite topical administration of antibiotics resulting in high tissue concentrations, poor clinical outcomes may occur partly from antibiotic resistance (Green et al., 2007, Tummanapalli and Willcox, 2020). The increase in resistance to prescribed antibiotic leads to more severe disease resulting in slow resolution or vision loss (Tummanapalli and Willcox, 2020, Borkar et al., 2014). The consequences of keratitis caused by multiple-drug resistant *P aeruginosa* can be severe and vision threatening given the limited choice of effective antimicrobials (Aloush et al., 2006).

There is limited information available on the antimicrobial and disinfectant susceptibility patterns of clinical ocular isolates of *P. aeruginosa* in Australia. Earlier studies have often used standard strains (Mohammadinia et al., 2012) or only limited numbers of clinical isolates (Subedi et al., 2018d). There is limited information about current trends of antibiotic susceptibilities of Australian ocular *P. aeruginosa* isolates.

Therefore, the aims of this study were:

- To Investigate the susceptibility of MPDS and disinfectants components present in the MPDS formulation.
- To analyse synergistic association between disinfectants.

- To compare antibiotic and MPDS susceptibility to historical and recent Australian *P. aeruginosa* isolates collected at different times.

2.2 Materials and methods

2.2.1 *Pseudomonas aeruginosa* isolates

Strains of *Pseudomonas aeruginosa* isolated from contact lens-related microbial keratitis (either from corneal scrapes or contact lenses) from Queensland and Sydney, Australia between the years 2001 to 2006 and 2018 to 2019 were retrieved from the culture collection of the School of Optometry and Vision Science, UNSW Sydney, Australia (Table 2-1). All the isolates were collected from different patients. The strains were stored at -80^{o}C and revived on nutrient agar (Oxoid Ltd., Basingstoke, Hampshire, UK). Isolates were then inoculated into Mueller-Hinton broth (MHB; Oxoid Ltd.) and grown at 37°C for 18 to 24 hours. The optical density of the bacterial suspension was adjusted 0.1 (1× 10^{8} CFU/ml) at 660nm using a spectrophotometer (FLUOstar Omega, BMG LABTECH, Germany) (Watanabe et al., 2014).

Table 2-1 Strains of *Pseudomonas aeruginosa* recovered from microbial keratitis.

***P. aeruginosa* Isolates**	**Source**	**Year of isolation**
115	Cornea	2004
116	Cornea	2004
121	Contact lens	2005
123	Cornea	2005
124	Cornea	2005
126	Cornea	2005
127	Cornea	2005
129	Cornea	2005
155	Cornea	2006
162	Cornea	2006
165	Cornea	2001
169	Cornea	2006
174	Cornea	2006
176	Contact lens	2004
179	Cornea	2006
181	Cornea	2006
182	Cornea	2004
223	Cornea	2018
224	Cornea	2018
225	Cornea	2018
226	Cornea	2018
227	Cornea	2018
228	Cornea	2018
229	Cornea	2018

P. aeruginosa Isolates	Source	Year of isolation
230	Cornea	2019
233	Cornea	2019
235	Cornea	2019

Strains isolated in the years 2001-2006 (historical isolates) and coloured in light blue and those isolated in the years 2018-2019 (recent isolates) and coloured in dark blue.

2.2.2 Susceptibility to multipurpose disinfecting solutions

Susceptibility of the bacterial strains to four commercially available MPDS; OPTI-FREE PureMoist (Alcon, Fort Worth, TX, USA), Complete RevitaLens OcuTec (Abbot Medical Optics Hangzhou ZJ, China), Biotrue and Renu Advanced Formula (Bausch + Lomb, Rochester, NY, USA) (Table 2-2) was measured using a previously described method (Watanabe et al., 2014). In brief, each MPDS was serially diluted in phosphate buffer saline (PBS; NaCl 80gm/L, Na_2HPO_4 11.5gm/L, KCl 2gm/L & KH_2PO_4 2gm/L, pH=7.2) to obtain final concentrations of 90.9%, 45.5%, 22.7%, 11.4%, 5.7% and 3.1%. The serially diluted MPDS (200 µl) was added to wells of a microtiter plate and 20µl of bacterial suspension was added to achieve a final concentration of 1×10^5 CFU/ml. The plates were incubated for 18 - 24 hours at 37°C. Growth turbidity was measured using a spectrophotometer (FLUOstar Omega, BMG LABTECH, Germany) at 660nm to obtain the minimum inhibitory concentration (MIC). Minimum inhibitory concentration was taken as the dilution of MPDS with no visible growth. To measure the minimum bactericidal concentration (MBC), viable counts were performed (on nutrient agar plates incubated at 37°C for 18 - 24 hours) from wells at the MIC and the two next lower dilutions of MPDS. The MBC was the concentration of MPDS that gave 99.99% (3 log units) bacterial killing (Kirven and Thornsberry, 1978) (Taylor et al., 1983).

For MPDS, strains with MIC greater than 10% were categorized as resistant. The 10% cut-off for MPDS is arbitrary and it cannot be used as a standard for reference for any other study. For disinfectants, those strains having MIC above what was present in the respective MPDS were considered resistant (if the disinfectant was present in more than one MPDS at different concentrations, its mean concentration was taken for this analysis). These experiments

comparing susceptibility to MPDS, disinfecting components and antibiotics were conducted for the larger group of historical isolates only to avoid influence of isolation time.

Table 2-2 Multipurpose disinfecting solutions.

MPDS	Manufacturer	Disinfectants	Surfactants	Other Ingredients
Opti-Free PureMoist	Alcon, Fort Worth, TX, USA	Polyquaternium-1 10ppm, Aldox 6ppm	Tectronic 1304, polyoxyethylene-polyoxybutylene copolymer	Sodium citrate, sodium chloride, boric acid, aminomethyl-propanol, sorbitol, ethylenediaminetriacetic acid
Complete RevitaLens OcuTec	Abbot Medical Optics, Hangzhou ZJ, China	Alexidine dihydrochloride 1.6ppm, polyquaternium-1 3ppm	Tetronic 904	Sodium citrate, sodium chloride, boric acid, sodium borate decahydrate, ethylenediaminetriacetic acid
Biotrue	Bausch +Lomb, Rochester, NY, USA	PAPB (PHMB) 1.3ppm, polyquaternium-1 1ppm	Poloxamine, sulfobetaine	Sodium chloride sodium borate, boric acid, ethylenediaminetriacetic acid, hyaluronan
Renu Advanced		PAPB (PHMB) 0.5ppm, polyquaternium-1 1.5ppm, alexidine 2ppm	Poloxamine, poloxamer 181	Sodium chloride, boric acid, sodium borate, ethylenediaminetriacetic acid, diglycine

Aldox = myristamidopropyl dimethylamine; PAPB = polyaminopropyl biguanide and is synonymous with PHMB = polyhexamethylene biguanide

2.2.3 Inhibition of *P. aeruginosa* by disinfectants

Polyaminopropyl biguanide (PAPB; Novachem Pty Ltd Heidelberg West, VIC, Australia), polyquaternium-1 (Toronto Research Chemicals Inc. Toronto, ON, CA), myristamidopropyl dimethylamine (Aldox; Toronto Research Chemicals Inc. Toronto, ON, CA) and alexidine dihydrochloride (Cayman Chemicals Ann Arbor, USA) were used. Inhibition of only a selection of isolates by disinfectants was analysed. Disinfectants were prepared as 10X stock solutions in phosphate-buffered saline. Dilutions ranging between 1% to 0.00004% were used such that the concentration of the disinfectants present in the MPDSs were in the range tested. Two hundred microlitres of disinfectant and 20 µl of bacterial cells (final concentration of 1×10^5 CFU/ml) were incubated in 96 well microtiter plates for 18 - 24 hours at 37 °C to determine the

MIC (as described previously in 2.2.2). Viable plate count was performed as described in section 2.2.2 to elucidate the MBC of each disinfectant.

2.2.4 Fractional inhibitory concentration of components of MPDS

Three isolates (123, 127 and 155) were selected because of the variation in their MICs to different disinfectants (higher and lower MICs for different disinfectants). The interactions between disinfectants were analysed through a modified checkerboard method (White et al., 1996). The dual combinations tested were selected from those used in the composition of each MPDS (Table 2). The triple combination of polyquaternium-1, polyhexamethylene biguanide (PHMB) and alexidine (present in Renu Advanced) was tested with a small modification to the checkerboard assay (Stein et al., 2015). Briefly, the three disinfectants were diluted in three different directions in order of increasing concentration in the 96-well microtiter plate, such that, the three disinfectants were combined in different concentrations in the wells. In the 3-dimensional assay, 11 dilution steps of disinfectant A, 7 dilution steps of disinfectant B, and 6 dilution steps of disinfectant C were tested. The experiment was repeated three times, changing the position of disinfectants to check different combinations of all three disinfectants in the Renu Advanced formula.

Fifty microliters of each disinfectant were used to give a total volume of 150 µL in every well. The concentration for each disinfectant ranged between 16× MIC to 0.24× MIC. Bacterial inocula were prepared as described previously and the plates were incubated for 18 to 24 hours at 37°C to determine the combined MIC. For the evaluation of the type of the interaction between different disinfectants the fractional inhibitory concentration index (FICI) was calculated using the formula (White et al., 1996):

$$FICI_{A/B} = \frac{MIC_{A\ (combination)}}{MIC_{A\ (alone)}} + \frac{MIC_{B\ (combination)}}{MIC_{B\ (alone)}}$$

For triple disinfectants, the FIC of disinfectant C was added to the above equation (Stein et al., 2015).

$$FICI_{A/B/C} = \frac{MIC_{A\ (combination)}}{MIC_{A\ (alone)}} + \frac{MIC_{B\ (combination)}}{MIC_{B(alone)}} + \frac{MIC_{C\ (combination)}}{MIC_{C(alone)}}$$

Synergy was defined when the FICI was ≤ 0.5, no interaction when the FICI was > 0.5 but <4 and antagonism when the FICI was >4 (Oo et al., 2010, Odds, 2003).

2.2.5 Antibiotic susceptibility testing

Susceptibility of antibiotics was assessed using MIC and MBC, performed following the standard protocol described by Clinical and Laboratory Standards Institute (Patel et al., 2014). The antibiotics used were ciprofloxacin, levofloxacin, gentamicin, ceftazidime (Sigma-Aldrich, USA), polymyxin B (Sigma-Aldrich, Denmark) tobramycin, piperacillin (Cayman, Chemical, Company, USA) and imipenem (LKT, Laboratories, Inc, USA). The concentrations tested ranged from 5120 µg/ml to 0.25 µg/ml.

The MIC for each antibiotic was determined in 96 wells plates with 100 µL serially diluted antibiotics and 100 µL of the bacterial inocula with a final concentration of 1×10^5 CFU/mL per well incubated 37°C for 18 to 24 hours. Antibiotics were diluted with Mueller-Hinton broth and bacterial cells were diluted with fresh media. The MIC and MBC were measured as described for MPDS previously. Interactions of strains with antibiotics can be described as susceptible intermediate or resistant based on Clinical and Laboratory Standards Institute (Patel et al., 2014) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2018) breakpoints. There are no standards for interpreting topical ocular treatment or efficacy with contact lens solutions, but the serum standards can be used if it is assumed that the antibiotic

concentrations in the ocular tissue and contact lens solutions are equal or greater than the antibiotic concentrations that can be attained in the serum (Kowalski et al., 2005).

2.2.6 Statistical analysis

The data were statistically analyzed using the Statistical Package for the IBM SPSS v25 (IBM Corp., Armonk, NY, USA). Differences between the distribution of the MICs of the bacterial isolates to MPDS and disinfectants were evaluated using Friedman's two-way analysis of variance. Briefly, mean ranks were calculated for each MPDS and disinfectant (a higher rank equates to a lower level of efficacy and vice versa). Based on a significant difference in the analysis of variance test, *post hoc* pairwise comparisons were conducted to identify the differences between the disinfectants and MPDSs. Medians were calculated and differences in the antibiotic resistant isolates were analysed using Fischer's exact test. *P* values less than 0.05 were considered as significant.

2.3 Results

2.3.1 Multipurpose solution susceptibilities

2.3.1.1 Historical Australian isolates

Historical Australian (contact lens-related) isolates (isolated between 2001 and 2006) of *P. aeruginosa* showed variations in their susceptibility to Renu Advanced Formula, OPTI-Free PureMoist, Complete RevitaLens OcuTec, and Biotrue (Table 2-3). When all four MPDSs were used at 100% concentration, they all reduced the bacterial growth to below the limit of detection (i.e., no bacteria grew on the agar plates). However, at other dilutions, there were differences in MICs and MBCs between the MPDSs. In general, the MBC of each MPDS was equivalent to twice its MIC. Overall, Renu Advanced formula had the lowest average MIC (7.9%) and MBC (15.8%), followed by OPTI-FREE PureMoist (average MIC 11%, MBC 22%) Complete RevitaLens OcuTec (average MIC 15.7%, MBC 32.7%), and Biotrue (average MIC 19.4%, MBC 38.7%; Figure 2-1).

A significant difference among MPDS types was found ($P = 0.0313$; OPTI-FREE PureMoist vs. RevitaLens, $P \leq 0.0001$; OPTI-FREE PureMoist vs. Biotrue, $P \leq 0.0001$; RevitaLens OcuTec vs. Renu Advanced formula and $P \leq 0.0001$; Biotrue vs. Renu Advanced Formula) except for OPTI-FREE PureMoist vs. Renu Advanced Formula ($P = 0.25$) and RevitaLens OcuTec versus Biotrue ($P = 0.12$).

2.3.1.2 Recent Australian isolates

Recent Australian (CL-related) isolates (isolated between 2018 and 2019) of *P. aeruginosa* also varied in their susceptibilities to the different MPDS (Table 2-3). Again, at 100% concentration of all MPDS no bacterial growth was recorded, but at dilutions lower than 100% all MPDS has different abilities to prevent growth of the bacteria. Renu Advanced Formula had the most consistent activity, with all strains having MIC of 5.6% and MBC of 11.4%; Figure 2-1).

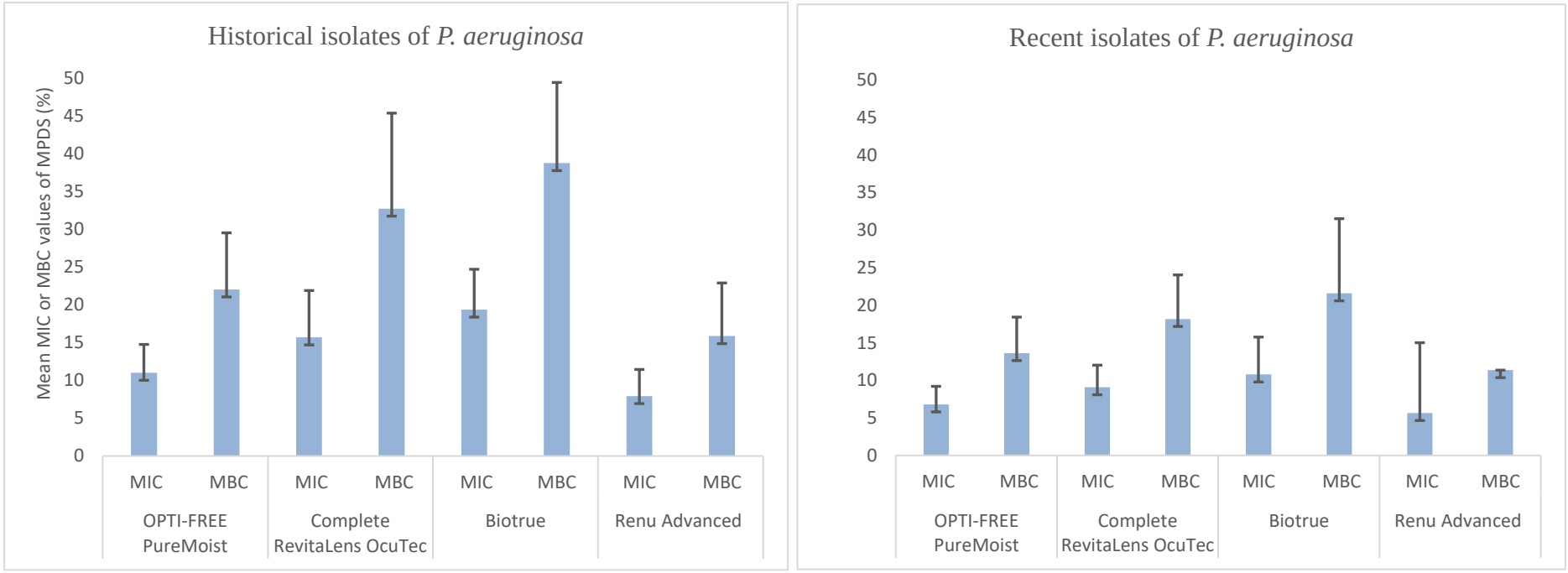

Figure 2-1 MICs and MBCs of the different MPDS (mean ± standard deviation) to historical and recent Australian *Pseudomonas aeruginosa* isolates.

Table 2-3 Minimum inhibitory (MIC) and minimum bactericidal concentration (MBC) (% of original) of MPDS.

Strains of *P. aeruginosa*	OPTI-FREE PureMoist (%)		Complete RevitaLens OcuTec (%)		Biotrue (%)		Renu Advanced Formula (%)	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
115	11.36	22.72	11.36	45.45	11.36	22.72	5.68	11.36
116	5.68	11.36	11.36	22.72	11.36	22.72	11.36	22.72
121	22.72	45.45	22.72	45.45	22.72	45.45	11.36	22.72
123	11.36	22.72	22.72	45.45	22.72	45.45	11.36	22.72
124	11.36	22.72	22.72	45.45	22.72	45.45	11.36	22.72
126	11.36	22.72	11.36	22.72	22.72	45.45	11.36	22.72
127	5.68	11.36	5.68	11.36	11.36	22.72	11.36	22.72
129	11.36	22.72	11.36	22.72	22.72	45.45	11.36	22.72
155	11.36	22.72	11.36	22.72	11.36	22.72	5.68	11.36
162	11.36	22.72	11.36	22.72	22.72	45.45	5.68	11.36
165	11.36	22.72	22.72	45.45	22.72	45.45	5.68	11.36
169	5.68	11.36	11.36	22.72	22.72	45.45	5.68	11.36
174	11.36	22.72	11.36	22.72	11.36	22.72	2.84	5.68
176	11.36	22.72	22.72	45.45	22.72	45.45	5.68	11.36
179	11.36	22.72	11.36	22.72	22.72	45.45	5.68	11.36
181	11.36	22.72	22.72	45.45	22.72	45.45	11.36	22.72
182	11.36	22.72	22.72	45.45	22.72	45.45	1.42	2.84
223	5.68	11.36	5.68	11.36	11.36	22.72	5.68	11.36
224	5.68	11.36	5.68	11.36	5.68	11.36	5.68	11.36
225	5.68	11.36	11.36	22.72	11.36	22.72	5.68	11.36
226	5.68	11.36	5.68	11.36	5.68	11.36	5.68	11.36
227	5.68	11.36	11.36	22.72	5.68	11.36	5.68	11.36
228	11.36	22.72	5.68	11.36	11.36	22.72	5.68	11.36
229	5.68	11.36	11.36	22.72	11.36	22.72	5.68	11.36
230	5.68	11.36	11.36	22.72	22.72	45.45	5.68	11.36
233	11.36	22.72	11.36	22.72	11.36	22.72	5.68	11.36
235	5.68	11.36	11.36	22.72	11.36	22.72	5.68	11.36

Strains isolated in the years 2001-2006 (historical isolates) and coloured in light blue and those isolated in the years 2018-2019 (recent isolates) and coloured in dark blue.

Overall, Renu Advanced Formula had the lowest average MIC (5.65%) and MBC (11.36%), followed by OPTI-FREE PureMoist (MIC= 6.7%, MBC=13.63%), Complete RevitaLens OcuTec (MIC= 9%, MBC=18%) then finally Biotrue (average MIC= 10.7%, MBC= 21.5%). In other words, Renu Advanced Formula was the most potent, Biotrue was the least potent. Although it was slightly more common for higher MICs and MBCs of MPDS to be associated

with the historical isolates, the MICS or MBCs were not statistically different when compared to the recent isolates.

2.3.2 Inhibition of *P. aeruginosa* by disinfectants

Analysis of the disinfectants in the MPDS individually showed that all the disinfectants gave higher MICs and MBCs than the concentrations in the dilutions of MPDS (Table 2-4), indicating that in isolation the disinfectants were less active against *P aeruginosa* than when they were formulated into MPDS. For example, OPTI-FREE with aldox and polyquaternium-1 was effective even when the concentrations of these were reduced to 6 and 10 ppm, respectively, upon diluting the MPDS even though the MICs of aldox alone ranged from 22.7 to 727.3 ppm. Generally, the MBC of each disinfectant was double the MIC.

Table 2-4 Minimum inhibitory and minimum bactericidal concentrations of disinfectants.

Strains of *P. aeruginosa*	PAPB (PHMB) (ppm)		Alexidine (ppm)		Polyquaternium-1 (ppm)		Aldox (ppm)	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
115	22.7	45.5	2.8	5.6	5.6	11.4	727.3	1454.5
116	45.5	90.9	2.8	5.6	11.4	22.7	727.3	1454.5
121	45.5	90.9	2.8	5.6	5.6	11.4	727.3	1454.5
123	45.5	90.9	2.8	5.6	11.4	22.7	90.9	181.8
124	22.7	45.5	2.8	5.6	11.4	22.7	90.9	181.8
126	22.7	45.5	2.8	5.6	5.6	11.4	181.8	363.6
127	45.5	90.9	2.8	5.6	2.8	5.6	90.9	181.8
129	45.5	90.9	2.8	5.6	5.6	11.4	45.5	90.9
155	22.7	45.5	2.8	5.6	11.4	22.7	22.7	45.5
162	22.7	45.5	1.4	2.8	5.6	11.4	181.8	363.6
165	22.7	45.5	5.6	11.4	5.6	11.4	90.9	181.8
169	45.5	90.9	2.8	5.6	5.6	11.4	181.8	363.6
174	22.7	45.5	0.7	1.4	2.8	5.6	90.9	181.8
176	22.7	45.5	0.7	1.4	2.8	5.6	90.9	181.8
179	22.7	45.5	2.8	5.6	1.4	2.8	90.9	181.8
181	22.7	45.5	2.8	5.6	5.6	11.4	181.8	363.6
182	45.5	90.9	2.8	5.6	5.6	11.4	90.9	181.8

Overall, alexidine had the lowest mean MIC (2.66 ppm) and MBC (5.31 ppm) followed by polyquaternium-1 (mean MIC = 6.2, MBC = 12.5) and the PAPB (mean MIC = 32 ppm; mean MBC = 64 ppm). Aldox had the highest mean MIC (217 ppm) and MBC (435 ppm) among all the disinfectants (Figure 2-2).

The comparative activities of each disinfectant based on the MIC and MBC were significantly different from each other ($P \leq 0.001$; alexidine vs. PAPB, $P \leq 0.001$; alexidine vs. Aldox, $P \leq 0.001$; polyquaternium-1 vs. PHMB, $P = 0.047$; polyquaternium-1 vs. Aldox ($P \leq 0.001$) except between Alexidine and polyquaternium-1 ($P = 0.505$) and PHMB and Aldox ($P = 0.278$). By Friedmann's two-way analysis of variance, alexidine was ranked lowest having the lowest MIC and therefore the highest antimicrobial activity. This was followed by polyquaternium-1 > PHMB > Aldox.

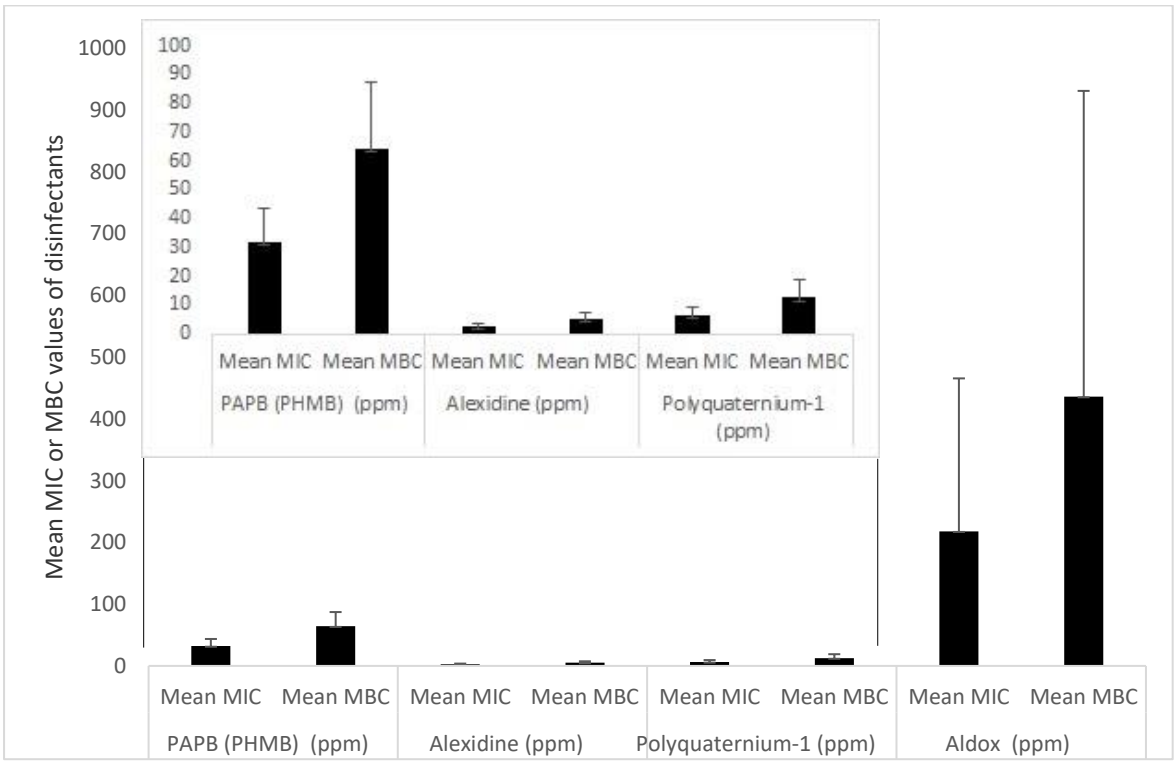

Figure 2-2 Mean (+ standard deviations) MICs and MBCs for disinfectants used in the study.

2.3.3 Fractional inhibitory concentration of components of MPDS

The combinations tested were selected based on their presence in the MPDSs. In dual combinations, none of the disinfectants showed synergistic activity. No interactions between the disinfectants were found for isolate 127 except for the triple combination of the disinfectants. For isolate 123, antagonism was found between polyquad-1 and PAPB; for isolate 155, antagonism occurred between polyquad-1 and alexidine or polyquad-1 and PAPB (Table 2-5). For the triple combination, synergy (FICI ≤ 0.5) occurred with all the isolates.

Table 2-5 Fractional Inhibitory concentration.

P. aeruginosa **strains**	**Disinfectants combination**	**MIC in the combination (ppm)**	**Checkerboard FICI**	**Interpretation**
P.aer 123	Polyquad-1+Aldox	5.64 + 90.9	1.5	No interaction
	Polyquad-1+PAPB	45.45 + 90.9	6	Antagonism
	Polyquad-1+Alexidine	5.64 + 5.64	2.5	No interaction
	Polyquad-1+PAPB+Alexidine	0.78 + 2.84 + 0.78	0.36	Synergy
P.aer 127	Polyquad-1+Aldox	2.84 + 11.36	1.125	No interaction
	Polyquad-1+PAPB	4.5 + 90.9	3.6	No interaction
	Polyquad-1+Alexidine	2.5 + 2 .84	1.2	No interaction
	Polyquad-1+PAPB+Alexidine	0.0097 + 5.64 + 0.39	0.25	Synergy
P.aer 155	Polyquad-1+Aldox	11.36 + 45.45	3	No interaction
	Polyquad-1+PAPB	45.45 + 90.9	8	Antagonism
	Polyquad-1+ALX	45.45+ 5	16.4	Antagonism
	Polyquad-1+PAPB+Alexidine	0.39+ 2.84+ 0.78	0.4	Synergy

Polyquad-1 = polyquaternium-1; PAPB = polyaminopropyl biguanide; ALDOX = myristamidopropyl dimethylamine; ALX = alexidine. Combined MIC is the MIC of each disinfectant when tested in combination with other disinfectants. FICI=fractional inhibitory concentration index (synergy: FICI ≤ 0.5; no interaction: 0.5< FICI ≤ 4; and antagonism: FICI > 4).

2.3.4 Antibiotic susceptibilities

Table 2-6 summarizes the MIC and MBC levels of historical and recent isolates.

2.3.4.1 Historical Australian isolates

All the historical isolates Australian isolates were susceptible to gentamicin and levofloxacin. 94% of the historical CL isolates were susceptible to tobramycin, polymyxin B, and piperacillin. Between 82% and 88% of isolates were susceptible to ceftazidime or ciprofloxacin. Fewer isolates were susceptible to imipenem (42%). Only 11% showed resistance (intermediate) to ciprofloxacin, one strain was resistant to tobramycin, a different strain was resistant to piperacillin. 58% of strains had intermediate or full resistance to imipenem. One isolate, 123, was resistant to polymyxin B with MIC and MBC values of 1280 µg/ml. Strain 127 was a resistant to two different beta-lactams and an aminoglycoside. Two isolates, strains 126 and 181, were resistant to two different beta-lactams.

2.3.4.2 Recent Australian isolates

Among the recent Australian isolates 70% were resistant to ciprofloxacin, 20% to levofloxacin, 40% to piperacillin, 90% to imipenem and 60% to ceftazidime. No isolate was resistant to gentamicin, tobramycin or polymyxin.

Overall, recent isolates showed more antibiotic resistant compared to the historical ones. There were significant differences between the resistance rates for historical and recent isolates for ciprofloxacin ($p= 0.0036$), piperacillin ($p=0.047$), imipenem ($p=0.0008$), ceftazidime ($p= 0.0042$), but not for tobramycin ($p=0.9$), polymyxin ($p=0.9$) and levofloxacin ($p=0.16$). None of the historical and recent isolates were resistant to gentamicin.

Table 2-6 Minimum inhibitory (MIC) and minimum bactericidal (MBC) concentration for contact lens-related keratitis *P. aeruginosa* isolates

Antibiotics Breakpoints	Ciprofloxacin µg/ml ≤ 1,2, ≥ 4	Levofloxacin µg/ml ≤ 2, 4, ≥ 8	Gentamicin µg/ml ≤ 4, 8, ≥ 16	Tobramycin µg/ml ≤ 4, 8, ≥ 16	Piperacillin µg/ml ≤ 16	Imipenem µg/ml ≤ 2, 4, ≥ 8	Ceftazidime µg/ml ≤ 8, 16, ≥ 32	Polymyxin B µg/ml ≤ 2, 4, ≥ 8
P. isolates	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC
115	0.25(S)/0.5	0.5 (S)/1	1 (S)/1	0.25 (S)/1	8 (S)/16	**4 (I)/8**	8 (S)/16	0.25 (S)/0.25
116	0.25 (S)/0.5	0.5 (S)/1	0.5 (S)/1	0.25 (S)/1	8 (S)/8	**4 (I)/4**	2 (S)/2	0.5 (S)/0.5
121	0.25 (S)/0.5	1 (S)/1	0.5 (S)/2	2 (S)/4	2 (S)/4	**8 (R)/8**	2 (S)/2	1 (S)/1
123	1 (S)/1	1 (S)/1	0.25 (S)/0.5	4 (S)/4	8 (S)/16	**4 (I)/8**	2 (S)/2	**1280 (R)/1280**
124	1 (S)/2	0.5 (S)/1	0.5 (S)/2	0.25 (S)/1	4 (S)/8	**8 (R)/16**	2 (S)/4	1 (S)/2
126	0.5 (S)/1	0.5 (S)/1	0.5 (S)/1	0.25 (S)/0.5	8 (S)/16	**8 (R)/16**	**128 (R)/256**	1 (S)/2
127	1 (S)/2	0.25 (S)/1	2 (S)/4	**32 (R)/128**	4 (S)/16	**4 (I)/8**	**128 (R)/256**	0.5 (S)/1
129	0.25 (S)/1	0.25 (S)/1	0.25 (S)/0.5	0.25 (S)/0.5	4 (S)/8	**4 (I)/8**	2 (S)/4	0.25 (S)/0.5
155	0.25 (S)/0.5	0.25 (S)/0.5	0.25 (S)/0.5	4 (S)/4	4 (S)/4	2 (S)/4	2 (S)/4	0.25 (S)/0.5
162	0.5 (S)/1	0.5 (S)/1	0.25 (S)/0.5	0.25 (S)/1	8 (S)/8	4 (S)/4	2 (S)/4	0.25 (S)/0.5
165	1 (S)/1	0.25 (S)/0.5	0.25 (S)/0.5	0.25 (S)/1	8 (S)/8	**16 (R)/16**	2 (S)/4	0.25 (S)/0.5
169	**2 (I)/4**	0.25 (S)/0.5	0.25 (S)/0.5	0.25 (S)/0.5	4 (S)/8	2 (S)/4	1 (S)/2	0.25 (S)/0.25
174	0.25 (S)/0.5	0.25 (S)/0.5	0.25 (S)/0.5	0.25 (S)/0.5	1 (S)/2	**8 (R)/16**	0.5 (S)/2	0.25 (S)/0.5
176	0.5 (S)/1	0.25 (S)/0.5	0.25 (S)/0.5	0.25 (S)/0.5	4 (S)/8	2 (S)/8	2 (S)/4	0.25 (S)/0.5
179	**2 (I)/4**	0.25 (S)/0.5	0.25 (S)/0.5	0.5 (S)/0.5	4 (S)/8	2 (S)/4	1 (S)/2	0.25 (S)/0.5
181	1 (S)/4	0.25 (S)/0.5	0.25 (S)/0.5	0.25 (S)/0.5	**32 (R)/64**	**4 (I)/8**	**16 (R)/32**	0.5 (S)/1
182	1 (S)/2	0.25 (S)/0.5	0.25 (S)/0.5	0.25 (S)/0.5	4 (S)/8	8 (S)/16	1 (S)/2	0.25 (S)/0.5

Antibiotics Breakpoints	Ciprofloxacin µg/ml ≤ 1,2, ≥ 4	Levofloxacin µg/ml ≤ 2, 4, ≥ 8	Gentamicin µg/ml ≤ 4, 8, ≥ 16	Tobramycin µg/ml ≤ 4, 8, ≥ 16	Piperacillin µg/ml ≤ 16	Imipenem µg/ml ≤ 2, 4, ≥ 8	Ceftazidime µg/ml ≤ 8, 16, ≥ 32	Polymyxin B µg/ml ≤ 2, 4, ≥ 8
P. isolates	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC
223	**64 (R)/128**	1(S) /2	0.5(S)/1	0.5(S)/1	**160 (R)/320**	1(S)/ 2	**16 (I)/32**	2(S)/ 4
224	**16 (R)/32**	1(S)/ 2	0.25(S)/0.5	0.25(S)/0.5	8(S)/ 16	**64 (R)/128**	**16 (I)/32**	1(S)/ 2
225	**64 (R)/128**	**16(R)/32**	0.5(S)/2	0.25(S)/1	16(S)/32	**64 (R)/128**	8(S)/ 16	0.25(S)/0.5
226	1(S)/ 2	1(S)/ 2	1(S)/2	1(S)/ 2	**32 (R)/64**	**160 (R)/320**	8(S)/ 16	1(S)/ 2
227	**64 (R)/128**	**64 (R)/128**	0.5(S)/1	0.25(S)/1	16(S)/32	**16 (R)/32**	**16 (I)/32**	0.25(S)/0.5
228	0.5(S)/2	0.25(S)/0.5	0.25(S)/0.5	0.25(S)/0.5	4(S)/ 8	**320(R)/640**	4(S)/ 8	0.25(S)/0.5
229	0.25(S)/0.5	0.25(S)/0.5	0.5(S)/1	0.25(S)/0.5	4(S)/ 8	**32 (R)/64**	8(S)/ 16	0.25(S)/0.5
230	**4 (R)/8**	2(S)/ 4	2(S)/4	0.5(S)/1	**32 (R)/64**	**32 (R)/64**	**64 (I)/128**	0.25(S)/0.5
233	**8 (R)/16**	1(S)/ 2	1(S)/ 2	0.5(S)/1	16(S)/32	**4 (I)/8**	**160 (I)/320**	0.5(S)/1
235	**16 (R)/32**	0.5(S)/1	2(S)/ 4	0.5(S)/1	**64 (R)/128**	**4 (I)/8**	**64 (I)/128**	0.25(S)/0.5

Strains isolated in the years 2001-2006 (historical isolates) and coloured in light blue and those isolated in the years 2018-2019 (recent isolates) and coloured in dark blue.

Antibiotics break points were ≤Sensitive, Intermediate, ≥Resistant. Bold font indicates those combinations that showed intermediate or complete resistance.

2.3.5 Comparison of sensitivity to Antibiotics, MPDS, and Disinfectants

Table 2-7 shows the comparison of susceptibilities of antibiotics, MPDS and disinfectants (taking any MIC greater than 10% as being resistant to MPDS or disinfectants, and the breakpoints of each antibiotic into account). Most historical isolates that were susceptible to antibiotics were not susceptible to MPDS or disinfectants. In contrast, for recent isolates there was an increase in susceptibility to Renu Advanced MPDS but an increase in resistance to ciprofloxacin, piperacillin, and ceftazidime

Table 2-7 Heat map for the comparative susceptibilities of antibiotics and disinfectants for *P. aeruginosa* isolates.

P. aeruginosa isolates	CIP	LEVO	GN	TOB	PIP	IMI	CEFTA	POLY-B	OPTI	REV	BIO	RENU	PAPB	Polyquad	Alexidine	Aldox
115	S	S	S	S	S	I	S	S	R	R	R	S	R	R	R	R
116	S	S	S	S	S	I	S	S	S	R	R	R	R	R	R	R
121	S	S	S	S	S	R	S	S	R	R	R	R	R	R	R	R
123	S	S	S	S	S	I	S	R	R	R	R	R	R	R	R	R
124	S	S	S	S	S	R	S	S	R	R	R	R	R	R	R	R
126	S	S	S	S	S	R	S	S	R	R	R	R	R	R	R	R
127	S	S	S	S	S	I	R	S	S	R	R	R	R	S	R	R
129	S	S	S	S	S	I	S	S	R	R	R	R	R	R	R	R
155	S	S	S	S	S	S	S	S	R	R	R	S	R	R	R	R
162	S	S	S	S	S	S	S	S	R	R	R	S	R	R	R	R
165	S	S	S	S	S	R	S	S	R	R	R	S	R	R	R	R
169	I	S	S	S	S	S	S	S	S	R	R	S	R	R	R	R
174	S	S	S	S	S	R	S	S	R	R	R	S	R	S	S	R
176	S	S	S	S	S	S	S	S	R	R	R	S	R	S	R	R
179	I	S	S	S	S	S	S	S	R	R	R	S	R	S	R	R
181	S	S	S	S	R	I	R	S	R	R	R	R	R	R	R	R
182	S	S	S	S	I	R	S	S	R	R	R	S	R	R	R	R
223	R	S	S	S	R	S	I	S	S	S	R	S	Not determined			
224	R	S	S	S	S	R	I	S	S	S	S	S				
225	R	R	S	S	S	R	S	S	S	R	R	S				
226	S	S	S	S	R	R	S	S	S	S	S	S				
227	R	R	S	S	S	R	I	S	S	R	S	S				
228	S	S	S	S	S	R	S	S	R	S	R	S				
229	S	S	S	S	S	R	S	S	S	R	R	S				
230	R	S	S	S	R	R	I	S	S	R	R	S				
233	R	S	S	S	S	I	I	S	R	R	R	S				
235	R	S	S	S	R	I	I	S	S	R	R	S				

S Sensitive I Intermediate R Resistant CIP; ciprofloxacin, LEVO; levofloxacin, GN; gentamicin, TOB; tobramycin, PIP; piperacillin, IMI; imipenem, CEFTA; ceftazidime, POLYB; polymyxin; B, PAPB; polyaminopropyl biguanide, Aldox; myristamidopropyl dimethylamine; polyquad, polyquaternium-1. Strains isolated in the years 2001-2006 (historical isolates) and coloured in light blue and those isolated in the years 2018-2019 (recent isolates) and coloured in dark blue

2.4 Discussion

This study reports the *in vitro* susceptibility of *P aeruginosa* Australian isolates from CL-related keratitis to various antimicrobials. The study has demonstrated that strains of *P aeruginosa* had different susceptibilities to diluted MPDS, but all strains were susceptible to all the MPDS used at 100% concentrations, indicating good activity overall for the MPDS against *P aeruginosa* isolates. The MIC for the disinfectants in the MPDS when tested alone were mostly higher than the concentrations of the disinfectants in the MPDS, yet combinations of the disinfectants found in different MPDSs did not show synergy, suggesting that it is the whole MPDS formulation that results in high antimicrobial activity.

There was a reduction in activity of MPDSs upon dilution (i.e., diluted MPDSs did not completely kill *P aeruginosa* strains compared with their 100% concentration). When diluted the efficacy of MPDS was in the order Renu Advance Formula > OPTI-FREE PureMoist > Complete RevitaLens > Biotrue. Dilution may occur in use, especially during topping off the MPDSs in lens cases. This concept was tested following an outbreak of CL-related *Fusarium* keratitis attributed to performance of the MPDS ReNu MoistureLoc (Levy et al., 2006). The data in the current investigation reinforce the need to instruct daily wear contact lens users in the proper use of MPDS and to avoid topping off.

The finding that Renu Advanced was associated with the lowest MICs and MBCs is perhaps not surprising given that this MPDS contains three different disinfectants. Renu Advanced contains alexidine as its highest concentration disinfectant, which is an efficient disinfectant against bacteria (McDonnell and Russell, 1999) and against the biofilms formed by bacteria (Ruiz-Linares et al., 2014). In the current study, alexidine had the lowest MIC of any other disinfectant. Polyquaternium-1 is the next most abundant disinfectant in Renu Advanced and it has been shown to have significant activity against *P. aeruginosa* (Codling et al., 2003). The third most abundant disinfectant present in Renu Advanced is PAPB (PHMB), which has also been proven to be effective against bacteria (Cazzaniga et al., 2002) particularly *P. aeruginosa*

(Gabriel et al., 2018), although in the current study PAPB was less effective than alexidine or polyquaternium-1. Even though the Renu Advanced formula was highly effective against *P. aeruginosa,* the individual disinfectants were not effective at the concentration found in Renu Advanced. However, this tri-disinfectant system was the only formulation to show synergy between the disinfectants which may have contributed to the overall better activity of this product.

The next most effective MPDS was OPTI-FREE PureMoist. This contrasts with the results in another study that compared OPTI-FREE PureMoist and Biotrue, with both MPDSs having similar results (Artini et al., 2015). OPTI-FREE PureMoist contains polyquaternium-1, which showed good activity when used alone, as well as aldox. Interestingly, aldox had a relatively high MIC and MBC (i.e., low activity). Aldox is believed to be more effective against fungi (Codling et al., 2003). The difference in activity of OPTI-FREE PureMoist compared with the individual disinfectants when used in combination was particularly marked with strain 155, which showed a high level of antagonism between polyquaternium-1 and aldox. This further reinforces the effect of the whole formulation on overall antimicrobial activity. The addition of the antimicrobial ethylenediaminetriacetic acid and surfactants (both known to be antimicrobial) (Guan et al., 2015) likely resulted in the relatively high antimicrobial activity of OPTI-FREE PureMoist.

Complete RevitaLens OcuTec had a lower efficacy than OPTI-Free PureMoist, although in a previous study, (Abjani et al., 2017) the MPDSs showed similar levels of efficacy. Biotrue has been shown to be more effective compared to OPTI-FREE PureMoist against certain gram-negative bacteria including *Achromobacter xylosoxidans*, *Delftia acidovorans* and *Stenotrophomonas maltophilia* (Callahan et al., 2017). However, this finding contrasts with the present study where Biotrue was the least effective of all the tested MPDS against the *P. aeruginosa* clinical strains tested. The findings of the current study are in general agreement with another study (Gabriel et al., 2018) on the most to least active MPDS, OPTI-

FREE PureMoist > Complete RevitaLens > Biotrue, against fungal and bacterial isolates including *P. aeruginosa*.

The higher MICs and MBCs of individual disinfectants compared with the concentration of these disinfectants at the MICs and MBCs of MPDS suggested that it was the combination of excipients in MPDS that contributed to the inhibition of growth and killing of bacteria. The current study examined whether this effect was due to the combination of disinfectants within the MPDS, but synergy was only observed for the combination of the three disinfectants in Renu Advanced. Indeed, for the combination of Polyquad-1+PAPB there was antagonism between the two disinfectants for all the three strains of *P aeruginosa* tested. All MPDS contain additional excipients to disinfectants. These include surfactants and ethylenediaminetriacetic acid which have known antimicrobial activity (Guan et al., 2015). The other components of MPDS including acids and alcohols may also have antimicrobial activity (Jeffrey, 1995). It is likely the combination of excipients with disinfectants in MPDS that contribute to the overall antimicrobial activity. It would be useful in future studies to test the efficacy of other components of MPDS in combination with the disinfectants and to add a possible comparator with common usage in ophthalmic solutions like benzalkonium chloride or chlorhexidine in MPDS for rigid gas-permeable lenses.

The development of resistance to antibiotics used for keratitis treatment in Australia would be alarming especially if the resistance is to multiple antibiotics. Treatment of multi-drug resistant *P. aeruginosa* is challenging because it increases antibiotics consumption (Golkar et al., 2014). The failure of first and second-line antibiotic treatment options adds considerable costs to treatment (Lushniak, 2014, CDC, 2013) due to prolonged treatment. However, this study showed that the majority of the historical *P aeruginosa* isolates were susceptible to most of the antibiotics except for imipenem, and only one strain showed multiantibiotic resistance (i.e., was resistant to two or more antibiotics from different classes). Fewer strains were susceptible to the first-generation fluoroquinolone ciprofloxacin (88%) compared with the later generation levofloxacin (100%). This is in contrast to previous reports where more strains were

susceptible to ciprofloxacin than levofloxacin (Kowalski et al., 2001) or where sensitivity of both the antimicrobials was equivalent (Thibodeaux et al., 2004). In contrast, in the recent isolates resistance to ciprofloxacin, piperacillin, imipenem and ceftazidime had increased. The lower rate of sensitivity to ciprofloxacin is important as ciprofloxacin is frequently prescribed as monotherapy for the treatment of corneal infections in Australia (Keay et al., 2006b). Therefore, careful evaluation of changes to the susceptibility of isolates is warranted. On the other hand, recent and historical isolates were as sensitive to levofloxacin. This lack of increase in resistance to levofloxacin might because it is not used in clinical practice in Australia. However, the increase in resistance to piperacillin in the recent isolates partly counters this argument as it is not prescribed for keratitis treatment in Australia. Imipenem was the least effective beta-lactam, with only 29% of recent and 40% of historical strains being susceptible to it. This level of resistance of *P. aeruginosa* keratitis isolates to imipenem has recently been reported (Spierer et al., 2018). Imipenem should not be recommended as a treatment for *P. aeruginosa* keratitis (Chatterjee and Agrawal, 2016).

Similar proportions of historical and recent isolates were susceptible to the aminoglycosides, gentamicin and tobramycin. These data are consistent with a surveillance study of keratitis isolates of *P. aeruginosa* from Sydney, Australia, that showed 100% of isolates (none of which were used in the current study) were susceptible to these two aminoglycosides (Watson et al., 2018). Similar susceptibility (>99%) to aminoglycoside has been reported from the USA (Alexandrakis et al., 2000), whereas susceptibility to aminoglycosides was lower in Pakistan (36% gentamicin) (Abidi et al., 2013) and India (~ 77% to gentamicin) (Lalitha et al., 2017).

Only the recent strain 123 was resistant to polymyxin B (which potentially shares the same mechanism of action to disinfectants in MPDS through targeting the cell membrane of bacteria) (Ikeda et al., 1984). This resistance may be due two component systems present in *P. aeruginosa* including PhoPQ, PmrAB, ParRS, CprRS and ColRS which, when mutated, result in the modification of lipopolysaccharide and efflux pumps (Olaitan et al., 2014) or due to

acquisition of the external *mcr-1* gene (Liu et al., 2016, Fernandes et al., 2016b). This strain also had relatively high MIC and MBC values for MPDS and disinfectants which may be due to the use of the same resistance mechanisms. This requires further investigation. However, overall, there was no relationship between resistance to antibiotics and relative resistance to MPDS or disinfectants, even though resistance to disinfectants can be mediated by *qac* genes (Boost et al., 2014) and these genes can be carried on mobile genetic elements that can also carry antibiotic resistance genes (Chapman, 2003). These findings may indicate that exposure to disinfectants does not contribute to the acquisition of antibiotic resistance genes in *P. aeruginosa*.

This study compared the susceptibilities of ocular *P. aeruginosa* isolates collected 10-15 years apart and found increased resistance to certain antibiotics, but not to multiplurpose disinfecting systems. Identification of the mechanisms behind the resistance to antibiotics in the recent Australian isolates is required.

3 Susceptibility of Indian *Pseudomonas aeruginosa* keratitis isolates to antibiotics compared with Australian isolates

3.1 Introduction

Microbial keratitis is considered one of the leading causes of blindness internationally (Resnikoff et al., 2004) and it is viewed as a neglected disease (Ung et al., 2019). *Pseudomonas aeruginosa* is a common cause of keratitis causing up to 65% of cases in contact lens wearers (Galentine et al., 1984, Stapleton et al., 2007, Stapleton et al., 2005, Schein et al., 2005) and, while less common in non-contact lens wearers, still causes 2-7% of cases per year (Hooi and Hooi, 2005). In developed countries, contact lens wear (Dart et al., 1991, Fong et al., 2004, Keay et al., 2006a) for cosmetic or therapeutic purposes is a major risk factor for microbial keratitis, while in developing countries ocular trauma due to injury (Bharathi et al., 2009, Kunimoto et al., 1998) is a more common.

The annual incidence rate of CL-related microbial keratitis was 0.63 in Hong Kong (Lam et al., 2002) 0.26 in Scotland (Seal et al., 1999) 4.8 in Australia (Stapleton et al., 2008a) per 10,000 population per year. In contrast, the prevalence of keratitis in developing countries, which was mostly due to ocular trauma, in Southeast Asian region ranged from 11.3 per 10,000 in India (Gonzales et al., 1996) to 79.9 per 10,000 in Nepal, (Upadhyay et al., 2001).

Fluoroquinolones or fortified antibiotics are prescribed as the first choice of treatment for keratitis (Kunimoto et al., 1999, Nixon, 2018, Green et al., 2019). Due to the resistance to earlier generation fluoroquinolones, later generations have also been used as a treatment option for bacterial keratitis. *P. aeruginosa* has also developed resistance to many of the recent fluoroquinolones including the fourth generation drug moxifloxacin (Chaudhry et al., 1999, Kunimoto et al., 1999, Alexandrakis et al., 2000) in different regions of the world (Oldenburg et al., 2013). *P. aeruginosa*-causing keratitis has also shown a reduced susceptibility to aminoglycosides (Alexandrakis et al., 2000) which indicates the increasing resistance of this bacterium to multiple antibiotics. Antibiotic sensitivity of Australian *P.*

aeruginosa isolates from Sydney, Australia (Watson et al., 2018) in a recent surveillance study and Queensland (Green et al., 2019), Australia was stable over time. However, the results of Chapter 2 indicate some increase in resistance to antibiotics of keratitis strains over time. The pattern of *P. aeruginosa* antibiotic susceptibility varies internationally (Ng et al., 2015, Watson et al., 2018, Garg et al., 1999, Kowalski et al., 2003, Green et al., 2008, Kowalski et al., 2001, Lomholt and Kilian, 2003, Willcox, 2011).

The aims of this study were to:

- To analyse the antibiotic susceptibilities of Indian *P. aeruginosa* isolates.
- To compare antibiotic susceptibility of Indian isolates and Australian isolates. Isolates examined in chapter 2 are included for comparison.

3.2 Materials and methods

The antibiotic susceptibility was investigated using the methodology outlined in chapter 2.

3.2.1 *Pseudomonas aeruginosa* isolates

Strains of *Pseudomonas aeruginosa* isolated from corneal scrapes in non-contact lens-related microbial keratitis from India between 2017 to 2018 (strains 188 to 222, Table 1) and historical isolates from 1997 (strains 31-37, Table 1) (Subedi et al., 2018b) were donated by the LV Prasad Eye Institute (Table 3-1). The strains were stored at -80^{o}C and revived on nutrient agar (Oxoid Ltd., Basingstoke, Hampshire, UK). Isolates were then inoculated into Mueller-Hinton broth (MHB; Oxoid Ltd.) and grown at 37°C for 18 to 24 hours. The optical density of the bacterial suspension was adjusted to 0.1 (1× 10^{8} CFU/ml) at 660nm using a spectrophotometer (FLUOstar Omega, BMG LABTECH, Germany) (Watanabe et al., 2014).

3.2.2 Statistical analysis

The statistical analysis was carried out using GraphPad Prism v8. The proportions of sensitive and resistant isolates from India or Australia or historical and recent isolates were compared using a Fischer's exact test. Minimum inhibitory and minimum bactericidal concentrations

were compared using the Kruskal-Wallis test. A P-value of less than 0.05 was considered significant.

3.3 Results

3.3.1 Antibiotic susceptibility

Table 3-1 summarizes the MIC and MBC levels of historical and recent strains.

3.3.1.1 Historical Indian isolates

All historical Indian isolates (6/6) were resistant to ciprofloxacin, gentamicin, tobramycin, imipenem. 83% (5/6) were reistant to levofloxacin, ceftazidime and polymyxin, and 33% (2/6) to piperacillin. The historical non-CL related *P.aeruginosa* isolates were all multi-drug resistant (resistant to three or more antibiotics of different classes).

3.3.1.2 Recent Indian isolates

Of the recent isolates, 44% (15/34) were resistant to ciprofloxacin, 11% (4/34) to gentamicin, 20% (7/34) to tobramycin, 73% (25/34) to imipenem, 23% (8/34) to levofloxacin, 14% (5/34) to polymyxin and 26% (9/34) to piperacillin. Excluding isolates 193, 199, 201 and 206, all other isolates were either resistant or showed intermediate resistance to at least one antibiotic. Isolates 198, 202, 210, 216, 217, 218, 219 and 220 were multi-drug resistant. MICs $\geq$ to 2560 µg/ml were found for ciprofloxacin, levofloxacin and gentamicin.

Table 3-1 Minimum inhibitory and minimum bactericidal concentration of antibiotics for Indian keratitis *P. aeruginosa* isolates

***P. aeruginosa* isolates**	**Antibiotics and Breakpoints**							
	Ciprofloxacin µg/ml ≤ 1,2, ≥ 4	**Levofloxacin µg/ml ≤ 2, 4, ≥ 8**	**Gentamicin µg/ml ≤ 4, 8, ≥ 16**	**Tobramycin µg/ml ≤ 4, 8, ≥ 16**	**Piperacillin µg/ml ≤ 16**	**Imipenem µg/ml ≤ 2, 4, ≥ 8**	**Ceftazidime µg/ml ≤ 8, 16, ≥ 32**	**Polymyxin B µg/ml ≤ 2, 4, ≥ 8**
	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC
31	**32 (R)/62**	**32 (R)/32**	**≥5120 (R)/5120**	**640 (R)/1280**	4/8	**4 (I)/16**	**16 (I)/32**	**4 (I)/4**
32	**64 (R)/128**	**32 (R)/32**	**2560 (R)/5120**	**640 (R)/1280**	16/32	**4 (I)/4**	**16 (I)/16**	**4 (I)/16**
33	**128 (R)/128**	**32 (R)/64**	**2560 (R)/5120**	**≥5120 (R)/5120**	**32 (R)/64**	**8 (R)/16**	**32 (R)/64**	2/4
34	**2 (I)/4**	2/4	**2560 (R)/2560**	**640 (R)/1280**	**32 (R)/64**	**16 (R)/16**	4/8	2/2
35	**2 (I)/4**	2/4	**2560 (R)/2560**	**1280 (R)60**	8/16	**16 (R)/16**	4/8	2/2
37	**64 (I)/128**	**32 (R)/32**	**2560 (R)/2560**	**1280 (R)2560**	8/16	**8 (R)/16**	**16 (I)/64**	2/4
188	**2 (I)/4**	1/2	0.5/1	**32 (R)/64**	16/64	0.5/1	4/8	2/4
189	0.25/1	1	0.25/0.5	**16(R)/32**	4/8	2/4	8/16	2/4
190	0.25/0.5	0.5/2	0.25/0.5	**8 (I)/16**	16/16	2/4	4/8	1/2
191	**1 (I)/2**	1/4	0.25/0.5	2/4	8/16	**16 (R)/64**	2/4	2/4
192	2/4	0.25/0.5	0.5/1	0.25/0.5	16/32	**32 (R)/64**	2/4	0.25/0.5
193	1/2	0.25/0.5	0.25/0.25	0.25/0.5	4/8	2/4	2/4	0.5/1
194	0.5/0.5	0.5/1	1/2	0.25/0.5	8/16	**4 (I)/8**	4/8	1/1
195	0.5/1	0.5/1	0.5/1	0.25/0.25	8/16	**8 (R)/16**	4/8	0.5/1
196	0.5	0.25	0.5/1	0.25/0.5	4/8	**8(R)/16**	4/8	0.25/0.5

***P. aeruginosa* isolates**	**Ciprofloxacin µg/ml ≤ 1,2, ≥ 4**	**Levofloxacin µg/ml ≤ 2, 4, ≥ 8**	**Gentamicin µg/ml ≤ 4, 8, ≥ 16**	**Tobramycin µg/ml ≤ 4, 8, ≥ 16**	**Piperacillin µg/ml ≤ 16**	**Imipenem µg/ml ≤ 2, 4, ≥ 8**	**Ceftazidime µg/ml ≤ 8, 16, ≥ 32**	**Polymyxin B µg/ml ≤ 2, 4, ≥ 8**
	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC
197	1/2	0.25/0.5	0.5/1	0.25/0.5	4/4	**16 (R)/16**	2/4	0.25/0.5
198	**1280 (R)**	**320 (R)**	**2560 (R)**	**16 (R)**	8/16	1/2	8/8	**4 (I)/4**
199	0.25/0.25	0.25/1	0.25/0.5	0.25/1	2/4	2/8	2/8	0.25/1
200	**32 (R)/32**	**16 (R)/64**	0.25/0.5	0.25/1	8/8	2/2	4/4	0.25/1
201	1/2	0.25/0.5	0.25/0.25	0.5/2	8/16	2/8	1/4	1/4
202	**640 (R)/1280**	**320 (R)/640**	**8 (I)/32**	**320 (R)/640**	16/64	**8 (R)/32**	8/32	0.25/0.25
203	0.25/0.25	1/4	0.25/0.25	0.5/1	8/16	**4 (I)/8**	8/32	0.25/1
204	0.5/2	0.5/1	0.5/1	0.25/1	4/16	**4 (I)/8**	4/8	0.25/0.5
205	**2 (I)/2**	0.5/0.5	0.25/0.25	0.25/0.25	4/16	**8 (R)/32**	4/16	0.25/0.5
206	1/1	0.5/0.5	1/1	0.25/0.5	8/8	2/4	2/4	0.25/0.5
207	0.25/0.5	0.5/2	1/2	0.25/1	8/8	**4 (I)/4**	0.5/1	**4 (I)4**
208	0.5/1	0.25/0.5	0.5/0.5	0.25/1	4/8	**4 (I)/8**	2/4	2/4
209	1/2	1/2	1/2	0.5/1	**64 (R)/128**	**4 (I)/8**	0.5/1	0.5/1
210	**≥5120 (R)/5120**	**≥5120 (R)/5120**	4/8	4/8	**1280 (R)/2560**	**32 (R)/64/**	**160 (R)320**	**4 (I)/16**
211	0.5/1	0.25/0.5	0.25/0.5	0.25/1	2/4	**4 (I)/8**	1/2	1/2
212	**8 (R)/16**	1/2	0.5/1	0.25/0.5	**32 (R)/16**	**8 (R)/16**	8/16	0.5/1
213	**2 (I)/4**	1/2	1/4	0.25/0.5	16/32	**4 (I)/8**	**16 (I)/32**	0.5/1
215	0.5/1	1/2	1/4	0.5/1	**32 (R)/64**	**4 (I)/8**	4/8	2/8
216	**64 (R)/128**	4/8	1/2	0.5/1	**160 (R)/320**	**16 (R)/32**	**64 (R)/128**	**64 (R)/128**
217	**64 (R)/128**	**32 (R)/64**	1/2	1/2	**64 (R)/128**	**8 (R)/16**	**32 (R)/64**	0.25/1
218	**8 (R)/16**	1/2	0.5/1	0.5/1	**160 (R)/320**	**8 (R)/16**	**64 (R)/128**	1/4
219	**≥5120 (R)/5120**	**640 (R)/1280**	**≥5120 (R)/5120**	**1280 (R)/2560**	**2560 (R)/5120**	**40 (R)/80**	**16 (I)/32**	0.25/1

***P. aeruginosa* isolates**	**Ciprofloxacin µg/ml ≤ 1,2, ≥ 4**	**Levofloxacin µg/ml ≤ 2, 4, ≥ 8**	**Gentamicin µg/ml ≤ 4, 8, ≥ 16**	**Tobramycin µg/ml ≤ 4, 8, ≥ 16**	**Piperacillin µg/ml ≤ 16**	**Imipenem µg/ml ≤ 2, 4, ≥ 8**	**Ceftazidime µg/ml ≤ 8, 16, ≥ 32**	**Polymyxin B µg/ml ≤ 2, 4, ≥ 8**
	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC
220	**2 (I)/4**	0.25/0.5	0.5/1	0.5/1	0.25/0.5	**8 (R)/16**	**160 (R)/320**	**8 (R)/16**
221	**2560 (R)/5120**	**2560 (R)/5120**	**2560 (R)/5120**	**2560 (R)/5120**	**64 (R)128**	**16 (R)/32**	**32 (R)/64**	0.25/0.5
222	0.25/1	2/4	2/8	0.5/1	4/8	**32 (R)/64**	**160 (R)/320**	1/2

Historical Indian (31-37) isolates were collected in 1997 and are coloured in light blue. Recent Indian isolates 188-210, 211-222 were collected between 2017 and 2018 and are coloured in dark blue. Antibiotics breakpoints were S, I, R = ≤ Sensitive, Intermediate, ≥ resistant. MIC= minimum inhibitory concentration, MBC= minimum bactericidal concentration. Bold font indicates intermediate or complete resistance.

There was a significant difference ($p<0.025$) between the susceptibility of recent and historical non-CL related isolates to ciprofloxacin, levofloxacin, gentamicin, tobramycin, ceftazidime (Table 3-2) but not for piperacillin, imipenem or polymyxin.

Table 3-2 Median minimum inhibitory concentration (MIC) for antibiotics to Indian *P. aeruginosa* isolates.

Antibiotics	**Median MIC (IQR, 25%-75%) for recent *P. aeruginosa* isolates**	**Median MIC (IQR, 25%-75%) for historical *P. aeruginosa* isolates**	**P-values**
Ciprofloxacin	1(0-14)	64 (24-80)	**0.021**
Levofloxacin	1 (0-2.5)	32 (2-32)	**0.0095**
Gentamicin	0 (0-1)	2560 (2560-3200)	**0.0001**
Tobramycin	0 (0-2.5)	960 (640-2240)	**0.0004**
Ceftazidime	4 (2-16)	16 (4-20)	**0.01**
Piperacillin	8 (4-32)	12 (7-32)	0.9
Imipenem	4 (2-10)	12 (4-16)	0.3
Polymyxin B	0.5 (0-2)	2 (2-4)	0.2

3.3.2 Comparative antibiotic susceptibilities of Indian and Australian isolates

Overall (combining the recent and historical isolates from each group), 32% of Indian isolates (N=40) but 14% of Australian isolates (N=27; Chapter 2) were resistant to at least one antibiotic. Among the Indian isolates (n=40), 77% were resistant to imipenem, 52% to ciprofloxacin, 32% to tobramycin and ceftazidime, 30% to levofloxacin, 27% to piperacillin, 25% to gentamicin and 17% to polymyxin. In contrast, in Australian isolates (n=27) 70% were resistant to imipenem, 33% to ciprofloxacin and ceftazidime, 18% to piperacillin, 7% to

levofloxacin, 3% to tobramycin and polymyxin, and no isolate was resistant to gentamicin. Indian isolates had higher resistance rates to levofloxacin (p=0.03), gentamicin (p=0.04) and tobramycin (p=0.005). Indian isolates tended to have higher MIC values to antibiotics. The median MIC values of antibiotics for Australian and Indian related isolates are shown in Table 3-3.

Table 3-3 Median minimum inhibitory concentration for Indian and Australian *P. aeruginosa* isolates.

Antibiotics	**Median MIC IQR (25%-75%) for *P. aeruginosa* isolates from India**	**Median MIC IQR (25%-75%) for *P. aeruginosa* isolates from Australia**	**P-values**
Ciprofloxacin	2 (0.5-64)	1 (0.2-4)	0.14
Levofloxacin	1 (0.5-28)	0.5 (0.2-1)	**0.03**
Gentamicin	0.7 (0.3-7)	0.5 (0.2-0.5)	**0.004**
Tobramycin	0.5 (0.2-28)	0.2 (0.2-0.5)	**0.005**
Ceftazidime	4 (2-16)	2 (2-16)	0.9
Piperacillin	8 (4-32)	8 (4-16)	0.5
Imipenem	6 (4-14)	4 (4-16)	0.5
Polymyxin B	1 (0.2-2)	0.2 (0.2-1)	0.1

The percentage antibiotic resistance of historical and recent Australian and Indian isolates are given in Figure 3-1. Several of the recent Indian isolates were MDR with resistance to seven antibiotics. Four isolates (201, 193, 199 and 206) were not resistant to any of the antibiotics.

The historical Indian isolates were resistant to 5 or 7 antibiotics and were categorized as MDR whereas no isolate was susceptible to all antibiotics (Figure 3-2).

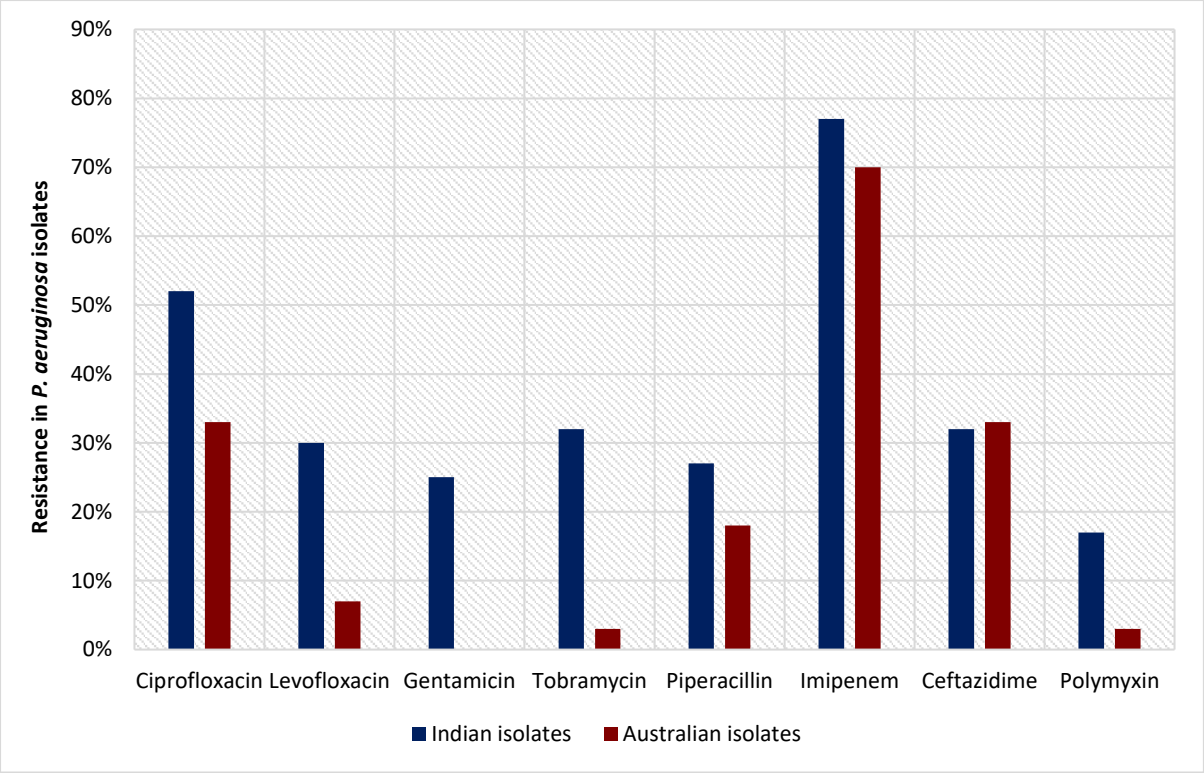

Figure 3-1 Percentage resistance in Indian and Australian *P. aeruginosa* isolates.

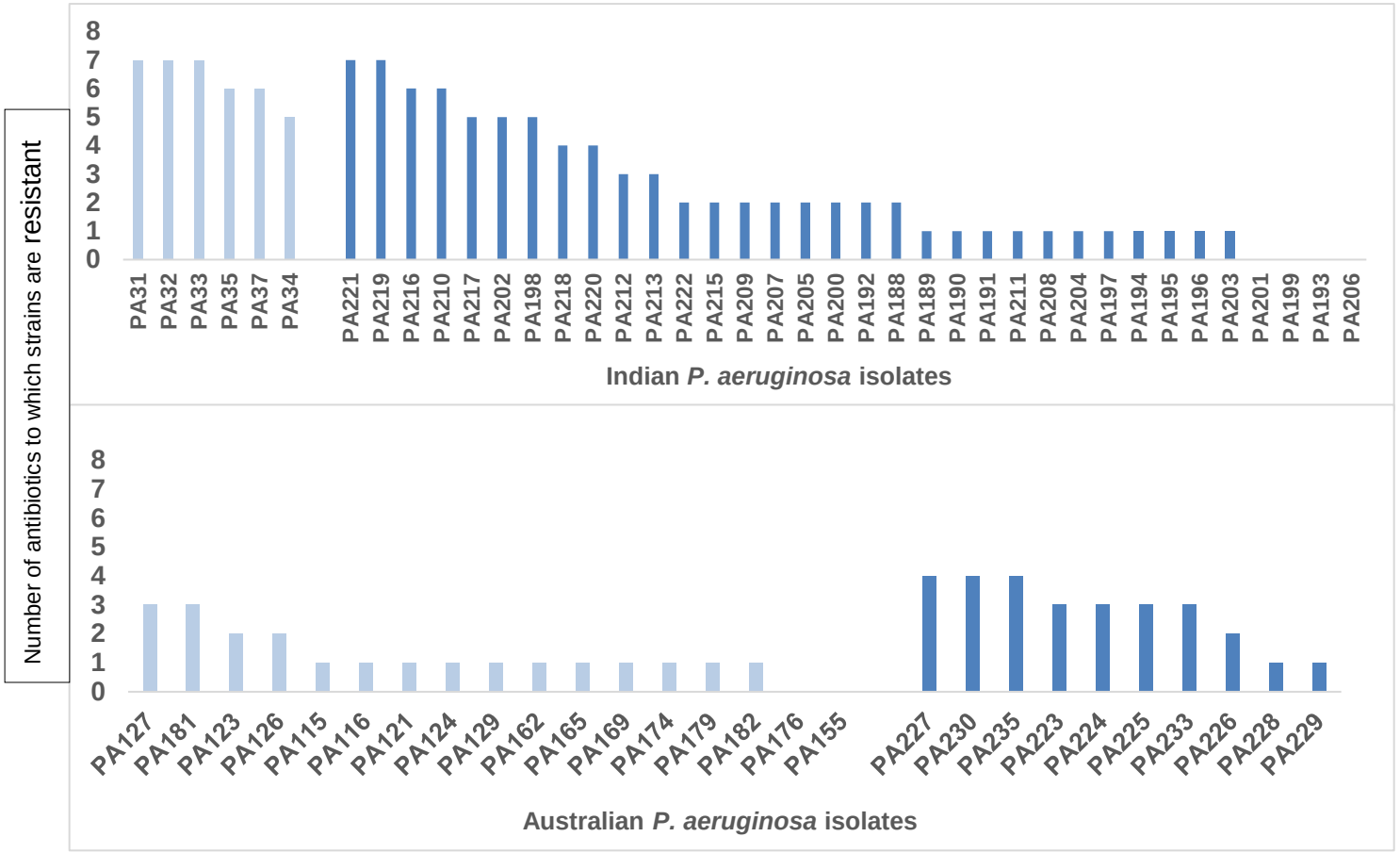

Figure 3-2 Frequency of antibiotic resistance in the Indian and Australian *P. aeruginosa* isolates.

Isolates shaded with light blue are historical and those shaded in dark blue are recent isolates.

The recent Australian keratitis *P. aeruginosa* isolates showed increased antibiotic resistant compared to historical ones (Chapter 2). Recent Australian *P. aeruginosa* isolates remained 100% sensitive to gentamicin and tobramycin. No recent Australian isolate was resistant to all tested antibiotics (Figure 3-2). The MDR isolates were resistant to four antibiotics. Among the ten most recent Australian isolates, no isolate was 100% susceptible to antibiotics. In the historical Australian *P. aeruginosa* isolates, none of the isolates was MDR. Two historical Australian isolates 127 and 181 were resistant to three antibiotics and two isolates 123 and 126 were resistant to two antibiotics.

3.4 **Discussion**

The historical MDR isolates had higher MICs and MBCs to ciprofloxacin, levofloxacin, gentamicin, tobramycin and ceftazidime compared to the recent isolates. A higher MIC might be related to additional or multiple resistance mechanisms to the antibiotics including mutations, antibiotic modification, efflux pumps, or acquired resistance genes (Park et al., 2006, Yoshida et al., 1990, Li et al., 1994, Chávez-Jacobo et al., 2018).

Susceptibility to polymyxin in the recent and historical non-CL isolates remained the same which indicated that polymyxin can still be a drug of choice to treat keratitis caused by *P. aeruginosa*, especially if the strains were resistant to other antibiotics. However, use of polymyxin B in the form of topical ointment in combination with neomycin can induce contact dermatitis which potentially limits the use of polymyxin B (Zug et al., 2009, Gehrig and Warshaw, 2008). On the other hand, resistance to imipenem was relatively high in recent and historical non-CL isolates which suggests it might not be a drug of choice to treat *P. aeruginosa* keratitis. Imipenem resistance can occur by the acquisition of specific resistance genes, or decreased expression of the outer membrane protein *oprD,* or overexpression of *mexB* which forms a multi-drug export system, mexAB-OprM, in the outer membrane of *P. aeruginosa* (Wi et al., 2017, Lynch et al., 1987). In a previous study of ocular strains, 93% of imipenem sensitive *P. aeruginosa* isolates were sensitive to ciprofloxacin, whereas 54% of imipenem

resistant isolates were ciprofloxacin resistant (Haas et al., 2011). The present study had similar findings where 35% of *P. aeruginosa* strains (Australia and India overall) were both ciprofloxacin and imipenem resistant.

None of the recent or historical CL-related *P. aeruginosa* isolates showed resistance to greater than four antibiotics and no isolate was resistant to polymyxin B except one historical isolate, 123. Isolate 123 was resistant to polymyxin B with a very high MIC 1280 µg/ml which might be due to acquired genes for polymyxin (Liu et al., 2016) or mutations in two component systems (phoPQ and pmrAB) (Owusu-Anim and Kwon, 2012, Gutu et al., 2013). There is also the possibility that this strain has acquired variants of the *mcr* gene that can cause polymyxin resistance, which has been reported in other bacterial species (Perreten et al., 2016) and recently identified in *P. aeruginosa* (Hameed et al., 2019). However, there are no published data related to the frequency of this gene in ocular *P. aeruginosa* isolates.

The antibiotic resistance of ocular *P. aeruginosa* isolates had been reported previously from India and other regions (Oldenburg et al., 2013, Marasini et al., 2016, Kowalski et al., 2001, Lomholt and Kilian, 2003, Subedi et al., 2018b). Reports from Australia and New Zealand have shown no change over time in *P. aeruginosa* resistance (Watson et al., 2018, Marasini et al., 2016, Green et al., 2019). This study has provided an updated information on the susceptibility of recent ocular *P. aeruginosa* isolates from India and Australia. It has also provided a comparison between isolates from CL and non-CL related keratitis, although further study should include isolates from same sources from both regions with an equal sample size.

In conclusion, the proportion of antibiotic resistance for CL isolates from Australia and non-CL related isolates from India had increased significantly over the past 10 years. MICs for non-CL were significantly higher than the CL isolates. These data suggest that environment is a major determinant in resistance. Poor antibiotic regulation and greater availability of antibiotics (Lushniak, 2014) are more common in India (Milder et al., 2012, Hsu et al., 2014, Ray et al., 2013) compared to Australia. In 2010, India was the largest consumer of antibiotics and the

rate of consumption had increased by ~22% for some antibiotics including carbapenems, beta-lactams, cephalosporins (Farooqui et al., 2018). The global consumption of antibiotics was 35% as of 2014 report (Van Boeckel et al., 2014). Countries with high antibiotic use generally have high rates of antibiotic resistance (Bell et al., 2014, Versporten et al., 2014). Better control of antibiotic resistance can be achieved with stronger management of antibiotic consumption, audit and surveillance, regulation of health care settings, improved governance, and development of new antibiotics.

4 Antibiotic resistance characteristics of *Pseudomonas aeruginosa* isolated from keratitis in Australia and India

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I certify that all co-authors of this work have agreed to submission of this work as a part of this thesis.

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Article

Antibiotic Resistance Characteristics of *Pseudomonas aeruginosa* Isolated from Keratitis in Australia and India

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Abstract: This study investigated genomic differences in Australian and Indian *Pseudomonas aeruginosa* isolates from keratitis (infection of the cornea). Overall, the Indian isolates were resistant to more antibiotics, with some of those isolates being multi-drug resistant. Acquired genes were related to resistance to fluoroquinolones, aminoglycosides, beta-lactams, macrolides, sulphonamides, and tetracycline and were more frequent in Indian (96%) than in Australian (35%) isolates ($p = 0.02$). Indian isolates had large numbers of gene variations (median 50,006, IQR = 26,967–50,600) compared to Australian isolates (median 26,317, IQR = 25,681–33,780). There were a larger number of mutations in the *mutL* and *uvrD* genes associated with the mismatch repair (MMR) system in Indian isolates, which may result in strains losing their efficacy for DNA repair. The number of gene variations were greater in isolates carrying MMR system genes or *exoU*. In the phylogenetic division, the number of core genes were similar in both groups, but Indian isolates had larger numbers of pan genes (median 6518, IQR = 6040–6935). Clones related to three different sequence types—ST308, ST316, and ST491—were found among Indian isolates. Only one clone, ST233, containing two strains was present in Australian isolates. The most striking differences between Australian and Indian isolates were carriage of *exoU* (that encodes a cytolytic phospholipase) in Indian isolates and *exoS* (that encodes for GTPase activator activity) in Australian isolates, large number of acquired resistance genes, greater changes to MMR genes, and a larger pan genome as well as increased overall genetic variation in the Indian isolates.

Keywords: antibiotic susceptibility; WGS; phylogenetic analysis; DNA mismatch repair system

1. Introduction

Pseudomonas aeruginosa is a ubiquitous bacterium which can cause opportunistic or nosocomial infections in immuno-compromised patients [1]. *P. aeruginosa* commonly causes corneal (keratitis) [2], respiratory, burn and wound infections, and infections related to medical or surgical devices including ventilator-associated pneumonia [3,4]. *P. aeruginosa* corneal infections are usually related to contact lens wear, but other risk factors for keratitis in non-contact lens wearers include ocular trauma, ocular surgery, and prior ocular surface disease [5–8].

The prevalence of multi-drug resistant (MDR) or extensively drug resistant strains of *P. aeruginosa* reduces treatment options, significantly increasing morbidity rates [9]. *P. aeruginosa* is naturally resistant to some antibiotics due to the possession of specific resistance genes such as *catB* that confers

4.1 Introduction

Pseudomonas aeruginosa is a ubiquitous bacterium which can cause opportunistic or nosocomial infections in immuno-compromised patients (Richards et al., 1999). *P. aeruginosa* commonly causes corneal (keratitis) (Abjani et al., 2017), respiratory, burn and wound infections, and infections related to medical or surgical devices, including ventilator-associated pneumonia (Trouillet et al., 2002). *P. aeruginosa* corneal infections are usually related to contact lens wear, but other risk factors for keratitis in non-contact lens wearers include ocular trauma, ocular surgery, and prior ocular surface disease (Hooi and Hooi, 2005, Parmar et al., 2006, Sharma et al., 2006, Green et al., 2008).

The prevalence of multi-drug resistant (MDR) or extensively drug resistant strains of *P. aeruginosa* reduces treatment options, significantly increasing morbidity rates (Chatterjee and Agrawal, 2016). *P. aeruginosa* is naturally resistant to some antibiotics due to the possession of specific resistance genes such as *catB* that confers chloramphenicol resistance and an inducible *ampC* which encodes for a beta-lactamase, that hydrolyses cephalothin and ampicillin, conferring resistance to beta-lactams (Livermore, 1995b). Additionally, the regulation of efflux pumps also contributes towards an elevated resistance to antibiotics (Li et al., 1994). For example, expression of the efflux pump MexAB-OprM contributes towards intrinsic resistance to a broad spectrum of antibiotics (Poole, 2011), whereas the efflux pump MexXY-OprM is involved in the adaptive resistance to aminoglycosides (Hocquet et al., 2003). Other resistance mechanisms in *P. aeruginosa* include the acquisition of transferrable resistance determinants, including those associated with transposons and integrons (Livermore, 2002). Antibiotic resistance of *P. aeruginosa* varies according to the region where the strains have been isolated (Lomholt and Kilian, 2003, Willcox, 2011) presumably due to the prescribing practices, availability of antibiotics, and perhaps their use in animal husbandry. Various epidemiological studies have identified MDR *P. aeruginosa* from different infections and these isolates have acquired different resistance characteristics. For example, aminoglycoside resistance (Poonsuk et al., 2013) and ciprofloxacin persistence (Diver et al.,

1991) are found in cystic fibrosis isolates of *P. aeruginosa.* Some of these MDR strains are clonal and such clonal strains are often the predominant global clinical MDR isolates (Guzvinec et al., 2014) which spread resistance characteristics into the wider population enabling clonal lineages to expand with time.

ExoU has been associated with virulence of *P. aeruginosa* at the ocular surface. *ExoU* is a phospholipase that causes mammalian cell death (Phillips et al., 2003) and *exoU* possession is common in strains isolated from ocular infections (Finck-Barbançon et al., 1997). There is a correlation between carriage of *exoU* and elevated resistance to fluoroquinolones and aminoglycosides (Subedi et al., 2018d). *ExoU* is carried by strains on a genomic island that also contains resistance genes for a range of antibiotics (Maciá et al., 2005).

In addition to the acquisition of resistance genes, bacteria can develop resistance through mutation of genes so that antibiotic targets are modified. Mutation rates are elevated in strains that carry mutations in DNA mismatch repair (MMR) systems and hence such mutator strains will normally carry more mutations than non-mutator strains (Modrich, 1991). In *P. aeruginosa*, the MMR system is composed of *mutS*, *mutL*, and *UvrD* genes (Oliver et al., 2002). Strains of *P. aeruginosa* isolated from the lungs of cystic fibrosis patients have alterations in the DNA MMR system and this has been correlated with multiple antimicrobial resistance (Maciá et al., 2005).

In Australia, there is a tight regulation of prescribing antibiotics, and antibiotics can only be obtained legally with a prescription from a qualified healthcare professional. In India, on the other hand, whilst branded antibiotics exist, other forms such as counterfeit, substandard, and 'spurious' antibiotics have been reported (Alsan et al., 2015), along the less strict antibiotic prescription regulation, making surveillance and regulation difficult (Sánchez and Sivaraman, 2010). While the antibiotic consumption per person in Australia and India in 2010 was approximately similar, there was a more rapid increase between 2000 and 2010 in India (Van Boeckel et al., 2014). These differences may affect antibiotic resistance development.

The aim of the current study was to compare the phenotypic resistance and genetic characteristics associated with resistance between strains isolated from Australia and India to

better understand the underlying factors that may lead to an increased antibiotic resistance in *P. aeruginosa* strains associated with ocular infection.

4.2 Materials and Methods

4.2.1 P. aeruginosa strains and susceptibility testing

Twenty-six *P. aeruginosa* keratitis isolates, eight isolated in Australia from 2004 to 2006, six from 2018 and 2019 (total 14 Australian isolates), and twelve isolated in India between 2017 and 2018, were included in this study. These isolates were selected from a larger collection of strains based on their antibiotic susceptibilities (those phenotypically resistant to multiple antibiotics, some resistant to one or multiple antibiotics, and some which were sensitive to all antibiotics). The susceptibilities of Australian strains (2004–2006) included in this study are reported in chapter 2 (Khan et al., 2020). Strains were selected after comparing their susceptibilities to antibiotics that are used to treat ocular infections. For genetic comparisons, the data of 34 *P. aeruginosa* isolates from eyes and other sources were also included. The general characteristics of these isolates are described in Supplementary Table 4. The genomes of these isolates were downloaded from the NCBI database and reannotated for this study using the same parameters as of the isolates of this study to avoid any bias in results.

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of various antibiotics which are commonly used to treat *P. aeruginosa* keratitis (Willcox, 2011) were assessed for the isolates (selected from chapter 2 &3) using the broth microdilution method in 96-well plates following the Clinical and Laboratory Standard Institute guidelines (Patel et al., 2014). The antibiotics tested were ciprofloxacin, levofloxacin, gentamicin, ceftazidime (Sigma-Aldrich, St. Louis, MO, USA), polymyxin B (Sigma-Aldrich, Vandtårnsvej, Søborg, Denmark), tobramycin, piperacillin (Cayman Chemical Company, Ann Arbor, Michigan, USA), and imipenem (LKT Laboratories Inc, St Paul, MN, USA). The susceptibility results were interpreted using the EUCAST v10 (EUCAST, 2020) and CLSI 2014 (Patel et al., 2014) breakpoints.

4.2.2 Genomic sequencing

DNeasy Blood and Tissue Kits (Qiagen, Hilden, Germany) were used for DNA extraction as per the manufacturer's recommendations. The Nextera XT DNA library preparation kit (Illumina, San Diego, CA, USA) was used to prepare paired-end libraries. All the libraries were multiplexed on one MiSeq run. FastQC version 0.117 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc) was used to assess the quality of sequenced genomes using raw reads. Version 0.38 of Trimmomatic (Bolger et al., 2014) was used for trimming the adapters from the reads following de novo assembly using Spades v3.13.0 (Nurk et al., 2013). Genomes were annotated using Prokka v1.12 (Seemann, 2014). Sequence types were investigated using PubMLST https://pubmlst.org/. Pan genomes of the *P. aeruginosa* isolates were analyzed using Roary v3.11.2 (Page et al., 2015) using PAO1 as a reference, while core genome phylogeny was constructed using Harvest Suite Parsnp v1.2 (Treangen et al., 2014) with strains PAO1, PA7, and PA14 used as reference strains. The output file 'genes_ presence_absence' was used to compare the *P. aeruginosa* isolates. Acquired resistance genes were identified using the online database Resfinder v3.1 (Centre for Genomic Epidemiology, DTU, Denmark) (Zankari et al., 2012). Integron Finder v1.5.1 was used to identify any integrons present in the isolates. Mutations in the genes were detected using Snippy V2 (Seeman, 2015). Isolates with same sequence types were compared for nucleotide similarities using the MUMmer online web tool (http://jspecies.ribohost.com/jspeciesws/#analyse).

Using the Pseudomonas genome database (http://www.pseudomonas.com) and comprehensive antibiotic resistance database (https://card.mcmaster.ca), 76 genes related to *P. aeruginosa* resistance were selected to investigate the presence of single nucleotide polymorphisms. All isolates were analyzed for the presence of the type III secretion system associated virulence factors *exoU* and *exoS* using the BlastN database.

4.2.3 Statistical analysis

The statistical analysis was performed using GraphPad Prism v8. Medians were calculated with the 'descriptive statistics' option during analysis of variance (ANOVA). P-values less than 0.05 were considered as significant. Fischer's Exact test was used to examine the difference between acquired genes. The Mann-Whitney test was used to analyse the difference between DNA mismatch repair genes in *exoU* and *exoS* isolates and genes variations in the isolates.

4.3 Results

4.3.1 Antibiotic Susceptibility

The minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of the *P. aeruginosa* isolates were determined (Table 4-1). Strains showing intermediate resistance (I) as well as full resistance to antibiotics were categorised as resistant (R) for subsequent analyses. Based on the Centers for Disease Control and Prevention (CDC, Atlanta, GA, USA) definition of multi-drug resistance as "an isolate that is resistant to at least one antibiotic in three or more drug classes", isolates 198, 202, 216, 217, 218, 219, 220 and 221 were deemed to be multi-drug resistant. Australian isolates 223, 224, 225, 227, 233, 235 were also resistant to three antibiotics but these antibiotics were not of different classes. Isolates 176, 193 and 206 were sensitive to all antibiotics, but all other isolates were resistant to at least one antibiotic. Overall, Indian isolates were more resistant to antibiotics compared to Australian isolates. In Australian isolates (n=14), 78% were resistant to imipenem, 57% to ceftazidime, 50% to ciprofloxacin, 21% to piperacillin, 14% to levofloxacin, 7% to tobramycin. No resistance was found to gentamicin or polymyxin. In contrast, in Indian isolates (n=12) 75% were resistant to ciprofloxacin, 58% to imipenem, 50% to levofloxacin, tobramycin, and ceftazidime, 41% to piperacillin, 40% to gentamicin, and 25% to polymyxin.

Table 4-1 MIC and MBC of antibiotics to *P. aeruginosa* keratitis isolates.

Strain number	Fluoroquinolones*		Aminoglycosides		Beta-lactams			Polypeptide
	2nd generation	3rd generation			Penicillin 4th generation	Carba-penem	Cephalosporin 3rd generation	
	Cipro µg/ml ≤1, 2, ≥4#	Levo µg/ml ≤2, 4, ≥8	Genta µg/ml ≤4, 8, ≥16	Tobra µg/ml ≤4, 8, ≥16	Pipera µg/ml ≤16	Imi µg/ml ≤2, 4, ≥8	Ceftaz µg/ml ≤8, 16, ≥32	PMB µg/ml ≤2, 4, ≥8
	MIC/ MBC	MIC/ MBC	MIC/ MBC	MIC/ MBC	MIC/ MBC	MIC/ MBC	MIC/ MBC	MIC/ MBC
123	1/1	1/1	0.25/0.5	4/4	8/16	**4(I)/8**	2/2	**1280(R)/1280**
126	0.5/1	0.5/1	0.5/1	0.25/0.5	8/16	**8(R)/16**	**128(R)/256**	1/1
127	1/2	0.25/1	2/4	**32(R)/128**	4/16	**4(I)/8**	**128(R)/256**	0.5/1
162	0.5/1	0.5/1	0.25/0.5	0.25/1	8/8	**4(I)/4**	2/4	0.25/0.5
169	**2(I)/4**	0.25/0.5	0.25/0.5	0.25/0.5	4/8	2/4	1/2	0.25/0.25
176	0.5/1	0.25/0.5	0.25/0.5	0.25/0.5	4/8	2/8	2/4	0.25/0.5
181	1/4	0.25/0.5	0.25/0.5	0.25/0.5	**32(R)/64**	**4(I)/8**	**16(I)/32**	0.5/1
182	1/2	0.25/0.5	0.25/0.5	0.25/0.5	4/8	**8(R)/16**	1/2	0.25/0.5
223	**64(R)/128**	1/2	0.5/1	0.5/1	**160(R)/320**	1/2	**16(I)/32**	2/4

Strain number	Fluoroquinolones		Aminoglycosides		Beta lactams			Polypeptide
	2nd generation	3rd generation			Penicillin 4th generation	Carba-penem	Cephalosporin 3rd generation	
	Cipro µg/ml ≤1, 2, ≥4#	Levo µg/ml ≤2, 4, ≥8	Genta µg/ml ≤4, 8, ≥16	Tobra µg/ml ≤4, 8, ≥16	Pipera µg/ml ≤16	Imi µg/ml ≤2, 4, ≥8	Ceftaz µg/ml ≤8, 16, ≥32	PMB µg/ml ≤2, 4, ≥8
	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC
224	**16(R)/32**	1/2	0.25/0.5	0.25/0.5	8/16	**64(R)/128**	**16(I)/32**	1/2
225	**64(R)/128**	**16(R)/32**	0.5/2	1/2	16/32	**64(R)/128**	8/16	0.25/0.5
227	**64(R)/128**	**64(R)/128**	0.5/1	0.25/1	16/32	**16(R)/32**	**16(I)/32**	0.25/0.5
233	**8(R)/16**	1/2	1/2	105/1	16/32	**4(I)/8**	**160(I)/320**	0.5/1
235	**16(R)/32**	0.5/1	2/4	0.5/1	**64(R)/128**	**4(I)/8**	**64(I)/128**	0.25
188	**2(I)/4**	1/2	0.5/1	**32(R)/64**	16/64	0.5/1	4/8	2/4
189	0.25/1	1/2	0.25/0.5	**16(R)/32**	4/8	1/ 2	8/16	2/4
193	1/1	0.25/1	0.250.25	0.25/0.5	4/8	2/4	2/2	0.5/1
198	**1280(R)/2560**	**320(R)/1280**	**2560(R)/5120**	**16(R)/16**	8/8	1/2	8/8	**4(I)/4**
202	**640(R)/1280**	**320(R)/640**	**8 (I)/32**	**320(R)/640**	16/64	**8(R)/32**	8/32	0.25/0.25
206	1/1	0.5/0.5	1/1	0.25/0.5	8/8	2/4	2/4	0.25/0.5

Strain number	Fluoroquinolones		Aminoglycosides		Beta-lactams			Polypeptide
	2nd generation	3rd generation			Penicillin 4th generation	Carba-penem	Cephalosporin 3rd generation	
	Cipro µg/ml ≤1, 2, ≥4#	Levo µg/ml ≤2, 4, ≥8	Genta µg/ml ≤4, 8, ≥16	Tobra µg/ml ≤4, 8, ≥16	Pipera µg/ml ≤16	Imi µg/ml ≤2, 4, ≥8	Ceftaz µg/ml ≤8, 16, ≥32	PMB µg/ml ≤2, 4, ≥8
	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC
216	**64(R)/128**	**4 (I)/8**	1/2	0.5/2	**160(R)/320**	**16(R)/32**	**64(R)/128**	**64(R)/128**
217	**64(R)/128**	**32(R)/64**	1/2	1/2	**64(R)/128**	**8(R)/16**	**32(R)/64**	0.25/1
218	**8(R)/16**	1/2	0.5/1	0.5/1	**160(R)/320**	**8(R)/16**	**64(R)/128**	1/4
219	**≥5120(R)/≥5120**	**640(R)/1280**	**≥5120(R) ≥5120**	**1280(R)/2560**	**2560(R)/5120**	**40(R)/80**	**16(I)/32**	0.25/1
220	**2(I)/4**	0.25/0.5	0.5/1	0.5/1	0.25/0.5	**8(R)/16**	**160(R)/320**	8(R)/16
221	**2560(R)/5120**	**2560(R)/5120**	**2560(R)/5120**	**2560(R)/5120**	**64(R)/128**	**16(R)/32**	**32(R)/64**	0.5/1

Data for Australian isolates (shaded in grey) 123-182 is from a previously published study (Khan et al., 2020). Strains 188-221 were Indian keratitis isolates. R= resistant, I= intermediate resistance. * Cipro= Ciprofloxacin, Levo= Levofloxacin, Genta= Gentamicin, Tobra= Tobramycin, Pipera= Piperacillin, Imi= Imipenem, Ceftaz= Ceftazidime, PMB= Polymyxin B; #=Antibiotic breakpoints for sensitive, intermediate, resistant classifications.

4.3.2 General features of the genomes

The isolates after *de novo* assembly consisted of different numbers of contigs ranging from 50 for isolate 169 to 1917 for isolate 216. The average number of coding sequences was 6162 ± 359.2 for the Australian isolates and 6544 ± 889 for the Indian isolates. Isolates had an average of 66.1% G + C content. The tRNA copy number for the isolates ranged from 57 to 86 (which may vary between studies that use different assembly methods). The general features of the isolates are provided in Supplementary Table 1.

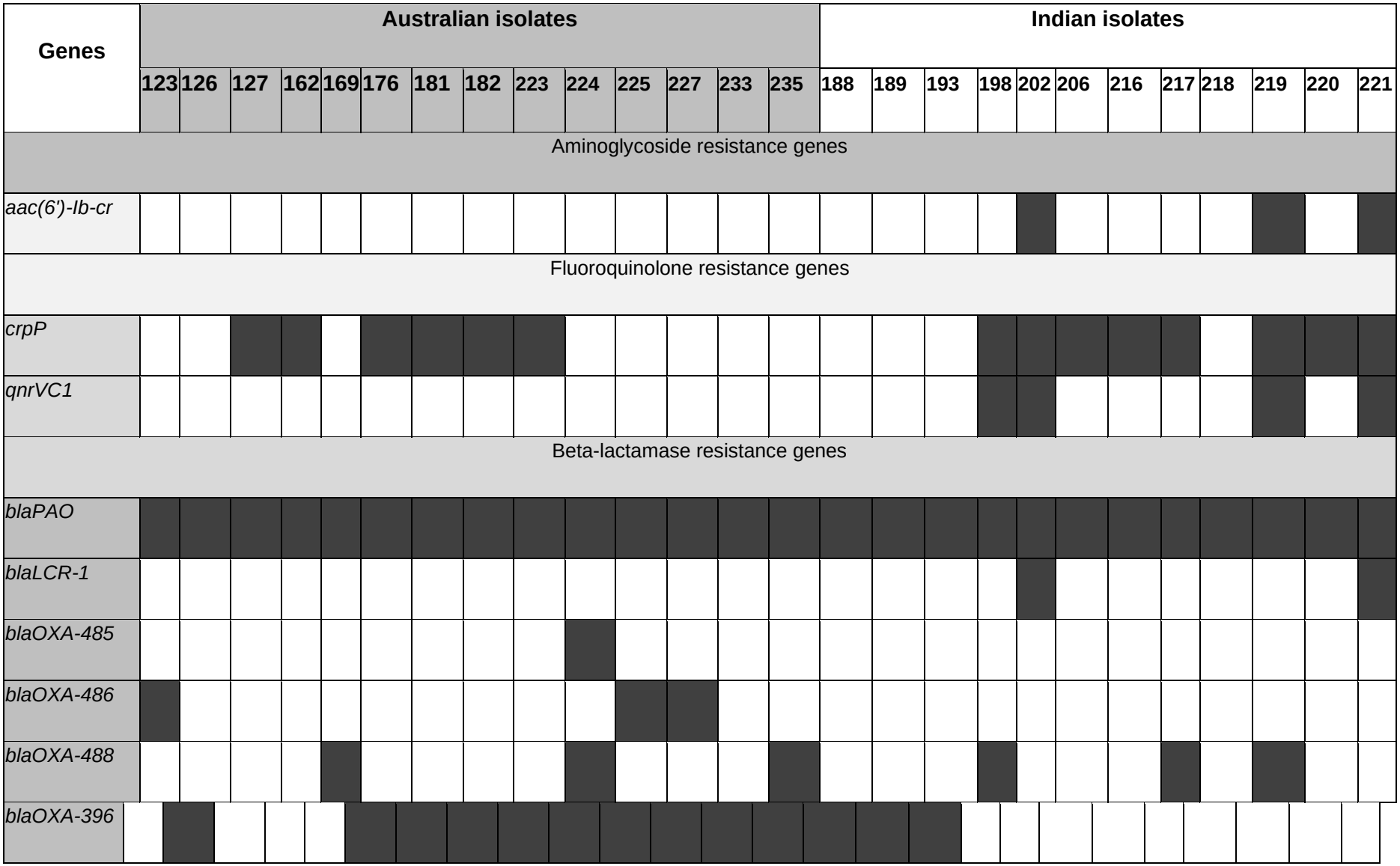
4.3.3 Acquired resistance genes

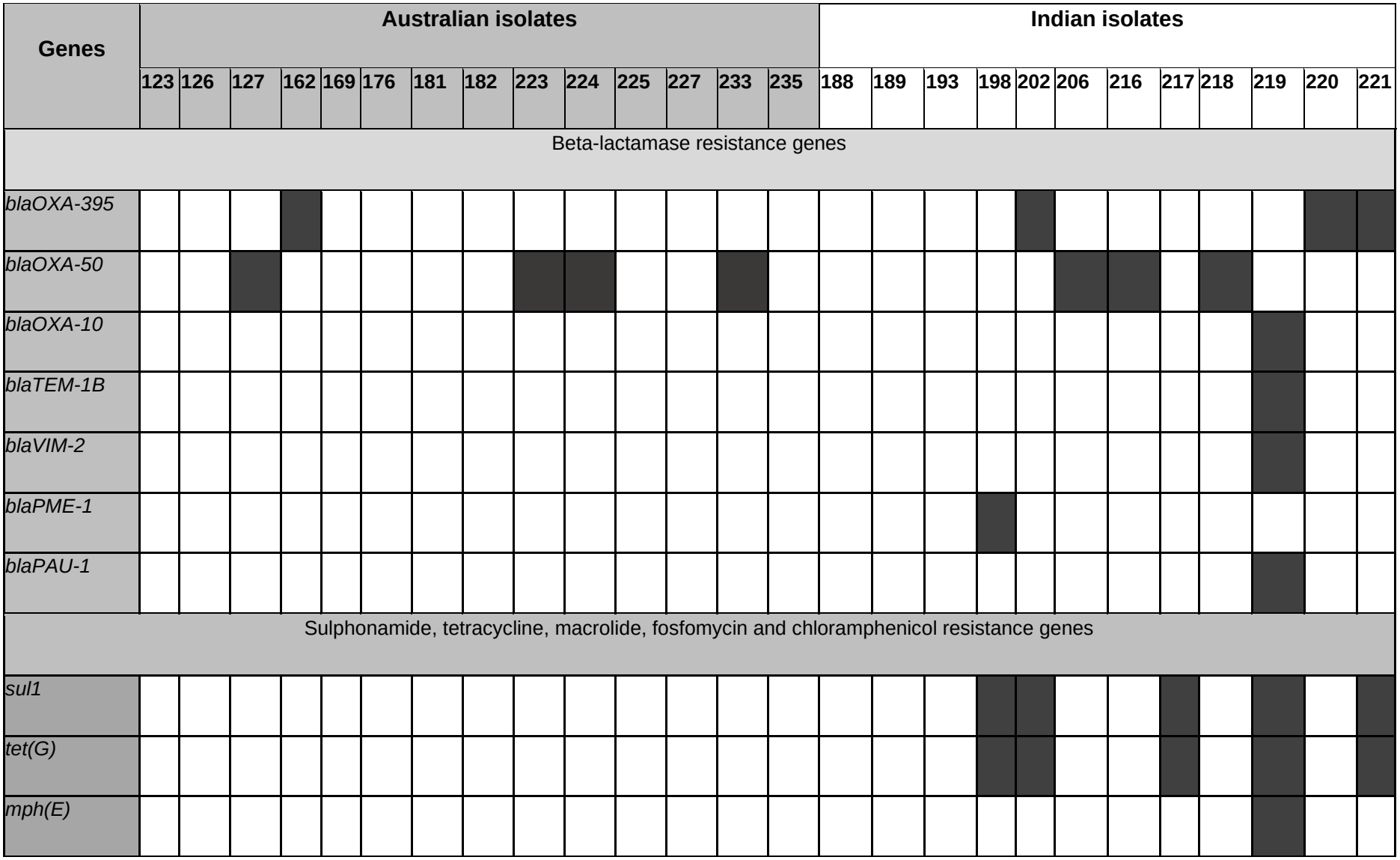
P. aeruginosa isolates were examined for horizontally acquired antibiotic resistance genes (Table 4-2) using the Resfinder database. Altogether, 33 different acquired antibiotic resistance genes for various classes of antibiotics including aminoglycosides, fluoroquinolones, beta-lactams were found in these isolates.

An aminoglycoside resistance gene (*aph(3')-IIb*), a beta-lactam resistance gene (*blaPAO*), a fosfomycin resistance gene (*fosA*), and a chloramphenicol resistance gene (*catB7*) were common to all isolates. The Australian isolates (123–182) had acquired only eight resistance genes, while the Indian isolates (188–221) had acquired 26 different resistance genes (Table 2). Five Indian isolates (198, 202, 217, 219, and 221, with large pan genomes) acquired the largest number of resistance genes. Of these five isolates, the pairs 198/219 and 202/221 had the most similar resistance gene profiles and each member of the pair were of the same sequence type, ST308 and ST316 respectively. As acquired resistance genes may be carried on integrons, the genomes of the *P. aeruginosa* isolates were analyzed for integrons using Integron Finder version 1.5.1. *qnrCV1* was associated with a class 1 integron in isolates 202 and 221 and a Tn3 transposon in isolates 198 and 219.

Table 4-2 Acquired resistance genes in *P. aeruginosa* isolates from India and Australia.

Genes	Australian isolates														Indian isolates										
	123	126	127	162	169	176	181	182	223	224	225	227	233	235	188	189	193	198	202	206	216	217	218	219	220	221
Aminoglycoside resistance genes																										
aph(3')-IIb	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
aph(6)- Id																		■	■			■		■	■	■
rmtD2																		■								■
rmtB																								■		
aph(3')-VI																		■								
aph(3')-IIb																						■				
aph(3'')-Ib							■											■	■			■		■	■	■
aac(6')-Ib3																			■							■
aac(3)-IId																			■							■
aadA1																			■					■		■


Genes
Australian isolates
Indian isolates
123 126 127 162 169 176 181 182 223 224 225 227 233 235
188 189 193 198 202 206 216 217 218 219 220 221
Aminoglycoside resistance genes
aac(6')-Ib-cr
Fluoroquinolone resistance genes
crpP
qnrVC1
Beta-lactamase resistance genes
blaPAO
blaLCR-1
blaOXA-485
blaOXA-486
blaOXA-488
blaOXA-396


Genes
Australian isolates
Indian isolates
123 126 127 162 169 176 181 182 223 224 225 227 233 235
188 189 193 198 202 206 216 217 218 219 220 221
Beta-lactamase resistance genes
blaOXA-395
blaOXA-50
blaOXA-10
blaTEM-1B
blaVIM-2
blaPME-1
blaPAU-1
Sulphonamide, tetracycline, macrolide, fosfomycin and chloramphenicol resistance genes
sul1
tet(G)
mph(E)

Genes	Australian isolates														Indian isolates											
	123	126	127	162	169	176	181	182	223	224	225	227	233	235	188	189	193	198	202	206	216	217	218	219	220	221
Sulphonamide, tetracycline, macrolide, fosfomycin and chloramphenicol resistance genes																										
mph(A)																										
*msr(E)**																										
fosA																										
catB																										

* msr(E) encodes macrolide and lincosamide resistance.

Several types of non-synonymous variations were found in the core genome of the keratitis *P. aeruginosa* isolates when compared with the reference genome of PAO1 (Table 4-3). These non-synonymous mutations included single nucleotide polymorphisms (SNPs), multi-nucleotide polymorphisms (MNPs), deletions, insertions, and complex variations (where more than one change occurred at one specific location compared to the reference strain). The total variations in the isolates ranged from 76,080 in isolate 206 to 22,536 in isolate 181. There was a median of 26,317 (IQR = 25,681–33,780) variations in the genomes of Australian isolates and a median of 50,006 (IQR = 26,967–50,600) in the Indian isolates ($p = 0.09$). Based on the grouping of core genome phylogeny, isolates within group 2 (198, 202, 219, 220, 221, 233) had the most variations. Isolate 206, which had a unique sequence type and was placed in a separate group by pan genome analysis, had an exceptionally high number of variations (76,080) and SNPs (67,271).

Non-synonymous mutations were assessed in resistance genes of the *P. aeruginosa* isolates (Supplementary Table 2). There were no large differences in the mutations in resistance genes of any of the isolates except the antibiotic efflux-related genes *opmH* and *rosC*. *opmH* had ≥ 9 mutations in all isolates except 127, 162,169, 202, 218, 220, and 221 (mostly isolates of group 2 of core and pan genome phylogenies except 127 and 218). *rosC* had 20 non-synonymous mutations including insertions/deletions in isolate 206, 11 in 233, and ≥ 5 mutations in isolates 162, 169, 176, 202, 216, 217, 219, 220, and 221 (mostly isolates of group 2 of core and pan genome phylogenies except 176, 216), but ≤ 3 mutations in isolates 123, 126, 123, 181, 182, 188, 189, 193, 198, and 218 (mostly isolates of group 1 of core and pan genome phylogenies except 198). Mutations in genes encoding efflux pumps were also found, including *mexX, mexT, mexD, mexM,* and *mexY*, although there was no significant difference between the two groups in the possession of mutations in these genes. All other mutations in the genes were random without any association to sequence type, phylogeny, or susceptibility to antibiotics.

Table 4-3 Frequency of different types of variation in the genes of *P. aeruginosa* isolates.

***P. aeruginosa* isolates**	**Total variants**	**complex**	**insertions**	**deletions**	**MNP**	**SNP**
123	28279	1593	187	163	398	25938
126	26258	1416	164	159	355	24164
127	25760	1362	163	176	391	23668
162	50999	3481	281	257	951	46029
169	50283	3359	269	245	922	45488
176	26065	1372	168	161	342	24022
181	22536	1063	162	133	283	20895
182	25684	1359	172	180	368	23605
223	25672	1358	167	176	402	23568
224	26376	1435	163	165	353	24260
225	28070	1566	167	156	385	25796
227	28000	1560	162	154	370	25754
233	52392	3590	285	263	956	47298
235	24919	1349	162	171	354	22883
188	25833	1435	164	154	351	23729

P. aeruginosa isolates	Total variants	complex	insertions	deletions	MNP	SNP
189	25910	1458	165	155	365	23767
193	26567	1445	180	147	389	24406
198	50631	3503	280	236	945	45667
202	49981	3461	257	236	902	45125
206	76180	6449	336	371	1653	67271
216	28166	1548	183	164	433	25838
217	51119	3575	290	226	944	46084
218	29161	1676	182	181	430	26692
219	50507	3484	273	237	925	45588
220	50180	3452	267	234	894	45332
221	50030	3477	260	237	906	45150

SNP= single nucleotide polymorphism; MNP= multi-nucleotide polymorphism. Isolate numbers highlighted in grey are from Australia.

4.3.4 Possession of *exoU* and mutations in the DNA mismatch repair system

ExoU was present in the genomes of all isolates in group 2 (core and pan genome phylogenetic group) as well as isolates 123 and 127 (Table 4-4). All other isolates possessed *exoS* with the exception of isolate 126 which possessed both *exoU* and *exoS*. To address differences in the numbers of sequence variants between the isolates, the genes involved in the DNA mismatch repair (MMR) system *mutS* (that encodes a protein which binds to errors in DNA), *mutL* (that encodes a protein that works in synergy with MutS and activates UvrD), and *uvrD* (a DNA helicase active in DNA replication) were examined. The mutations in the MMR system included SNPs, indels, and complex variants. The number of mutations in *mutL* ranged from 1 to 2 and mutations in *mutS* (which ranged between 0 and 2) were found in seven isolates (Table 4-4). In *uvrD*, the number of mutations ranged between 0 and 5 (Table 4-4). *exoU* containing isolates possessed a median of two (IQR = 1–3) mutations in *mutL*, zero (IQR = 0–2) mutations in *mutS*, and four (IQR = 2–5) mutations in *uvrD*, whereas *exoS* containing isolates possessed a median of zero (IQR = 0–1) mutations in *mutL*, zero (IQR = 0–1) mutations in *mutS*, and two (IQR = 0–2) median mutations in *uvrD*. There were significant differences in the number of *mutL* (p = 0.0021) and *uvrD* (p = 0.02) mutations in *exoS* and *exoU* isolates but not with *mutS* (p = 0.3). Isolate 206, an *exoS* strain and an outlier in the core genome analysis, had one mutation in *mutL*. Details of mutations occurring in nucleotide and respective proteins are provided in Supplementary Table 3.

Table 4-4 Possession of exoU and exoS and number and type of non-synonymous mutations in the mismatch repair system genes in *P. aeruginosa* isolates

***P. aeruginosa* isolates**	**Type III secretion system genes**	***mutL***	***mutS***	***uvrD***
123	*exoU*	1 SNP	0	1 complex
126	*exoU/exoS*	0	0	0
127	*exoU*	0	1 MNP	1 MNP, 1 complex
162	*exoU*	1 SNP	0	2 SNP, 1 MNP, 2 complex
169	*exoU*	1 SNP	1 complex	2 SNP, 2 MNP, 1 complex
176	*exoS*	1 SNP	0	1 SNP
181	*exoS*	0	0	0
182	*exoS*	0	0	1 MNP 1complex
223	*exoS*	0	1 SNP	1 MNP, 1 complex
224	*exoS*	1 SNP	0	1 MNP, 1 complex
225	*exoS*	0	0	2 SNP, 2 MNP, 1 complex
227	*exoS*	0	0	2 SNP, 2 MNP, 1 complex
233	*exoU*	0	0	1 MNP, 1 complex
235	*exoS*	0	0	0
188	*exoS*	0	0	1 MNP, 1 complex
189	*exoS*	1 SNP	0	1 MNP, 1 complex
193	*exoS*	0	0	0
198	*exoU*	2 SNP	0	1 SNP, 3 complexes
202	*exoU*	1 SNP	1 complex	1 SNP, 2 MNP, 2 complex
206	*exoS*	1 MNP	1 complex	0
216	*exoS*	0	0	0
217	*exoU*	2 SNP	1 complex	1 SNP, 2 MNP, 1 complex
218	*exoS*	0	0	0
219	*exoU*	2 SNP	0	1 SNP, 1 MNP, 2 complex
220	*exoU*	1 SNP	1 complex	1 SNP, 2 MNP, 2 complex
221	*exoU*	1 SNP	1 complex	1 SNP, 2 MNP, 2 complex

(SNP = single nucleotide polymorphism, MNP = multinucelotide polymorphism). Isolates shaded in grey indicate Australian isolates.

4.3.5 Sequence type analysis and phylogenetics

All Australian isolates were of different sequence types (ST), except 225 and 227 which belonged to ST233. Among the 12 Indian isolates, one isolate was designated as belonging to a new sequence type, two isolates (198 and 219) belonged to ST308, two others (188 and 189) belonged to ST491, and three isolates (202, 220, and 221) belonged to ST316 (Table 4-5).

Table 4-5 Sequence types of *P. aeruginosa* isolates.

***P. aeruginosa* isolates**	**Sequence types**	**Core genes**	**Shell genes**	**Pan/total genes**
123	ST218	5496	508	6004
126	ST2726	5483	712	6195
127	ST845	5483	938	6421
162	ST298	5439	905	6344
169	ST1027	5456	694	6150
176	ST709	5547	1112	6659
181	ST244	5588	1047	6662
182	ST27	5486	1096	6582
223	ST17	5471	1232	6703
224	ST168	5483	607	6090
225¤	ST233	5515	1338	6853
227¤	ST233	5493	1304	6797
233	NEWST	5440	624	6064
235	ST262	5470	540	6010
188*	ST491	5490	535	6025
189*	ST491	5492	531	6023
193	ST760	5490	594	6084
198†	ST308	5454	1428	6882
202#	ST316	5425	1505	6930
206	NEWST	5331	1084	6415
216	ST1527	5480	1488	6968
217	ST1047	5448	1173	6621
218	ST3083	5513	488	6001
219†	ST308	5451	1796	7247
220#	ST316	5430	948	6378

P. aeruginosa isolates	Sequence types	Core genes	Shell genes	Pan/total genes
221#	ST316	5425	1511	6936
PA7	ST1196	3599	4586	8185
PA14	ST253	5436	790	6226

Grey shading denotes Australian isolates. *, **†, #,** ¤ indicates strains belong to the same sequence types (STs).

The number of core and total or pan (or total) genes were reported from the statistical summary of Roary v3.11.2. The core genomes of the isolates were aligned using PA7 (Accession number NC_009656.1), PA14 (Accession number NC_004863.1), and PAO1 (Accession number NC_002516.1) as reference strains. The eight published genomes of *P. aeruginosa* isolates from eye as well as strains from other sources were also included. The core genes of published isolates are provided in Supplementary Table S4. The isolates were sub-grouped based on the number of core genes; isolates with a similar number of core genes were closely aligned and isolates with the same sequence type were grouped together. The core genomes formed two groups in the phylogenetic tree (Figure 4-1). Isolates in group 1 tended to have a larger number of core genes than isolates in group 2. Isolate 206, PA57, and PA7 were outliers based on core genome phylogeny. The Australian and Indian isolates had a similar number of core genes, whereas the Indian isolates had a larger number of pan genes (10,889) due to the acquisition of shell genes (genes present in two or more strains) (Table 4-5).

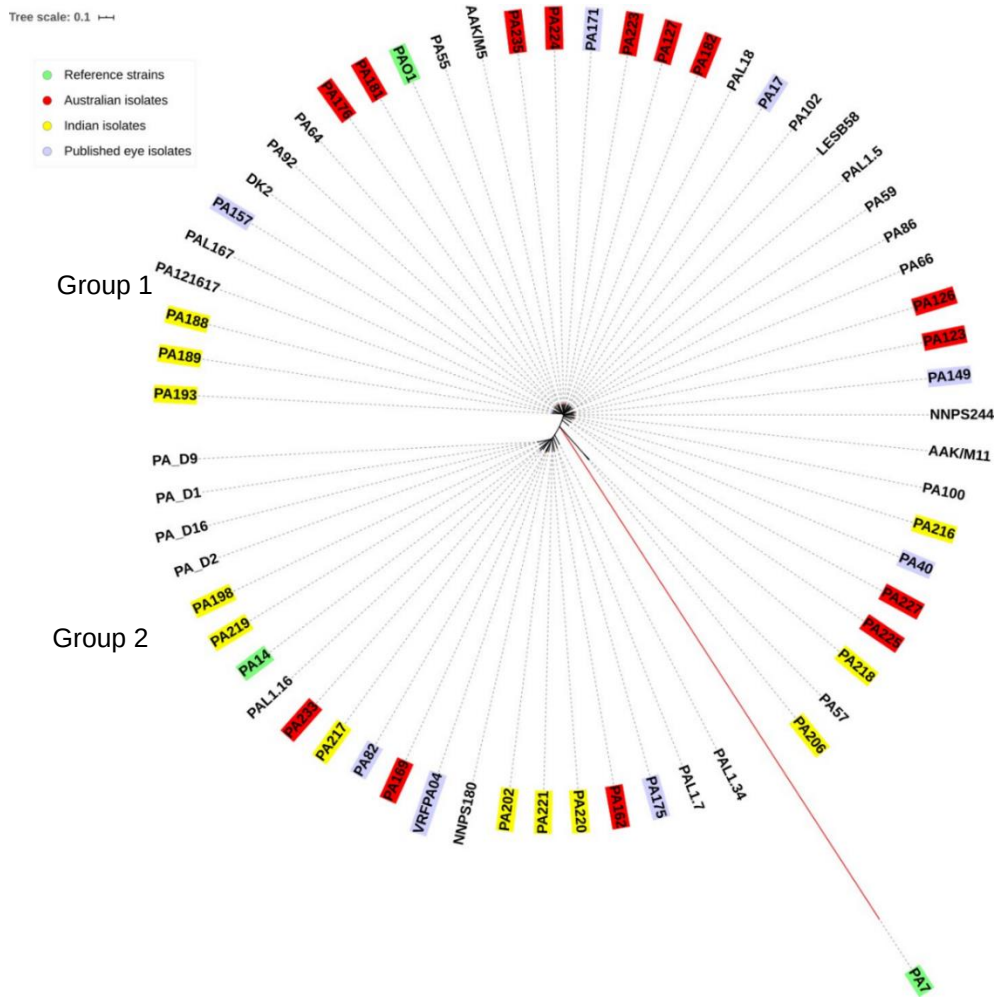


Figure 4-1. Core genome phylogeny of P. aeruginosa isolates using Parsnp.

The phylogenetic relationships of these *P. aeruginosa* isolates were assessed by aligning their pan genome against PAO1 as a reference. The output generated using Roary showing the gene presence or absence in all isolates is provided in Supplementary Figure 1. This again divided the *P. aeruginosa* isolates into two major groups. Six multi-drug resistant Indian isolates (198, 202, 217, 219, 220, 221) and the VRFPA04 isolate (isolated from the cornea) were clustered in one group, which also contained the two Australian isolates 162 and 169. The Indian isolate 216 was categorized in a separate sub-group due to the large number of shell genes and possession of *exoS*.

The second group (group 2 of the pan genome analysis) included most of the Australian (123, 126, 127, 162, 176, 181, 182, 223, 224, 225, 227, 235) and Indian (188, 189, 193, 216 218) isolates along with reference strain PAO1 (Figure 4-2). Overall, the multi-drug resistant Indian

isolates had a large pan genome (total of 10,889 genes obtained from the statistical summary in Roary v3.11.2). The pan genome grouping of isolates was broadly based on the number of pan (or total) genes and possession of either *exoU* or *exoS* in each group, except for the two Australian isolates 123 and 127 which were in group 2 but possessed *exoU*. The other exception to this grouping pattern was isolates 181 and 182 which had large pan genomes and were clustered into group 1 but carried *exoS*.

The isolates of group 2 usually had a large number of pan genes and were *exoU*+. Isolates having similar numbers of pan genes were sub-grouped together. For example, isolate 193 (pan genes = 6084) and 218 (pan genes = 6001) were sub-grouped together. Isolate 218 had a similar number of pan genes to isolate 123 (pan genes = 6001), but isolate 218 possessed *exoS*, while 123 possessed *exoU*, and thus these were not grouped together. Isolates belonging to the same sequence type were also grouped together. The MDR isolates, the isolates with same STs, and isolates with large gene variations were clustered in one pan genome group. The previously published isolates PA_D1, PA_D2, PA_D9, and PA_D16 with the same ST and those with a large number of shell genes were grouped with the MDR isolates of the current study.

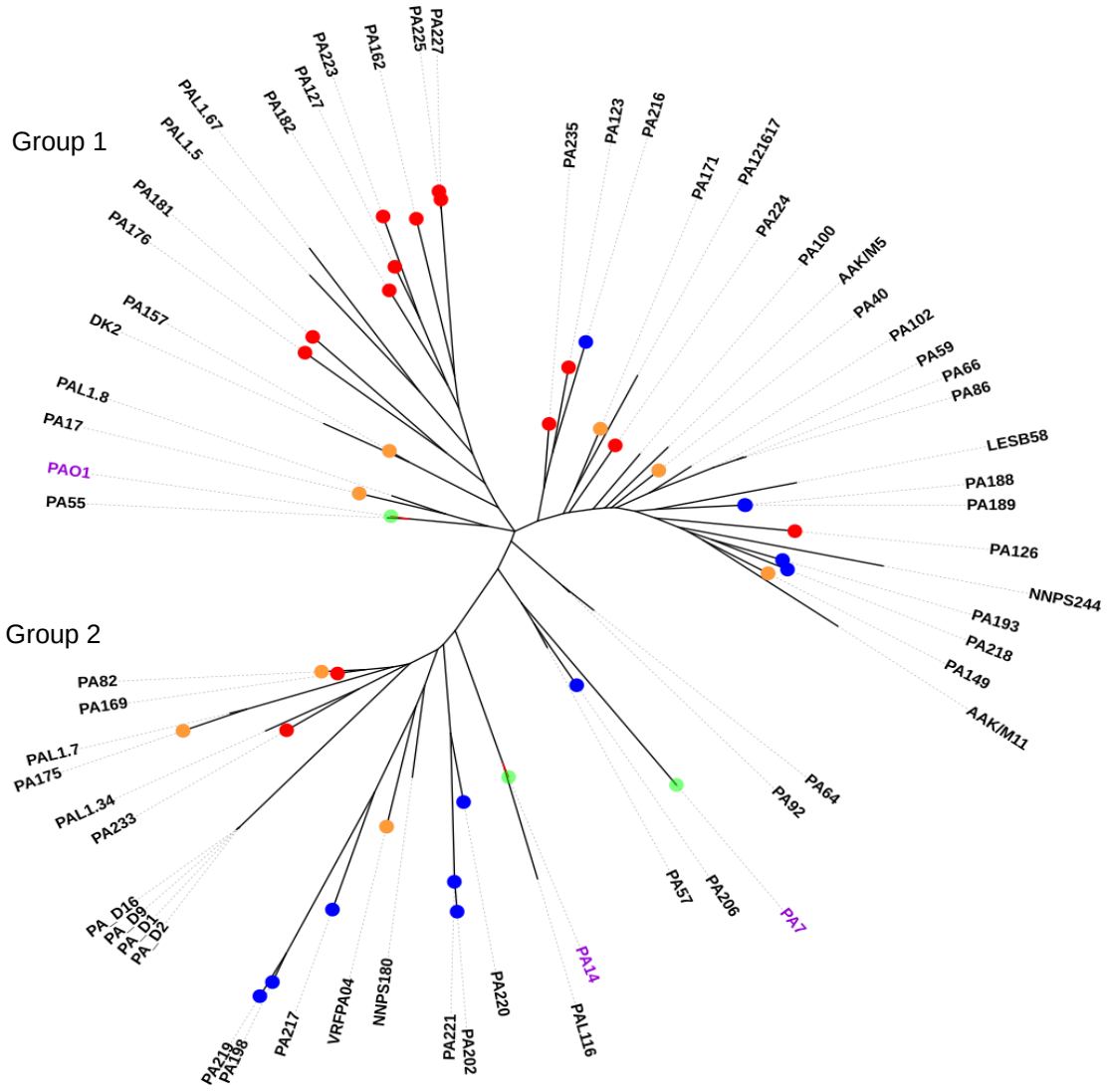


Figure 4-2 Pan genome phylogeny of *P. aeruginosa* isolates.

Branches without colour representation indicate non-ocular isolates. Red indicates Australian, blue represents Indian and orange represents published eye isolates. Green represents reference strains. Purple represents reference strains

4.4 Discussion

This study investigated genomic differences in *P. aeruginosa* keratitis isolates from Australia and India. Phenotypically, more resistance was found in Indian isolates compared to Australian isolates as has been shown in previous study (Subedi et al., 2018b). Unregulated antibiotic use in India has been linked to increased antibiotic resistance (Porter and Grills, 2015). Resistance to antibiotics is problematic even in the treatment of keratitis, where topical application of antibiotics is used. Infection with antibiotic resistant strains results in prolonged infection (Wilhelmus et al., 2003), more severe keratitis (Green et al., 2008), and an increase in the cost of treatment (Keay et al., 2006b, Keay et al., 2008).

Indian *P. aeruginosa* strains harbored more resistance genes compared to Australian isolates, although *aph(3')-IIb, blaPAO1 (fosA)*, and *catB7* were found in all isolates, which was consistent with previous studies (N et al., 2016, Subedi et al., 2018b). *qnrVC1* was found in four Indian isolates but no Australian isolates. This fluoroquinolone resistance gene has not been previously reported in *P. aeruginosa* ocular isolates (N et al., 2016, Subedi et al., 2018b), but it has been reported in burns isolates and has been identified as carried on an integron (Belotti et al., 2015). Similarly, in the current study *qnrVC1* was carried on a class 1 integron in isolates 202 and 221 but integrated into a Tn3 transposon in isolates 198 and 219. This gene has also been isolated from the high risk ST773 clone of *P. aeruginosa* from urine in Hungary (Kocsis et al., 2019). High risk clones are isolates with high mutational rates in resistance genes and those that have acquired a large number of resistance genes. As previously described, resistance to fluoroquinolones in keratitis *P. aeruginosa* isolates was also due to mutations in the quinolone resistance determining regions of *gyrA* and *parC* (Lomholt and Kilian, 2003). Possession of *qnrVC1* and mutations in *gyrA* and *parC* were associated with high levels of fluroquinolone resistance. The possession of large numbers of acquired resistance genes by Indian isolates likely contributed to the higher rates of resistance of these isolates. The Indian isolates 198, 202, 217, 219, 220, and 221 also had a high number of gene variations which is an independent mechanism of resistance.

The aminoglycoside resistance gene *aph(6)-Id* which encodes for streptomycin resistance was found in six Indian isolates, including the four that carried *qnrVC1,* but in no Australian isolates. Previously, *aph(6)-Id* was found in only one Indian ocular isolate from 1997 (Subedi et al., 2018b), but has been found in cystic fibrosis *P. aeruginosa* isolates (Tauch et al., 2003) and has been associated with the transposon Tn5393 on a plasmid in one strain of *P. aeruginosa* (Sundin and Bender, 1996). As streptomycin is no longer used in topical treatment (Sundin and Bender, 1996), this resistance may not be clinically relevant but does suggest environmental selection for the persistence of this gene.

The total number of gene variants found in the Indian isolates 198, 202, 219, and 221 were greater than Australian isolates. However, there were a small number of SNPs found in the genes associated with resistance for these isolates. The Indian isolate 206 (NEWST) had a high number of SNPs in antibiotic resistance genes *mexC, mexD, mexM, mexX, mexS, opmE, mexP, mexK, oprJ, ampC, rosC,* and *mprF*. There was no difference in the mutations of other *mex* genes including *mexX, mexT, mexD, mexM*, and *mexY* between Australian and Indian isolates. Given that most isolates from both countries, whether they were sensitive or resistant, had a similar number of mutations in the resistance genes, it is likely that the resistance to antibiotics was related to the possession of acquired resistance genes rather than mutations in chromosomal genes.

In the Australian isolates, four out of the eight isolates (50%) carried *exoU*, while one isolate was both *exoU+/exoS+* and three (38%) were *exoS+*. In Indian isolates, 50% carried *exoU* and 50% carried *exoS*. A previous study has also shown an equal ratio of both genes (Zhu et al., 2006) in keratitis isolates. The possession of the *exoU* genotype in *P. aeruginosa* ocular isolates has been related to elevated resistance to disinfectants (Lakkis and Fleiszig, 2001), fluoroquinolones (Borkar et al., 2014), and multiple antibiotics (Zhu et al., 2006). Furthermore, one study reported worst clinical outcomes and more resistance by *exoU* carrying isolates (Borkar et al., 2014). The findings in this study were consistent with these results where *exoU+* isolates 198, 202, 217, 219, 220, 221, and 233 were also MDR.

The DNA mismatch repair system (MMR) in *P. aeruginosa* is based on the protein trimer MutS-MutL-UvrD and functions to correct errors and preserve the integrity of the genome (Modrich, 1991, Tiraby and Fox, 1973). The *mutH* component of MMR, which is important in other gram-negative bacteria, such as *E. coli* (Acharya et al., 2003), has not been found previously in *P. aeruginosa* (Oliver et al., 2002) and was not present in the isolates of the current study. Mutations in *mutS*, *mutL* and *uvrD* can reduce the ability of the bacterium to repair DNA lesions (Rayssiguier et al., 1989). Strong mutator strains have defects in their MMR system and mutations in *mutS* predominate (Chopra et al., 2003). Mutations in the MMR can cause hypermutation in the isolates. In cystic fibrosis, hypermutations were a key factor in the development of MDR resistant *P. aeruginosa* strains (Maciá et al., 2005). Similar findings were found in this study where isolates 198, 202, 206, 219, and 221 had mutations in the MMR genes, and these isolates had an overall larger variation in their genomes. In the current study, isolates had more mutations in *mutL* and *uvrD,* suggesting the strains may not be strong mutators (which is usually associated with mutation in *mutS*), but nevertheless can undergo uncorrected genetic changes. Indeed, the *P. aeruginosa* isolates in the current study which had mutations in *mutL* and *uvrD* had greater numbers of SNPs, insertions and deletions, acquired genes, and had large pan genomes. Among these isolates, 198, 202, 219, and 221 possessed either the transposon Tn3 or class 1 integrons which carried the acquired genes. This also might be due to mutated MMR, as mutations in MMR genes increase the chances of horizontal gene transfer in mutator isolates (Chopra et al., 2003). The number of mutations in MMR was greater in *exoU* possessing isolates with large gene variations. *exoU* is carried on genomic islands (Sato et al., 2003, Kulasekara et al., 2006) and these *exoU* carrying isolates had larger pan genomes with possession of mobile genetic elements. Therefore, the isolates with the mutated MMR systems may have a greater ability of strains to accumulate gene variations and the acquisition of *exoU*. Isolate 206, on the other hand, possessed *exoS* and was not MDR but possessed a large number of SNPs and a large pan genome with one mutation in each of *mutS* and *mutL*. Further studies are required to understand the influence of the MMR system on genomic changes in *P. aeruginosa*.

Analysis of the sequence types of the *P. aeruginosa* ocular isolates revealed the presence of three clones, two in the Indian and one in the Australian isolates. The isolates with the same STs had mostly the same phenotypic and genotypic features. The exception to this was isolate 220 that had acquired fewer resistance genes compared to the other two isolates 202 and 221 of ST316 isolated at the same time. Previously, five ocular *P. aeruginosa* strains from India isolated in 1997 were of sequence type ST308 (Subedi et al., 2018b). The two isolates of ST308 in the current study, isolated in 2017 and 2018, had acquired more resistance genes compared to isolates from 1997 (Subedi et al., 2018b). This indicates that the clonal isolates have continued to evolve over this period, although the specific selection factors driving those changes are yet to be elucidated. None of the isolates were collected from the same patient. The majority of the isolates with the same STs grouped in the same phylogeny including previously published isolates (PA_D1, PA_D2, PA_D9, P_D16) with ST1971).

Core and pan genome phylogenies of the isolates produced two almost identical groups, which was in agreement with previously published studies (Freschi et al., 2015b, Subedi et al., 2018b). Both phylogenies included isolates from either Australia or India, but those in group 2 tended to be the MDR Indian isolates and possessed higher numbers of antibiotic resistance genes. About 65% of all ocular isolates grouped together which indicated less diversity in the ocular *P. aeruginosa* isolates (Winstanley et al., 2009). The grouping of MDR strains from this study with PA14 along with a MDR ocular isolate VRFPA04 (N et al., 2016) in both core genome and pan genome analysis, and the grouping of the sensitive strains with PAO1 along with the commonly studied cystic fibrosis isolates DK2 and LESB58, was similar to a previous study examining older isolates from India and Australia (Subedi et al., 2018b). Isolate 206, which had the smallest number of core genes and was of a new sequence type, was an outlier in the core genome phylogeny similar to the taxonomic outlier PA7 (Roy et al., 2010). However, isolate 206 was grouped together with other isolates in the pan genome because it had acquired a large number of genes. Acquired genes are part of the pan rather than the core genome (Klockgether et al., 2011) and the presence of larger pan genomes in MDR *P. aeruginosa* isolates points towards the acquisition of new genes (Freschi et al., 2019).

Previously, a smaller core genome size of 4910 genes has been reported in ocular *P. aeruginosa* isolates (Subedi et al., 2018b). However, the current study found a core genome similar to *P. aeruginosa* from different sources, comprising 5316–5233 genes (Ozer et al., 2014, Valot et al., 2015). The core genome (which is almost 90% of the total genome) refers to the conserved genes present in a species (Wolfgang et al., 2003) which might differ in each individual strain within that species. Additionally, SNPs can be a result of poor sequencing quality and hence it is important to have a good sequencing depth at those positions to identify them as a mutation rather than sequencing error (Smits, 2019). Grouping of all the isolates including ocular and non-ocular remained the same in both core and pan genome phylogeny. Indian isolates and Australian isolates were clearly distinct in carrying type III secretion system related *exoU* and *exoS*. There was an association in the isolates between acquired resistance genes and a large number of pan genes. Indian isolates were more resistant to antibiotics compared to Australian isolates. Additionally, isolates of *P. aeruginosa* from ocular infection had a large number of genetic variations (mutations) and mutated mismatch repair system. However, further study using a larger sample size will give a clearer idea of these differences.

Supplementary information

Supplementary Table 4-6 Details of the *Pseudomonas aeruginosa* isolates used in the current study.

P. aeruginosa Isolates	Source	Region	Year of isolation	% GC	No. of contigs	Total sequence length (bp)	CDSs (total)	tRNAs
123	Cornea	Australia	2005	66.4	86	6,357,971	5858	57
126	Cornea	Australia	2005	66.4	81	6,470,807	6007	57
127	Cornea	Australia	2005	66.4	86	6,357,971	5858	57
162	Cornea	Australia	2006	66.2	87	6,639,257	6131	59
169	Cornea	Australia	2006	66.4	50	6,397,665	5918	57
176	Contact lens	Australia	2004	65.8	305	7,065,610	6672	59
181	Cornea	Australia	2006	65.7	122	7,109,511	6619	63
182	Cornea	Australia	2004	66.1	58	6,836,159	6392	63
223	Cornea	Australia	2018	66.6	108	6,923,150	6408	70
224	Cornea	Australia	2018	66.4	151	6,418,520	5811	67
225	Cornea	Australia	2018	65.8	294	7,287,087	6607	68
227	Cornea	Australia	2018	65.6	102	7,141,983	6529	68
233	Cornea	Australia	2019	66.3	92	6,349,374	5745	65
235	Cornea	Australia	2019	66.1	56	6,279,891	5719	68
188	Cornea	India	2017	66.5	56	6,329,075	5818	58
189	Cornea	India	2017	66.5	59	6,329,723	5820	58
193	Cornea	India	2017	66.3	66	6,369,452	5888	61
198	Cornea	India	2017	66	119	7,101,775	6727	59
202	Cornea	India	2017	66	368	7,192,476	6883	61
206	Cornea	India	2017	66.4	55	6,535,880	6047	58
216	Cornea	India	2018	66.1	1917	8,328,371	8943	86
217	Cornea	India	2018	66.1	132	6,886,566	6482	58
218	Cornea	India	2018	66.4	77	6,375,153	5840	58
219	Cornea	India	2018	65.9	166	7,456,853	7112	59
220	Cornea	India	2018	66.3	90	6,653,669	6144	57
221	Cornea	India	2018	65.6	294	7,205,091	6829	59

Supplementary Table 4-7 Gene variations of resistance genes in *Pseudomonas aeruginosa* isolates.

Gene name	Mechanism	*Pseudomonas aeruginosa*																									
		Number of Single Nucleotide polymorphisms																									
		123	126	127	162	169	176	181	182	223	224	225	227	233	235	188	189	193	198	202	206	216	217	218	219	220	221
triA	Antibiotic efflux	1	1	1	2	2	1	0	0	1	0	0	0	3	0	0	0	3	5	3	1	1	4	0	5	3	3
triB		0	0	1	0	0	1	1	0	1	0	0	0	1	1	1	1	1	0	0	1	2	0	0	0	0	0
triC		0	0	0	1	1	2	0	0	0	0	0	0	2	0	0	0	0	2	2	0	0	2	0	2	2	3
mexR		0	0	0	1	1	0	0	2	0	1	0	0	1	0	0	0	1	1	1	2	0	1	1	1	1	1
mexA		0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
mexB		0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	1	2	1	1	0	1	0	2	1	1
oprM		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
farB		1	0	0	0	0	1	0	0	0	0	1	1	0	2	1	1	0	1	1	0	0	0	0	1	1	1
lrfA		3	4	4	6	6	5	6	7	3	3	5	4	5	6	3	3	3	8	5	8	3	7	5	8	5	5
lrfA		3	2	3	3	3	3	2	2	3	4	3	3	1	3	2	0	2	2	3	4	4	2	3	2	2	3
mexM		4	4	6	3	3	3	2	5	6	6	6	6	3	3	6	6	5	4	3	8	4	4	4	4	3	3
mdtC		3	3	3	1	2	1	0	2	3	2	3	3	1	3	3	3	4	2	1	3	2	3	0	2	1	1
cysB		0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0
mexY		2	1	1	5	2	2	2	1	1	3	1	1	4	2	3	3	3	5	2	4	1	2	1	5	2	2
mexX		3	3	3	4	3	3	3	3	3	3	2	2	4	3	2	2	3	4	4	7	3	4	3	4	4	4
macA		1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1
macB		1	2	1	3	1	2	1	1	1	1	1	1	2	1	2	2	2	1	2	2	2	0	2	1	2	2
opmQ		3	3	1	4	4	1	2	1	1	1	3	3	3	4	1	1	2	5	2	4	2	4	3	5	2	2
mexS		1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	2	1	7	2	1	1	2	1	1
mexE		0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	1
mexF		0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	1
mexT		2	2	2	2	1	3	3	2	3	2	1	3	2	2	2	2	2	1	2	3	3	2	1	1	2	2

Gene name	Mechanism	*Pseudomonas aeruginosa*																									
		Number of Single Nucleotide polymorphisms																									
		123	126	127	162	169	176	181	182	223	224	225	227	233	235	188	189	193	198	202	206	216	217	218	219	220	221
oprN	Antibiotic efflux	0	0	0	1	1	0	0	0	0	0	0	0	1	1	1	1	0	1	1	5	2	1	0	1	1	1
adeC		0	6	0	5	4	1	5	0	0	7	0	0	4	0	0	0	5	0	0	6	0	1	4	0	0	0
muxC		0	1	0	1	0	0	2	2	0	1	1	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0
muxB		0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0
muxA		1	2	1	1	1	0	1	1	1	1	3	3	1	1	2	2	1	1	1	1	1	1	1	1	1	1
opmA		2	1	1	6	5	0	5	1	1	0	1	1	3	0	0	0	3	3	3	5	3	4	1	3	5	5
taeA		1	3	1	2	3	1	1	1	1	2	2	2	4	1	2	2	2	1	3	4	2	3	2	1	3	3
farB		1	0	0	1	0	0	0	1	0	0	2	2	1	1	1	1	2	0	0	2	1	0	3	0	0	0
opmE		3	3	4	0	4	5	0	4	4	3	3	3	3	6	4	4	5	3	3	9	5	5	4	3	3	3
mexQ		4	5	2	2	2	1	0	2	2	2	1	1	2	4	1	1	2	4	2	3	4	5	0	4	2	2
mexP		0	2	1	1	2	2	0	2	1	2	1	1	1	0	1	1	0	2	2	5	2	1	0	2	2	2
nalD		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1
mexK		0	0	1	2	4	0	0	2	1	0	1	1	1	2	1	1	0	1	1	8	1	1	1	1	1	1
mexZ		0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	2	1	0	0	1	0	1
mexJ		2	0	0	2	2	1	0	1	0	0	0	0	3	1	0	0	0	2	2	3	0	3	2	2	2	2
mexL		1	1	0	1	1	0	0	1	0	0	0	0	1	0	0	0	0	1	1	1	0	1	1	1	1	1
adeC		1	2	2	2	3	0	2	1	0	1	2	2	2	2	0	0	0	1	2	2	0	0	0	0	2	2
mexG		0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	3	0	0	0	1	0	0
mexH		0	0	0	2	0	0	1	0	0	0	0	0	2	1	0	0	0	1	1	2	0	1	2	1	1	1
mexI		1	0	0	1	1	0	2	0	0	2	0	0	1	0	0	0	0	2	1	0	0	0	1	1	1	1
opmD		1	1	1	2	2	0	1	0	1	0	0	0	3	0	0	0	2	2	2	4	1	3	3	2	2	2
mexV		3	2	1	3	3	1	1	2	1	2	2	2	2	1	2	2	2	2	2	4	1	3	1	2	2	2
mexW		0	4	1	1	3	1	1	0	2	1	1	1	3	2	2	2	1	2	2	2	0	3	2	2	2	2
Yjjk		2	2	2	2	2	2	2	2	2	2	2	2	1	2	2	2	2	0	1	3	2	2	2	0	1	1
oprJ		1	1	1	2	0	2	1	1	1	0	1	1	0	1	1	1	0	0	0	8	1	1	0	0	0	0
mexD		1	3	2	2	2	3	1	1	2	1	1	2	4	2	2	2	2	2	2	11	3	4	2	2	2	2

Gene name	Mechanism	*Pseudomonas aeruginosa*																									
		Number of Single Nucleotide polymorphisms																									
		123	126	127	162	169	176	181	182	223	224	225	227	233	235	188	189	193	198	202	206	216	217	218	219	220	221
mexC	**Antibiotic efflux**	2	1	0	4	6	1	4	0	0	1	2	2	5	1	1	1	3	7	5	10	3	4	2	7	5	5
nfxB		0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0
opmH		10	10	1	0	0	10	10	11	1	0	11	11	12	0	10	10	10	10	1	9	10	11	1	10	1	1
emrE		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0
msbA		4	4	0	4	4	0	2	0	0	1	2	2	4	1	0	0	0	4	5	3	1	4	0	4	5	5
adeC		2	4	2	3	3	0	1	0	3	4	1	1	2	1	4	4	0	3	4	4	0	5	1	3	4	4
farB		3	3	4	5	4	3	3	3	4	3	3	3	4	3	4	4	0	3	3	4	3	3	3	3	3	3
rosB		1	0	2	1	1	1	1	0	2	1	1	1	3	1	3	3	1	2	2	1	0	1	2	2	2	2
catB7	**Antibiotic inactivation**	6	4	0	4	3	2	0	2	0	2	3	3	4	4	2	2	3	4	4	2	3	3	4	4	4	4
fosA		0	0	1	0	0	1	0	0	1	0	1	1	0	1	1	1	0	0	0	0	0	1	0	0	0	0
ampR		2	0	0	3	0	0	0	0	0	0	0	1	2	0	0	0	1	2	3	2	0	2	0	2	3	3
ampC		1	1	2	4	6	3	0	3	2	1	1	0	4	2	0	0	2	5	4	12	2	4	4	5	4	4
Aph(3′)-IIb		0	0	0	0	1	1	1	1	0	1	1	1	1	1	1	1	1	2	1	4	0	2	1	2	1	1
OXA-50		3	1	0	3	2	1	4	2	0	1	2	1	2	1	1	1	2	2	2	4	0	2	0	2	2	2
gyrB	**Antibiotic target alternation**	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0
alaS		2	0	0	1	0	0	0	0	0	1	0	0	2	2	0	0	0	1	0	1	2	0	2	1	0	0
pmrC		3	3	3	3	3	3	3	2	3	2	3	3	3	3	1	1	2	3	4	3	4	3	2	3	4	4
mfd		2	2	0	3	1	0	1	0	0	0	2	2	1	1	0	0	0	2	1	3	0	1	1	2	1	1
gyrA		0	0	1	1	0	0	0	0	1	2	1	1	1	0	0	0	0	1	1	0	0	1	0	1	1	1
rocS		1	1	2	8	10	7	2	1	2	1	2	2	11	1	0	0	2	8	8	20	8	5	3	8	8	8
tufA		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0
tufB		0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ileS		1	1	1	2	2	1	1	0	1	4	1	0	3	2	3	3	2	3	2	2	2	2	2	2	2	2
parC		1	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	2	2	1	1	1	0	2	1	2

Gene name	Mechanism	*Pseudomonas aeruginosa*																								
		Number of Single Nucleotide polymorphisms																								
		123	126	127	162	169	176	181	182	223	224	225	227	233	235	188	189	193	198	202	206	216	217	218	219	220	221
parE	**Antibiotic target alternation**	0	0	1	1	1	0	0	0	1	0	0	0	1	3	0	0	0	1	1	1	2	1	0	1	2	1
pmrF		0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0
arnA		2	3	1	2	3	1	2	1	1	0	3	3	4	0	2	2	1	3	3	6	1	3	3	3	3	3
mprF		0	4	1	2	8	1	1	1	1	1	3	3	5	1	3	3	5	6	2	9	6	6	2	6	2	2

Supplementary Table 3. Types of mutations in the mismatch repair system.

P. aeruginosa isolates	Mutation in MMR genes		
	mutL	***mutS***	***uvrD***
123	1SNP(missense_variant c.1034C>T p.Pro345Leu)	1SNP(missense_variant c.232G>A p.Val78Ile)	1 complex(missense_variant c.705_709delGCGGAinsACGGG p.Ile237Val)
126	0	0	0
127	0	1 MNP (missense_variant c.559G>T p.Ala187Ser)	1 MNP (missense_variant c.1985_1986delGTinsAC p.Ser662Asn) (1 Complex (missense_variant c.1985_1986delGTinsAC p.Ser662Asn)
162	1 SNP (missense_variant c.1784A>G p.Asn595Ser)	0	2 SNP (c.1031A>G p.Lys344Arg),)c.1666G>A p.Val556Ile), 1 mnp (c.1985_1986delGTinsAC p.Ser662Asn), 2 variant complex (c.1917_1921delGCCGGinsACCGT p.Ala641Ser), (c.1997_2001delACCTCinsGCCTG p.Asn666Ser)
169	1 SPN(c.10G>A p.Ala4Thr)	1 complex (c.2341_2343delGCCinsACT p.Ala781Thr)	2 SNP (c.1666G>A p.Val556Ile), (c.1921G>T p.Ala641Ser), 2 MNP(c.1031_1032delAGinsGA p.Lys344Arg), (c.1985_1986delGTinsAC p.Ser662Asn), 1 complex (c.1997_2001delACCTCinsGCCTG p.Asn666Ser)
176	1 SNP (c.1284G>T p.Glu428Asp)	0	1 SNP (c.1970G>C p.Gly657Ala)

P. aeruginosa isolates	Mutation in MMR genes		
	mutL	*mutS*	*mutL*
181	0	0	0
182	0	0	1 MNP(c.1985_1986delGTinsAC p.Ser662Asn), 1 complex (c.1997_2001delACCTCinsGCCTG p.Asn666Ser)
223	0	1 SNP (c.559G>T p.Ala187Ser)	1 MNP (c.1985_1986delGTinsAC p.Ser662Asn), 1 complex (c.1997_2001delACCTCinsGCCTG p.Asn666Ser)
224	1 SNP (c.1276G>A p.Ala426Thr)	0	1 MNP (c.1985_1986delGTinsAC p.Ser662Asn), 1 complex (c.1997_2001delACCTCinsGCCTG p.Asn666Ser)
225	0	0	2 SNP (c.1666G>A p.Val556Ile), (c.1921G>T p.Ala641Ser), 2MNP (c.1031_1032delAGinsGA p.Lys344Arg), (c.1985_1986delGTinsAC p.Ser662Asn), 1 complex (c.1997_2001delACCTCinsGCCTG p.Asn666Ser)

***P. aeruginosa* isolates**	**Mutation in MMR genes**		
	mutL	***mutS***	***mutL***
227	0	0	2 SNP (c.1666G>A p.Val556Ile), (c.1921G>T p.Ala641Ser), 2 MNP (c.1031_1032delAGinsGA p.Lys344Arg) (c.1985_1986delGTinsAC p.Ser662Asn), 1 complex(c.1997_2001delACCTCinsGCCTG p.Asn666Ser)
233	0	0	1 MNP (c.1985_1986delGTinsAC p.Ser662Asn), 1 complex (c.1997_2001delACCTCinsGCCTG p.Asn666Ser)
235	0	0	0
188	0	0	1 MNP (c.1985_1986delGTinsAC p.Ser662Asn), 1 complex (c.1997_2001delACCTCinsGCCTG p.Asn666Ser), 1 complex (c.1997_2001delACCTCinsGCCTG p.Asn666Ser)
189	1 SNP (c.265A>G p.Lys89Glu)	0	1 MNP (c.1985_1986delGTinsAC p.Ser662Asn), 1 complex (c.1997_2001delACCTCinsGCCTG p.Asn666Ser)
193	0	0	0

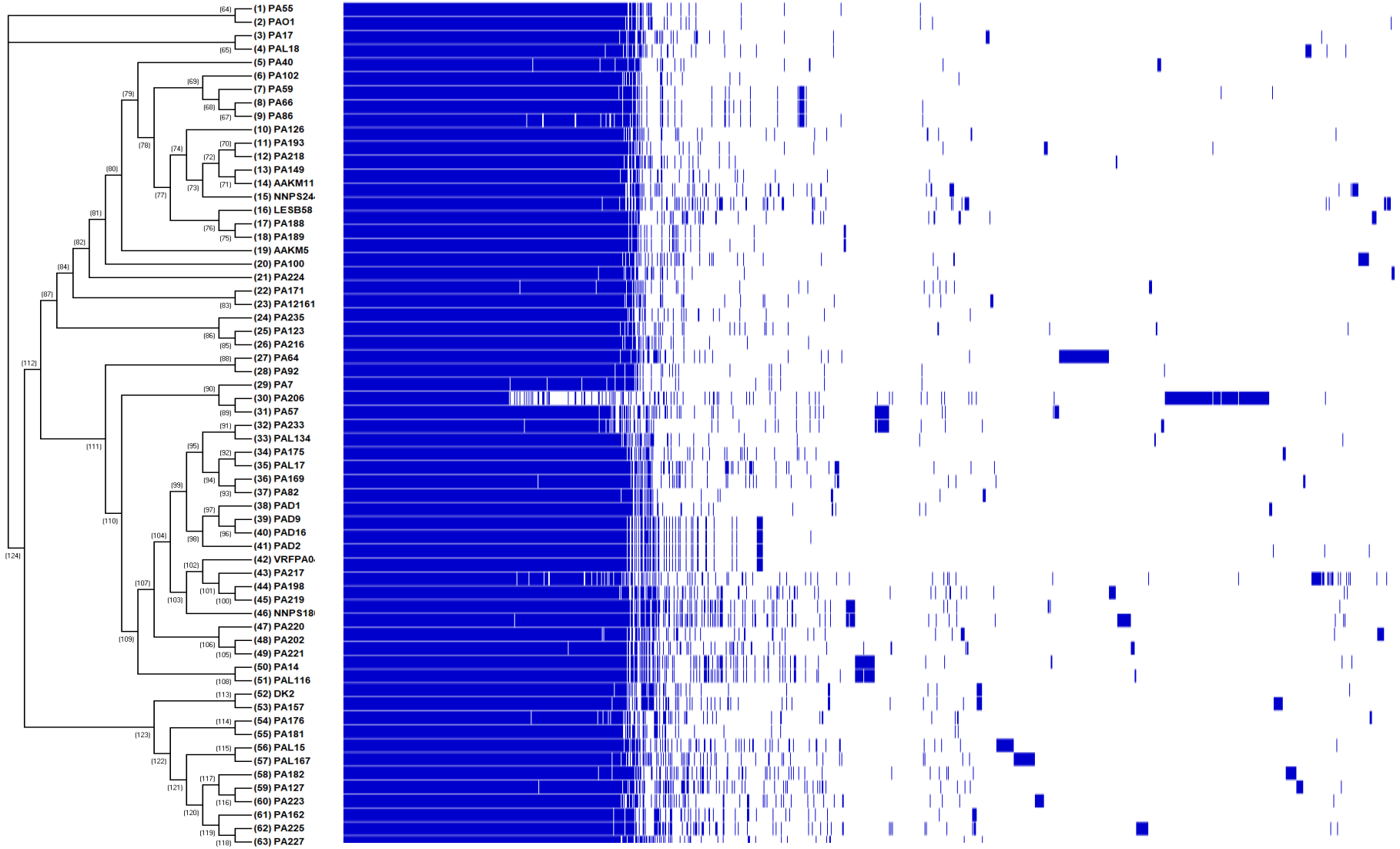
P. aeruginosa isolates	Mutation in MMR genes		
	mutL	***mutS***	***mutL***
193	0	0	0
198	2 SNP (c.1172C>T p.Ala391Val), (c.10G>A p.Ala4Thr)	0	1 SNP (c.1666G>A p.Val556Ile), 3 complex (c.1917_1921delGCCGGinsACCGT p.Ala641Ser), (c.1985_1986delGTinsAC p.Ser662Asn), (c.1997_2001delACCTCinsGCCTG p.Asn666Ser)
202	1 SNP (c.1172C>T p.Ala391Val)	1 complex (c.2341_2343delGCCinsACT p.Ala781Thr)	1 SNP (c.1666G>A p.Val556Ile), 2 MNP (c.1031_1032delAGinsGA p.Lys344Arg), (c.1985_1986delGTinsAC p.Ser662Asn), 2 complexes (c.1917_1921delGCCGGinsACCGT p.Ala641Ser), (c.1997_2001delACCTCinsGCCTG p.Asn666Ser)
206	1 MPN (c.5_6delGTinsAC p.Ser2Asn)	1 complex (c.837_841delCCTCAinsTCTCG p.Ser281Gly)	0
216	0	0	0
217	2 SNPS (.1172C>T p.Ala391Val), (c.10G>A p.Ala4Thr)	1 complex (c.2341_2343delGCCinsACT p.Ala781Thr)	1 SNP (c.1249G>A p.Ala417Thr), 2 MNP (c.1031_1032delAGinsGA p.Lys344Arg), (c.1985_1986delGTinsAC p.Ser662Asn), 1 complex (c.1997_2001delACCTCinsGCCTG p.Asn666Ser)
218	0	0	0

P. aeruginosa isolates	Mutation in MMR genes		
	mutL	***mutS***	***mutL***
219	2 SNP (c.1172C>T p.Ala391Val), (c.10G>A p.Ala4Thr)	0	1 SNP (c.1666G>A p.Val556Ile), 1 MNP (c.1985_1986delGTinsAC p.Ser662Asn), 2 complex (c.1917_1921delGCCGGinsACCGT p.Ala641Ser), (c.1997_2001delACCTCinsGCCTG p.Asn666Ser)
220	1 SNP (c.1172C>T p.Ala391Val)	1 complex (c.2341_2343delGCCinsACT p.Ala781Thr)	1 SNP (c.1666G>A p.Val556Ile), 2 MNP (c.1031_1032delAGinsGA p.Lys344Arg), (c.1985_1986delGTinsAC p.Ser662Asn), 2 complexes (c.1917_1921delGCCGGinsACCGT p.Ala641Ser), (c.1985_1986delGTinsAC p.Ser662Asn), 2 complex (c.1917_1921delGCCGGinsACCGT), (c.1997_2001delACCTCinsGCCTG p.Asn666Ser) p.Ala641Ser), (
221	1 SNP (c.1172C>T p.Ala391Val)	1 complex (c.2341_2343delGCCinsACT p.Ala781Thr)	1 SNP (c.1666G>A p.Val556Ile), 2 MNP (c.1031_1032delAGinsGA p.Lys344Arg), (c.1985_1986delGTinsAC p.Ser662Asn), 2 complex (c.1917_1921delGCCGGinsACCGT p.Ala641Ser), (c.1997_2001delACCTCinsGCCTG p.Asn666Ser)

Supplementary table 4. Genomic features of *P. aeruginosa* isolates.

P. aeruginosa isolates	Accession number	Source	Genome size (bp)	GC %	Contigs	Core genes	Shell genes	Pan/total genes
PA17	NZ_QDGR00000000.1	Eye isolate	6360407	66.4	58	5524	545	6069
PA40	NZ_QDGW00000000.1	Eye isolate	6281273	66.4	97	5465	532	5997
PA55	NZ_QDGH00000000.1	Cystic fibrosis	6235426	66.6	76	5682	66	5748
PA57	NZ_QDGS00000000.1	Cystic fibrosis	6332663	66.5	70	5278	999	6277
PA59	NZ_CP024630.1	Cystic fibrosis	6289631	66.5	76	5485	561	6046
PA64	NZ_QDGF00000000.1	Cystic fibrosis	6264146	66.6	85	5470	533	6003
PA66	NZ_QDGE00000000.1	Cystic fibrosis	6337182	66.5	92	5478	637	6115
PA82	NZ_QDGM00000000.1	Eye isolate	6386862	66.5	60	5452	667	6119
PA86	NZ_QDGD00000000.1	Cystic fibrosis	6170765	66.5	75	5346	758	6104
PA92	NZ_QDGC00000000.1	Cystic fibrosis	6144291	66.6	79	5424	497	5921
PA100	NZ_QDGV00000000.1	Cystic fibrosis	6309141	66.5	78	5435	623	6058
PA102	NZ_QDGU00000000.1	Cystic fibrosis	6245346	66.5	61	5493	489	5982
PA149	NZ_QDGL00000000.1	Eye isolate	6314561	66.5	57	5491	526	6017
PA157	NZ_QDGK00000000.1	Eye isolate	6248837	66.5	51	5456	560	6016
PA171	NZ_QDGJ00000000.1	Eye isolate	6339042	66.5	58	5498	582	6080

P. aeruginosa isolates	Accession number	Source	Genome size (bp)	GC %	Contigs	Core genes	Shell genes	Pan/total genes
PA175	NZ_QDGI00000000.1	Eye isolate	6757369	66.2	60	5446	1050	6496
PA121617	CP016214.1	Sputum	6430493	66.4	1	5497	589	6086
PA_D1	CP012585.1	Sputum	6643823	66.2	1	5437	924	6361
PA_D2	CP012578.1	Sputum	6642996	66.2	1	5435	924	6359
PA_D9	CP012580.1	Sputum	6645477	66.2	1	5435	935	6370
PA_D16	CP012581.1	Sputum	6681975	66.2	1	5437	961	6398
AAK/M5	NZ_JACGTC000000000.1	Environmental	6595587	65.3	155	5518	688	6206
AAK/M11	NZ_JACFOK000000000.1	Environmental	6737177	65.9	164	5486	935	6421
PAL1.16	NZ_JACEVD000000000.1	Pneumonia	6946945	65.9	130	5435	1270	6705
PAL1.34	NZ_JACEVA000000000.1	Pneumonia	6404462	66.5	40	5454	724	6178
PAL1.67	NZ_JACEUY000000000.1	Pneumonia	6926712	66.1	58	5468	1266	6734
PAL1.7	NZ_JACEQP000000000.1	Pneumonia	6625472	66.3	74	5437	899	6336
PAL1.5	NZ_JACEQR000000000.1	Pneumonia	7017998	66	63	5508	1243	6751
PAL1.8	NZ_JACEQO000000000.1	Pneumonia	6361965	66.5	38	5527	533	6060
NNPS180	NZ_JACCIH000000000.1	Soft tissue infection	6602334	66.2	140	5392	1023	6415
NNPS244	NZ_JACCII000000000.1	Soft tissue infection	6909183	66	141	5484	1175	6659
LESB58	NC_011770.1	Sputum	6601757	66.3	1	5493	831	6324
DK2	CP003149.1	Sputum	6402658	66.3	1	5386	851	6237
VRFPA04	CP008739.2	Corneal button	6818030	66.5	1	5137	1622	6759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(8) PA66
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(10) PA126
(11) PA193
(12) PA218
(13) PA149
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(15) NNPS24
(16) LESB58
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(18) PA189
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(20) PA100
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(23) PA12161
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(29) PA7
(30) PA206
(31) PA57
(32) PA233
(33) PAL134
(34) PA175
(35) PAL17
(36) PA169
(37) PA82
(38) PAD1
(39) PAD9
(40) PAD16
(41) PAD2
(42) VRFPA0
(43) PA217
(44) PA198
(45) PA219
(46) NNPS18
(47) PA220
(48) PA202
(49) PA221
(50) PA14
(51) PAL116
(52) DK2
(53) PA157
(54) PA176
(55) PA181
(56) PAL15
(57) PAL167
(58) PA182
(59) PA127
(60) PA223
(61) PA162
(62) PA225
(63) PA227

Supplementary Figure 4-3.

Pan-genome phylogenetic tree. The data on the right of the figure shows the presence and absence of genes. The tree was built using the genome of PAO1 as a reference.

5 Acquired fluoroquinolone resistance genes in corneal isolates of *Pseudomonas aeruginosa*

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I certify that all co-authors of this work have agreed to submission of this work as a part of this thesis.

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Research Paper

Acquired fluoroquinolone resistance genes in corneal isolates of *Pseudomonas aeruginosa*

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ABSTRACT

Fluoroquinolones are widely used as an empirical therapy for pseudomonal ocular infections. Based on increasing reports on acquired fluoroquinolone resistance genes in clinical isolates of *Pseudomonas aeruginosa*, we investigated 33 strains of *P. aeruginosa* isolated from the cornea of microbial keratitis patients in India and Australia between 1992 and 2018 to understand the prevalence of acquired fluoroquinolone resistance genes in ocular isolates and to assess whether the possession of those genes was associated with fluoroquinolone susceptibility. Fourteen out of 33 strains were resistant to at least one fluoroquinolone. We obtained the whole genome sequence of 33 isolates using Illumina MiSeq platform and investigated the prevalence of two fluoroquinolone resistance genes *crpP* and *qnrVC1*. To examine the associated mobile genetic elements of *qnrVC1* positive strains, we obtained long read sequences using Oxford Nanopore MinION and performed hybrid assembly to combine long reads with Illumina short sequence reads. We further assessed mutations in quinolone resistance determining regions (QRDRs) and antibiotic susceptibilities to ciprofloxacin, levofloxacin and moxifloxacin to examine the association between resistance genes and phenotype. Twenty strains possessed *crpP* in genetic islands characterised by possession of integrative conjugative elements. The *qnrVC1* gene was carried by four isolates on class I integrons and Tn*3* transposons along with aminoglycoside and beta-lactam resistance genes. We did not observe any evidence of plasmids carrying fluoroquinolone resistance genes. Resistance to fluoroquinolones was observed in those strains which possessed *crpP*, *qnrVC1* and that had QRDRs mutations. The presence of *crpP* on its own was not associated with increased resistance to fluoroquinolones.

1. Introduction

Pseudomonas aeruginosa is a highly adaptable opportunistic pathogen which is ubiquitously present in the environment. This bacterium is naturally resistant to many antimicrobials and can acquire antibiotic resistance through mutations in chromosomal genes and lateral gene transfer (Blair et al., 2015; Livermore, 2002). *P. aeruginosa* is associated with different types of human infections and because of emerging multidrug-resistant strains, these infections are major global public health concerns (World Health Organization (WHO), 2017).

Fluoroquinolones are broad spectrum and widely prescribed antibiotics to treat pseudomonal infections including ocular infections (Blondeau, 2004; Linder et al., 2005; Smith et al., 2001). Fluoroquinolone resistance in various clinical isolates is on the rise (Neuhauser et al., 2003). For example, a single centre study has shown that the prevalence of fluoroquinolone resistant *P. aeruginosa* increased from 15% to 41% in ten years (Gasink et al., 2006). This increase in fluoroquinolone resistance has been linked to the excessive use of the antibiotics (Werner et al., 2011). The rate of isolation of fluoroquinolone resistant strains also depends on the type of infections; nosocomial isolates are more resistant than isolates from non-nosocomial sources (Yayan et al., 2015). In general, fluoroquinolone resistance is relatively low in ocular isolates of *P. aeruginosa* compared to other infections (Subedi et al., 2018c). However, higher resistance rates have been reported in ocular isolates in certain regions of the world and like other systemic infections this rate has been increasing over time

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5.1 Introduction

Pseudomonas aeruginosa is a highly adaptable opportunistic pathogen which is ubiquitously present in the environment. This bacterium is naturally resistant to many antimicrobials and can acquire antibiotic resistance through mutations in chromosomal genes and lateral gene transfer (Blair et al., 2015, Livermore, 2002). *P. aeruginosa* is associated with different types of human infections and because of emerging multi-drug-resistant strains, these infections are major global public health concerns (World Health Organization (WHO), 2017).

Fluoroquinolones are broad spectrum and widely prescribed antibiotics to treat pseudomonal infections including ocular infections (Linder et al., 2005, Blondeau, 2004, Smith et al., 2001). Fluoroquinolone resistance in various clinical isolates is on the rise (Neuhauser et al., 2003). For example, a single centre study has shown that the prevalence of *P. aeruginosa* resistance to fluoroquinolone increased from 15% to 41% in ten years from 1991 to 2000 (Gasink et al., 2006). This increase in fluoroquinolone resistance has been linked to the excessive use of the antibiotics (Werner et al., 2011). The proportion of fluoroquinolone resistant strains also depends on the type of infections; nosocomial isolates are more resistant than isolates from non-nosocomial sources (Yayan et al., 2015). In general, fluoroquinolone resistance is relatively low in ocular isolates of *P. aeruginosa* compared to other infections (Subedi et al., 2018d). However, higher resistance rates have been reported in ocular isolates in certain regions of the world (Willcox, 2011) and like other systemic infections this rate has been increasing over time (Smitha et al., 2005). This has raised the concern that horizontal transfer of fluoroquinolone resistance genes can be associated with spread of fluoroquinolone resistance in ocular isolates.

Mutations that alter target sites (DNA gyrase [*gyrA/gyrB*] and topoisomerase IV [*parC/parE*]) and increased membrane permeability due to an upregulated efflux pump are common mechanisms of fluoroquinolone resistance in *P. aeruginosa* (Higgins et al., 2003, Nakano et al., 1997, Hooper, 1999, Rehman et al., 2019, Livermore, 2002). In other gram-negative

bacteria, fluoroquinolone resistance genes such as *qnr* are carried on plasmids (Martinez-Martinez et al., 1998, Jacoby, 2005). The gene encodes a pentapeptide repeat protein which protects DNA gyrase and topoisomerase from the action of fluoroquinolones (Robicsek et al., 2006a). In contrast, carriage of *qnr* on plasmids is very rare in *P. aeruginosa* (Cayci et al., 2014). Despite this low carriage, recent studies have identified fluoroquinolone resistance genes in certain mobile genetic elements. Amongst six families of Qnr protein, QnrVC, which was first described in *Vibrio* spp., has been reported, carried on the *P. aeruginosa* mega plasmids pBM413 (Liu et al., 2018). The *qnrVC* gene was found to be associated with a class I integron, which is carried together with other beta-lactamase genes (Kocsis et al., 2019). In addition, CrpP is a ciprofloxacin modifying enzyme encoded by *crpP*, which has been recently shown to be carried on a mega plasmid pUM505 in *P. aeruginosa* (Chávez-Jacobo et al., 2018). Another study has shown that 46% of *P. aeruginosa* isolates carried *crpP* on the chromosome and this was integrated into genomic islands (Ortiz de la Rosa et al., 2020). These studies were based on strains from different infections and the prevalence of acquired fluoroquinolone resistance genes in ocular isolates of *P. aeruginosa* remains unclear. This led to the examination of mobile fluoroquinolone resistance genes in *P. aeruginosa* isolated from cases of keratitis. Given the concern of increasing fluoroquinolone resistance in ocular isolates, we have undertaken sequence analysis of 33 ocular isolates of *P. aeruginosa*, isolated from corneal ulcers in the last 25 years to assess whether the possession of fluoroquinolone resistance genes was associated with fluoroquinolone susceptibility and whether resistance genes carrying strains had any genetic commonalities.

5.2 Materials and Methods

5.2.1 *Pseudomonas aeruginosa* strains

Isolates in this study were collected from the cornea of microbial keratitis patients at the LV Prasad Eye Institute, Hyderabad, India and multiple centres in Australia between 1992 and 2018 (Supplementary Table 1). They comprise 20 isolates collected for this study and used for the antibiotic susceptibility testing and whole genome sequencing. Information on antibiotic

susceptibility and the whole genome data of 13 isolates was obtained from a previous study (Subedi et al., 2018b) carried out at the same institution. All isolates were collected from different patients, but no patient data were available to include in this study.

5.2.2 Ethical approval

All isolates were retrieved from the culture collection of the School of Optometry and Vision Science, University of New South Wales, Australia without identifiable patient data and all experiments followed the institutional guidelines which were in place at the time.

5.2.3 Antibiotic susceptibility testing of *Pseudomonas aeruginosa* strains

Three fluoroquinolones from two different generations, which are used to treat eye infections were selected for the antibiotic susceptibility testing. Susceptibility of *P. aeruginosa* isolates to ciprofloxacin (Sigma-Aldrich, Inc., St. Louis Missouri, USA), levofloxacin (Sigma-Aldrich) and moxifloxacin (European Pharmacopoeia, Strasbourg, Cedex France) was investigated using the broth microdilution method, following the protocol of the Clinical and Laboratory Standard Institute (Patel et al., 2014). The minimum inhibitory concentration (MIC) was taken as the lowest concentration of an antibiotic in which no noticeable growth (turbidity) was observed and the break point was established according to published standards . Based on MICs, the isolates were categorised into four groups, susceptible ($\leq$ resistance break point), resistant (> resistance break point – 32 μg/mL), highly resistant (> 32 – 128 μg/mL) and very highly resistant (>128 μg/mL) for the analysis.

5.2.4 DNA extraction and Illumina sequencing

Bacteria were revived from the -80°C glycerol stock into tryptone-soya broth (TSB; Oxoid Ltd, Basingstoke, UK). Bacterial DNA was extracted from overnight cultures using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The extracted DNA was quantified and purity-checked using Nanodrop (NanoDrop Technologies, Wilmington, DE, USA), Qubit fluorometer (Life Technologies, Carlsbad, CA, USA), and 1%

agarose gel electrophoresis. The extracted DNA was dried for transport to the sequencing facility at Singapore Centre for Environmental Life Sciences Engineering, Singapore. DNA was sequenced on MiSeq (Illumina, San Diego, CA, USA) generating 300 base pairs paired end reads. The paired-end library was prepared using Nextera XT DNA library preparation kit (Illumina, San Diego, CA, USA). All the libraries were multiplexed on one MiSeq run.

5.2.5 Bioinformatics analysis of short read sequences and construction of phylogenies

The quality of raw reads was analysed using the online tool FastQC v0.117 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc). Adaptor sequences were removed using Trimmomatic v0.38 (Bolger et al., 2014) with quality and length filtering (SLIDINGWINDOW:4: 15 MINLEN:36) . The reads were *de novo* assembled using Spades v3.13.0 with the programs' default setting (Bankevich et al., 2012) followed by annotation using Prokka v1.12 (Seemann, 2014). To examine acquired resistance genes, the genomes were uploaded into online database ResFinder v3.1 of Centre for Genomic Epidemiology, DTU, Denmark (Zankari et al., 2012). The contigs carrying *crpP* and *qnrVC1* genes were selected and analysed for mobile genetic elements using BLAST, Integron finder v1.5.1 (Cury et al., 2016) and IS finder (Siguier et al., 2006). The genome was visualised and manually annotated based on BLAST search using Geneious prime v2019.2 (Kearse et al., 2012). A figure of BLAST comparison was generated using EasyFig v2.2.2 (Sullivan et al., 2011). To identify mutations in the QRDRs (*gyrA, gyrB, parC* and *parE*), the genome sequences were analysed using Snippy v4.2 with the program's default settings (https://github.com/tseemann/snippy). The QRDRs were assigned to amino acid positions 83 to 87 of the GyrA protein, positions 429 to 585 of the GyrB protein, positions 82 to 84 of the ParC protein, and positions 357 to 503 of the ParE protein. The genomes were also examined for the presence of Type III secretion genes (*exoU* and *exoS*) using BLAST searches. Codon adaptation index (CAI) was examined to understand the possible expression of different orthologues of *crpP* using CAIcal (Puigbò et al., 2008, Sharp and Li, 1987). 50s ribosomal protein L19 (*rplS*), which is a highly expressed

chromosomal gene was included as the reference to show the difference in expression level between chromosomal gene and acquired genes.

Core genome single nucleotide polymorphisms (SNPs) were identified using Parsnp v1.2 from Harvest Suite (Treangen et al., 2014) using settings to exclude SNPs identified in regions that had arisen by recombination. The core genome SNPs were used to construct a maximum likelihood phylogenetic tree. All genomes were examined for multi-locus sequence type (MLST) using the MLST database https://pubmlst.org/. Nucleotide sequences of all MLST loci were extracted and aligned, which were then used in jModelTest 2 with Bayesian information criteria (BIC) (Darriba et al., 2012) to select the best model of nucleotide substitution for evolutionary analysis. The model with the lowest BIC (10650.89) and delta (0) was selected. Accordingly, Bayesian phylogenetic analyses were performed with the following parameters: gamma site heterogeneity model, Hasegawa-Kishino-Yano (HKY) substitution model and relaxed-clock log-normal using BEAST2 v2.4.7 (Drummond and Rambaut, 2007). BEAST 2 output was summarised using TreeAnnotator with a 5% burn in. The phylogenetic trees were visualised using iTol v4 (Letunic and Bork, 2019).

5.2.6 MinION sequencing and analysis

Wizard Promega DNA extraction kits (Promega, Madison, WI) was used to extract DNA from overnight broth culture. DNA was quantified and transported as mentioned above. Long read libraries were prepared using a rapid sequencing kit (RAD-SQK004, ONT, Oxford, UK) and subsequent sequencing was conducted using the MinIon flow cell (R9.4.1) for 48 hours. Long reads were base-called using Guppy v3.3.0 and adapters removed using Porechop v0.2.4. Assembly of both long and short reads into a hybrid genome assembly was achieved with Unicycler v0.4.3 (Wick et al., 2017), opting for default parameters and using only short reads with merging pairs. Following assembly, all assemblies were assessed for quality using Quast v5.0.2.

5.2.7 Nucleotide accession

The nucleotide sequences are available in the GenBank under the Bio project accession number PRJNA590804 and PRJNA431326.

5.3 Results

5.3.1 Population structure, phylogeny and fluoroquinolone resistance of *P. aeruginosa* strains

Whole genomes were analysed from 33 corneal isolates of *P. aeruginosa*, 20 of which were sequenced as a part of this study and 13 genomes that were published previously (Subedi et al., 2018b). All strains were isolated between 1992 and 2018 in India (19 isolates) or Australia (14 isolates) (Supplementary Table 1). Draft genomes were mapped against the reference genome *P. aeruginosa* PAO1 and a total of 202,232 SNPs were observed among the 33 isolates, which were used to construct core genome phylogeny using Parsnp v1.2 (Treangen et al., 2014). Similar to previous reports (Stewart et al., 2014, Jeukens et al., 2017, Freschi et al., 2015b, Subedi et al., 2018b), the phylogenetic tree based on the core genome revealed two major clades (Figure 5-1). These clades followed a similar pattern as for other previously described strains of *P. aeruginosa,* where *exoU* carrying strains clustered together in a single clade (Phylogroup 2) (Subedi et al., 2018b). Our results showed that 18 (of 33) isolates carried *exoU*, of which 16 were clustered together in phylogroup 2, which contained predominantly Indian isolates. The detection rate of *exoU* was 68% in Indian isolates and 36% in Australian isolates.

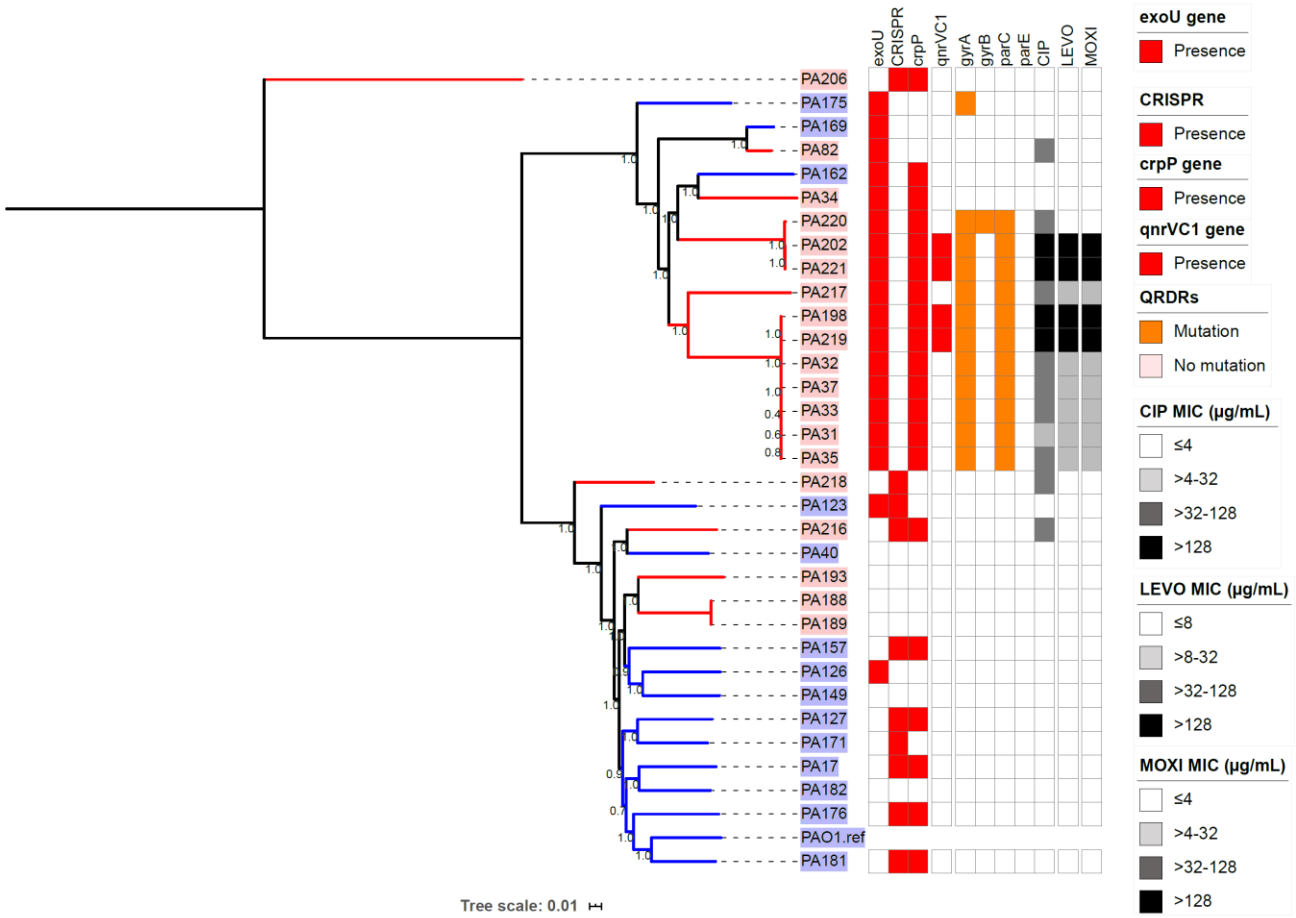


Figure 5-1 Maximum likelihood phylogenetic tree based on core genome SNPs.

Analysis was conducted using *Pseudomonas aeruginosa* PAO1 as the reference, excluding SNPs identified in regions that had arisen by recombination, using the default parameters of Parsnp v1.2 (Treangen et al., 2014). Isolates from India are labelled red and Australian isolates are labelled blue. Numbers given at the nodes represent bootstrap values. The presence of *crpP, exoU,* and *qnrVC1* are given by red squares. Orange squares represent the presence of mutations in the quinolone resistance determining regions (QRDRs). Fluoroquinolone (CIP = Ciprofloxacin; LEVO = Levofloxacin; and MOX = Moxifloxacin) susceptibilities are shown as a heat map with the ranges indicated in the figure. The figure was drawn using iTol v4 (Letunic and Bork, 2019).

Acquired fluoroquinolone resistance genes, mutations in quinolone resistance determining region (QRDRs) and susceptibility to three different fluoroquinolones were examined (Figure 5-1). None of the Australian isolates regardless of phylogenic grouping were resistant to fluoroquinolones. In contrast, 75% of isolates of phylogroup 2 were resistant to at least one fluoroquinolone. Of the 33 strains, 73.7 % (14 of 19) of Indian and 42.8% (6 of 14) of Australian isolates possessed *crpP*, which has been recently shown to be on a plasmid (pUM505) and associated with ciprofloxacin resistance (Chávez-Jacobo et al., 2018). However, eight (40%) *crpP* carrying strains in the current study regardless of country of isolation were not resistant to the fluoroquinolones, including ciprofloxacin (Figure 5-1). Eleven out of 14 fluoroquinolone resistance strains had mutations in both *gyrA* (T81I) and *parC* (S87L) and all except one carried the *exoU* gene. In addition, four strains from the latter cohort of 11 strains carried another fluoroquinolone resistance gene, *qnrVC1,* in combination with mutations in *gyrA* and *parC*, and this was associated with a very high MIC (>128 µg/mL) to all three fluoroquinolones (Figure 5-1).

5.3.2 Bayesian phylogenetic reconstruction

To examine the evolutionary trends of isolates, a Bayesian analysis was performed based on MLST sequences. The evolutionary tree was constructed by BEAST, which uses a molecular clock model to estimate time-measured phylogenies (Drummond and Rambaut, 2007). The result suggested that the most recent common ancestor of isolates of this study appeared approximately 50 years ago and the branching of fluoroquinolone resistant strains (except PA82), which had mutations in QRDRs and/or had acquired resistance *qnrVC1* within each subclade, occurred between 1988 and 2000 (Figure 5-2). This coincided with the time when ciprofloxacin and ofloxacin were introduced as antibiotic treatments (Emmerson and Jones, 2003). The BEAST analysis showed that acquisition of *crpP* and *exoU* occurred in the strains that diverged from a common ancestor at least 50 years ago. However larger sample sets with a wider sampling date is required to confirm the result.

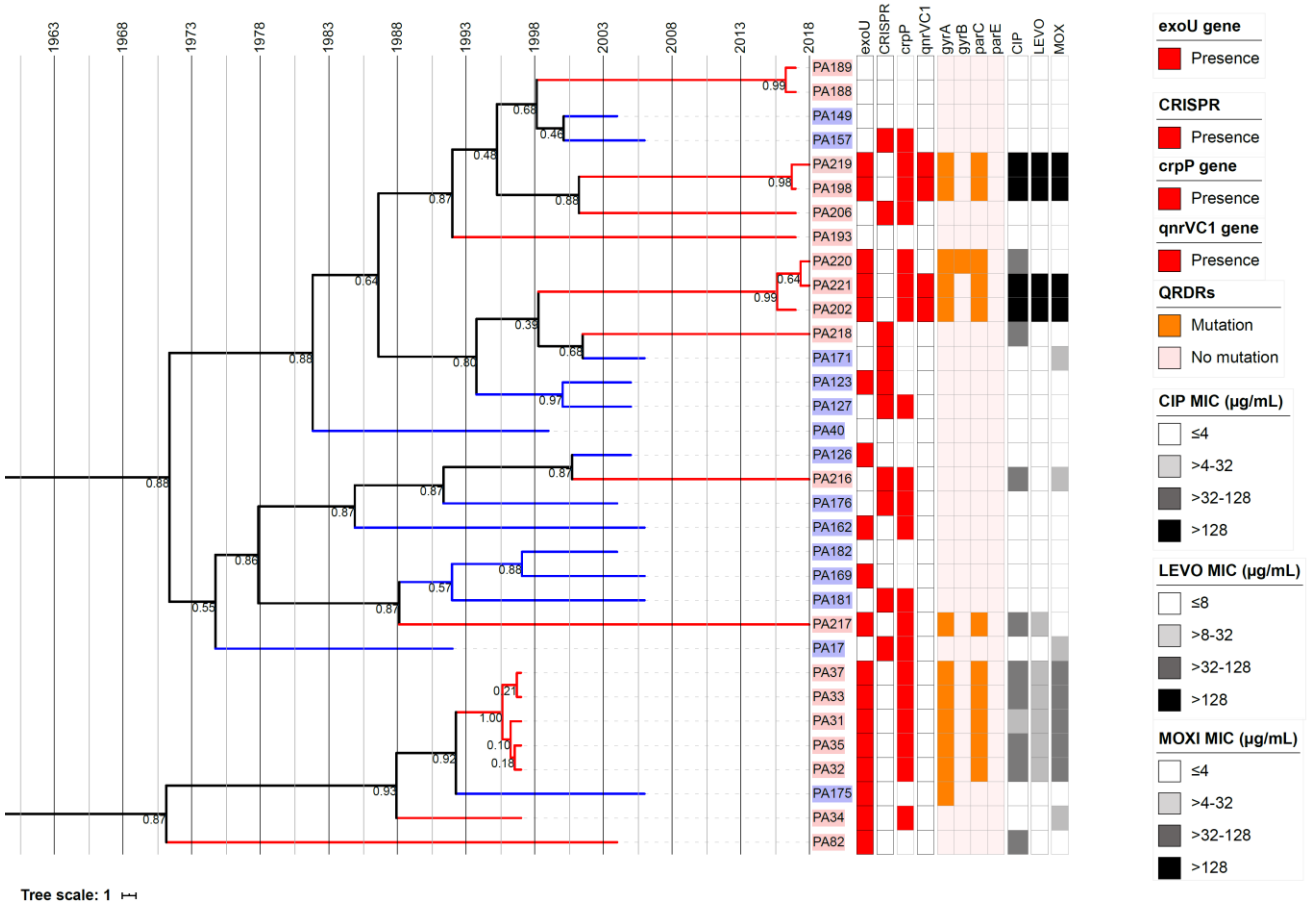


Figure 5-2 Consensus tree of 33 *P. aeruginosa* isolates, based on Bayesian evolutionary analysis by sampling trees (BEAST).

Tree was constructed using concatenated multi-locus sequence type (MLST) under strict clock analysis (Drummond and Rambaut, 2007). The tip of the tree was constrained by date of isolation. The time scale is shown in years at the top and each internal node is labelled with posterior probability limit. Isolates from India are labelled red and Australian isolates are labelled blue. The presence of genes *crpP, exoU,* and *qnrVC1* are represented by red squares. Orange square represents presence of mutations in quinolone resistance determining region (QRDRs). Fluoroquinolone (CIP = Ciprofloxacin; LEVO = Levofloxacin; and MOX = Moxifloxacin) susceptibilities are shown as heat maps in the grey scale indicated in the figure. The figure was drawn using iTol v4 (Letunic and Bork, 2019). (Tree with the confidence interval for identification of year is available in supplementary figure 2**)**

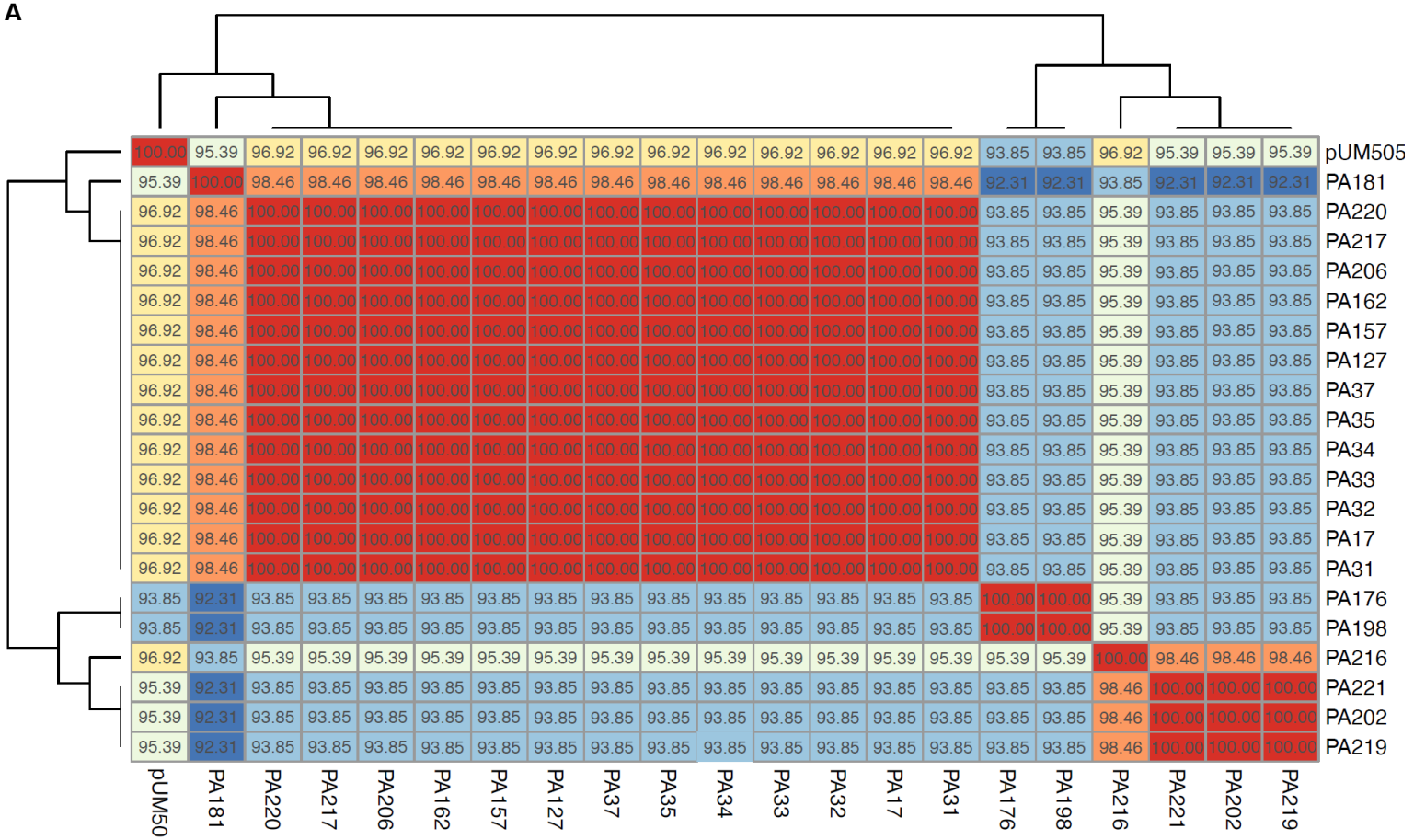
5.3.3 Prevalence of the *crpP* gene

A recent study has shown that *crpP* encodes for a protein that is associated with increased resistance to ciprofloxacin (Chávez-Jacobo et al., 2018). In the current study, 63% of isolates possessed *crpP*, however nine out of the 20 *crpP* carrying strains were not resistant to ciprofloxacin (Figure 5-1). Nucleotide and amino acid sequences of *crpP* from all strains were compared to the sequence of *crpP* in pUM505 using local BLASTn. The BLAST matrix showed that the nucleotide sequences of *crpP* genes varied between 100% and 93.43% and amino acids sequence varied between 100% to 93.85% (Figure 5-3), indicating the possibility of differences in the activity of the encoded protein. We observed six variants of CrpP exhibiting amino acid substitution at positions 4, 5, 7, 11, 13, 26, and 55. Others have reported an even higher number of variants in CrpP (Ruiz, 2019). None of the CrpP variants of the studied strains matched 100% with CrpP of pUM505. Codon usage, presented as codon adaptation index (CAI) (Figure 5-4) was examined to understand the possible expression of different variants of *crpP* (Sharp and Li, 1987). The 50s ribosomal protein L19 (*rplS*), which is a highly expressed chromosomal gene was included in this analysis to show the difference in expression between chromosomal and acquired genes. A CAI between 0.36 and 0.39 was observed in different orthologues of *crpP*, which while low, corresponded to divergence of sequences. However, the sequence divergence was not associated with resistance to fluoroquinolones. For example, fluoroquinolone resistant strains PA32, PA33 and PA35 and sensitive strains PA17, PA127, PA157 and PA162 carried the same variants of CrpP protein (Figure 5-3).

We also investigated the presence of the *crpP* homologues in NCBI databases using local BLAST with a cut off of 80% coverage and 90% sequence identity to examine the distribution of *crpP* gene in the bacterial database including plasmids as of 2019-06-15. The BLAST search against *P. aeruginosa* complete genome databases returned 52% matches and this rate of detection is broadly comparable to *crpP* prevalence observed in the current strains. No BLAST matches other than *P. aeruginosa* were observed with this search parameter. Within

the *P. aeruginosa* plasmid database, two matches were observed against 45 complete plasmids using the parameters mentioned above. A CrpP-like protein, which has a sequence identity of 10-46% with *P. aeruginosa crpP,* has been observed in species of the *Enterobacteriaceae* (Chávez-Jacobo et al., 2019). This result suggested that *crpP* or its orthologue can be transferred between species or strains.

A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PA221 95.39 92.31 93.85 93.85 93.85 93.85 93.85 93.85 93.85 93.85 93.85 93.85 93.85 93.85 93.85 93.85 93.85 98.46 100.00 100.00 100.00
PA202 95.39 92.31 93.85 93.85 93.85 93.85 93.85 93.85 93.85 93.85 93.85 93.85 93.85 93.85 93.85 93.85 93.85 98.46 100.00 100.00 100.00
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pUM50
PA181
PA220
PA217
PA206
PA162
PA157
PA127
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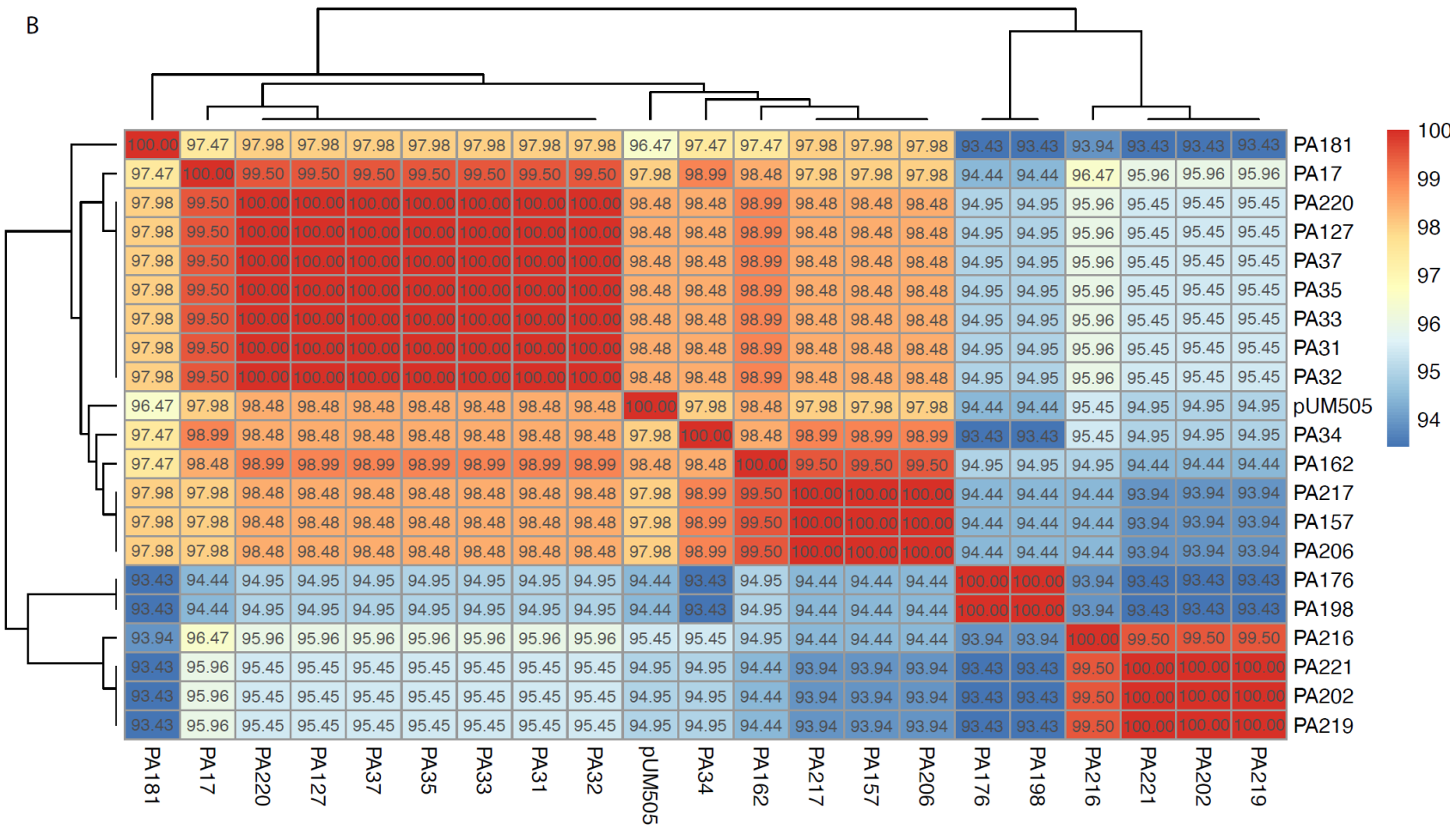


Figure 5-3 Heat map of BLAST matrix.

BLAST matrix showing A. Amino acid identity (in percentage) and B. Nucleotide identity (in percentage) of *crpP* sequences of each isolate. The dendrograms represent similarity between strains based on *crpP* sequence.

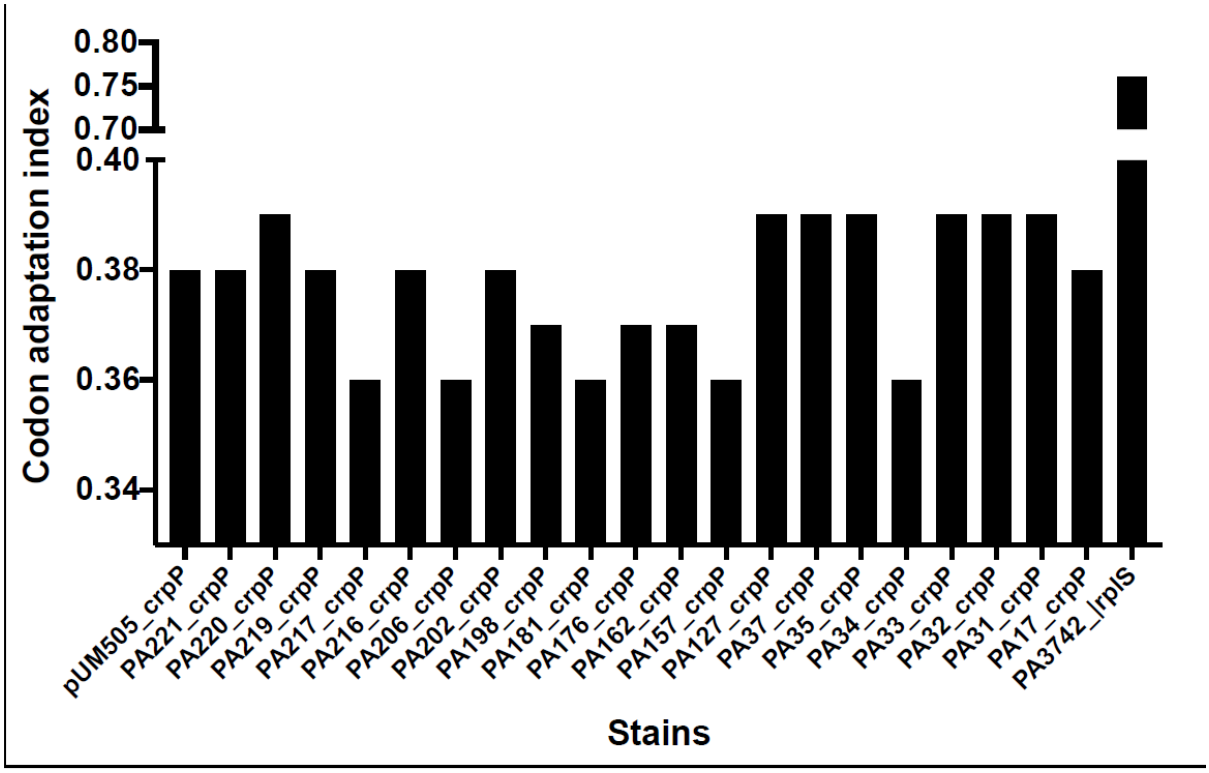


Figure 5-4 Codon adaptation index of *crpP* orthologues of *P. aeruginosa* strains.

PA3742_rplS denotes 50s ribosomal protein L19 (rplS) of *P. aeruginosa* PAO1.

5.3.4 *crpP* associated mobile genetic elements

Although *crpP* was first reported in plasmid pUM505 of a clinical strain of *P. aeruginosa* (Chávez-Jacobo et al., 2018), BLAST analysis, as mentioned above, revealed this gene was mostly associated with the chromosome and its presence in plasmids was rare. The *crpP* containing contigs of each isolate were assessed to determine similarities between associated genomic islands. The *crpP* flanking region of pUM505 is homologous to the pathogenicity island PAPI-1 of *P. aeruginosa* PA14 (He et al., 2004) and pKLC102 of *P. aeruginosa* C (Kung et al., 2010b, Ramirez-Diaz et al., 2011), which is an integrative conjugative element and is associated with carriage of virulence genes. A PAPI-1-like genomic island was also reported in a complete genome of the ocular isolate PA34 (Subedi et al., 2019). Using PAPI-1 of PA34

as a reference, the contigs of each strain were compared and this showed that there were similarities between contigs. Those common regions were extracted and annotated manually using Geneious Prime v2019.1.3 (Kearse et al., 2012). After annotation, all of the extracted regions were compared using Easyfig (Sullivan et al., 2011). The region that has been shared amongst all isolates is presented in Figure 5-5, where the colour code represents open reading frames. While the size of *crpP* carrying genomic islands differed between strains, the exact size of islands could not be ascertained because of the use of draft genomes for analysis. Nevertheless, three genes (integrative conjugative element, an unknown protein and single-strand binding protein) upstream and one gene (type I DNA topoisomerase) downstream of *crpP* were common in all isolates and this indicates that these genes may be involved in the transfer of *crpP* gene. A characteristic type IV pili synthesis operon was associated with 75% of the *crpP* positive strains. There was no association between size of *crpP* islands and fluoroquinolone resistance.

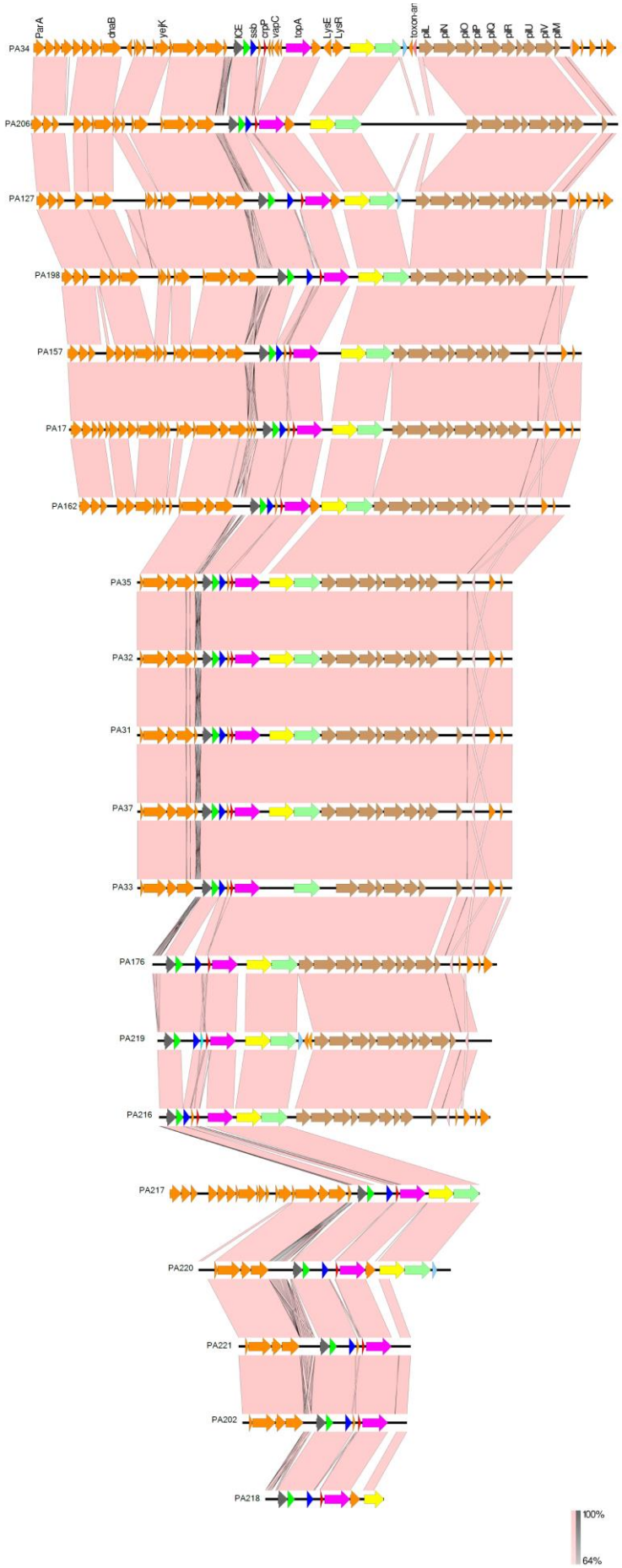

ParA
dnaB
yejK
ICE
ssb
cnpP
vapC
topA
LysE
LysR
toxon-ari
pilL
pilN
pilO
pilP
pilQ
pilR
pilU
pilV
pilM
PA34
PA206
PA127
PA198
PA157
PA17
PA162
PA35
PA32
PA31
PA37
PA33
PA176
PA219
PA216
PA217
PA220
PA221
PA202
PA218
100%
64%

Figure 5-5 Comparison of crpP-associated genomic islands of *P. aeruginosa* strains.

Protein-coding regions are represented by the orange arrows and common key features/associated genes among all strains are shown in various coloured arrows. The gradient pink and grey shading represent regions of nucleotide sequence identity (100% to 64%) in forward and reverse directions respectively, determined by BLASTn analysis. Figures are drawn to scale using Easyfig (Sullivan et al., 2011). (parA= plasmid partition protein A, dnaB= replicative DNA helicase, yejK= nucleoid-associated protein, ICE= integrating conjugative element protein, ssb= single-stranded DNA-binding protein, crpP= ciprofloxacin resistance protein, vapC= tRNA(fMet)-specific endonuclease, topA= type I DNA topoisomerase, LysE= LysE family translocator, LysR= LysR family transcriptional regulator, toxin-anti= type II toxin-antitoxin system RelE/ParE family toxin, pilL-pilM= type IV pilus synthesis operon).

5.3.5 Prevalence of quinolone resistance protein (*qnr* gene)

The *qnr* is commonly carried on plasmids, especially in the *Enterobacteriaceae*. A transferable variant of the *qnr* gene, *qnrVC1* was first identified in a class I integron in *Vibrio cholerae* 01 (Fonseca et al., 2008). Several *qnrVC* alleles have also been reported to be carried in various bacteria including *P. aeruginosa* (Belotti et al., 2015, Robicsek et al., 2006a). For example, a recent study had reported that 2.3% of clinical strains of *P. aeruginosa* carried *qnrVC* (Lin et al., 2020). In the current study, 12% (four of 33) of isolates possessed *qnrVC1*. All these four were isolated in 2017 and 2018. We further examined the prevalence of *qnrVC1* homologues in complete chromosomes and plasmids of other bacterial species in NCBI database using BLAST search. We observed that *qnrVC1*-like proteins were present in several bacterial species. Upon limiting the BLAST criteria to 100% coverage and 98% identity, the search returned 19 matches from species of *Pseudomonas, Vibrio* and *Aeromonas*. (Supplementary Figure 1). This suggests that the *qnrVC1* gene is prevalent in various bacterial families. Curiously, the *qnrVC1* gene was not identified in the plasmid database of NCBI. Furthermore, *qnrVC1* had a G + C content of 37%, which is considerably different from that of the *P. aeruginosa* genome (66%). This evidence along with the presence of *qnrVC1* homologues in several bacterial species indicates that *qnrVC1* is a horizontally acquired gene in *P. aeruginosa*, which is potentially acquired through inter-species or inter-genera transfer.

5.3.6 *qnrVC1* associated mobile genetic elements

We further examined the flanking nucleotide sequences of *qnrVC1* to understand the mobile genetic elements associated with transfer of the gene. Our initial analysis found that *qnrVC1* was present in small contigs (contig size <5000 bp) and this limited our aim to examine mobile genetic elements. The four *qnrVC1* positive strains were then analysed using the long-read sequencing technique ONT minION (ONT, Oxford). With hybrid assembly of long and short sequencing reads, we archived long contigs ranging in size from 400 Kbp to 2000 Kbp (PA202 N_{50} = 680,290; PA219 N_{50} = 2,159,014; PA221 N_{50} = 2,110,541) in three isolates and a complete and closed genome of one strain PA198 (N_{50} = 7,188,952).

The *qnrVC1* gene was present in two different types of mobile genetic elements. In strains PA198 and PA219, the gene was integrated into Tn*3* transposons, which have 99% identity with one another (Figure 5-6). In addition, the Tn*3* transposon carried other antibiotic resistance genes associated with aminoglycoside 3'-phosphotransferase and tetracycline resistance. BLAST comparison revealed recombination in various places within the transposon, most notably in the areas flanking the *qnrVC1-VOC* and *tetR-tetA* genes (Figure 5-6), which represents the site of integration of these genes. The role of VOC protein in antibiotic resistance is not well known. However, evidence suggests that proteins of the VOC family include beomycin and fosfomycin resistance proteins (He and Moran, 2011). Integration of VOC with other antibiotic resistance genes leads to the hypothesise that VOC may be associated with antibiotic resistance. Further research is required to investigate its role in antibiotic resistance in *P. aeruginosa*.

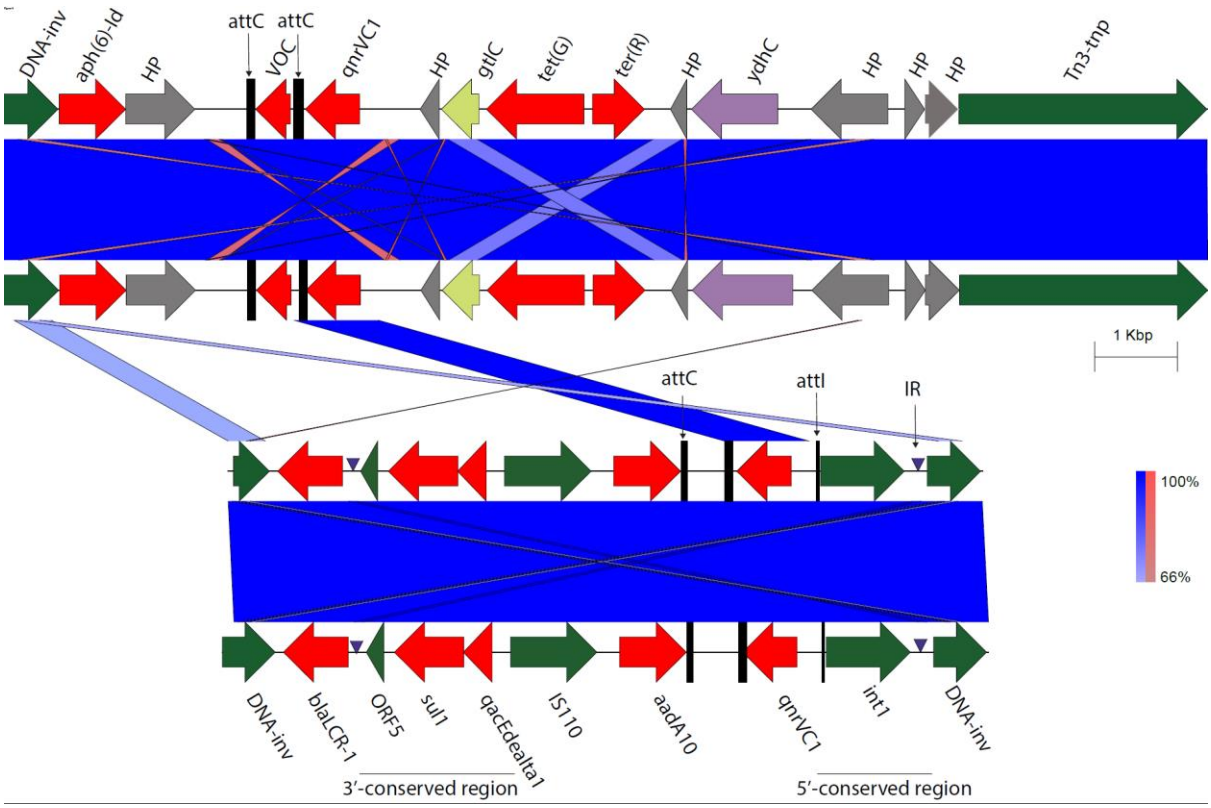


Figure 5-6 Comparison of *qnrVC1*-associated genomic islands of *P. aeruginosa* strains.

Protein-coding regions are represented by the arrows and common key features/associated genes among all strains are shown in various coloured arrows. The gradient blue and red shading represent regions of nucleotide sequence identity (100% to 65%) in forward and reverse directions, respectively, determined by BLASTn analysis. The sequences are from strains top to bottom; PA198, PA219, PA202 and PA221. Figures are drawn to scale using Easyfig (Sullivan et al., 2011). (Tn3-tnp = Tn3-transposase HP= hypothetical protein, ydhC= Inner membrane transport protein, tet(R)-tet(g) = tetracycline resistance genes, qnrVC1=quinolone resistance gene, VOC = VOC family protein, aph(6)-Id = aminoglycoside resistance protein, DNA-inv = DNA invertase, aadA10= aminoglycoside resistance protein, IS110= IS110 family transposase, qacEdealta1= quaternary ammonium compound-resistance protein, sul1= Dihydropteroate synthase, blaLCR-1=beta-lactamase gene, attC = recombination sites of gene cassette, attI = integron recombination site, IR=invert repeat).

In the other two strains, PA202 and PA221, *qnrVC1* was carried by identical class 1 integrons (Figure 5-6), along with two additional antibiotic resistance genes; the aminoglycoside gene *aadA10* which was within the integron and the beta-lactam gene *blaLCR-1* in the flanking region. Other studies have also shown that *qnrVC1* is a part of integrons that carry multiple resistance genes (Belotti et al., 2015, Kocsis et al., 2019). The class 1 integron observed here had a typical structure containing promoter sequences and an attachment site (*attI*) required for integration and transcription of gene cassettes (Stokes and Hall, 1989). The *qnrVC1* gene

in all four strains was associated with a perfectly matched gene recombination site (*attC*). The *attC* sequence of *qnrVC1* gene cassette had 100% identity with that of *Vibrio parahaemolyticus* (GenBank accession number EU436855), supporting the likelihood that the *Vibrionaceae* is a source of *qnr* in *P. aeruginosa* (Poirel et al., 2005c).

5.4 Discussion

While fluoroquinolones are the preferred empirical therapy for contact lens related corneal ulcers, which are often caused by *P. aeruginosa*, increasing resistance in this bacterium raises concern about the efficacy of these antibiotics (Linder et al., 2005, Blondeau, 2004, Smith et al., 2001, Neuhauser et al., 2003, Gasink et al., 2006, Werner et al., 2011, Yayan et al., 2015, Subedi et al., 2018d). Acquired fluoroquinolone resistance genes are starting to be reported in clinical isolates of *P. aeruginosa* (Chávez-Jacobo et al., 2019, Liu et al., 2018, Kocsis et al., 2019). However, the rate of carriage of acquired fluoroquinolone resistance genes in ocular isolates has not been previously reported. This study examined the prevalence of acquired fluoroquinolone resistance genes, *crpP* and *qnrVC1,* which have recently been shown to be quinolone-associated acquired resistance genes in *P. aeruginosa*. Whole genomes of 33 strains isolated from corneal ulcers in the last 25 years isolates from Australia and India were studied. Fluoroquinolone resistance was common in Indian isolates and mutations in QRDRs were associated with this increased MIC to all three fluoroquinolones (2^{nd}, 3^{rd}, 4^{th} generation) tested. Possession of the acquired gene *qnrVC1* substantially increased the MICs whereas *crpP* was not associated with increased fluoroquinolone resistance in those isolates that carried *crpP* but lacked QRDRs mutations or *qnrVC1*. Both quinolone resistance genes were integrated into the chromosome and their associated genomic islands were broadly common between strains.

Fluoroquinolone resistance in *P. aeruginosa* is mediated by several mechanisms including, various mutations that lead to overexpression of efflux pumps and QRDRs of *gyrA, gyrB, parC* and *parE* that alter drug target sites (Higgins et al., 2003, Nakano et al., 1997, Hooper, 1999,

Livermore, 2002). In addition, horizontally transferred fluoroquinolone resistance genes such as *crpP* and *qnr*-variants have been recently reported in clinical strains of *P. aeruginosa* (Liu et al., 2018, Lin et al., 2020, Chávez-Jacobo et al., 2018, Kocsis et al., 2019). *CrpP* encodes a ciprofloxacin modifying enzyme, which is specifically involved in phosphorylation of ciprofloxacin and hence confers resistance to it (Chávez-Jacobo et al., 2018). Our BLAST analysis against the NCBI database showed that more than half of the strains in the *P. aeruginosa* complete genome database contained homologues of *crpP* and this corresponded to the detection frequency of *crpP* in the ocular strains reported in this study and in *P. aeruginosa* strains isolated from various sources in Europe (Ortiz de la Rosa et al., 2020). *CrpP* carriage rate was higher in Indian isolates (74%) compared to Australian isolates (43%). However, more data on strains from multiple centres will be required to verify this. Nearly half of the *crpP* carrying strains regardless of region of isolation were not resistant to any of the fluoroquinolones tested. Furthermore, fluoroquinolone resistant *crpP*+ strains also had target site mutations *gyrA* (T81I) and *parC* (S87L) and had acquired the quinolone resistance gene *qnrVC1*. Therefore, the role of *crpP* alone on fluoroquinolone resistance could not be ascertained for these strains. Given that *crpP* is ciprofloxacin specific and associated with low-level resistance, its presence may not necessarily be responsible for the MICs that exceeded the clinical breakpoint (Chávez-Jacobo et al., 2018).

The *crpP* gene was associated with similar genomic islands in all strains. These genomic islands were characterised by the possession of integrative conjugative elements and DNA replication factors together with virulence genes *(pil).* This finding is supported by an *in silico* analysis of the Genbank database, which has shown that CrpP-like protein is integrated into various types of genomic islands (PAPI-1 and PAGI-5) in *Pseudomonas* spp. (Ruiz, 2019). These genomic islands are closely related to transferable pKCL102 (Kung et al., 2010b), highlighting the mobility of *crpP* across the species. Furthermore, a most recent study has proved that *crpP* carrying PAGI-like element is functionally mobilizable (Ortiz de la Rosa et al., 2020). The *crpP* gene was originally reported in the plasmid pUM505 (Ramirez-Diaz et al.,

2011) but there are no other studies showing plasmid associated *crpP* in *P. aeruginosa*. Therefore, the BLAST search against the NCBI plasmid database specific to *P. aeruginosa* was performed and observed two matches (plasmids pKLC102 and pY89) out of 45 complete plasmids. Furthermore, the most recent ancestors of *crpP* positive strains appeared at least 50 years ago, which is earlier than the appearance of resistant subclades in the phylogeny. This result, together with finding of high divergence in *crpP* orthologues between strains and lower CAI compared to a highly expressed chromosomal gene (*rlpS*), (Dötsch et al., 2010) suggests that acquisition of the *crpP* gene was a relatively old evolutionary event in *P. aeruginosa.*

The higher frequency of *crpP*+ strains in the Indian cohort of our study might be linked to the close similarities between the strains. Although the phylogenetic analysis showed that strains PA202/220/221 and strains PA31/35/33/37/32/219/198 were identical, each strain was isolated at different time points (from 1997 to 2018). It is therefore possible that these represent clones circulating in the local Indian environment. However, a recent study has found that about half of the clonally unrelated *P. aeruginosa* strains carried c*rpP* gene, which was likely mediated by the acquisition of a PAGI-like element (Ortiz de la Rosa et al., 2020). Our analysis of genomes sequences available from GenBank also revealed that CrpP-like genes were frequently identified within *P. aeruginosa*, which is in line with this study and others observations (Ruiz, 2019).

The plasmid-mediated quinolone resistance *qnr* gene was first reported in 1998 (Martinez-Martinez et al., 1998) and this corresponds with increased usage of fluoroquinolones in the 1980s (Jacoby et al., 2003). Our analysis also revealed that resistant *P. aeruginosa* strains were phylogenetically separated from susceptible strains from the 1990s. The *qnr* genes protect DNA-gyrase and topoisomerase IV, to prevent the action of quinolones and confer low-level fluoroquinolone resistance, often below the clinical breakpoint (Tran and Jacoby, 2002, Nordmann and Poirel, 2005). The *qnrVC1* gene is a mobile quinolone resistance gene that is associated with class I integrons (Fonseca et al., 2008). Orthologues of *qnrVC1* have been

reported in a variety of bacterial species (Fonseca et al., 2008). More recently, *qnrVC1* and another variant *qnrVC6* have been reported from clinical *P. aeruginosa* strains isolated between 2007 and 2012 (Liu et al., 2018, Belotti et al., 2015). In the current study, the acquired fluoroquinolone resistance gene *qnrVC1* was observed in isolates sampled in 2017 and 2018, indicating that transferable fluoroquinolone resistance genes may be recently acquired or have passed unnoticed in previous ocular isolates of *P. aeruginosa.* No strains contained *qnrVC6.*

This study revealed that *qnrVC1* was associated with a Tn*3* transposon, which has not been previously reported in *P. aeruginosa*. Integration of several other antibiotic resistance genes in these mobile genetic elements suggests these elements may concentrate antibiotic resistance genes. *QnrVC* is reported predominantly in water bacteria including *Aeromonas* spp., and *Acinetobacter* spp (Fonseca and Vicente, 2013). In this study, the recombination site (*attC)* of all four *qnrVC1* alleles had 100% identity with the aquatic bacterium *Vibrio parahaemolyticus.* This corresponds with our previous study in ocular isolates of *P. aeruginosa*, where acquired resistance genes closely matched with that of environmental isolates (Subedi et al., 2018b).

The current study demonstrated that *qnrVC1* carrying strains also had QRDRs mutations and were highly resistant to all three fluoroquinolones compared to those lacking *qnrVC1*. This indicates that possession of *qnrVC1* potentially facilitates selection of strains with high-level fluoroquinolone resistance. Given that *qnrVC1* has been reported as being responsible for low-level fluoroquinolone resistance (Robicsek et al., 2006a), the high MICs observed for these isolates is not clear but may indicate synergistic activity between the QRDR mutations and *qnrVC1.*

Keratitis isolates of *P. aeruginosa* predominantly possess a cytotoxic factor, ExoU, which is associated with mobile genetic elements (Subedi et al., 2019). Many studies have shown that fluoroquinolone resistant strains were also associated with possession of *exoU* (Heidary et al., 2016, Georgescu et al., 2016, Sawa et al., 2014, Tran et al., 2011, Agnello et al., 2016, Agnello

and Wong-Beringer, 2012, Stewart et al., 2011, Finck-Barbançon et al., 1997, Garey et al., 2008, Cho et al., 2014, Choy et al., 2008). Correspondingly, these data suggested the predominance of *exoU* in fluroquinolones resistance isolates. Although the reason behind this predominance is not completely understood, the concurrent occurrence of transferable fluoroquinolone resistance genes and *exoU* may heighten the concern for the selection of more virulent strains during antibiotic therapy.

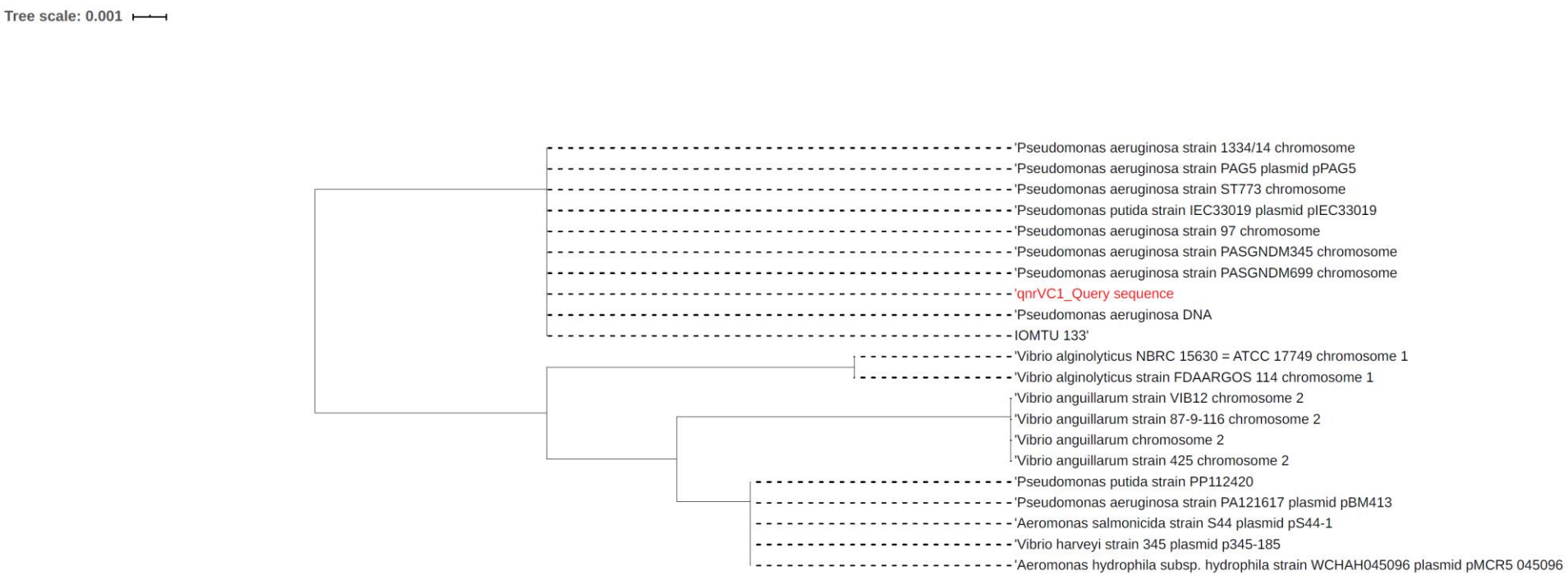
Supplementary information

Supplementary Table 5-1 *P. aeruginosa* isolates.

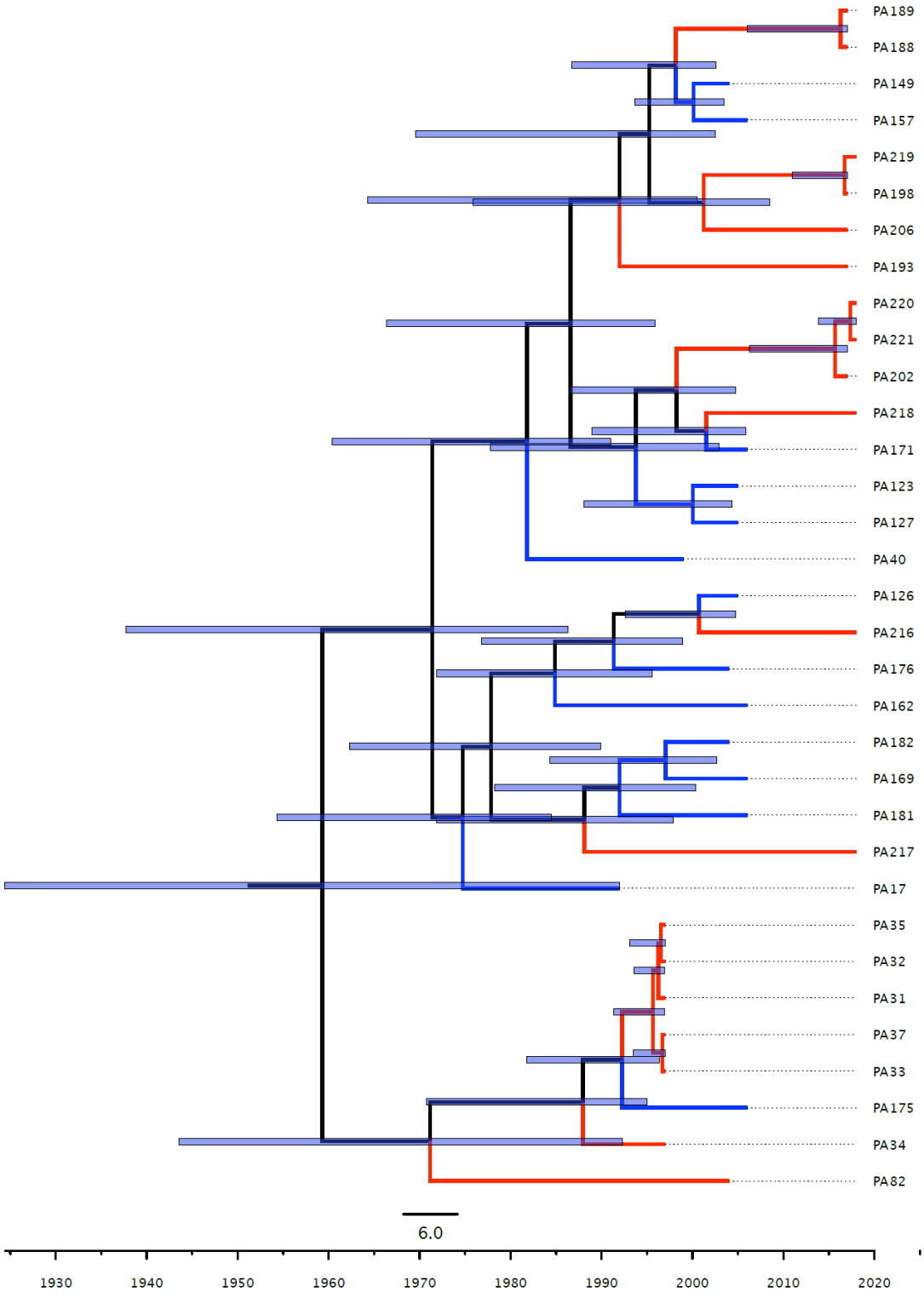
P.aeruginosa isolate designation	Year of isolation	Region of isolation
PA17*	1992	Flinders University, Adelaide, Australia
PA40*	1999	SEH, Sydney, Australia
PA 176	2004	Australia
PA182	2004	PAH Brisbane Australia
PA149*	2004	Flinders University, Adelaide, Australia
PA123	2005	PAH Brisbane Australia
PA126	2005	PAH Brisbane Australia
PA127	2005	PAH Brisbane Australia
PA 162	2006	PAH Brisbane Australia
PA169	2006	PAH Brisbane Australia
PA181	2006	PAH Brisbane Australia
PA157*	2006	PAH Brisbane Australia
PA171*	2006	PAH Brisbane Australia
PA175*	2006	PAH Brisbane Australia
PA31*	1997	LVPEI, Hyderabad, India
PA32*	1997	LVPEI, Hyderabad, India
PA33*	1997	LVPEI, Hyderabad, India
PA34*	1997	LVPEI, Hyderabad, India
PA35*	1997	LVPEI, Hyderabad, India
PA37*	1997	LVPEI, Hyderabad, India
PA82*	2004	LVPEI, Hyderabad, India
PA188	2017	LVPEI, Hyderabad, India
PA189	2017	LVPEI, Hyderabad, India
PA193	2017	LVPEI, Hyderabad, India
PA198	2017	LVPEI, Hyderabad, India
PA202	2017	LVPEI, Hyderabad, India
PA206	2017	LVPEI, Hyderabad, India
PA216	2018	LVPEI, Hyderabad, India
PA217	2018	LVPEI, Hyderabad, India
PA218	missing	LVPEI, Hyderabad, India
PA219	2018	LVPEI, Hyderabad, India
PA220	2018	LVPEI, Hyderabad, India
PA221	2018	LVPEI, Hyderabad, India

Isolates with asterisk (*) are from previous study (Subedi, Vijay, Kohli, Rice, & Willcox, 2018), LVPEI= LV Prasad Eye Institute, PAH=Princes Alexandra Hospital, SEH= Sydney Eye Hospital.

Subedi, D., Vijay, A. K., Kohli, G. S., Rice, S. A., & Willcox, M. (2018). Comparative genomics of clinical strains of *Pseudomonas aeruginosa* strains isolated from different geographic sites. *Sci Rep, 8*(1), 15668. doi:10.1038/s41598-018-34020-7



Supplementary Figure 5-7 Tree View of the blast comparison of qnrVC1 gene.



Supplementary Figure 5-8 Phylogenetic tree based on Bayesian evolutionary analysis by sampling trees.

Tree was constructed using concatenated multi locus sequences (MLST) under strict clock analysis. The tip of the tree was constrained by date of isolation. The time scale is shown in years at the bottom and the bars represented the confidence interval. Isolates from India are labelled red and Australian isolates are labelled blue. The tree was visualized using Figtree v1.4.

6 Changes to the resistome of *Pseudomonas aeruginosa* clone ST308 associated with corneal infection over time

This work has been submitted as:

- Mahjabeen Khan, Mark D P Willcox, Scott A Rice, Savitri Sharma, Fiona Stapleton, Changes to the resistome of *Pseudomonas aeruginosa* clone ST308 associated with corneal infection over time. Experimental Eye Research.

Date of submission: 06.09.2020

Status: Under review (addressing 1st revision).

Student contribution: 70%, design, laboratory work, analysis and writing.

I certify that all co-authors of this work have agreed to submission of this work as a part of this thesis.

6.1 Introduction

Pseudomonas aeruginosa causes a variety of infections, including lung infections in patients with cystic fibrosis, skin infections after burns and corneal infections (microbial keratitis). The increasing prevalence of multi-drug resistant (MDR) *P. aeruginosa* isolates reduces treatment options and complicates the management of these infections. Antibiotic resistance occurs mainly due to chromosomal gene mutations and possession of transferrable resistance determinants (Lister et al., 2009). MDR isolates can be clonal, particularly those associated with hospital acquired infections (Woodford et al., 2011).

Clones of *P. aeruginosa* isolated from different environments may vary. Clones may cause infection outbreaks when they enter a new environment, for example, *P. aeruginosa* isolated from water sources can also be isolated from cystic fibrosis patients (Römling et al., 1994). Diverse clonal lineages can cause disease in the lungs of cystic fibrosis patients (Romling et al., 1992) Clones of ocular isolates of *P. aeruginosa* have rarely been reported (Lomholt et al., 2001, Subedi et al., 2018b). However, five multi-drug resistant *P. aeruginosa* isolates, collected in 1997 from microbial keratitis cases in India, have been reported to be clonal and of sequence type 308 (Subedi et al., 2018b). ST308 is a multi-drug resistant epidemic clone that has been associated with sporadic outbreaks of hospital infections (Jeanvoine et al., 2019). *P. aeruginosa* ST308 has been identified as a major contaminant in the hospital water plumbing networks where it was identified as a copper tolerant clone due to the presence of genomic island number 7 (Jeanvoine et al., 2019). This clone shows intraclonal diversity as it adapts to different environments and acquires genes such as those on mobile genetic elements and acquires mutations in its genome (Abdouchakour et al., 2017).

The current study investigated the genomes of MDR *P. aeruginosa* corneal ST 308 isolates collected in 2018 with those recovered from the same location in India and collected in 1997. The aim of the study was to investigate whether this clone had changed its resistome during this period.

6.2 Materials and methods

6.3 *P. aeruginosa* strains

All strains had been isolated from the corneas of patients with keratitis that presented at the LV Prasad Eye Institute in 1997 or 2018. Isolates from 1997 were included from a previous study (Subedi et al., 2018b). Isolates from 2018 were selected from a larger group of isolates described in chapter 4. All isolates were obtained from different patients. Sequence types were investigated using PubMLST (https://pubmlst.org/).

6.4 *P. aeruginosa* genomic sequencing and analysis

DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen, Hilden Germany) as per the manufacturer's recommendations from two keratitis *P. aeruginosa* strains 198 and 219 isolated in India in 2018 for cases of microbial keratitis. A paired-end library was prepared using Nextera XT DNA library preparation kit (Illumina, San Diego, CA, USA). All the libraries were multiplexed on one MiSeq run. The raw reads of the sequenced genomes were analysed for their quality using FastQC version 0.117 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc). Version 0.38 of the Trimmomatic (Bolger et al., 2014) was used for trimming the adapters from the reads following *de-novo* assembly using Spades v3.13.0 (Nurk et al., 2013). Genomes were annotated using Prokka v1.12 (Seemann, 2014). Resistance genes were identified using the online database Resfinder v3.1 (Centre for Genomic Epidemiology, DTU, Denmark) (Zankari et al., 2012). Mutations in the genes were detected using Snippy V2 (Seeman, 2015) with PAO1 as the reference genome. Core genome and pan genomes were analysed using Harvest Suite Parsnp v1.2 and Roary v3.11.2 respectively. Integrons were located using Integron Finder in Galaxy v1.5.1 (Cury et al., 2016). The genes possessed by strains 198 and 219 were then compared to those from other ST308 isolates that had been previously examined (Subedi et al., 2018b). Genomes of other *P. aeruginosa* isolates (Bio project PRJNA381838) were also included for comparison. Several non-clonal isolates were also added for comparison, including isolate PA17 (Accession number NZ_QDGR00000000.1), PA57 (Accession number

NZ_QDGS00000000.1), PA86 (Accession number NZ_QDGD00000000.1), PA_D1 (Accession number CP012585.1) and PA_D2 (Accession number CP012578.1). These genomes were re-annotated in the current study to avoid any bias. Pairwise genome comparison of the isolates was conducted using web-based programme JSpeciesWS (http://jspecies.ribohost.com/jspeciesws/#analyse).

6.5 Antibiotic susceptibility

Two strains (PA198 and PA219) isolated in 2018 and five strains (PA31, PA32, PA33, PA35 and PA37) isolated in 1997 (Subedi et al., 2018b) were screened for resistance to a variety of antibiotics which are commonly used to treat microbial keratitis (Willcox, 2011). The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of ciprofloxacin, levofloxacin, gentamicin, ceftazidime (Sigma-Aldrich, St. Louis Missouri, USA), polymyxin B (Sigma-Aldrich, Vandtårnsvej, Søborg, Denmark) tobramycin, piperacillin (Cayman Chemical Company, Ann Arbor, Michigan, USA) and imipenem (LKT Laboratories Inc, Minnesota, USA) were determined using the broth microdilution method in 96-wells plates following the Clinical and Laboratory Standard Institute CLSI guidelines (CLSI). The concentrations of antibiotics tested ranged from 5120 µg/ml to 0.25 µg/ml. Susceptibilities were interpreted using the European Committee on Antimicrobial Susceptibility Testing v9 (EUCAST, 2020) and CLSI (Patel et al., 2014) breakpoints for antibiotics.

6.6 Results

Isolates PA198 and PA219 had sequence type 308 indicating that these strains were clonally related to each other and the five ST308 strains isolated in 1997 at the same hospital.

6.6.1 Whole genome comparison and phylogenetics of ST308

Isolate PA219 had the largest genome of 7456853 base pairs (bp) (Table 6-1). The number of core genes and total genes were similar for all isolates, except isolate PA219 which had a larger number of total genes but a similar number of core genes to all other isolates. The recent isolates PA198 and PA219 had acquired a large number of additional (accessory) genes.

Table 6-1 General features of ST308 of *P. aeruginosa* isolates.

***P. aeruginosa* isolates**	**Core genes**	**Total genes**	**Accessory genes**	**Number of coding sequences**	**Number of contigs**	**Length of genome (bp)**	**% GC**	**tRNA**
PA31	5445	6937	1709	6619	137	7100450	66	69
PA32	5447	6927	1701	6611	155	7101461	66	69
PA33	5440	6932	1699	6609	166	7092296	66	69
PA35	5442	6932	1701	6611	156	7094655	66	69
PA37	5450	6958	1735	6645	241	7154299	65.9	69
PA198	5454	6882	1817	6727	118	7101775	66	59
PA219	5451	7247	2202	7112	163	7456853	65.	59

Accessory genes were determined by subtracting number of core genes (4910) of the reference strain PAO1 from total number of CDS of each isolate. Grey shaded isolates are the recent clones.

Pan genome phylogeny was constructed using PAO1 (Accession number NC_002516.1) as a reference genome. The recently published *P. aeruginosa* genome sequences of ST308 strains on NCBI with Bio project number PRJNA381838 were also aligned for both core and pan genome phylogeny. These reference isolates were from non-ocular sources. In the pan genome phylogeny, all the ocular isolates clustered together in one group which included the non-ocular isolates PAO1 and PASGNDM590. The majority of non-ocular isolates were clustered into two separate groups, separated from the cluster containing the ocular ST308 isolates (Figure 6-1).

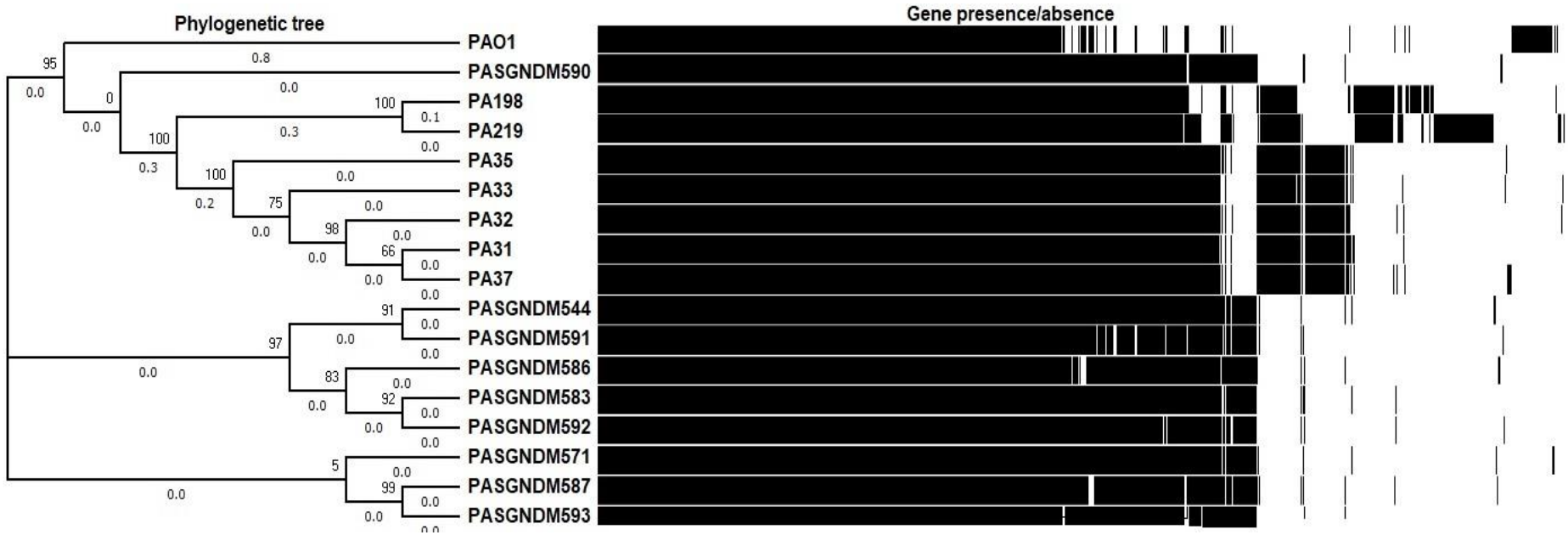


Figure 6-1 Pan genome phylogeny of *P. aeruginosa isolates* using Roary.

PAO1 was used as reference to build the tree. The number (ranging between 66-100) at each node (vertical line of the tree) represents bootstrap values. A higher bootstrap indicates higher relatedness or similarity. The number displayed on each horizontal line is the branch length, which represents an evolutionary lineage changing over time.

When comparing the genomes of strains, isolate PA219 was used as a reference because it had the largest genome of 7456853 base pairs. Comparison of the whole genomes of the two 2018 ST308 isolates with previous isolates from cases of keratitis (Subedi et al., 2018b) showed that they were > 94% similar and they were > 89.9% similar to other non-ocular ST308 isolates within Bio project PRJNA381838 (Table 6-2). Non-ST308 isolates were all less than 84% similar.

Table 6-2 Pairwise comparison of the genomes of *P. aeruginosa* isolates compared to strain PA219.

***P. aeruginosa* isolates**	**Isolation source**	**Aligned %**	**Aligned base pairs**
ST308 isolates			
PA31	Cornea	94.09	7016166
PA32	Cornea	94.09	7015918
PA33	Cornea	94.09	7015041
PA35	Cornea	94.08	7015534
PA37	Cornea	94.09	7017651
PA198	Cornea	95.77	7141329
PASGNDM544	Endotracheal tube	90.73	6765394
PASGNDM571	Urine	90.72	6765003
PASGNDM583	Urine	90.76	6767894
PASGNDM586	Urine	89.92	6705216
PASGNDM587	Foot wound	90.14	6721892
PASGNDM590	Urine	90.69	6762596
PASGNDM591	Urine	89.93	6705872
PASGNDM592	Urine	90.74	6766496
PASGNDM593	Urine	90.42	6742830
Non-ST308 isolates			
PA17	Eye	81.34	6064371
PA57	Cystic fibrosis	77.89	5808417
PA86	Cystic fibrosis	78.15	5827469
PA_D1	Sputum	83.99	6263071
PA_D2	Sputum	83.99	6263071

PASGNDM isolates are those from Bio project PRJNA381838.

6.6.2 Antibiotic susceptibility of *P. aeruginosa* isolates

Isolate PA219 was resistant to all antibiotics except polymyxin B (MIC= 0.5 µg/ml, MBC=1 µg/ml). Isolate PA198 was resistant to ciprofloxacin, levofloxacin, tobramycin and gentamicin, and showed intermediate susceptibility to polymyxin B. The isolates from 1997 were all resistant to gentamicin and tobramycin and showed either intermediate or definite resistance to imipenem. All five isolates from 1997 were resistant or had intermediate resistance to ciprofloxacin and four were resistant to levofloxacin. Two isolates from 1997 showed intermediate resistance to polymyxin B. Overall, the MIC and MBC values to ciprofloxacin and levofloxacin of PA198 and PA219 were higher than those recorded for the historical isolates (Supplementary Table 1).

6.6.3 Possession of horizontally-acquired resistance genes

In total 24 acquired resistance genes were present in the ST308 isolates of *P. aeruginosa* (Table 6-3). The isolates from 1997 all possessed the same 11 resistance genes. However, the isolates from 2018 had acquired additional resistance genes. Isolate PA198 carried 15 and PA219 carried 20 resistance genes. Ten resistance genes were common to all seven isolates (Table 6-3).

The ten genes included three aminoglycoside resistance genes *(aph(6)-Id*, *aph(3')-IIb, aph(3'')-Ib),* a beta-lactam resistance gene (*blaPAO),* a tetracycline resistance gene (*tet(G)),* a fosfomycin resistance gene *(fosA)*, a chloramphenicol resistance gene (*catB7)* a sulphonamide resistance gene (*sul1),* and a quaternary ammonium compound resistance gene (*qacEdelta1)*. The recent isolates lacked one beta-lactam gene (*blaOXA-50)* which was present in all the historical isolates.

Strain PA198 had acquired a 16S rRNA methylase gene *(rmtD2)* and three aminoglycoside modifying enzyme genes *aph(6)-Id*, *aph(3')-IIb,* and *aph(3'')-Ib)*. Strain PA219 had acquired three different aminoglycoside resistance genes, a 16S rRNA methylase (*rmtB),* a streptomycin adenylyltransferase gene *(aadA1)* and an aminoglycoside acetyltransferase

gene *(aac(6')-Ib-cr).* Strain PA219 had also acquired the plasmid related aminoglycoside and fluoroquinolone resistance gene *aac(6')-Ib-cr*.

One fluoroquinolone resistance gene, *crpP,* was present in all isolates from 1997 and 2018. An integron-related fluoroquinolone resistance gene *qnrVC1* (Belotti et al., 2015) was only present in isolates from 2018 (Table 6-3). The plasmid related aminoglycoside and fluoroquinolone resistance gene *aac(6')-Ib-cr* was found in strain PA219 and this strain had higher MICs for ciprofloxacin and levofloxacin compared to strain PA198 and the 1997 isolates.

The metallo beta-lactamase gene class B metallo beta-lactamase *blaVIM-2* and a transposon (Tn2) encoded gene *blaTEM-1B* had been acquired by isolate PA219. An extended spectrum plasmid-related class A beta-lactamase gene *blaPME-1* had been acquired by PA198. A class-D beta-lactamase gene *blaOXA-488* had been acquired by both PA198 and PA219.

6.6.4 Non-synonymous mutations in the ST308 resistome

Non-synonymous mutations leading to changes in the nucleic acid sequence in the resistance genes of these *P. aeruginosa* isolates, including those related to efflux pumps, antibiotic-inactivating enzymes and drug target alterations were examined (Supplementary table 2). These non-synonymous mutations were made in comparison to the reference genome of strain PAO1. The number of mutations in almost all of the genes remained the same in the 1997 and 2018 isolates. However, the efflux pump gene *opmH* contained 10 SNPs in the isolates from 2018, but only 1-5 SNPS in the isolates from 1997. Similarly, *oprD* also contained 8 SNPs in the two 2018 isolates. Furthermore, non-synonymous insertions/deletions and frame-shift mutations were found in the two isolates from 2018 (Supplementary Table 3) whereas the ST308 isolates from 1997 had no insertions/deletions or frame-shift mutations (Subedi et al., 2018b).

6.6.5 Mobile genetic elements

Due to the large number of acquired resistance genes in the recent isolates, the genes locations were extracted using Geneious. *QnrVC1* in the recent clones was integrated on Tn3

transposons. *QnrVC1* was accompanied by other genes on the transposon including aminoglycoside 6'-phosphotransferase *(aph(6')-Id*) and tetracycline (*tetG*) resistance genes. *BlaTEM-1B* in isolate PA219 was also integrated into the Tn3 transposon. *BlaTEM-1B* and *qnrVC1* carrying transposons were located on contig 2 and 4 respectively and so were considered to be different Tn3 transposons. In isolate PA219, a class 1 integron was also identified on contig 2 which was carried *blaOXA-10* (Supplementary Figure 1). All the other acquired resistance genes appeared to be randomly integrated into the chromosome.

Table 6-3 Presence of acquired antibiotic resistance genes in *P. aeruginosa* ST308 ocular isolates.

Antibiotic classes	Resistance genes	PA198	PA219	PA31	PA32	PA33	PA35	PA37
Amino- glycoside	*aph(6)-Id*	■	■	■	■	■	■	■
	aph(3')-VI	■						
	aph(3')-IIb	■						
	aph(3'')-Ib	■	■	■	■	■	■	■
	aadA1		■					
	rmtD2	■						
	rmtB		■					
Fluoroquinolone + Aminoglycoside	*aac(6')-Ib-cr*		■					
Fluoroquinolone	*crpP*	■	■	■	■	■	■	■
	qnrVC1	■	■					
Beta-lactam	*blaOXA-488*	■	■					
	blaPAO	■	■	■	■	■	■	■
	blaOXA-50			■	■	■	■	■
	blaPAU-1		■					
	blaOXA-10		■					
	blaTEM-1B		■					
	blaVIM-2		■					
	blaPME-1	■						
Quaternary ammonium compound	*qacEdelta1*	■	■	■	■	■	■	■
Sulphonamide	*sul1*	■	■	■	■	■	■	■
Tetracycline	*tet(G)*	■	■	■	■	■	■	■
Macrolide	*mph(E)*		■					
	mph(A)		■					
Macrolide, Lincosamide and Streptogramin B	*msr(E)*		■					
Chloramphenicol	*catB7*	■	■	■	■	■	■	■
Fosfomycin	*fosA*	■	■	■	■	■	■	■

Black denotes gene presence and white colour shows gene absence

6.7 Discussion

This study examined whether the resistome of ST308 clones of ocular isolates of *P. aeruginosa* had changed over time. Previously, *P. aeruginosa* ST308 clones had been reported as multidrug-resistant isolates from nosocomial (Fournier et al., 2012), ocular (Subedi et al., 2018b) and canine (Wang et al., 2014) infections. The ocular isolates of clone ST308 from 2018 had acquired additional resistance genes compared to those isolated in 1997 at the same hospital in India. Also, the recent 2018 isolates of ST308 showed changes in the mutational patterns of the resistance genes compared to ocular isolates from 1997.

There was >94% similarity in the genomes of the ocular ST308 isolates, indicating that these isolates were indeed very similar and can be considered to be clonally related. Of all the ocular ST308 isolates, the recent isolate PA219 had the largest genome which contained more accessory genes and coding sequences. All the ST308 isolates, whether ocular or non-ocular, had a similar number of core genes, perhaps indicating a conserved genome of these *P. aeruginosa* isolates (Römling et al., 1995, Klockgether et al., 2011). However, the larger pan genome of isolate PA219 indicated that this isolate had acquired a number of genes (Klockgether et al., 2011). In the pan genome phylogeny, all ocular isolates clustered as one group and all non-ocular isolates grouped separately, which was similar to the previous findings (Freschi et al., 2015a, Subedi et al., 2018b).

Two different variants of 16S rRNA methylase, *rmtD2* and *rmtB,* related to aminoglycoside resistance were found in the 2018 isolates. These genes have not been reported previously in ST308 but other variants of the same genes have been identified in clones ST316 and ST235 (Tada et al., 2018). The latter clone being identified as a widespread multi-drug resistance clone. The presence of a large group of beta-lactam resistance genes, specifically those acquired on mobile genetic elements, is a unique finding related to ST308 in the current study. For example, a metallo beta-lactam gene including *blaTEM-1B* and a FQ resistance gene *qnrVC1* on a transposon, and *blaOXA-10* on an integron. These beta-lactam resistance

genes have not been reported previously in strains of this clone (Subedi et al., 2018b). However, the possession of the *sul1* gene in isolates of the present study was similar to a previous report (Wang et al., 2014). The possession of *blaVIM-2* and *blaTEM-1B* may have been responsible for the high MIC to piperacillin and imipenem of PA219. Previously, these genes were associated with increased MIC of imipenem and piperacillin/tazobactam in *P. aeruginosa* isolates (Quinones-Falconi et al., 2010).

Metallo beta-lactam genes are usually found on class-1 integrons along with other antibiotic resistance determinants (Yu et al., 2006). This finding is similar to the present study but identification of a class 1 integron carrying resistance genes in ocular *P. aeruginosa* isolates is a novel finding. Sequence type ST308 has previously been reported to carry metallo beta-lactam *blaIMP-13* (Wang et al., 2014, Fournier et al., 2012). However, *blaTEM-1B, blaVIM-2* and *blaPME-1* had not been previously reported in ST308. Acquired genes within the mobile genetic elements of ST308 clones were not identified in an earlier report (Subedi et al., 2018b). Both recent isolates PA198 and PA219 had acquired genes associated with mobile genetic elements in the current study. Additional beta-lactam genes were found in the recent clones compared to the historical ones.

The presence of the plasmid-related fluoroquinolone resistance gene *qnrVC1* (Kocsis et al., 2019) and the recently reported plasmid-related gene *crpP* (Chávez-Jacobo et al., 2018) are also novel findings in ocular ST308 *P. aeruginosa* isolates. All isolates contained the fluoroquinolone resistance gene *crpP,* but this had not been identified as a potential plasmid related fluoroquinolone resistance gene prior to the publication of resistance genes in the 1997 isolates (Subedi et al., 2018b). Usually, fluoroquinolone resistance is due to mutation in DNA gyrase and topoisomerase IV genes (Livermore, 2002). However, the very high MICs to ciprofloxacin and levofloxacin in the 2018 isolates of ST308 might be due to the acquisition of *qnrVC1*. Strain PA219 had also acquired the plasmid related fluoroquinolone resistance gene *aac(6')-Ib-cr* (Ma et al., 2009), which can confer resistance to both fluoroquinolones and aminoglycosides (Robicsek et al., 2006b). This gene was responsible for the 16 to 128-fold

higher MICs for ciprofloxacin in transconjugants of *Enterobacteriaceae* (Yang et al., 2008) and MIC of 64 µg/ml for ciprofloxacin of MDR *P. aeruginosa* non-ocular isolates (Araujo et al., 2016). These resistance elements for fluoroquinolones suggest that alternative treatments for keratitis other than fluoroquinolone monotherapy might need to be considered in the future. A poorer clinical outcome of *P. aeruginosa* corneal infection is associated with resistance to the fluoroquinolone moxifloxacin (Lalitha et al., 2012). Furthermore, acquisition of a larger number of aminoglycoside and beta-lactam resistance genes is alarming because, where first line therapy such as monotherapy with fluoroquinolones fails, fortified antibiotics (Nixon, 2018) such as gentamicin plus cephalosporins are often prescribed.

Indels (insertion/deletion polymorphisms due to non-synonymous mutations) in the 2018 isolates of *P. aeruginosa* ST308 clones were not present in the strains isolated in 1997 (Subedi et al., 2018b). Indels or frame shift mutations can potentially trigger changes in the amino acid sequence of proteins resulting in unstable protein expression or expression of non-functional proteins. Genetic mutations can lead to a change in the binding ability of ligands (McCready et al., 2019) and a potential increase in antibiotic resistance due to target alteration (Juan et al., 2005, Amin et al., 2005). Additionally, the increased number of SNPs suggests that an increase in selection pressure in the environment may have selected for these mutations. Overall, large mutations were present for *opmH* and *rocS*. OpmH is part of an efflux pump responsible for the resistance of *P. aeruginosa* to the biocide triclosan that is often used in hospitals (Jones et al., 2000). RocS is a sensor kinase involved in the transduction pathway for fimbrial adhesion during biofilm formation (Kulasekara et al., 2005). No other differences were found in mutation of other genes in the ocular ST308 isolates in the present study.

The emergence of genetic variants in cystic fibrosis infection strains has been related to mutation during prolonged colonization and antibiotic administration (Bragonzi et al., 2009, Jelsbak et al., 2007). The ocular and the non-ocular isolates used for comparison in the current study were different to each other in the phylogenetic evolution. The recent ocular clones were different from the historical ones in acquisition of genes and showed different patterns of gene

mutations. Furthermore, the coeval (isolated at same time) isolates PA198 and PA219 were even different from each other in the number and patterns of mutations. Genomic variations occur as a result of mutations in the genes even in the *Pseudomonas aeruginosa* isolates causing the same infection (Chung et al., 2012). The recent clones 198 and 219 with distinct features were different in their characteristics from rest of the isolates. This differentiation indicated the intraclonal diversity of isolates of this particular sequence type (Abdouchakour et al., 2017).

The acquisition of resistance genes, at least in the Indian environment, may result in high levels of antibiotic resistance and this may in turn result in vision loss and blindness. Furthermore, the increasing levels of antibiotics needed to kill strains (higher MICs) suggest that the treatment of keratitis might be becoming more problematic. Studies which link genetic and phenotypic resistance to clinical outcome would help to identify the reasons for poor outcomes.

Supplementary information

Supplementary Table 1. Antibiotic susceptibility of *P. aeruginosa* isolates

Breakpoints	**Ciprofloxacin µg/ml ≤1,2, ≥4**		**Levofloxacin µg/ml ≤2, 4, ≥8**		**Gentamicin µg/ml ≤4, 8, ≥16**		**Tobramycin µg/ml ≤4, 8, ≥16**		**Piperacillin µg/ml ≤16**		**Imipenem µg/ml ≤2, 4, ≥8**		**Ceftazidime µg/ml ≤8, 16, ≥32**		**Polymyxin µg/ml ≤2, 4, ≥8**	
***P. aeruginosa* Strains of**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
PA198	1280 (R)	2560	320 (R)	1280	2560 (R)	5120	16 (R)	16	8	8	1	2	8	8	4 (I)	4
PA219	≥5120 (R)	≥5120	640 (R)	1280	≥5120 (R)	≥5120	1280 (R)	2560	2560 (R)	5120	32 (R)	64	16 (I)	32	0.25	1
PA31	32 (R)	64	32 (R)	32	5120 (R)	5120	640 (R)	1280	4	8	4 (I)	16	16 (I)	32	4 (I)	4
PA32	64 (R)	128	32 (R)	32	2560 (R)	5120	640 (R)	1280	16	32	4 (I)	4	16 (I)	16	4 (I)	16
PA33	128 (R)	128	32 (R)	64	2560 (R)	5120	≥5120 (R)	≥5120	32 (R)	64	8 (R)	16	32 (R)	64	2	4
PA35	2 (I)	4	2	4	2560 (R)	2560	1280 (R)	2560	8	16	16 (R)	16	4	8	2	2
PA37	64 (R)	128	32 (R)	32	2560 (R)	2560	1280 (R)	2560	8	16	8 (R)	8	16 (I)	64	2	4

P. aeruginosa isolates in the light shade are recent and those with dark shading are historical isolates. MIC and MBC values of historical isolates were included from previously published data (Subedi et al., 2018b) for all antibiotics except tobramycin and piperacillin. MICs and MBCs of these two antibiotics for the historical isolates and all antibiotics for the recent isolates have been are examined in this study.

Supplementary Table 2. The number of non-synonymous single nucleotide polymorphisms in the genes of *P. aeruginosa* encoding for antibiotic resistance

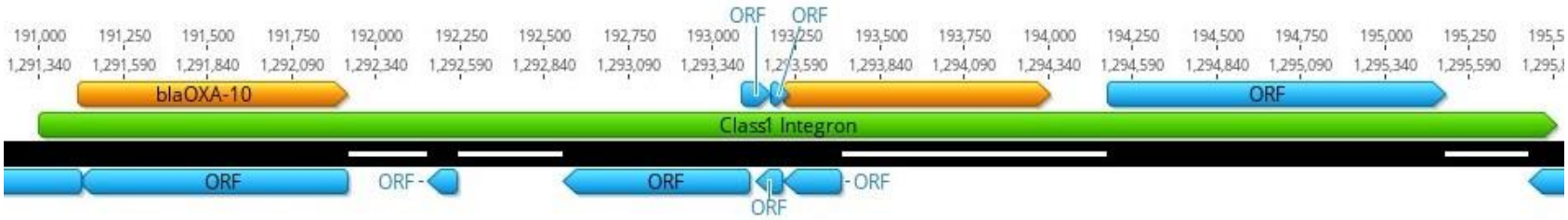
Gene locus	Gene name	*P. aeruginosa*/number of SNPs						
			PA31	PA32	PA33	PA35	PA37	PA198	PA219
PA0156	*triA*	Antibiotic Efflux	4	5	5	6	5	5 *	5 *
PA0157	*triB*		0	0	1	0	0	0	0
PA0158	*triC*		2	2	2	2	2	2 *	2 *
PA0424	*mexR*		2	1	1	1	1	1	1
PA0426	*mexB*		1	1	2	1	4	2 *	2 *
PA1236	*farB*		1	1	1	1	1	1	1
PA1282	*lrfA*		6	6	9	6	8	8 *	8 *
PA1316	*lrfA*		2	2	2	2	2	2	2
PA1435	*mexM*		4	4	4	4	4	4	4
PA1436	*mdtC*		2	2	2	2	2	2	2
PA2018	*mexY*		5	5	5	5	5	5 *	5 *
PA2019	*mexX*		4	4	4	4	4	4	4
PA2389	*macA*		2	1	1	1	1	1	1
PA2390	*macB*		1	1	1	1	1	1	1
PA2391	*opmQ*		6	5	6	6	6	5 *	5 *
PA2491	*mexS*		2	2	2	2	2	2	2
PA2495	*oprN*		1	1	1	1	1	1	1
PA2837	*opmA*		3	3	3	3	3	3	3
PA3019	*taeA*		1	1	1	1	1	1	1
PA3137	*farB*		1	1	1	1	1	0	0
PA3521	*opmE*		3	3	3	3	3	3 *	3 *
PA3522	*mexQ*		4	4	4	4	4	4	4
PA3523	*mexP*		2	2	2	2	2	2	2
PA3676	*mexK*		1	1	1	1	1	1	1
PA3677	*mexJ*		2	2	2	2	2	2	2
PA3678	*mexL*		1	1	1	1	1	1	1
PA4205	*mexG*		1	1	1	1	1	1	1
PA4206	*mexH*		1	1	1	1	1	1	1
PA4207	*mexI*		1	1	1	1	1	2	1
PA4208	*opmD*		3	3	3	3	3	2 *	2 *
PA4374	*mexV*		2	2	2	2	2	2	2
PA4375	*mexW*		2	2	2	2	2	2	2
PA4598	*mexD*		2	2	2	2	2	2	2
PA4599	*mexC*		7	8	8	8	8	7 *	7 *
PA4974	*opmH*		2	1	1	5	5	10 *	10 *

Gene locus	Gene name		*P. aeruginosa*l number of SNPs						
			PA31	PA32	PA33	PA35	PA37	PA198	PA219
PA4990	*emrE*		1	1	1	1	2	1	1
PA4997	*msbA*		2	3	3	2	3	4 *	4 *
PA5158	*adeC*		3	3	3	3	3	3 *	3 *
PA5160	*farB*		4	3	3	4	3	3	3
PA5518	*rosB*		3	3	3	3	3	2	2
PA0706	*catB7*	Antibiotic inactivation	4	4	4	4	4	4	4
PA4109	*ampR*		2	2	2	2	2	2 *	2 *
PA4110	*ampC*		5	5	5	5	5	5 *	5 *
PA4119	*Aph(3')-IIb*		2	2	2	2	2	2	2
PA5514	*OXA-50*		1	2		3	2	2	2
PA0903	*alaS*	Antibiotic target alteration	1	1	1	1	1	1	1
PA1972	*pmrC*		3	3	3	3	3	3	3
PA3002	*mfd*		1	2	2	2	2	2	2
PA3168	*gyrA*		1	1	1	1	1	1	1
PA3946	*rosC*		6	8	8	7	6	8 *	8 *
PA4265	*tufA*		1	0	0	0	1	0	0
PA4560	*ileS*		2	2	2	2	2	3	2
PA4964	*parC*		2	2	2	2	2	2	2
PA4967	*parE*		1	1	1	1	1	1	1
PA3554	*arnA*		2	4	4	4	4	3 *	3 *
PA0920	*mprF*		6	6	6	6	6	6	6
PA0958	*oprD*		2	1	1	1	4	8 *	8 *
PA2492	*mexT*		3	2	3	5	4	2†	2 †
PA2020	*mexZ*		0	1	1	1	1	1	1

(*) represents insertions or deletions in the genes, (†) represents frame-shift mutations in the genes.

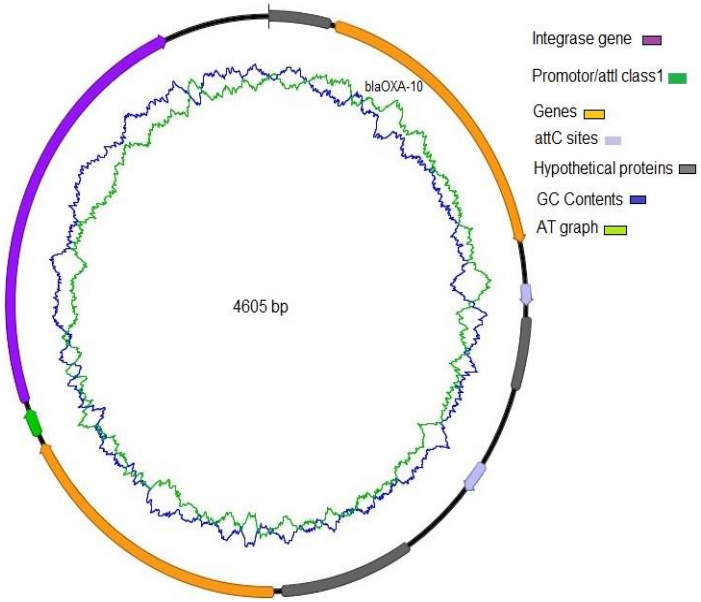
Supplementary Table 3. Insertion/deletion and frame-shift mutation in the resistance genes of the 2018 isolates

Gene locus	Genes	Number of deletions and insertions in the genes	
		PA198	**PA219**
PA0156	*triA*	2	2
PA0158	*triC*	2	1
PA0426	*mexB*	1	1
PA1282	*lrfA*	2	2
PA2018	*mexY*	1	1
PA2391	*opmQ*	3	3
PA3521	*opmE*	1	1
PA4208	*opmD*	1	1
PA4599	*mexC*	1	1
PA4974	*opmH*	7	8
PA4997	*msbA*	2	2
PA5518	*adeC*	1	1
PA4109	*ampR*	1	1
PA4110	*ampC*	2	2
PA3946	*rosC*	1	1
PA3554	*arnA*	2	2
PA0958	*oprD*	6	6
PA2492	*mexT*	1Frame-shift deletion	1Frame-shift deletion



Position of integron in the contig. Upper numbering represents position of contigs starting from 1. Lower is numbering of nucleotides using positions from source sequence.

Circular image of integron with annotations



Supplementary Figure 1. Class 1 integron carrying blaOXA-10 in isolate 219.

Integron Finder Galaxy version 1.5.1 was used to identify the type and location of integron. Prokka Galaxy version 1.12 was used for integron annotations. The integron and its annotations were visualized using Geneious v2020.2 Some of the annotations were manually edited in geneious.

7 Summary and Future Directions

This thesis analysed phenotypic and genotypic characteristics of numerous ocular *Pseudomonas aeruginosa* isolates to identify the patterns of resistance and underlying resistance mechanisms. The isolates were collected from two different regions: Australia (12 historical, 10 recent) and India (non-CL) (34 recent). Isolates collected at different time points were analysed to examine changes in resistance patterns over time. Furthermore, genomes of 14 (eight historical and six recent) Australian isolates and 18 (six historical from a previously published study and 12 recent) (Subedi et al., 2018b) Indian isolates were analysed. The genomes were analysed using computational analysis with different bioinformatics tools. The major findings of the thesis are listed below:

I. Antibiotic susceptibility of *P. aeruginosa* isolates has changed over time but there was no correlation between antibiotic and MPDS susceptibility.
II. *P. aeruginosa* keratitis isolates from India carried more acquired resistance genes.
III. *P. aeruginosa* isolates from both regions had distinct genetic features which included differences in their core and pan genomes, and TTSS virulence genes (*exoS* and *exoU).*
IV. There was evidence of clones circulating in the Indian population of strains and these showed changes in the carriage of antibiotic resistance genes over time.
V. *P. aeruginosa* isolates with a high number of gene mutations had larger mutations in their mismatch repair system.

7.1 Antibiotic susceptibility of *Pseudomonas aeruginosa* isolates changed over time and there was no correlation between antibiotic and MPDS susceptibility.

P. aeruginosa isolates from both regions showed variations in their susceptibility to antibiotics. Overall, Indian isolates were more resistant to antibiotics and some of these were MDR. Both historical and recent Indian isolates were resistant to antibiotics, but the historical Indian isolates had higher resistance levels to ciprofloxacin, levofloxacin, gentamicin and tobramycin. Increased antibiotic resistance in India might be due to the ease of availability of antibiotics (Porter and Grills, 2015). The antibiotic susceptibility of Australian isolates changed over time and again the more recent isolates showed higher resistant to antibiotics. The recent Australian *P. aeruginosa* isolates showed higher resistance to ciprofloxacin, imipenem, piperacillin and ceftazidime. Other reports from Australia however, have found stability in the antibiotic susceptibility of ocular *P. aeruginosa* isolates (Watson et al., 2018, Green et al., 2019) and this difference may be due to the use of strains collected from different populations in the current study and those previous studies. This increase in the antibiotic resistance in the Australian isolates is of concern and may indicate that alternative treatment options for microbial keratitis should be considered. A convenience sample of Australian *P. aeruginosa* isolates were collected from two different locations Brisbane (historical) and Sydney (recent) which may impact the results due to climatic variations in the regions. Further studies to explore isolates collected over time from the same region may show a clearer pattern of antibiotic susceptibilities.

The Australian strains of *P. aeruginosa* were compared for their MPDS susceptibility but no change in their susceptibilities to MPDS were found. Also, there was no correlation between antibiotic and MPDS susceptibility. Although, a recent report from Taiwan suggested an increased resistance to MPDS (Shen et al., 2020) but different studies reported stability in the antibiotic susceptibility of *P. aeruginosa* isolates (Hsiao et al., 2016). This is important when considering the comparison of the Australian and Indian isolates as the Australian isolates had been all isolated from contact lens-related microbial keratitis, whereas the India isolates had

all been isolated from non-contact lens-associated microbial keratitis. The lack of relationship between resistance to antibiotics and resistance to MPDS indicates that exposure to MPDS is unlikely to induce transfer of disinfectant resistant genes such as *qac* along with antibiotic resistance genes. This makes the comparison between the Australian and Indian isolates more valid, especially as strains from both countries had been isolated, predominantly, from the corneas of microbial keratitis patients.

7.2 *P. aeruginosa* keratitis isolates from India carried more acquired resistance genes

The bacterial genome can be divided into two type of genes, core (present in the majority of isolates) and accessory genes (present in select isolates and usually associated with MGE) (Tenover, 2006). These accessory genes together with core genes are part of the pan (total) genome. Further classification of accessory genes can be made, with those unique to one strain called cloud genes (or unique genes) and those shared by two or more strains known as shell genes. These acquired genes can be related to specific virulence or resistance traits of *P. aeruginosa*. *P. aeruginosa* acquires various resistance genes conferring resistance to different antimicrobials from the other strains of same or different species. In this study, out of total of 33 acquired resistance genes in these isolates, 24 were present in Indian and 13 were present in Australian isolates. The acquired resistance genes contribute towards genome evolution. Once acquired genes stabilize in the host, even in the absence of antibiotics, their loss can be slow (Salyers and Amábile-Cuevas, 1997). Despite them not being needed with cessation of their cognate antibiotic, once antibiotic use is resumed these genes can become active again (Salyers and Amábile-Cuevas, 1997). However, different acquired resistance genes contribute differently and have different stabilities in the bacterial genome due to antibiotic selection pressure (Marciano et al., 2007).

Once infection with a to multi-drug resistant strains has been initiated in the eye it is likely to take longer to resolve (CDC, 2013), the treatment cost is higher and it may result in permanent loss of vision. In the present study the MDR isolates from India mostly carried acquired

resistance genes. Although resistant *P. aeruginosa* ocular isolates have been reported previously, the genomic causes behind the antibiotic resistance have not been fully explored.

The current research found, for the first time in ocular isolates that, the acquisition of the fluoroquinolone resistant gene *qnrVC1* was related to increased fluoroquinolone resistance. Four of the Indian isolates harbouring *qnrVC1* had exceedingly high MICs for fluoroquinolones. This gene was carried on a Tn3 transposon and a class 1 integron in the Indian isolates. Similarly, another FQ related gene *crpP* which has been shown to be carried on a plasmid (Chávez-Jacobo et al., 2018) was found in the *P. aeruginosa* ocular isolates but it was not found to be carried on any MGE or related to elevated resistance levels to FQ in the present study.

Beta-lactam resistance genes *blaOXA-10*, *blaVIM-2, blaTEM-1, blaPSE-2, blaLCR-1* and aminoglycoside resistance genes *rmtD2, rmtB* and *aadA1* were also identified in the *P. aeruginosa* isolates. However, there was a disconnect between genotypic and phenotypic characteristics. Of the 20 *P. aeruginosa* isolates analysed for genomic changes, four isolates carried a large number of aminoglycoside resistance genes. The same isolates were phenotypically resistant to aminoglycosides. These findings indicate that ocular isolates of *P. aeruginosa* acquired specific antibiotic resistance genes but not all of those contributed to the antibiotic resistance. The current study did not analyse resistance to all antibiotics to which the genes may mediate resistance. For example, the presence of *aadA1* modifies streptomycin and spectinomycin (Shaw et al., 1993) but these antibiotics were not used in the present study as they are not used in the treatment of eye infection. The presence of these genes may indicate that the strains have been exposed to these antibiotics in the environment, or that the genes entered the strains as bystanders along with other genes of MGE that the strains required for survival.

In conclusion, MGEs play a key role in the rapid spread of antibiotic resistance genes. This study provides evidence for carriage of fluoroquinolone resistance genes *qnrVC1* and *crpP* on

MGEs in ocular isolates of *P. aeruginosa*. The *qnrVC1* gene was mobilised by a class I integron and Tn3 transposon and was associated with other antibiotics resistance genes. The *crpP* gene may have evolved prior to other transferable fluoroquinolone resistance genes. Although possession of these genes has not been shown to be associated with high level of fluoroquinolone resistance, *qnrVC1* in strains with *gyrA* (T81I) and *parC* (S87L) mutations were associated with very high fluoroquinolone resistance in this study. Further studies on a larger number of fluoroquinolone resistant strains, including molecular analysis of combined effects of mutations in resistance, should be undertaken to provide further insights into the role of acquired fluoroquinolone resistance gene in clinically significant resistance in *P. aeruginosa*. Additionally, the contribution of the exact acquired gene present in the MDR isolates requires further investigation. Future work using gene 'knock out' assays to analyse the effect of presence or absence of the acquired gene in the isolate will be of larger interest.

7.3 *Pseudomonas aeruginosa* isolates from both regions had distinct genetic features including core genome, pan genomes, sequence types, presence of TTSS *exoS* and *exoU* and gene mutations

P. aeruginosa isolates from two distinct regions were similar in some genetic traits, such as in their core genome and pan genomes. The core genome was composed of a similar number of genes in Australian and Indian isolates. However, the Indian isolates had a larger number of pan genes (average pan genes=10882), driven in part by the fact that the Indian isolates had 1634 and Australian isolates had 1272 average accessory genes. The acquisition of a larger number of accessory genes in Indian isolates suggests a survival advantage for gaining antibiotic resistance and virulence genes, consistent with an environmental origin of the ocular isolates (Shankar et al., 2012).

The large number of accessory genes included genes responsible for resistance and virulence. The Indian isolates with the largest number of accessory genes were *exoU*+ and were multi-drug resistant. This suggest several options for their acquisition of genes. *exoU* might be

acquired through horizontal genes transfer on genomic islands which bring along with *exoU* many other genes (Kulasekara et al., 2006, Subedi et al., 2018b), or the MDR isolates had been exposed to greater overall selection pressure leading to acquisition of larger numbers of genes that they needed to survive.

The finding of this study were in contrast to the previous findings of more frequent *exoU* possession in contact lens-related keratitis isolates (Shen et al., 2012, Choy et al., 2008). Instead the MDR isolates were *exoU*+ irrespective of being recovered from CL or non-CL keratitis. Interestingly, the identification of recently isolated CL-related isolates from Australia as *exoS*+ except for isolate 233, may indicate changes to selection pressures of contact lens-related microbial keratitis isolates, such as the increasing use of daily disposable contact lenses.

7.4 Different sequence types were found in the ocular isolates and clones had genotypically changed over time.

Clonality was evident in the ocular isolates of *P. aeruginosa* in the multilocus sequence type analysis and this was confirmed by analysing their whole genomes. Three different sequence types were found in the Indian isolates: ST316, ST308 and ST491. Two of the recent Australian isolates were clones belonging to sequence type ST233. Among these clones, ST308 was previously reported in ocular *P. aeruginosa* isolates (Subedi et al., 2018b). The ST308 isolates in the current study and those reported previously from 1997 were isolated from same location in India (Subedi et al., 2018b). However, the ST308 strains isolated in 2018 had evolved genotypically by acquiring more resistance and accessory genes compared with the 1997 clones. Additionally, the pattern of mutation also changed from historical to recent isolates. The changes in the resistance patterns of the recent clones indicated the evolution of the isolates over time. However, it is not certain what the specific selection factors behind those changes were. These changes in the resistome of ST308 over time are novel findings in ocular *P. aeruginosa* isolates. The use of animal models to see whether these

genetic changes influenced disease severity and outcome would be interesting for future studies.

7.5 *Pseudomonas aeruginosa* isolates with a higher number of gene mutations had larger numbers of mutations in their mismatch repair system

P. aeruginosa ocular isolates from India and Australia were analysed for non-synonymous single nucleotide polymorphisms (SNPs), multi-nucleotide polymorphisms (MNPs), deletions, insertions and complex variations (where more than one change occurred at one specific location compared to the reference strain) in their genes. Overall, more mutations in the Indian isolates were observed with an average of 42855 ±15665 compared to Australian isolates with an average of 31521 ± 10783 mutations. This may reflect that mutations in the isolates occur due to antibiotic exposure which might be more common in India than Australia due to differences in antibiotic prescribing regulation in both regions (Laxminarayan and Chaudhury, 2016, Porter and Grills, 2015). However, there were no differences in the mutations related to antibiotic resistance genes among the isolates. The largest number of mutations occurred in *opmH* which is part of the TriABC-OpmH efflux pump responsible for resistance to triclosan biocide, and *rocS* which encodes RocS a sensor kinase involved in the transduction pathway for fimbriae adhesion during biofilm formation (Kulasekara et al., 2005).

The *P. aeruginosa* DNA mismatch repair (MMR) system is a highly conserved system which repairs the bacterial genome by correcting mismatch nucleotide base pairs. It also restricts genetic evolution by inhibiting recombination in the isolates by preventing the addition of new genes. The MMR system in *P. aeruginosa* is composed of a triplicate of three proteins mutS-mutL-uvrD which are controlled by the *mutS, mutL and uvrD* respectively. The activity of the MMR system is affected, with mutations in the genes regulating the MMR system proteins. In the ocular *P. aeruginosa* isolates, the number of mutations was higher in *mutL* > *uvrD* >*mutS*. The pattern of mutations in the present isolates was different to that reported previously (Chopra et al., 2003), and more mutations were found in the *mutS*. The mutations in the MMR

system in isolates in this thesis indicates that they may be more able to mutate their genes to survive in their environment, and these mutations may alter the expression or acquisition of genes or activity of proteins involved in resistance to biocides and virulence. Indeed, the isolates with a mutated MMR had the largest pan genomes, and among those were four isolates (198, 202, 219 and 221) with either a transposon or integron in their genomes.

7.6 *Conclusion and future research*

This thesis describes the identification of MDR isolates from microbial keratitis from India and Australia. The existence of MDR strains indicates that alternative treatment options, either in new drug developments or combination therapy with existing antibiotics, might be needed in the future to treat microbial keratitis caused by *P. aeruginosa*. It may be that combinations of existing antibiotics can partly overcome the resistance shown in the current study and use of, for instance, fractional inhibitory concentration assays may help to identify such combinations. The development of new antibiotics such as antimicrobial peptides may offer alternative treatments (Tummanapalli and Willcox, 2020) against MDR ocular *P. aeruginosa* isolates. Evaluation of the synergy of these peptides with traditional antibiotics may also be useful.

References

ABDELKADER, A. 2014. Cosmetic soft contact lens associated ulcerative keratitis in southern Saudi Arabia. *Middle East Afr J Ophthalmol,* 21**,** 232-235.

ABDOUCHAKOUR, F., AUJOULAT, F., LICZNAR-FAJARDO, P., MARCHANDIN, H., TOUBIANA, M., PARER, S., LOTTHÉ, A. & JUMAS-BILAK, E. 2017. Intraclonal variations of resistance and phenotype in Pseudomonas aeruginosa epidemic high-risk clone ST308: A key to success within a hospital? *Int J Med Microbiol,* 308**,** 279-289.

ABIDI, S. H., SHERWANI, S. K., SIDDIQUI, T. R., BASHIR, A. & KAZMI, S. U. 2013. Drug resistance profile and biofilm forming potential of Pseudomonas aeruginosa isolated from contact lenses in Karachi-Pakistan. *BMC Ophthalmo,* 13**,** 57-62.

ABJANI, F., KHAN, N. A., JUNG, S. Y. & SIDDIQUI, R. 2017. Status of the effectiveness of contact lens disinfectants in Malaysia against keratitis-causing pathogens. *Exp Parasitol,* 183**,** 187-193.

ABRIL, D., MARQUEZ-ORTIZ, R. A., CASTRO-CARDOZO, B., MONCAYO-ORTIZ, J. I., OLARTE ESCOBAR, N. M., CORREDOR ROZO, Z. L., REYES, N., TOVAR, C., SÁNCHEZ, H. F., CASTELLANOS, J., GUACA-GONZÁLEZ, Y. M., LLANOS-URIBE, C. E., VANEGAS GÓMEZ, N. & ESCOBAR-PÉREZ, J. 2019. Genome plasticity favours double chromosomal Tn4401b-blaKPC-2 transposon insertion in the Pseudomonas aeruginosa ST235 clone. *BMC Microbiol,* 19**,** 45.

ACHARYA, S., FOSTER, P. L., BROOKS, P. & FISHEL, R. 2003. The coordinated functions of the E. coli MutS and MutL proteins in mismatch repair. *Mol Cell,* 12**,** 233-46.

AGNELLO, M., FINKEL, S. E. & WONG-BERINGER, A. 2016. Fitness cost of fluoroquinolone resistance in clinical isolates of *Pseudomonas aeruginosa* differs by type III secretion genotype. *Front Microbiol,* 7**,** 1591.

AGNELLO, M. & WONG-BERINGER, A. 2012. Differentiation in quinolone resistance by virulence genotype in *Pseudomonas aeruginosa*. *PLoS One,* 7**,** e42973.

AIRES, J. R., KÖHLER, T., NIKAIDO, H. & PLÉSIAT, P. 1999. Involvement of an active efflux system in the natural resistance of Pseudomonas aeruginosa to aminoglycosides. *Antimicrob Agents Chemother,* 43**,** 2624-2628.

AKPAKA, P. E., SWANSTON, W. H., IHEMERE, H. N., CORREA, A., TORRES, J. A., TAFUR, J. D., MONTEALEGRE, M. C., QUINN, J. P. & VILLEGAS, M. V. 2009. Emergence of KPC-producing Pseudomonas aeruginosa Trinidad and Tobago. *J Clin Microbiol,* 47**,** 2670-2671.

AL-NAYYEF, H., GUYEUX, C., PETITJEAN, M., HOCQUET, D. & BAHI, J. M. Relation between insertion sequences and genome rearrangements in Pseudomonas aeruginosa. 2015 Cham. Springer International Publishing, 426-437.

AL NAIEMI, N., DUIM, B. & BART, A. 2006. A CTX-M extended-spectrum beta-lactamase in Pseudomonas aeruginosa and Stenotrophomonas maltophilia. *J Med Microbiol,* 55**,** 1607-1608.

ALEXANDRAKIS, G., ALFONSO, E. C. & MILLER, D. 2000. Shifting trends in bacterial keratitis in South Florida and emerging resistance to fluoroquinolones11Received October 25, 1999. Accepted March 28, 2000. *Ophthalmol,* 107**,** 1497-1502.

ALONSO, A., CAMPANARIO, E. & MARTíNEZ, J. L. 1999. Emergence of multidrug-resistant mutants is increased under antibiotic selective pressure in Pseudomonas aeruginosa. *Microbiol,* 145**,** 2857-2862.

ALOUSH, V., NAVON-VENEZIA, S., SEIGMAN-IGRA, Y., CABILI, S. & CARMELI, Y. 2006. Multidrug-resistant Pseudomonas aeruginosa: risk factors and clinical impact. *Antimicrob Agents Chemother,* 50**,** 43-8.

ALSAN, M., SCHOEMAKER, L., EGGLESTON, K., KAMMILI, N., KOLLI, P. & BHATTACHARYA, J. 2015. Out-of-pocket health expenditures and antimicrobial

resistance in low-income and middle-income countries: an economic analysis. *Lancet Infect Dis,* 15**,** 1203-1210.

AMER AWAN, M., REEKS, G., RAHMAN, M. Q., BUTCHER, I. & RAMAESH, K. 2010. The patterns of in vitro antimicrobial susceptibility and resistance of bacterial keratitis isolates in Glasgow, United Kingdom. *Clin Exp Optom,* 93**,** 354-9.

AMIN, N. E., GISKE, C. G., JALAL, S., KEIJSER, B., KRONVALL, G. & WRETLIND, B. 2005. Carbapenem resistance mechanisms in Pseudomonas aeruginosa: Alterations of porin OprD and efflux proteins do not fully explain resistance patterns observed in clinical isolates. *APMIS : acta pathologica, microbiologica, et immunologica Scandinavica,* 113**,** 187-196.

ARAUJO, B. F., FERREIRA, M. L., CAMPOS, P. A., ROYER, S., BATISTÃO, D. W., DANTAS, R. C., GONÇALVES, I. R., FARIA, A. L., BRITO, C. S., YOKOSAWA, J., GONTIJO-FILHO, P. P. & RIBAS, R. M. 2016. Clinical and olecular epidemiology of multidrug-resistant P. aeruginosa carrying aac(6')-Ib-cr, qnrS1 and blaSPM genes in Brazil. *PLoS One,* 11**,** e0155914.

ARBER, W. 2014. Horizontal gene transfer among bacteria and its role in biological evolution. *Life,* 4**,** 217-224.

ARTINI, M., CELLINI, A., SCOARUGHI, G. L., PAPA, R., TILOTTA, M., PALMA, S. & SELAN, L. 2015. Evaluation of contact lens multipurpose solutions on bacterial biofilm development. *Eye Contact Lens,* 41**,** 177-182.

ASLAM, B., WANG, W., ARSHAD, M. I., KHURSHID, M., MUZAMMIL, S., RASOOL, M. H., NISAR, M. A., ALVI, R. F., ASLAM, M. A., QAMAR, M. U. J. I. & RESISTANCE, D. 2018. Antibiotic resistance: a rundown of a global crisis. 11**,** 1645.

AUBERT, D., POIREL, L., CHEVALIER, J., LEOTARD, S., PAGES, J.-M. & NORDMANN, P. 2001. Oxacillinase-mediated resistance to cefepime and susceptibility to ceftazidime in Pseudomonas aeruginosa. *Antimicrob Agents Chemother,* 45**,** 1615-1620.

BALABAN, N. Q., GERDES, K., LEWIS, K. & MCKINNEY, J. D. 2013. A problem of persistence: still more questions than answers? *Nat Rev Microbiol,* 11**,** 587-591.

BANKEVICH, A., NURK, S., ANTIPOV, D., GUREVICH, A. A., DVORKIN, M., KULIKOV, A. S., LESIN, V. M., NIKOLENKO, S. I., PHAM, S., PRJIBELSKI, A. D., PYSHKIN, A. V., SIROTKIN, A. V., VYAHHI, N., TESLER, G., ALEKSEYEV, M. A. & PEVZNER, P. A. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol,* 19**,** 455-77.

BARBIERI, J. & SUN, J. 2004. Pseudomonas aeruginosa exos and exot. *Rev Physiol, Biochemist Pharmacol.* Springer.

BEBRONE, C., BOGAERTS, P., DELBRÜCK, H., BENNINK, S., KUPPER, M. B., REZENDE DE CASTRO, R., GLUPCZYNSKI, Y. & HOFFMANN, K. M. 2013. GES-18, a new carbapenem-hydrolyzing GES-type β-lactamase from Pseudomonas aeruginosa that contains Ile80 and Ser170 residues. *Antimicrob Agents Chemother,* 57**,** 396-401.

BELL, B. G., SCHELLEVIS, F., STOBBERINGH, E., GOOSSENS, H. & PRINGLE, M. 2014. A systematic review and meta-analysis of the effects of antibiotic consumption on antibiotic resistance. *BMC Infect Dis,* 14**,** 13.

BELOTTI, P. T., THABET, L., LAFFARGUE, A., ANDRÉ, C., COULANGE-MAYONNOVE, L., ARPIN, C., MESSADI, A., M'ZALI, F., QUENTIN, C. & DUBOIS, V. 2015. Description of an original integron encompassing blaVIM-2, qnrVC1 and genes encoding bacterial group II intron proteins in Pseudomonas aeruginosa. *J Antimicrob Chemother,* 70**,** 2237-2240.

BENNETT, J. V. 1974. Nosocomial Infections Due to Pseudomonas. *J Infect Dis,* 130**,** S4-S7.

BERNASCONI, O. J., KUENZLI, E., PIRES, J., TINGUELY, R., CARATTOLI, A., HATZ, C., PERRETEN, V. & ENDIMIANI, A. 2016. Travelers can import colistin-resistant Enterobacteriaceae, including those possessing the plasmid-mediated mcr-1 gene. *Antimicrob Agents Chemother,* 60**,** 5080-5084.

BHADANGE, Y., DAS, S., KASAV, M. K., SAHU, S. K. & SHARMA, S. 2015. Comparison of culture-negative and culture-positive microbial keratitis: cause of culture negativity, clinical features and final outcome. *BJO,* 99**,** 1498-1502.

BHARATHI, M. J., RAMAKRISHNAN, R., MEENAKSHI, R., SHIVAKUMAR, C. & RAJ, D. L. 2009. Analysis of the risk factors predisposing to fungal, bacterial & Acanthamoeba keratitis in south India. *Indian J Med Res,* 130**,** 749-757.

BIANCO, N., NESHAT, S. & POOLE, K. 1997. Conservation of the multidrug resistance efflux gene oprM in Pseudomonas aeruginosa. *Antimicrob Agents Chemother,* 41**,** 853-856.

BLAIR, J. M., WEBBER, M. A., BAYLAY, A. J., OGBOLU, D. O. & PIDDOCK, L. J. 2015. Molecular mechanisms of antibiotic resistance. *Nat Rev Microbiol,* 13**,** 42-51.

BLONDEAU, J. M. 2004. Fluoroquinolones: mechanism of action, classification, and development of resistance. *Surv Ophthalmol,* 49 Suppl 2**,** S73-8.

BODEY, G. P., BOLIVAR, R., FAINSTEIN, V. & JADEJA, L. 1983. Infections caused by Pseudomonas aeruginosa. *Rev Infect Dis,* 5**,** 279-313.

BOLGER, A. M., LOHSE, M. & USADEL, B. 2014. Trimmomatic: a flexible trimmer for illumina sequence data. *Bioinformatics,* 30**,** 2114-2120.

BOONKERD, N., PIBALPAKDI, P., TILOKLURS, M. & NIUMSUP, P. R. 2009. Class 1 integron containing metallo beta-lactamase gene blaIMP-1 in carbapenem-resistant Pseudomonas aeruginosa in Thailand. *J Infect Chemother,* 15**,** 257-61.

BOOST, M. V., CHAN, J., SHI, G. S. & CHO, P. 2014. Effect of multipurpose solutions against Acinetobacter carrying QAC genes. *Optom Vis Sci,* 91**,** 272-7.

BOOST, M. V. & CHO, P. 2005. Microbial flora of tears of orthokeratology patients, and microbial contamination of contact lenses and contact lens accessories. *Optom Vis Sci,* 82.

BORKAR, D. S., ACHARYA, N. R., LEONG, C., LALITHA, P., SRINIVASAN, M., OLDENBURG, C. E., CEVALLOS, V., LIETMAN, T. M., EVANS, D. J. & FLEISZIG, S. M. J. 2014. Cytotoxic clinical isolates of Pseudomonas aeruginosaidentified during the Steroids for Corneal Ulcers Trial show elevated resistance to fluoroquinolones. *BMC Ophthalmol,* 14**,** 54.

BORKAR, D. S., FLEISZIG, S. J., LEONG, C. & ET AL. 2013. Association between cytotoxic and invasive pseudomonas aeruginosa and clinical outcomes in bacterial keratitis. *JAMA Ophthalmology,* 131**,** 147-153.

BOTELHO, J., GROSSO, F. & PEIXE, L. 2017. Characterization of the pJB12 Plasmid from Pseudomonas aeruginosa Reveals Tn6352, a novel putative transposon associated with mobilization of the blaVIM-2-harboring In58 integron. *Antimicrob Agents Chemother,* 61**,** e02532-16.

BRAGONZI, A., PARONI, M., NONIS, A., CRAMER, N., MONTANARI, S., REJMAN, J., DI SERIO, C., DÖRING, G. & TÜMMLER, B. 2009. Pseudomonas aeruginosa microevolution during cystic fibrosis lung infection establishes clones with adapted virulence. *Am J Respir Crit Care Med,* 180**,** 138-45.

BREIDENSTEIN, E. B. M., DE LA FUENTE-NÚÑEZ, C. & HANCOCK, R. E. W. 2011. Pseudomonas aeruginosa: all roads lead to resistance. *Trends Microbiol,* 19**,** 419-426.

BRINKMAN, F. S., BAINS, M. & HANCOCK, R. E. 2000. The amino terminus of Pseudomonas aeruginosa outer membrane protein OprF forms channels in lipid bilayer membranes: correlation with a three-dimensional model. *J Bacteriol,* 182**,** 5251-5255.

BRÍZIO, A., CONCEIÇÃO, T., PIMENTEL, M., DA SILVA, G. & DUARTE, A. 2006. High-level expression of IMP-5 carbapenemase owing to point mutation in the -35 promoter region of class 1 integron among Pseudomonas aeruginosa clinical isolates. *Int J Antimicrob Agents,* 27**,** 27-31.

BROOUN, A., LIU, S. & LEWIS, K. 2000. A dose-response study of antibiotic resistance in Pseudomonas aeruginosa biofilms. *Antimicrob Agents Chemother,* 44**,** 640-646.

BRUINSMA, G. M., RUSTEMA-ABBING, M., VAN DER MEI, H. C., LAKKIS, C. & BUSSCHER, H. J. 2006. Resistance to a polyquaternium-1 lens care solution and isoelectric points of Pseudomonas aeruginosa strains. *J Antimicrob Chemother,* 57**,** 764-766.

BUSH, K. 2010. Bench-to-bedside review: The role of beta-lactamases in antibiotic-resistant Gram-negative infections. *Crit Care (London, England),* 14**,** 224-224.

CALLAHAN, D., KOVACS, C., LYNCH, S. & RAH, M. 2017. Biocidal efficacy of multipurpose solutions against Gram-negative organisms associated with corneal infiltrative events. *Clin Exp Optom,* 100**,** 357-364.

CASTANHEIRA, M., BELL, J. M., TURNIDGE, J. D., MATHAI, D. & JONES, R. N. 2009. Carbapenem resistance among Pseudomonas aeruginosa strains from India: Evidence for nationwide endemicity of multiple metallo-β-lactamase clones (VIM-2, -5, -6, and -11 and the newly characterized VIM-18). *Antimicrob Agents Chemother,* 53**,** 1225-1227.

CASTANHEIRA, M., DESHPANDE, L. M., COSTELLO, A., DAVIES, T. A. & JONES, R. N. 2014. Epidemiology and carbapenem resistance mechanisms of carbapenem-non-susceptible Pseudomonas aeruginosa collected during 2009–11 in 14 European and Mediterranean countries. *J Antimicrob Chemother,* 69**,** 1804-1814.

CASTANHEIRA, M., MENDES, R. E., WALSH, T. R., GALES, A. C. & JONES, R. N. 2004. Emergence of the extended-spectrum beta-lactamase GES-1 in a Pseudomonas aeruginosa strain from Brazil: report from the SENTRY antimicrobial surveillance program. *Antimicrob Agents Chemother,* 48**,** 2344-2345.

CAYCI, Y. T., COBAN, A. Y. & GUNAYDIN, M. 2014. Investigation of plasmid-mediated quinolone resistance in *Pseudomonas aeruginosa* clinical isolates. *Indian J Med Microbiol,* 32**,** 285-9.

CAZZANIGA, A., SERRALTA, V., DAVIS, S., ORR, R., EAGLSTEIN, W. & MERTZ, P. M. 2002. The effect of an antimicrobial gauze dressing impregnated with 0.2-percent polyhexamethylene biguanide as a barrier to prevent Pseudomonas aeruginosa wound invasion. *Wounds,* 14**,** 169-176.

CDC 2013. Centers for Disease Control and Prevention, Office of infectious disease antibiotic resistance threats in the United States, 2013. Apr, 2013. Available at: http://www.cdc.gov/drugresistance/threat-report-2013.

CELENZA, G., PELLEGRINI, C., CACCAMO, M., SEGATORE, B., AMICOSANTE, G. & PERILLI, M. 2006. Spread of bla(CTX-M-type) and bla(PER-2) beta-lactamase genes in clinical isolates from Bolivian hospitals. *J Antimicrob Chemother,* 57**,** 975-978.

CHANAWONG, A., M'ZALI, F. H., HERITAGE, J., LULITANOND, A. & HAWKEY, P. M. 2001. SHV-12, SHV-5, SHV-2a and VEB-1 extended-spectrum beta-lactamases in Gram-negative bacteria isolated in a university hospital in Thailand. *J Antimicrob Chemother,* 48**,** 839-852.

CHAPMAN, J. S. 2003. Disinfectant resistance mechanisms, cross-resistance, and co-resistance. *Int Biodeter Biodegrad,* 51**,** 271-276.

CHATTERJEE, S. & AGRAWAL, D. 2016. Multi-drug resistant Pseudomonas aeruginosa keratitis and its effective treatment with topical colistimethate. *Indian J Ophthalmol,* 64**,** 153-157.

CHAUDHRY, N. A., FLYNN, H. W., JR., MURRAY, T. G., TABANDEH, H., MELLO, M. O., JR. & MILLER, D. 1999. Emerging ciprofloxacin-resistant Pseudomonas aeruginosa. *Am J Ophthalmol,* 128**,** 509-510.

CHÁVEZ-JACOBO, V. M., HERNÁNDEZ-RAMÍREZ, K. C., ROMO-RODRÍGUEZ, P., PÉREZ-GALLARDO, R. V., CAMPOS-GARCÍA, J., GUTIÉRREZ-CORONA, J. F., GARCÍA-MERINOS, J. P., MEZA-CARMEN, V., SILVA-SÁNCHEZ, J. & RAMÍREZ-DÍAZ, M. I. 2018. CrpP Is a novel ciprofloxacin-modifying enzyme encoded by the Pseudomonas aeruginosa pUM505 Plasmid. *Antimicrob Aents Chemother,* 62**,** e02629-17.

CHÁVEZ-JACOBO, V. M., HERNÁNDEZ-RAMÍREZ, K. C., SILVA-SÁNCHEZ, J., GARZA-RAMOS, U., BARRIOS-CAMACHO, H., ORTIZ-ALVARADO, R., CERVANTES, C.,

MEZA-CARMEN, V. & RAMÍREZ-DÍAZ, M. I. 2019. Prevalence of the crpP gene conferring decreased ciprofloxacin susceptibility in enterobacterial clinical isolates from Mexican hospitals. *Journal of Antimicrobial Chemotherapy,* 74**,** 1253-1259.

CHEN, Y., SUN, M., WANG, M., LU, Y. & YAN, Z. 2014. Dissemination of IMP-6-producing Pseudomonas aeruginosa ST244 in multiple cities in China. *Euro J Clin Microbiol Inf Dis,* 33**,** 1181-1187.

CHENG, K. H., LEUNG, S. L., HOEKMAN, H. W., BEEKHUIS, W. H., MULDER, P. G. H., GEERARDS, A. J. M. & KIJLSTRA, A. 1999. Incidence of contact-lens-associated microbial keratitis and its related morbidity. *The Lancet,* 354**,** 181-185.

CHIDAMBARAM, J. D., VENKATESH PRAJNA, N., SRIKANTHI, P., LANJEWAR, S., SHAH, M., ELAKKIYA, S., LALITHA, P. & BURTON, M. J. 2018. Epidemiology, risk factors, and clinical outcomes in severe microbial keratitis in South India. *Ophthal Epidemiol,* 25**,** 297-305.

CHO, H. H., KWON, K. C., KIM, S. & KOO, S. H. 2014. Correlation between virulence genotype and fluoroquinolone resistance in carbapenem-resistant Pseudomonas aeruginosa. *Annals of laboratory medicine,* 34**,** 286-292.

CHOPRA, I., O'NEILL, A. J. & MILLER, K. 2003. The role of mutators in the emergence of antibiotic-resistant bacteria. *Drug Res Updates,* 6**,** 137-145.

CHOY, M. H., STAPLETON, F., WILLCOX, M. D. & ZHU, H. 2008. Comparison of virulence factors in Pseudomonas aeruginosa strains isolated from contact lens-and non-contact lens-related keratitis. *J Med Microbiol,* 57**,** 1539-1546.

CHUNG, J. C. S., BECQ, J., FRASER, L., SCHULZ-TRIEGLAFF, O., BOND, N. J., FOWERAKER, J., BRUCE, K. D., SMITH, G. P. & WELCH, M. 2012. Genomic variation among contemporary Pseudomonas aeruginosa isolates from chronically infected cystic fibrosis patients. *J Bacteriol,* 194**,** 4857-4866.

CLAEYS, G., VERSCHRAEGEN, G., DE BAERE, T. & VANEECHOUTTE, M. 2000. PER-1 beta-lactamase-producing Pseudomonas aeruginosa in an intensive care unit. *J Antimicrob Chemother,* 45**,** 924-925.

CODLING, C. E., MAILLARD, J. Y. & RUSSELL, A. D. 2003. Aspects of the antimicrobial mechanisms of action of a polyquaternium and an amidoamine. *J Antimicrob Chemother,* 51**,** 1153-8.

CORVEC, S., POIREL, L., DECOUSSER, J.-W., ALLOUCH, P.-Y., DRUGEON, H. & NORDMANN, P. 2006. Emergence of carbapenem-hydrolysing metallo-β-lactamase VIM-1 in Pseudomonas aeruginosa isolates in France. *Clin Microbiol Infect,* 12**,** 941-942.

COSTERTON, J. W., STEWART, P. S. & GREENBERG, E. P. 1999. Bacterial biofilms: a common cause of persistent infections. *Science,* 284**,** 1318-1322.

CURY, J., JOVÉ, T., TOUCHON, M., NÉRON, B. & ROCHA, E. P. 2016. Identification and analysis of integrons and cassette arrays in bacterial genomes. *Nucleic Acids Res,* 44**,** 4539-4550.

CUZON, G., NAAS, T., VILLEGAS, M.-V., CORREA, A., QUINN, J. P. & NORDMANN, P. 2011. Wide dissemination of Pseudomonas aeruginosa producing beta-lactamase blaKPC-2 gene in Colombia. *Antimicrob Agents Chemother,* 55**,** 5350-5353.

DAHLGREN, M. A., LINGAPPAN, A. & WILHELMUS, K. R. 2007. The clinical diagnosis of microbial keratitis. *Ame J Ophthalmol,* 143**,** 940-944.e1.

DANEL, F., HALL, L. M., GUR, D. & LIVERMORE, D. M. 1997. OXA-15, an extended-spectrum variant of OXA-2 beta-lactamase, isolated from a Pseudomonas aeruginosa strain. *Antimicrobial Agents and Chemotherapy,* 41**,** 785-790.

DANIELL, M. 2003. Overview: Initial antimicrobial therapy for microbial keratitis. *BJO,* 87**,** 1172-1174.

DARRIBA, D., TABOADA, G. L., DOALLO, R. & POSADA, D. 2012. jModelTest 2: more models, new heuristics and parallel computing. *Nat Methods,* 9**,** 772.

DART, J. K. G., RADFORD, C. F., MINASSIAN, D., VERMA, S. & STAPLETON, F. 2008. Risk factors for microbial keratitis with contemporary contact lenses. *Ophthalmol,* 115**,** 1647-1654.e3.

DART, J. K. G., STAPLETON, F., MINASSIAN, D. & DART, J. K. G. 1991. Contact lenses and other risk factors in microbial keratitis. *The Lancet,* 338**,** 650-653.

DESHPANDE, L. M., DAVIES, T. A., BLANDINO, G., NICOLETTI, G., JONES, R. N. & CASTANHEIRA, M. 2013. IMP-33, a New IMP variant detected in Pseudomonas aeruginosa from Sicily. *Antimicrob Agents Chemother,* 57**,** 6401-6403.

DIONISIO, F., ZILHÃO, R. & GAMA, J. A. 2019. Interactions between plasmids and other mobile genetic elements affect their transmission and persistence. *Plasmid,* 102**,** 29-36.

DIVER, J. M., SCHOLLAARDT, T., RABIN, H. R., THORSON, C. & BRYAN, L. E. 1991. Persistence mechanisms in Pseudomonas aeruginosa from cystic fibrosis patients undergoing ciprofloxacin therapy. *Antimicrob Agents Chemother,* 35**,** 1538-46.

DÖTSCH, A., KLAWONN, F., JAREK, M., SCHARFE, M., BLÖCKER, H. & HÄUSSLER, S. 2010. Evolutionary conservation of essential and highly expressed genes in *Pseudomonas aeruginosa*. *BMC Genomics,* 11**,** 234.

DRIFFIELD, K., MILLER, K., BOSTOCK, J., O'NEILL, A. & CHOPRA, I. 2008. Increased mutability of Pseudomonas aeruginosa in biofilms. *J Antimicrob Chemother,* 61**,** 1053-1056.

DRUMMOND, A. J. & RAMBAUT, A. 2007. BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evol Biol,* 7**,** 214.

DULJASZ, W., GNIADKOWSKI, M., SITTER, S., WOJNA, A. & JEBELEAN, C. 2009. First organisms with acquired metallo-β-lactamases (IMP-13, IMP-22, and VIM-2) reported in Austria. *Antimicrob Agents Chemother,* 53**,** 2221-2222.

DUNHAM, S., MCPHERSON, C. & MILLER, A. 2010. The relative contribution of efflux and target gene mutations to fluoroquinolone resistance in recent clinical isolates of Pseudomonas aeruginosa. *Euro J Clin Microbiol Infect Dis,* 29**,** 279-288.

DUTTA, D., COLE, N. & WILLCOX, M. 2012. Factors influencing bacterial adhesion to contact lenses. *Mol Vis,* 18**,** 14-21.

EFRON, N., MORGAN, P. B., WOODS, C. A. & CONSORTIUM, T. I. C. L. P. S. 2013. An international survey of daily disposable contact lens prescribing. *Clin Exp Optom,* 96**,** 58-64.

EGRILMEZ, S. & YILDIRIM-THEVENY, Ş. 2020. Treatment-resistant bacterial keratitis: Challenges and solutions. *Clin Ophthalmol (Auckland, N.Z.),* 14**,** 287-297.

EL GARCH, F., BOGAERTS, P., BEBRONE, C., GALLENI, M. & GLUPCZYNSKI, Y. 2011. OXA-198, an acquired carbapenem-hydrolyzing class D β-lactamase from Pseudomonas aeruginosa. *Antimicrob Agents Chemother,* 55**,** 4828-4833.

EMMERSON, A. M. & JONES, A. M. 2003. The quinolones: decades of development and use. *J Antimicrob Chemother,* 51 Suppl 1**,** 13-20.

EMPEL, J., FILCZAK, K., MRÓWKA, A., HRYNIEWICZ, W., LIVERMORE, D. M. & GNIADKOWSKI, M. 2007. Outbreak of Pseudomonas aeruginosa Infections with PER-1 extended-spectrum β-lactamase in Warsaw, Poland: Further evidence for an international clonal complex. *J Clin Microbiol,* 45**,** 2829-2834.

ERBAY, H., YALCIN, A. N., SERIN, S., TURGUT, H., TOMATIR, E., CETIN, B. & ZENCIR, M. 2003. Nosocomial infections in intensive care unit in a Turkish university hospital: a 2-year survey. *Intens Care Med,* 29**,** 1482-1488.

EUCAST 2020. The European committee on antimicrobial susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 10.0, 2020. http://www.eucast.org,.

EVANS, J. C. & SEGAL, H. 2007. A Novel insertion sequence, IS Pa26, in oprD of Pseudomonas aeruginosa is associated with carbapenem resistance. *Antimicrob Agents Chemother,* 51**,** 3776-3777.

FAROOQUI, H. H., SELVARAJ, S., MEHTA, A. & HEYMANN, D. L. 2018. Community level antibiotic utilization in India and its comparison vis-à-vis European countries: Evidence from pharmaceutical sales data. *PloS one,* 13**,** e0204805-e0204805.

FELTMAN, H., SCHULERT, G., KHAN, S., JAIN, M., PETERSON, L. & HAUSER, A. R. 2001. Prevalence of type III secretion genes in clinical and environmental isolates of Pseudomonas aeruginosa. *Microbiol,* 147**,** 2659-2669.

FERNANDES, M., VIRA, D., MEDIKONDA, R. & KUMAR, N. 2016a. Extensively and pan-drug resistant Pseudomonas aeruginosa keratitis: clinical features, risk factors, and outcome. *Graefe's Arch Clin Exp Ophthalmol,* 254**,** 315-322.

FERNANDES, M. R., MOURA, Q., SARTORI, L., SILVA, K. C., CUNHA, M. P., ESPOSITO, F., LOPES, R., OTUTUMI, L. K., GONÇALVES, D. D. & DROPA, M. 2016b. Silent dissemination of colistin-resistant Escherichia coli in South America could contribute to the global spread of the mcr-1 gene. *Eurosurveillance,* 21.

FERNÁNDEZ, L. & HANCOCK, R. E. J. C. M. R. 2012. Adaptive and mutational resistance: role of porins and efflux pumps in drug resistance. *Clin Microbiol Rev,* 25**,** 661-681.

FINCK-BARBANÇON, V., GORANSON, J., ZHU, L., SAWA, T., WIENER-KRONISH, J. P., FLEISZIG, S. M. J., WU, C., MENDE-MUELLER, L. & FRANK, D. W. 1997. ExoU expression by Pseudomonas aeruginosa correlates with acute cytotoxicity and epithelial injury. *Mol Microbiol,* 25**,** 547-557.

FLEISZIG, S. M., WIENER-KRONISH, J. P., MIYAZAKI, H., VALLAS, V., MOSTOV, K. E., KANADA, D., SAWA, T., YEN, T. S. & FRANK, D. W. 1997. Pseudomonas aeruginosa-mediated cytotoxicity and invasion correlate with distinct genotypes at the loci encoding exoenzyme S. *Infect Immun,* 65**,** 579-586.

FLEISZIG, S. M. J. 2006. The pathogenesis of contact lens-related keratitis. *Optom Vis Sci,* 83**,** E866-E873.

FONG, C. F., HU, F. R., TSENG, C. H., WANG, I. J., CHEN, W. L. & HOU, Y. C. 2007. Antibiotic susceptibility of bacterial isolates from bacterial keratitis cases in a university hospital in Taiwan. *Am J Ophthalmol,* 144**,** 682-689.

FONG, C. F., TSENG, C. H., HU, F. R., WANG, I. J., CHEN, W. L. & HOU, Y. C. 2004. Clinical characteristics of microbial keratitis in a university hospital in Taiwan. *Am J Ophthalmol,* 137**,** 329-36.

FONSECA, E. L., DOS SANTOS FREITAS, F., VIEIRA, V. V. & VICENTE, A. C. 2008. New qnr gene cassettes associated with superintegron repeats in Vibrio cholerae O1. *Emerg Infect Dis,* 14**,** 1129-31.

FONSECA, E. L. & VICENTE, A. C. P. 2013. Epidemiology of qnrVC alleles and emergence out of the Vibrionaceae family. *J Med Microbiol,* 62**,** 1628-1630.

FOURNIER, D., JEANNOT, K., ROBERT-NICOUD, M., MULLER, E., CHOLLEY, P., MEE-MARQUET, N. & PLÉSIAT, P. 2012. Spread of the blaIMP-13 gene in French Pseudomonas aeruginosa through sequence types ST621, ST308 and ST111. *Int J Antimicrob Agents,* 40**,** 571-573.

FOWLER, R. C. & HANSON, N. D. 2014. Emergence of carbapenem resistance due to the novel insertion sequence IS Pa 8 in Pseudomonas aeruginosa. *PLoS One,* 9**,** e91299.

FRANK, D. W. 1997. The exoenzyme S regulon of Pseudomonas aeruginosa. *Mole Microbiol,* 26**,** 621-629.

FRESCHI, L., JEUKENS, J., KUKAVICA-IBRULJ, I., BOYLE, B., DUPONT, M.-J., LAROCHE, J., LAROSE, S., MAAROUFI, H., FOTHERGILL, J. L. & MOORE, M. 2015a. Clinical Utilization of Genomics Data Produced by the International Pseudomonas Aeruginosa Consortium. *Front Microbiol,* 6**,** 1036.

FRESCHI, L., JEUKENS, J., KUKAVICA-IBRULJ, I., BOYLE, B., DUPONT, M. J., LAROCHE, J., LAROSE, S., MAAROUFI, H., FOTHERGILL, J. L., MOORE, M., WINSOR, G. L., AARON, S. D., BARBEAU, J., BELL, S. C., BURNS, J. L., CAMARA, M., CANTIN, A., CHARETTE, S. J., DEWAR, K., DEZIEL, E., GRIMWOOD, K., HANCOCK, R. E., HARRISON, J. J., HEEB, S., JELSBAK, L., JIA, B., KENNA, D. T., KIDD, T. J., KLOCKGETHER, J., LAM, J. S., LAMONT, I. L., LEWENZA, S., LOMAN, N., MALOUIN, F., MANOS, J., MCARTHUR, A. G., MCKEOWN, J., MILOT, J., NAGHRA, H., NGUYEN, D., PEREIRA, S. K., PERRON, G. G., PIRNAY, J. P., RAINEY, P. B., ROUSSEAU, S., SANTOS, P. M.,

STEPHENSON, A., TAYLOR, V., TURTON, J. F., WAGLECHNER, N., WILLIAMS, P., THRANE, S. W., WRIGHT, G. D., BRINKMAN, F. S., TUCKER, N. P., TUMMLER, B., WINSTANLEY, C. & LEVESQUE, R. C. 2015b. Clinical utilization of genomics data produced by the international Pseudomonas aeruginosa consortium. *Front Microbiol,* 6**,** 1036.

FRESCHI, L., VINCENT, A. T., JEUKENS, J., EMOND-RHEAULT, J. G., KUKAVICA-IBRULJ, I., DUPONT, M. J., CHARETTE, S. J., BOYLE, B. & LEVESQUE, R. C. 2019. The Pseudomonas aeruginosa Pan-Genome Provides New Insights on Its Population Structure, Horizontal Gene Transfer, and Pathogenicity. *Genome Biol Evol,* 11**,** 109-120.

FROST, L. S., LEPLAE, R., SUMMERS, A. O. & TOUSSAINT, A. 2005. Mobile genetic elements: the agents of open source evolution. *Nat Rev Microbiol,* 3**,** 722-732.

GABRIEL, M. M., MCANALLY, C. & BARTELL, J. 2018. Antimicrobial Efficacy of Multipurpose Disinfecting Solutions in the Presence of Contact Lenses and Lens Cases. *Eye & contact lens,* 44**,** 125-131.

GALENTINE, P. G., COHEN, E. J., LAIBSON, P. R., ADAMS, C. P., MICHAUD, R. & ARENTSEN, J. J. 1984. Corneal ulcers associated with contact lens wear. *Arch Ophthalmol,* 102**,** 891-894.

GAREY, K., VO, Q., LAROCCO, M., GENTRY, L. & TAM, V. 2008. Prevalence of type III secretion protein exoenzymes and antimicrobial susceptibility patterns from bloodstream isolates of patients with Pseudomonas aeruginosa bacteremia. *Journal of chemotherapy,* 20**,** 714-720.

GARG, P., SHARMA, S. & RAO, G. N. 1999. Ciprofloxacin-resistant Pseudomonas keratitis. *Ophthalmol,* 106**,** 1319-23.

GARZA-RAMOS, U., TINOCO, P., SILVA-SANCHEZ, J., MORFIN-OTERO, R., RODRIGUEZ-NORIEGA, E., LEON-GARNICA, G., SADER, H. & JONES, R. 2008. Metallo-β-lactamase IMP-18 is located in a class 1 integron (In96) in a clinical isolate of Pseudomonas aeruginosa from Mexico. *Int J Antimicrob Agents,* 31**,** 78-80.

GASINK, L. B., FISHMAN, N. O., WEINER, M. G., NACHAMKIN, I., BILKER, W. B. & LAUTENBACH, E. 2006. Fluoroquinolone-resistant Pseudomonas aeruginosa: assessment of risk factors and clinical impact. *Am J Med,* 119**,** 526.e19-25.

GE, C., WEI, Z., JIANG, Y., SHEN, P., YU, Y. & LI, L. 2011. Identification of KPC-2-producing Pseudomonas aeruginosa isolates in China. *J Antimicrob Chemother,* 66**,** 1184-1186.

GEHRIG, K. A. & WARSHAW, E. M. 2008. Allergic contact dermatitis to topical antibiotics: Epidemiology, responsible allergens, and management. *J Am Acad Dermatol,* 58**,** 1-21.

GEORGESCU, M., GHEORGHE, I., CURUTIU, C., LAZAR, V., BLEOTU, C. & CHIFIRIUC, M. C. 2016. Virulence and resistance features of *Pseudomonas aeruginosa* strains isolated from chronic leg ulcers. *BMC Infect Dis,* 16 Suppl 1**,** 92.

GHOSH, P. 2004. Process of protein transport by the type III secretion system. *Microbiol Mol Biol Rev,* 68**,** 771-795.

GIBB, A. P., TRIBUDDHARAT, C., MOORE, R. A., LOUIE, T. J., KRULICKI, W., LIVERMORE, D. M., PALEPOU, M.-F. I. & WOODFORD, N. 2002. Nosocomial outbreak of carbapenem-resistant Pseudomonas aeruginosa with a new blaIMP allele, blaIMP-7. *Antimicrob Agents Chemother,* 46**,** 255-258.

GILARRANZ, R., JUAN, C., CASTILLO‐VERA, J., CHAMIZO, F., ARTILES, F., ALAMO, I. & OLIVER, A. 2013. First detection in E urope of the metallo‐β‐lactamase IMP‐15 in clinical strains of Pseudomonas putida and Pseudomonas aeruginosa. *Clin Microbiol Infect,* 19**,** E424-E427.

GIWERCMAN, B., LAMBERT, P. A., ROSDAHL, V. T., SHAND, G. H. & HØIBY, N. 1990. Rapid emergence of resistance in Pseudomonas aeruginosa in cystic fibrosis patients due to in-vivo selection of stable partially derepressed beta-lactamase producing strains. *J Antimicrob Chemother,* 26**,** 247-259.

GOKHALE, N. S. 2008. Medical management approach to infectious keratitis. *Indian J Ophthalmol,* 56**,** 215-220.
GOLKAR, Z., BAGASRA, O. & PACE, D. G. 2014. Bacteriophage therapy: a potential solution for the antibiotic resistance crisis. *J Infect Dev Ctries,* 8**,** 129-36.
GONZALES, C. A., SRINIVASAN, M., WHITCHER, J. P. & SMOLIN, G. 1996. Incidence of corneal ulceration in Madurai district, South India. *Ophthalmic Epidemiol,* 3**,** 159-66.
GOPINATHAN, U., SHARMA, S., GARG, P. & RAO, G. N. 2009. Review of epidemiological features, microbiological diagnosis and treatment outcome of microbial keratitis: experience of over a decade. *Indian J Ophthalmol,* 57**,** 273-279.
GREEN, M., APEL, A. & STAPLETON, F. 2008. Risk factors and causative organisms in microbial keratitis. *Cornea,* 27**,** 22-27.
GREEN, M., CARNT, N., APEL, A. & STAPLETON, F. 2019. Queensland microbial Keratitis database: 2005–2015. *BJO,* 103**,** 1481-1486.
GREEN, M. D., APEL, A. J., NADUVILATH, T. & STAPLETON, F. J. 2007. Clinical outcomes of keratitis. *Clin Exp Ophthalmol,* 35**,** 421-426.
GUAN, A., LI, Z. & PHILLIPS, K. S. 2015. The effects of non-ionic polymeric surfactants on the cleaning of biofouled hydrogel materials. *Biofouling,* 31**,** 689-697.
GUTU, A. D., SGAMBATI, N., STRASBOURGER, P., BRANNON, M. K., JACOBS, M. A., HAUGEN, E., KAUL, R. K., JOHANSEN, H. K., HØIBY, N. & MOSKOWITZ, S. M. 2013. Polymyxin resistance of Pseudomonas aeruginosa phoQ mutants is dependent on additional two-component regulatory systems. *Antimicrob Agents Chemother,* 57**,** 2204-2215.
GUZVINEC, M., IZDEBSKI, R., BUTIC, I., JELIC, M., ABRAM, M., KOSCAK, I., BARANIAK, A., HRYNIEWICZ, W., GNIADKOWSKI, M. & TAMBIC ANDRASEVIC, A. 2014. Sequence types 235, 111, and 132 predominate among multidrug-resistant pseudomonas aeruginosa clinical isolates in Croatia. *Antimicrob Agents Chemother,* 58**,** 6277-83.
HAAS, W., PILLAR, C. M., TORRES, M., MORRIS, T. W. & SAHM, D. F. 2011. Monitoring antibiotic resistance in ocular microorganisms: Results from the antibiotic resistance monitoring in ocular microrganisms (ARMOR) 2009 Surveillance study. *Am J Ophthalmol,* 152**,** 567-574.e3.
HACKER, J. & CARNIEL, E. 2001. Ecological fitness, genomic islands and bacterial pathogenicity. *EMBO reports,* 2**,** 376-381.
HALL, L., LIVERMORE, D., GUR, D., AKOVA, M. & AKALIN, H. 1993. OXA-11, an extended-spectrum variant of OXA-10 (PSE-2) beta-lactamase from Pseudomonas aeruginosa. *Antimicrob Agents Chemother,* 37**,** 1637-1644.
HAMEED, F., KHAN, M. A., MUHAMMAD, H., SARWAR, T., BILAL, H. & REHMAN, T. U. 2019. Plasmid-mediated mcr-1 gene in Acinetobacter baumannii and Pseudomonas aeruginosa: first report from Pakistan. *Revista da Sociedade Brasileira de Medicina Tropical,* 52**,** e20190237-e20190237.
HAMMERUM, A. M., JAKOBSEN, L., HANSEN, F., STEGGER, M., AGERSNAP, L., ANDERSEN, P. S. & WANG, M. 2015. Characterization of an IMP-7 producing ST357 Pseudomonas aeruginosa detected in Denmark using Whole Genome Sequencing. *Int J Antimicrob Agents,* 45**,** 200-201.
HANCOCK, R. E. 1998. Resistance mechanisms in Pseudomonas aeruginosa and other nonfermentative gram-negative bacteria. *Clin Infect Dis : an official publication of the Infectious Diseases Society of America,* 27 Suppl 1**,** S93-S99.
HANCOCK, R. E. & BELL, A. 1988. Antibiotic uptake into gram-negative bacteria. *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology,* 7**,** 713-720.
HANCOCK, R. E. & SPEERT, D. P. 2000. Antibiotic resistance in Pseudomonas aeruginosa: mechanisms and impact on treatment. *Drug Resist Updat,* 3**,** 247-255.
HANSON, N. D., HOSSAIN, A., BUCK, L., MOLAND, E. S. & THOMSON, K. S. 2006. First occurrence of a Pseudomonas aeruginosa isolate in the United States producing an IMP metallo-β-lactamase, IMP-18. *Antimicrob Agents Chemother,* 50**,** 2272-2273.

HAUSER, A. R. 2009. The type III secretion system of Pseudomonas aeruginosa: infection by injection. *Nat Rev Microbiol,* 7**,** 654-665.

HE, J., BALDINI, R. L., DEZIEL, E., SAUCIER, M., ZHANG, Q., LIBERATI, N. T., LEE, D., URBACH, J., GOODMAN, H. M. & RAHME, L. G. 2004. The broad host range pathogen Pseudomonas aeruginosa strain PA14 carries two pathogenicity islands harboring plant and animal virulence genes. *Proc Natl Acad Sci U S A,* 101**,** 2530-5.

HE, P. & MORAN, G. R. 2011. Structural and mechanistic comparisons of the metal-binding members of the vicinal oxygen chelate (VOC) superfamily. *J Inorg Biochem,* 105**,** 1259-72.

HEDAYATI, H., GHADERPANAH, M., RASOULINEJAD, S. A. & MONTAZERI, M. 2015. Clinical presentation and antibiotic susceptibility of contact lens associated microbial keratitis. *J Pathogen,* 2015**,** 152767.

HEIDARY, Z., BANDANI, E., EFTEKHARY, M. & JAFARI, A. A. 2016. Virulence genes profile of multidrug resistant *Pseudomonas aeruginosa* isolated from Iranian children with UTIs. *Acta Med Iran,* 54**,** 201-10.

HENRIQUES, M., SOUSA, C., LIRA, M., ELISABETE, M., OLIVEIRA, R., OLIVEIRA, R. & AZEREDO, J. 2005. Adhesion of Pseudomonas aeruginosa and Staphylococcus epidermidis to silicone–hydrogel contact lenses. *Optom Vis Sci,* 82**,** 446-450.

HIGGINS, P. G., FLUIT, A. C., MILATOVIC, D., VERHOEF, J. & SCHMITZ, F. J. 2003. Mutations in GyrA, ParC, MexR and NfxB in clinical isolates of *Pseudomonas aeruginosa*. *Int J Antimicrob Agents,* 21**,** 409-13.

HIRAI, K., SUZUE, S., IRIKURA, T., IYOBE, S. & MITSUHASHI, S. 1987. Mutations producing resistance to norfloxacin in Pseudomonas aeruginosa. *Antimicrob Agents Chemother,* 31**,** 582-586.

HO, S. E., SUBRAMANIAM, G., PALASUBRAMANIAM, S. & NAVARATNAM, P. 2002. Carbapenem-resistant Pseudomonas aeruginosa in Malaysia producing IMP-7 β-lactamase. *Antimicrob Agents Chemother,* 46**,** 3286-3287.

HOCQUET, D., VOGNE, C., EL GARCH, F., VEJUX, A., GOTOH, N., LEE, A., LOMOVSKAYA, O. & PLÉSIAT, P. 2003. MexXY-OprM efflux pump is necessary for adaptive resistance of Pseudomonas aeruginosa to aminoglycosides. *Antimicrob Agents Chemother,* 47**,** 1371-1375.

HODDENBACH, J. G., BOEKHOORN, S. S., WUBBELS, R., VREUGDENHIL, W., VAN ROOIJ, J. & GEERARDS, A. J. 2014. Clinical presentation and morbidity of contact lens–associated microbial keratitis: a retrospective study. *Graefe's Arch Clin Exp Ophthalmol,* 252**,** 299-306.

HONG, D. J., BAE, I. K., JANG, I.-H., JEONG, S. H., KANG, H.-K. & LEE, K. 2015. Epidemiology and characteristics of metallo-β-lactamase-producing Pseudomonas aeruginosa. *Infect Chemother,* 47**,** 81-97.

HOOI, S. H. & HOOI, S. T. 2005. Culture-proven bacterial keratitis in a Malaysian general hospital. *Med J Malaysia,* 60**,** 614-23.

HOOPER, D. C. 1999. Mechanisms of fluoroquinolone resistance. *Drug Resist Updat,* 2**,** 38-55.

HORNA, G., AMARO, C., PALACIOS, A., GUERRA, H. & RUIZ, J. 2019. High frequency of the exoU+/exoS+ genotype associated with multidrug-resistant “high-risk clones” of Pseudomonas aeruginosa clinical isolates from Peruvian hospitals. *Scientific Reports,* 9**,** 10874.

HRABÁK, J., FRIDRICHOVÁ, M., ŠTOLBOVÁ, M., BERGEROVÁ, T., ZEMLICKOVA, H. & URBASKOVA, P. 2009. First identification of metallo-beta-lactamase-producing Pseudomonas aeruginosa in the Czech Republic. *Eurosurveillance,* 14**,** 19102.

HSIAO, C.-H., SUN, C.-C., YEH, L.-K., MA, D. H., CHEN, P. Y., LIN, H.-C., TAN, H.-Y., CHEN, H.-C., CHEN, S.-Y. & HUANG, Y.-C. 2016. Shifting trends in bacterial keratitis in Taiwan: a 10-year review in a tertiary-care hospital. *Cornea,* 35**,** 313-317.

HSU, J., GERSTENBLITH, A. T., GARG, S. J. & VANDER, J. F. 2014. Conjunctival flora antibiotic resistance patterns after serial intravitreal injections without postinjection topical antibiotics. *Am J Ophthalmol,* 157**,** 514-518.e1.

IKEDA, T., LEDWITH, A., BAMFORD, C. H. & HANN, R. A. 1984. Interaction of a polymeric biguanide biocide with phospholipid membranes. *Biochimica et Biophysica Acta (BBA) - Biomembranes,* 769**,** 57-66.

IRAZ, M., DUZGUN, A. O., CICEK, A. C., BONNIN, R. A., CEYLAN, A., SARAL, A., NORDMANN, P. & SANDALLI, C. 2014. Characterization of novel VIM carbapenemase, VIM-38, and first detection of GES-5 carbapenem-hydrolyzing β-lactamases in Pseudomonas aeruginosa in Turkey. *Diagnost Microbiol Infect Dis,* 78**,** 292-294.

IYOBE, S., KUSADOKORO, H., TAKAHASHI, A., YOMODA, S., OKUBO, T., NAKAMURA, A. & O'HARA, K. 2002. Detection of a variant metallo-beta-lactamase, IMP-10, from two unrelated strains of Pseudomonas aeruginosa and an alcaligenes xylosoxidans strain. *Antimicrob Agents Chemother,* 46**,** 2014-2016.

JACKSON, D. M., LOWBURY, E. J. L. & TOPLEY, E. 1951. Pseudomonas pyocyanea in burns: Its a role as a pathogen, and the value of local polymyxin therapy. *The Lancet,* 258**,** 137-147.

JACOBY, G. A. 2005. Mechanisms of resistance to quinolones. *Clin Infect Dis,* 41 Suppl 2**,** S120-6.

JACOBY, G. A., CHOW, N. & WAITES, K. B. 2003. Prevalence of plasmid-mediated quinolone resistance. *Antimicrob Agents Chemother,* 47**,** 559-562.

JÁCOME, P. R. L. D. A., ALVES, L. R., CABRAL, A. B., LOPES, A. C. S. & MACIEL, M. A. V. 2012. First report of KPC-producing Pseudomonas aeruginosa in Brazil. *Antimicrob Agents Chemother,* 56**,** 4990-4990.

JALAL, S. & WRETLIND, B. 1998. Mechanisms of quinolone resistance in clinical strains of Pseudomonas aeruginosa. *Microb Drug Resis,* 4**,** 257-261.

JALAL, S., WRETLIND, G., GOTOH, N. & WRETLIND, B. 1999. Rapid identification of mutations in a multidrug efflux pump in Pseudomonas aeruginosa. *APMIS : acta pathologica, microbiologica, et immunologica Scandinavica,* 107**,** 1109-1116.

JEANNOT, K., POIREL, L., ROBERT-NICOUD, M., CHOLLEY, P., NORDMANN, P. & PLÉSIAT, P. 2012. IMP-29, a novel IMP-type metallo-β-lactamase in Pseudomonas aeruginosa. *Antimicrob Agents Chemother,* 56**,** 2187-2190.

JEANVOINE, A., MEUNIER, A., PUJA, H., BERTRAND, X., VALOT, B. & HOCQUET, D. 2019. Contamination of a hospital plumbing system by persister cells of a copper-tolerant high-risk clone of Pseudomonas aeruginosa. *Water Research,* 157**,** 579-586.

JEFFREY, D. 1995. Chemicals used as disinfectants: active ingredients and enhancing additives. *Revue Scientifique et Technique-Office International des Epizooties,* 14**,** 57-57.

JELSBAK, L., JOHANSEN, H. K., FROST, A. L., THØGERSEN, R., THOMSEN, L. E., CIOFU, O., YANG, L., HAAGENSEN, J. A., HØIBY, N. & MOLIN, S. 2007. Molecular epidemiology and dynamics of Pseudomonas aeruginosa populations in kungs of cystic Fibrosis Patients. *Infect Immun,* 75**,** 2214-24.

JEUKENS, J., KUKAVICA-IBRULJ, I., EMOND-RHEAULT, J. G., FRESCHI, L. & LEVESQUE, R. C. 2017. Comparative genomics of a drug-resistant *Pseudomonas aeruginosa* panel and the challenges of antimicrobial resistance prediction from genomes. *FEMS Microbiol Lett,* 364.

JIANG, X., ZHANG, Z., LI, M., ZHOU, D., RUAN, F. & LU, Y. 2006. Detection of extended-spectrum beta-lactamases in clinical isolates of Pseudomonas aeruginosa. *Antimicrob Agents Chemother,* 50**,** 2990-2995.

JIN, J., KWON, K., MOON, D. & LEE, J. 2009. Emergence of 16S rRNA methylase rmtA in colistin-only-sensitive Pseudomonas aeruginosa in South Korea. *Int J Antimicrob Agents,* 33**,** 490-491.

JONES, A. M., DODD, M. E., DOHERTY, C. J., GOVAN, J. R. W. & WEBB, A. K. 2002. Increased treatment requirements of patients with cystic fibrosis who harbour a highly transmissible strain of Pseudomonas aeruginosa. *Thorax,* 57**,** 924-925.

JONES, D. B. 1981. Decision-making in the management of microbial keratitis. *Ophthalmol,* 88**,** 814-820.

JONES, R. D., JAMPANI, H. B., NEWMAN, J. L. & LEE, A. S. 2000. Triclosan: A review of effectiveness and safety in health care settings. *Am J Infect Control,* 28**,** 184-196.

JUAN, C., MACIÁ, M. D., GUTIÉRREZ, O., VIDAL, C., PÉREZ, J. L. & OLIVER, A. 2005. Molecular mechanisms of β-lactam resistance mediated by ampC hyperproduction in Pseudomonas aeruginosa clinical strains. *Antimicrob Agents Chemother,* 49**,** 4733-4738.

JUAN, C., ZAMORANO, L., PÉREZ, J. L., GE, Y., OLIVER, A., SPANISH GROUP FOR THE STUDY OF, P. & SPANISH NETWORK FOR RESEARCH IN INFECTIOUS, D. 2010. Activity of a new antipseudomonal cephalosporin, CXA-101 (FR264205), against carbapenem-resistant and multidrug-resistant Pseudomonas aeruginosa clinical strains. *Antimicrob Agents Chemother,* 54**,** 846-851.

KALIAMURTHY, J., KALAVATHY, C. M., PARMAR, P., JESUDASAN, N., ARUL, C. & THOMAS, P. A. 2013. Spectrum of bacterial keratitis at a tertiary eye care centre in India. *BioMed research international,* 2013.

KEARSE, M., MOIR, R., WILSON, A., STONES-HAVAS, S., CHEUNG, M., STURROCK, S., BUXTON, S., COOPER, A., MARKOWITZ, S., DURAN, C., THIERER, T., ASHTON, B., MEINTJES, P. & DRUMMOND, A. 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics,* 28**,** 1647-9.

KEAY, L., EDWARDS, K., DART, J. & STAPLETON, F. 2008. Grading contact lens-related microbial keratitis: relevance to disease burden. *Optom Vis Sci,* 85**,** 531-537.

KEAY, L., EDWARDS, K., NADUVILATH, T., FORDE, K. & STAPLETON, F. 2006a. Factors affecting the morbidity of contact lens–related microbial keratitis: A population study. *IOVS,* 47**,** 4302-4308.

KEAY, L., EDWARDS, K., NADUVILATH, T., TAYLOR, H. R., SNIBSON, G. R., FORDE, K. & STAPLETON, F. 2006b. Microbial Kkeratitis: Predisposing factors and morbidity. *Ophthalmol,* 113**,** 109-116.

KEAY, L., EDWARDS, K. & STAPLETON, F. 2009. Signs, symptoms, and comorbidities in contact lens-related microbial keratitis. *Optom Vis Sci,* 86**,** 803-809.

KEAY, L., STAPLETON, F. & SCHEIN, O. 2007. Epidemiology of contact lens–related inflammation and microbial keratitis: a 20-year perspective. *Eye & contact lens,* 33**,** 346-353.

KHAN, M., STAPLETON, F. & WILLCOX, M. D. P. 2020. Susceptibility of contact lens-related Pseudomonas aeruginosa keratitis isolates to multipurpose disinfecting solutions, disinfectants, and antibiotics. *TVST,* 9**,** 2-2.

KHOSRAVI, Y., TAY, S. T. & VADIVELU, J. 2011. Analysis of integrons and associated gene cassettes of metallo-β-lactamase-positive Pseudomonas aeruginosa in Malaysia. *J Med Microbiol,* 60**,** 988-994.

KHOSRAVI, Y., TEE TAY, S. & VADIVELU, J. 2010. Metallo-beta-lactamase-producing imipenem-resistant Pseudomonas aeruginosa clinical isolates in a university teaching hospital in Malaysia: detection of IMP-7 and first identification of IMP-4, VIM-2, and VIM-11. *Diagn Microbiol Infect Dis,* 67**,** 294-6.

KIM, M. J., BAE, I. K., JEONG, S. H., KIM, S. H., SONG, J. H., CHOI, J. Y., YOON, S. S., THAMLIKITKUL, V., HSUEH, P.-R. & YASIN, R. M. 2013. Dissemination of metallo-β-lactamase-producing Pseudomonas aeruginosa of sequence type 235 in Asian countries. *J Antimicrob Chemother,* 68**,** 2820-2824.

KIRVEN, L. A. & THORNSBERRY, C. 1978. Minimum bactericidal concentration of sulfamethoxazole-trimethoprim for Haemophilus influenzae: correlation with prophylaxis. *Antimicrob Agents Chemother,* 14**,** 731-736.

KLOCKGETHER, J., CRAMER, N., WIEHLMANN, L., DAVENPORT, C. F. & TÜMMLER, B. J. F. I. M. 2011. Pseudomonas aeruginosa genomic structure and diversity. *Front Microbiol,* 2**,** 150.

KOCSIS, B., TOTH, A., GULYAS, D., LIGETI, B., KATONA, K., ROKUSZ, L. & SZABO, D. 2019. Acquired qnrVC1 and blaNDM-1 resistance markers in an international high-risk Pseudomonas aeruginosa ST773 clone. *J Med Microbiol,* 68**,** 336-338.

KOH, T. H., KHOO, C. T., TAN, T. T., ARSHAD, M. A. B. M., ANG, L. P., LAU, L. J., HSU, L.-Y. & OOI, E. E. 2010. Multilocus sequence types of carbapenem-resistant Pseudomonas aeruginosa in Singapore carrying metallo-β-lactamase genes, including the novel blaIMP-26 gene. *J Clin Microbiol,* 48**,** 2563-2564.

KOH, T. H., WANG, G. C. Y. & SNG, L.-H. 2004. Clonal spread of IMP-1-producing Pseudomonas aeruginosa in two hospitals in Singapore. *J Clin Microbiol,* 42**,** 5378-5380.

KÖHLER, T., EPP, S. F., CURTY, L. K. & PECHÈRE, J. C. 1999. Characterization of MexT, the regulator of the MexE-MexF-OprN multidrug efflux system of Pseudomonas aeruginosa. *J Bacteriol,* 181**,** 6300-6305.

KÖHLER, T., KOK, M., MICHEA-HAMZEHPOUR, M., PLESIAT, P., GOTOH, N., NISHINO, T., CURTY, L. K. & PECHERE, J. C. 1996. Multidrug efflux in intrinsic resistance to trimethoprim and sulfamethoxazole in Pseudomonas aeruginosa. *Antimicrob Agents Chemother,* 40**,** 2288-2290.

KÖHLER, T., MICHÉA-HAMZEHPOUR, M., HENZE, U., GOTOH, N., CURTY, L. K. & PECHÈRE, J. C. 1997. Characterization of MexE-MexF-OprN, a positively regulated multidrug efflux system of Pseudomonas aeruginosa. *Mol Microbiol,* 23**,** 345-354.

KOLAYLI, F., GACAR, G., KARADENIZLI, A., SANIC, A., VAHABOGLU, H. & GROUP, T. S. 2005. PER-1 is still widespread in Turkish hospitals among Pseudomonas aeruginosa and Acinetobacter spp. *FEMS Microbiol Letters,* 249**,** 241-245.

KOLLEF, M. H., SHORR, A., TABAK, Y. P., GUPTA, V., LIU, L. Z. & JOHANNES, R. 2005. Epidemiology and outcomes of health-care–associated pneumonia: results from a large US database of culture-positive pneumonia. *Chest,* 128**,** 3854-3862.

KOTSAKIS, S. D., PAPAGIANNITSIS, C. C., TZELEPI, E., LEGAKIS, N. J., MIRIAGOU, V. & TZOUVELEKIS, L. S. 2010. GES-13, a β-lactamase variant possessing Lys-104 and Asn-170 in Pseudomonas aeruginosa. *Antimicrob Agents Chemother,* 54**,** 1331-1333.

KOUDA, S., OHARA, M., FUJIWARA, T., KOMATSUZAWA, H., SUGAI, M., KUWAHARA, R., SHIGETA, M. & USUI, T. 2007. First isolation of blaIMP-7 in a Pseudomonas aeruginosa in Japan. *J Infect Chemother,* 13**,** 276-277.

KOWALSKI, R., PANDYA, A. N., KARENCHAK, L. M., ROMANOWSKI, E. G., HUSTED, R. C., RITTERBAND, D. C., SHAH, M. K. & GORDON, Y. J. 2001. An in vitro resistance study of levofloxacin, ciprofloxacin, and ofloxacin using keratitis isolates of Staphylococcus aureus and Pseudomonas aeruginosa. *Ophthalmol,* 108**,** 1826-9.

KOWALSKI, R. P., DHALIWAL, D. K., KARENCHAK, L. M., ROMANOWSKI, E. G., MAH, F. S., RITTERBAND, D. C. & GORDON, Y. J. 2003. Gatifloxacin and moxifloxacin: an in vitro susceptibility comparison to levofloxacin, ciprofloxacin, and ofloxacin using bacterial keratitis isolates. *Am J Ophthalmol,* 136**,** 500-505.

KOWALSKI, R. P., YATES, K. A., ROMANOWSKI, E. G., KARENCHAK, L. M., MAH, F. S. & GORDON, Y. J. 2005. An ophthalmologist's guide to understanding antibiotic susceptibility and minimum inhibitory concentration data. *Ophthalmol,* 112**,** 1987.e1-1987.e6.

KTARI, S., MNIF, B., ZNAZEN, A., REKIK, M., MEZGHANI, S., MAHJOUBI-RHIMI, F. & HAMMAMI, A. 2011. Diversity of β-lactamases in Pseudomonas aeruginosa isolates producing metallo-β-lactamase in two Tunisian hospitals. *Microb Drug Resist (Larchmont, N.Y.),* 17**,** 25-30.

KULASEKARA, B. R., KULASEKARA, H. D., WOLFGANG, M. C., STEVENS, L., FRANK, D. W. & LORY, S. 2006. Acquisition and evolution of the exoU locus in Pseudomonas aeruginosa. *J Bacteriol,* 188**,** 4037-50.

KULASEKARA, H. D., VENTRE, I., KULASEKARA, B. R., LAZDUNSKI, A., FILLOUX, A. & LORY, S. 2005. A novel two-component system controls the expression of Pseudomonas aeruginosa fimbrial cup genes. *Mol Microbiol,* 55**,** 368-380.

KUNG, V. L., OZER, E. A. & HAUSER, A. R. 2010a. The accessory genome of Pseudomonas aeruginosa. *Microbiol. Mol. Biol. Rev.,* 74**,** 621-641.

KUNG, V. L., OZER, E. A. & HAUSER, A. R. 2010b. The accessory genome of Pseudomonas aeruginosa. *Microbiol Mol Biol Rev,* 74**,** 621-41.
KUNIMOTO, D. Y., SHARMA, S., GARG, P. & RAO, G. N. 1999. In vitro susceptibility of bacterial keratitis pathogens to ciprofloxacin. *Ophthalmol,* 106**,** 80-85.
KUNIMOTO, D. Y., SHARMA, S., REDDY, M. K., GOPINATHAN, U., JYOTHI, J., MILLER, D. & RAO, G. N. 1998. Microbial keratitis in children. *Ophthalmol,* 105**,** 252-257.
LABUSCHAGNE, C. D. J., WELDHAGEN, G. F., EHLERS, M. M. & DOVE, M. G. 2008. Emergence of class 1 integron-associated GES-5 and GES-5-like extended-spectrum beta-lactamases in clinical isolates of Pseudomonas aeruginosa in South Africa. *Int J Antimicrob Agents,* 31**,** 527-530.
LAI, S., TREMBLAY, J. & DÉZIEL, E. 2009. Swarming motility: a multicellular behaviour conferring antimicrobial resistance. *Environment Microbiol,* 11**,** 126-136.
LAKKIS, C. & FLEISZIG, S. M. J. 2001. Resistance of Pseudomonas aeruginosa isolates to hydrogel contact lens disinfection correlates with cytotoxic activity. *J Clin Microbiol,* 39**,** 1477-1486.
LALITHA, P., MANOHARAN, G., KARPAGAM, R., PRAJNA, N. V., SRINIVASAN, M., MASCARENHAS, J., DAS, M., PORCO, T. C., LIETMAN, T. M., CEVALLOS, V. & KEENAN, J. D. 2017. Trends in antibiotic resistance in bacterial keratitis isolates from South India. *Br J Ophthalmol,* 101**,** 108-113.
LALITHA, P., SRINIVASAN, M., MANIKANDAN, P., BHARATHI, M. J., RAJARAMAN, R., RAVINDRAN, M., CEVALLOS, V., OLDENBURG, C. E., RAY, K. J., TOUTAIN-KIDD, C. M., GLIDDEN, D. V., ZEGANS, M. E., MCLEOD, S. D., ACHARYA, N. R. & LIETMAN, T. M. 2012. Relationship of in vitro susceptibility to moxifloxacin and in vivo clinical outcome in bacterial keratitis. *Clin Infect Dis,* 54**,** 1381-1387.
LAM, D. S. C., HOUANG, E., FAN, D. S. P., LYON, D., SEAL, D. & WONG, E. 2002. Incidence and risk factors for microbial keratitis in Hong Kong: comparison with Europe and North America. *Eye,* 16**,** 608.
LAMBERT, P. A. 2002. Mechanisms of antibiotic resistance in Pseudomonas aeruginosa. *Journal of the Royal Society of Medicine,* 95 Suppl 41**,** 22-26.
LAURETTI, L., RICCIO, M. L., MAZZARIOL, A., CORNAGLIA, G., AMICOSANTE, G., FONTANA, R. & ROSSOLINI, G. M. 1999. Cloning and characterization of bla VIM, a new integron-borne metallo-β-lactamase gene from a Pseudomonas aeruginosa clinical isolate. *Antimicrob Agents Chemother,* 43**,** 1584-1590.
LAXMI NARAYANA, B., RAO, P., BHAT, S. & VIDYALAKSHMI, K. 2018. Comparison of the antimicrobial efficacy of various contact lens solutions to inhibit the growth of Pseudomonas aeruginosa and Staphylococcus aureus. *Int J Microbiol,* 2018**,** 5916712.
LAXMINARAYAN, R. & CHAUDHURY, R. R. 2016. Antibiotic Resistance in India: Drivers and Opportunities for Action. *PLOS Medicine,* 13**,** e1001974.
LEE, K., LEE, W. G., UH, Y., HA, G. Y., CHO, J., CHONG, Y. & KOREAN NATIONWIDE SURVEILLANCE OF ANTIMICROBIAL RESISTANCE, G. 2003. VIM- and IMP-type metallo-beta-lactamase-producing Pseudomonas spp. and Acinetobacter spp. in Korean hospitals. *Emerg Infect Dis,* 9**,** 868-871.
LEE, W.-I., JAING, T.-H., HSIEH, M.-Y., KUO, M.-L., LIN, S.-J. & HUANG, J.-L. 2006. Distribution, infections, treatments and molecular analysis in a large cohort of patients with primary immunodeficiency diseases (PIDs) in Taiwan. *J Clin Immunol,* 26**,** 274-283.
LETUNIC, I. & BORK, P. 2019. Interactive Tree Of Life (iTOL) v4: recent updates and new developments. *Nucleic Acids Res*.
LEVY, B., HEILER, D. & NORTON, S. 2006. Report on testing from an investigation of fusarium keratitis in contact lens wearers. *Eye Contact Lens,* 32**,** 256-61.
LEWIS, K. 2010. Persister cells. *Ann Rev Microbiol,* 64**,** 357-372.
LI, X.-Z., MA, D., LIVERMORE, D. M. & NIKAIDO, H. 1994. Role of efflux pump (s) in intrinsic resistance of Pseudomonas aeruginosa: active efflux as a contributing factor to beta-lactam resistance. *Antimicrob Agents Chemother,* 38**,** 1742-1752.

LI, X. Z., NIKAIDO, H. & POOLE, K. 1995. Role of mexA-mexB-oprM in antibiotic efflux in Pseudomonas aeruginosa. *Antimicrob Agents Chemother,* 39**,** 1948-1953.

LI, X. Z. & POOLE, K. 2001. Mutational analysis of the OprM outer membrane component of the MexA-MexB-OprM multidrug efflux system of Pseudomonas aeruginosa. *J Bacteriol,* 183**,** 12-27.

LIBISCH, B., POIREL, L., LEPSANOVIC, Z., MIROVIC, V., BALOGH, B., PÁSZTI, J., HUNYADI, Z., DOBÁK, A., FÜZI, M. & NORDMANN, P. 2008a. Identification of PER-1 extended-spectrum beta-lactamase producing Pseudomonas aeruginosa clinical isolates of the international clonal complex CC11 from Hungary and Serbia. *FEMS Immunol Med Microbiol,* 54**,** 330-338.

LIBISCH, B., WATINE, J., BALOGH, B., GACS, M., MUZSLAY, M., SZABÓ, G. & FÜZI, M. 2008b. Molecular typing indicates an important role for two international clonal complexes in dissemination of VIM-producing Pseudomonas aeruginosa clinical isolates in Hungary. *Res Microbiol,* 159**,** 162-168.

LICHTINGER, A., YEUNG, S. N., KIM, P., AMIRAN, M. D., IOVIENO, A., ELBAZ, U., KU, J. Y. F., WOLFF, R., ROOTMAN, D. S. & SLOMOVIC, A. R. 2012. Shifting Trends in Bacterial Keratitis in Toronto: An 11-Year Review. *Ophthalmology,* 119**,** 1785-1790.

LIESEGANG, T. J. 1997. Contact lens-related microbial keratitis: Part I: Epidemiology. *Cornea,* 16**,** 125-131.

LIM, C. H. L., CARNT, N. A., FAROOK, M., LAM, J., TAN, D. T., MEHTA, J. S. & STAPLETON, F. 2016. Risk factors for contact lens-related microbial keratitis in Singapore. *Eye,* 30**,** 447-455.

LIN, J., CHEN, D. Q., HONG, J., HUANG, H. & XU, X. 2020. Prevalence of qnrVC Genes in Pseudomonas aeruginosa Clinical Isolates from Guangdong, China. *Curr Microbiol*.

LINDER, J. A., HUANG, E. S., STEINMAN, M. A., GONZALES, R. & STAFFORD, R. S. 2005. Fluoroquinolone prescribing in the United States: 1995 to 2002. *Am J Med,* 118**,** 259-68.

LISTER, P. D., WOLTER, D. J. & HANSON, N. D. 2009. Antibacterial-Resistant Pseudomonas aeruginosa: Clinical Impact and Complex Regulation of Chromosomally Encoded Resistance Mechanisms. *Clinical Microbiology Reviews,* 22**,** 582-610.

LIU, J., YANG, L., CHEN, D., PETERS, B. M., LI, L., LI, B., XU, Z. & SHIRTLIFF, M. E. 2018. Complete sequence of pBM413, a novel multidrug resistance megaplasmid carrying qnrVC6 and blaIMP-45 from *Pseudomonas aeruginosa*. *Int J Antimicrob Agents,* 51**,** 145-150.

LIU, W. E., LIU, X., LIAO, J., ZHANG, Y. & LIANG, X. 2010. Identification of bla OXA‐128 and bla OXA‐129, two novel OXA‐type extended‐spectrum‐β‐lactamases in Pseudomonas aeruginosa, in Hunan Province, China. *J Basic Microbiol,* 50**,** S116-S119.

LIU, Y.-Y., WANG, Y., WALSH, T. R., YI, L.-X., ZHANG, R., SPENCER, J., DOI, Y., TIAN, G., DONG, B., HUANG, X., YU, L.-F., GU, D., REN, H., CHEN, X., LV, L., HE, D., ZHOU, H., LIANG, Z., LIU, J.-H. & SHEN, J. 2016. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *The Lancet Infectious Diseases,* 16**,** 161-168.

LIVERMORE, D. M. 1984. Penicillin-binding proteins, porins andouter-membrane permeability of carbenicillin-resistant and -susceptible strains of Pseudomonas aeruginosa. *J Med Microbiol,* 18**,** 261-270.

LIVERMORE, D. M. 1995a. beta-Lactamases in laboratory and clinical resistance. *Clin Microbiol Rev,* 8**,** 557-584.

LIVERMORE, D. M. 1995b. beta-Lactamases in laboratory and clinical resistance. *Clin Microbiol Rev,* 8**,** 557-84.

LIVERMORE, D. M. 2002. Multiple mechanisms of antimicrobial resistance in Pseudomonas aeruginosa: Our worst nightmare? *Clin Infect Dis,* 34**,** 634-640.

LIZIOLI, A., PRIVITERA, G., ALLIATA, E., BANFI, E. A., BOSELLI, L., PANCERI, M., PERNA, M., PORRETTA, A., SANTINI, M. & CARRERI, V. 2003. Prevalence of nosocomial infections in Italy: result from the Lombardy survey in 2000. *J Hosp Infect,* 54**,** 141-148.

LOMHOLT, J. & KILIAN, M. 2003. Ciprofloxacin susceptibility of Pseudomonas aeruginosa isolates from keratitis. *Br J Ophthalmol,* 87**,** 1238-1240.

LOMHOLT, J. A., POULSEN, K. & KILIAN, M. 2001. Epidemic population structure of Pseudomonas aeruginosa: Evidence for a clone that is pathogenic to the eye and that has a distinct combination of virulence factors. *Infect immun,* 69**,** 6284-6295.

LUSHNIAK, B. D. 2014. Antibiotic resistance: a public health crisis. *Public Health Rep,* 129**,** 314-6.

LUZZARO, F., MANTENGOLI, E., PERILLI, M., LOMBARDI, G., ORLANDI, V., ORSATTI, A., AMICOSANTE, G., ROSSOLINI, G. M. & TONIOLO, A. 2001. Dynamics of a nosocomial outbreak of multidrug-resistant Pseudomonas aeruginosa producing the PER-1 extended-spectrum β-lactamase. *J Clin Microbiol,* 39**,** 1865-1870.

LYNCH, M. J., DRUSANO, G. L. & MOBLEY, H. L. 1987. Emergence of resistance to imipenem in Pseudomonas aeruginosa. *Antimicrob Agents Chemother,* 31**,** 1892-1896.

MA, J., ZENG, Z., CHEN, Z., XU, X., WANG, X., DENG, Y., LÜ, D., HUANG, L., ZHANG, Y., LIU, J. & WANG, M. 2009. High prevalence of plasmid-mediated quinolone resistance determinants aac(6)Ib-cr, and qepA among ceftiofur-resistant Enterobacteriaceae isolates from companion and food-producing animals. *Antimicrob Agents Chemother,* 53**,** 519-524.

MACIÁ, M. D., BLANQUER, D., TOGORES, B., SAULEDA, J., PÉREZ, J. L. & OLIVER, A. 2005. Hypermutation Is a key factor in development of multiple-antimicrobial resistance in Pseudomonas aeruginosa strains causing chronic lung infections. *Antimicrob Agents Chemother,* 49**,** 3382-3386.

MANSOUR, W., DAHMEN, S., POIREL, L., CHARFI, K., BETTAIEB, D., BOUJAAFAR, N. & BOUALLEGUE, O. 2009a. Emergence of SHV-2a extended-spectrum beta-lactamases in clinical isolates of Pseudomonas aeruginosa in a university hospital in Tunisia. *Microb Drug Res (Larchmont, N.Y.),* 15**,** 295-301.

MANSOUR, W., POIREL, L., BETTAIEB, D., BOUALLEGUE, O., BOUJAAFAR, N. & NORDMANN, P. 2009b. Metallo-β-lactamase–producing Pseudomonas aeruginosa isolates in Tunisia. *Diagn Microbiol Infect Dis,* 64**,** 458-461.

MARASINI, S., SWIFT, S., DEAN, S. J., ORMONDE, S. E. & CRAIG, J. P. 2016. Spectrum and sensitivity of bacterial keratitis isolates in Auckland. *J Ophthalmol,* 2016**,** 3769341-3769341.

MARCHANDIN, H., JEAN-PIERRE, H., DE CHAMPS, C., SIROT, D., DARBAS, H., PERIGAULT, P. F. & CARRIERE, C. 2000. Production of a TEM-24 plasmid-mediated extended-spectrum β-lactamase by a clinical isolate of Pseudomonas aeruginosa. *Antimicrob Agents Chemother,* 44**,** 213-216.

MARCIANO, D. C., KARKOUTI, O. Y. & PALZKILL, T. 2007. A fitness cost associated with the antibiotic resistance enzyme SME-1 beta-lactamase. *Genetics,* 176**,** 2381-2392.

MARTINEZ-MARTINEZ, L., PASCUAL, A. & JACOBY, G. A. 1998. Quinolone resistance from a transferable plasmid. *Lancet,* 351**,** 797-9.

MARTINS, A. F., ZAVASCKI, A. P., GASPARETO, P. B. & BARTH, A. L. 2007. Dissemination of Pseudomonas aeruginosa producing SPM-1-like and IMP-1-like metallo-beta-lactamases in hospitals from southern Brazil. *Infection,* 35**,** 457-60.

MASUDA, N., SAKAGAWA, E. & OHYA, S. 1995. Outer membrane proteins responsible for multiple drug resistance in Pseudomonas aeruginosa. *Antimicrob Agents Chemother,* 39**,** 645-9.

MASUDA, N., SAKAGAWA, E., OHYA, S., GOTOH, N., TSUJIMOTO, H. & NISHINO, T. 2000. Contribution of the MexX-MexY-oprM efflux system to intrinsic resistance in Pseudomonas aeruginosa. *Antimicrob Agents Chemother,* 44**,** 2242-2246.

MAVROIDI, A., TZELEPI, E., TSAKRIS, A., MIRIAGOU, V., SOFIANOU, D. & TZOUVELEKIS, L. S. 2001. An integron-associated β-lactamase (IBC-2) from Pseudomonas aeruginosa is a variant of the extended-spectrum β-lactamase IBC-1. *J Antimicrob Chemother,* 48**,** 627-630.

MAYO, M. S., SCHLITZER, R. L., WARD, M. A., WILSON, L. A. & AHEARN, D. G. 1987. Association of Pseudomonas and Serratia corneal ulcers with use of contaminated solutions. *J Clin Microbiol,* 25**,** 1398-400.

MAZEL, D. 2004. Integrons and the origin of antibiotic resistance gene cassettes. *ASM News-American Society for Microbiology,* 70**,** 520-525.

MAZEL, D., DYCHINCO, B., WEBB, V. A. & DAVIES, J. 1998. A distinctive class of integron in the Vibrio cholerae genome. *Science,* 280**,** 605-608.

MCALLUM, P. J., MCGHEE, C. N. J. C. & OPHTHALMOLOGY, E. 2003. Prescribing trends in infectious keratitis: a survey of New Zealand ophthalmologists. 31**,** 496-504.

MCCREADY, A. R., PACZKOWSKI, J. E., HENKE, B. R. & BASSLER, B. L. 2019. Structural determinants driving homoserine lactone ligand selection in the Pseudomonas aeruginosa lasR quorum-sensing receptor. *PNAS,* 116**,** 245-254.

MCDONNELL, G. & RUSSELL, A. D. 1999. Antiseptics and disinfectants: Activity, action, and resistance. *Clin Microbiol Rev,* 12**,** 147-179.

MCDONNELL, P. J. 1996. Empirical or culture-guided therapy for microbial keratitis?: A plea for data. *Arch Ophthalmol,* 114**,** 84-87.

MCLAUGHLIN, B., STAPLETON, MATHESON & DART 1998. Bacterial biofilm on contact lenses and lens storage cases in wearers with microbial keratitis. *Journal of Applied Microbiology,* 84**,** 827-838.

MCLEOD, S. D., LABREE, L. D., TAYYANIPOUR, R., FLOWERS, C. W., LEE, P. P. & MCDONNELL, P. J. 1995. The importance of initial management in the treatment of severe infectious corneal ulcers. *Ophthalmol,* 102**,** 1943-1948.

MENDES, R. E., TOLEMAN, M. A., RIBEIRO, J., SADER, H. S., JONES, R. N. & WALSH, T. R. 2004. Integron carrying a novel metallo-β-lactamase gene, blaIMP-16, and a fused form of aminoglycoside-resistant gene aac (6′)-30/aac (6′)-Ib′ : report from the SENTRY Antimicrobial Surveillance Program. *Antimicrob Agents Chemother,* 48**,** 4693-4702.

MILDER, E., VANDER, J., SHAH, C. & GARG, S. 2012. Changes in antibiotic resistance patterns of conjunctival flora due to repeated use of topical antibiotics after intravitreal injection. *Ophthalmol,* 119**,** 1420-1424.

MINE, T., MORITA, Y., KATAOKA, A., MIZUSHIMA, T. & TSUCHIYA, T. 1999. Expression in Escherichia coli of a New Multidrug Efflux Pump, MexXY, from Pseudomonas aeruginosa. *Antimicrob Agents Chemother,* 43**,** 415-417.

MIRSALEHIAN, A., FEIZABADI, M., NAKHJAVANI, F. A., JABALAMELI, F., GOLI, H. & KALANTARI, N. 2010. Detection of VEB-1, OXA-10 and PER-1 genotypes in extended-spectrum β-lactamase-producing Pseudomonas aeruginosa strains isolated from burn patients. *Burns,* 36**,** 70-74.

MODRICH, P. 1991. Mechanisms and biological effects of mismatch repair. *Annu Rev Genet,* 25**,** 229-53.

MOHAMMADINIA, M., RAHMANI, S., ESLAMI, G., GHASSEMI-BROUMAND, M., AGHAZADH AMIRI, M., AGHAIE, G., TABATABAEE, S. M., TAHERI, S. & BEHGOZIN, A. 2012. Contact lens disinfecting solutions antibacterial efficacy: comparison between clinical isolates and the standard ISO ATCC strains of Pseudomonas aeruginosa and Staphylococcus aureus. *Eye (London, England),* 26**,** 327-330.

MOREAU-MARQUIS, S., STANTON, B. A. & O'TOOLE, G. A. 2008. Pseudomonas aeruginosa biofilm formation in the cystic fibrosis airway. *Pulm Pharmacol Ther,* 21**,** 595-599.

MOSHIRFAR, M., MIRZAIAN, G., FEIZ, V. & KANG, P. C. 2006. Fourth-generation fluoroquinolone-resistant bacterial keratitis after refractive surgery. *J Cataract Refract Surg,* 32**,** 515-518.

MUGNIER, P., DUBROUS, P., CASIN, I., ARLET, G. & COLLATZ, E. 1996. A TEM-derived extended-spectrum beta-lactamase in Pseudomonas aeruginosa. *Antimicrob Agents Chemother,* 40**,** 2488-2493.

MUSCH, D. C., SUGAR, A. & MEYER, R. F. 1983. Demographic and predisposing factors in corneal ulceration. *Arch Ophthalmol,* 101**,** 1545-1548.

N, M., J, M., V, U. & H.N, M. 2016. Unraveling genomic and phenotypic nature of multidrug-resistant (MDR) Pseudomonas aeruginosa VRFPA04 isolated from keratitis patient. *Microbiol Res,* 193**,** 140-149.

NAAS, T., BOGAERTS, P., KOSTYANEV, T., CUZON, G., HUANG, T.-D., OZSU, S., NORDMANN, P. & GLUPCZYNSKI, Y. 2011. Silent spread of IMP-13-producing Pseudomonas aeruginosa belonging to sequence type 621 in Belgium. *J Antimicrob Chemother,* 66**,** 2178-2179.

NAAS, T., PHILIPPON, L., POIREL, L., RONCO, E. & NORDMANN, P. 1999. An SHV-derived extended-spectrum beta-lactamase in Pseudomonas aeruginosa. *Antimicrob Agents Chemother,* 43**,** 1281-1284.

NAKANO, M., DEGUCHI, T., KAWAMURA, T., YASUDA, M., KIMURA, M., OKANO, Y. & KAWADA, Y. 1997. Mutations in the gyrA and parC genes in fluoroquinolone-resistant clinical isolates of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother,* 41**,** 2289-91.

NEUHAUSER, M. M., WEINSTEIN, R. A., RYDMAN, R., DANZIGER, L. H., KARAM, G. & QUINN, J. P. 2003. Antibiotic resistance among gram-negative bacilli in US intensive care units: implications for fluoroquinolone use. *Jama,* 289**,** 885-8.

NG, A. L.-K., TO, K. K.-W., CHOI, C. C.-L., YUEN, L. H., YIM, S.-M., CHAN, K. S.-K., LAI, J. S.-M. & WONG, I. Y.-H. 2015. Predisposing factors, microbial characteristics, and clinical outcome of microbial keratitis in a tertiary centre in Hong Kong: A 10-year experience. *J Ophthalmol,* 2015**,** 769436.

NICHOLS, J. J., WILLCOX, M. D., BRON, A. J., BELMONTE, C., CIOLINO, J. B., CRAIG, J. P., DOGRU, M., FOULKS, G. N., JONES, L., NELSON, J. D., NICHOLS, K. K., PURSLOW, C., SCHAUMBERG, D. A., STAPLETON, F. & SULLIVAN, D. A. 2013. The TFOS International Workshop on Contact Lens Discomfort: executive summary. *Invest Ophthalmol Vis Sci,* 54**,** Tfos7-tfos13.

NIKAIDO, H. 1998. Multiple antibiotic resistance and efflux. *Curr Opin Microbiol,* 1**,** 516-523.

NIKAIDO, H. & HANCOCK, R. 1986. Outer membrane permeability of Pseudomonas aeruginosa. *The bacteria,* 10**,** 145-193.

NIXON, H. 2018. Preparation of Fortified Antimicrobial Eye Drops. *Kerala J Ophthalmol,* 30**,** 152-154.

NORDMANN, P. & NAAS, T. 1994. Sequence analysis of PER-1 extended-spectrum beta-lactamase from Pseudomonas aeruginosa and comparison with class A beta-lactamases. *Antimicrobial Agents and Chemotherapy,* 38**,** 104-114.

NORDMANN, P. & POIREL, L. 2005. Emergence of plasmid-mediated resistance to quinolones in Enterobacteriaceae. *J Antimicrob Chemother,* 56**,** 463-469.

NORDMANN, P., RONCO, E., NAAS, T., DUPORT, C., MICHEL-BRIAND, Y. & LABIA, R. 1993. Characterization of a novel extended-spectrum beta-lactamase from Pseudomonas aeruginosa. *Antimicrob Agents Chemother,* 37**,** 962-969.

NORINA, T., RAIHAN, S., BAKIAH, S., EZANEE, M., LIZA-SHARMINI, A. & WAN HAZZABAH, W. 2008. Microbial keratitis: aetiological diagnosis and clinical features in patients admitted to Hospital Universiti Sains Malaysia. *Singapore Med J,* 49**,** 67-71.

NOURI, R., AHANGARZADEH REZAEE, M., HASANI, A., AGHAZADEH, M. & ASGHARZADEH, M. 2016. The role of gyrA and parC mutations in fluoroquinolones-resistant Pseudomonas aeruginosa isolates from Iran. *Braz J Microbiol : [publication of the Brazilian Society for Microbiology],* 47**,** 925-930.

NURK, S., BANKEVICH, A., ANTIPOV, D., GUREVICH, A., KOROBEYNIKOV, A., LAPIDUS, A., PRJIBELSKY, A., PYSHKIN, A., SIROTKIN, A., SIROTKIN, Y., STEPANAUSKAS, R., MCLEAN, J., LASKEN, R., CLINGENPEEL, S. R., WOYKE,

T., TESLER, G., ALEKSEYEV, M. A. & PEVZNER, P. A. Assembling genomes and mini-metagenomes from highly chimeric reads. 2013// 2013 Berlin, Heidelberg. Springer Berlin Heidelberg, 158-170.

OCHS, M. M., MCCUSKER, M. P., BAINS, M. & HANCOCK, R. E. W. 1999. Negative regulation of the Pseudomonas aeruginosa outer membrane porin OprD selective for imipenem and basic amino acids. *Antimicrob Agents Chemother,* 43**,** 1085-1090.

ODDS, F. C. 2003. Synergy, antagonism, and what the chequerboard puts between them. *J Antimicrob Chemother,* 52**,** 1-1.

OHARA, M., KOUDA, S., ONODERA, M., FUJIUE, Y., SASAKI, M., KOHARA, T., KASHIYAMA, S., HAYASHIDA, S., KADONO, M., KOMATSUZAWA, H., GOTOH, N., USUI, T., ITAHA, H., KUWABARA, M., YOKOYAMA, T. & SUGAI, M. 2007. Molecular characterization of imipenem-resistant Pseudomonas aeruginosa in Hiroshima, Japan. *Microbiol Immun,* 51**,** 271-277.

OHLASOVA, D., KMET, V. & NIKS, M. 2007. First report of the carbapenem-resistant Pseudomonas aeruginosa producing IMP-7 metallo-β-lactamase in Slovakia. *Int J Antimicrob Agents,* 30**,** 370-371.

OLAITAN, A. O., MORAND, S. & ROLAIN, J.-M. 2014. Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. *Front Microbiol,* 5.

OLDENBURG, C. E., LALITHA, P., SRINIVASAN, M., RAJARAMAN, R., RAVINDRAN, M., MASCARENHAS, J., BORKAR, D. S., RAY, K. J., ZEGANS, M. E., MCLEOD, S. D., PORCO, T. C., LIETMAN, T. M. & ACHARYA, N. R. 2013. Emerging moxifloxacin resistance in Pseudomonas aeruginosa keratitis isolates in South India. *Ophthalmic Epidemiol,* 20**,** 155-158.

OLIVER, A., BAQUERO, F. & BLAZQUEZ, J. 2002. The mismatch repair system (mutS, mutL and uvrD genes) in Pseudomonas aeruginosa: molecular characterization of naturally occurring mutants. *Mol Microbiol,* 43**,** 1641-50.

OLIVER, A., CANTÓN, R., CAMPO, P., BAQUERO, F. & BLÁZQUEZ, J. 2000. High frequency of hypermutable Pseudomonas aeruginosa in cystic fibrosis lung infection. *Science,* 288**,** 1251-1253.

OO, T. Z., COLE, N., GARTHWAITE, L., WILLCOX, M. D. P. & ZHU, H. 2010. Evaluation of synergistic activity of bovine lactoferricin with antibiotics in corneal infection. *J Antimicrob Chemother,* 65**,** 1243-1251.

ORTIZ DE LA ROSA, J. M., NORDMANN, P. & POIREL, L. 2020. Pathogenicity Genomic Island-Associated CrpP-Like Fluoroquinolone-Modifying Enzymes among Pseudomonas aeruginosa Clinical Isolates in Europe. *Antimicrob Agents Chemother,* 64.

OVERHAGE, J., BAINS, M., BRAZAS, M. D. & HANCOCK, R. E. 2008. Swarming of Pseudomonas aeruginosa is a complex adaptation leading to increased production of virulence factors and antibiotic resistance. *J Bacteriol,* 190**,** 2671-2679.

OWUSU-ANIM, D. & KWON, D. H. 2012. Differential role of two-component regulatory systems (phoPQ and pmrAB) in polymyxin B susceptibility of Pseudomonas aeruginosa. *Adv Microbiol,* 2.

OZER, E. A., ALLEN, J. P. & HAUSER, A. R. 2014. Characterization of the core and accessory genomes of Pseudomonas aeruginosa using bioinformatic tools Spine and AGEnt. *BMC Genomics,* 15**,** 737.

OZGUMUS, O. B., CAYLAN, R., TOSUN, I., SANDALLI, C., AYDIN, K. & KOKSAL, I. 2007. Molecular epidemiology of clinical Pseudomonas aeruginosa Isolates carrying IMP-1 metallo-β-lactamase gene in a university hospital in Turkey. *Microb Drug Resis,* 13**,** 191-198.

PAGANI, L., COLINON, C., MIGLIAVACCA, R., LABONIA, M., DOCQUIER, J.-D., NUCLEO, E., SPALLA, M., BERGOLI, M. L. & ROSSOLINI, G. M. 2005. Nosocomial outbreak caused by multidrug-resistant Pseudomonas aeruginosa producing IMP-13 metallo-β-lactamase. *Journal of Clinical Microbiology,* 43**,** 3824-3828.

PAGE, A. J., CUMMINS, C. A., HUNT, M., WONG, V. K., REUTER, S., HOLDEN, M. T., FOOKES, M., FALUSH, D., KEANE, J. A. & PARKHILL, J. 2015. Roary: rapid large-scale prokaryote pan genome analysis. *Bioinformatics,* 31**,** 3691-3.

PANG, Z., RAUDONIS, R., GLICK, B. R., LIN, T.-J. & CHENG, Z. 2019. Antibiotic resistance in Pseudomonas aeruginosa: mechanisms and alternative therapeutic strategies. *Biotechnology Advances,* 37**,** 177-192.

PANTHI, S., PAUDEL, P., CHAUDHARY, M., SAPKOTA, K. & SHAH, D. N. 2014. Microbial contamination of contact lens care accessories and compliance with care regimens in Nepal. *Contact Lens and Anterior Eye,* 37**,** 2-10.

PARK, C. H., ROBICSEK, A., JACOBY, G. A., SAHM, D. & HOOPER, D. C. 2006. Prevalence of aac (6') Ib-cr encoding a ciprofloxacin-modifying enzyme in the United States. *Antimicrobial Agents and Chemotherapy*.

PARMAR, P., SALMAN, A., KALAVATHY, C. M., KALIAMURTHY, J., THOMAS, P. A. & JESUDASAN, C. A. 2006. Microbial keratitis at extremes of age. *Cornea,* 25**,** 153-8.

PARTRIDGE, S. R., KWONG, S. M., FIRTH, N. & JENSEN, S. O. 2018. Mobile genetic elements associated with antimicrobial resistance. *Clin Microbiol Rev,* 31**,** e00088-17.

PASTERAN, F., FACCONE, D., PETRONI, A., RAPOPORT, M., GALAS, M., VÁZQUEZ, M. & PROCOPIO, A. 2005. Novel variant (bla(VIM-11)) of the metallo-{beta}-lactamase bla(VIM) family in a GES-1 extended-spectrum-{beta}-lactamase-producing Pseudomonas aeruginosa clinical isolate in Argentina. *Antimicrob Agents Chemother,* 49**,** 474-475.

PATEL, J., COCKERILL 3RD, F. & ALDER, J. 2014. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fourth Informational Supplement. M100-S24. *Clinical and Laboratory Standards Institute (CLSI), Wayne, PA, USA*.

PEGG, K. M., LIU, E. M., LACURAN, A. E. & OELSCHLAEGER, P. 2013. Biochemical characterization of IMP-30, a metallo-β-lactamase with enhanced activity toward ceftazidime. *Antimicrob Agents Chemother,* 57**,** 5122-5126.

PELEG, A. Y., FRANKLIN, C., BELL, J. M. & SPELMAN, D. W. 2005. Dissemination of the Metallo-β-lactamase gene blaIMP-4 among gram-negative pathogens in a clinical setting in Australia. *Clin Infect Dis,* 41**,** 1549-1556.

PELLEGRINI, C., MERCURI, P. S., CELENZA, G., GALLENI, M., SEGATORE, B., SACCHETTI, E., VOLPE, R., AMICOSANTE, G. & PERILLI, M. 2009. Identification of bla IMP-22 in Pseudomonas spp. in urban wastewater and nosocomial environments: biochemical characterization of a new IMP metallo-enzyme variant and its genetic location. *J Antimicrob Chemother,* 63**,** 901-908.

PENS, C. J., DA COSTA, M., FADANELLI, C., CAUMO, K. & ROTT, M. 2008. Acanthamoeba spp. and bacterial contamination in contact lens storage cases and the relationship to user profiles. *Parasitol Res,* 103**,** 1241.

PERRETEN, V., STRAUSS, C., COLLAUD, A. & GERBER, D. 2016. Colistin resistance gene mcr-1 in avian-pathogenic Escherichia coli in South Africa. *Antimicrob Agents Chemother,* 60**,** 4414-4415.

PHILIPPON, L. N., NAAS, T., BOUTHORS, A.-T., BARAKETT, V. & NORDMANN, P. 1997. OXA-18, a class D clavulanic acid-inhibited extended-spectrum beta-lactamase from Pseudomonas aeruginosa. *Antimicrob Agents Chemother,* 41**,** 2188-2195.

PHILLIPS, R. M., SIX, D. A., DENNIS, E. A. & GHOSH, P. 2003. In vivo phospholipase activity of the Pseudomonas aeruginosa cytotoxin ExoU and protection of mammalian cells with phospholipase A2 inhibitors. *J Biol Chem,* 278**,** 41326-32.

PICÃO, R. C., POIREL, L., GALES, A. C. & NORDMANN, P. 2009a. Diversity of beta-lactamases produced by ceftazidime-resistant Pseudomonas aeruginosa isolates causing bloodstream infections in Brazil. *Antimicrob Agents Chemother,* 53**,** 3908-3913.

PICÃO, R. C., POIREL, L., GALES, A. C. & NORDMANN, P. 2009b. Further identification of CTX-M-2 extended-spectrum β-lactamase in Pseudomonas aeruginosa. *Antimicrob Agents Chemother,* 53**,** 2225-2226.

PITOUT, J., REVATHI, G., CHOW, B., KABERA, B., KARIUKI, S., NORDMANN, P. & POIREL, L. 2008. Metallo-β-lactamase-producing Pseudomonas aeruginosa isolated from a large tertiary centre in Kenya. *Clin Microbiol Infect,* 14**,** 755-759.

PIYAKUL, C., TIYAWISUTSRI, R. & BOONBUMRUNG, K. 2012. Emergence of metallo-β-lactamase IMP-14 and VIM-2 in Pseudomonas aeruginosa clinical isolates from a tertiary-level hospital in Thailand. *Epidemiol Infect,* 140**,** 539-541.

POGGIO, E. C., GLYNN, R. J., SCHEIN, O. D., SEDDON, J. M., SHANNON, M. J., SCARDINO, V. A. & KENYON, K. R. 1989. The incidence of ulcerative keratitis among users of daily-wear and extended-wear soft contact lenses. *N Engl J Med,* 321**,** 779-83.

POIREL, L., BRINAS, L., FORTINEAU, N. & NORDMANN, P. 2005a. Integron-encoded GES-type extended-spectrum beta-lactamase with increased activity toward aztreonam in Pseudomonas aeruginosa. *Antimicrob Agents Chemother,* 49**,** 3593-3597.

POIREL, L., BRINAS, L., VERLINDE, A., IDE, L. & NORDMANN, P. 2005b. BEL-1, a novel clavulanic acid-inhibited extended-spectrum beta-lactamase, and the class 1 integron In120 in Pseudomonas aeruginosa. *Antimicrob Agents Chemother,* 49**,** 3743-3748.

POIREL, L., DOCQUIER, J.-D., DE LUCA, F., VERLINDE, A., IDE, L., ROSSOLINI, G. M. & NORDMANN, P. 2010a. BEL-2, an extended-spectrum β-lactamase with increased activity toward expanded-spectrum cephalosporins in Pseudomonas aeruginosa. *Antimicrob Agents Chemother,* 54**,** 533-535.

POIREL, L., LAMBERT, T., TÜRKOGLÜ, S., RONCO, E., GAILLARD, J.-L. & NORDMANN, P. 2001a. Characterization of class 1 integrons from Pseudomonas aeruginosa that contain the bla VIM-2 carbapenem-hydrolyzing β-lactamase gene and of two novel aminoglycoside resistance gene cassettes. *Antimicrob Agents Chemother,* 45**,** 546-552.

POIREL, L., LEBESSI, E., CASTRO, M., FÈVRE, C., FOUSTOUKOU, M. & NORDMANN, P. 2004. Nosocomial outbreak of extended-spectrum beta-lactamase SHV-5-producing isolates of Pseudomonas aeruginosa in Athens, Greece. *Antimicrob Agents Chemother,* 48**,** 2277-2279.

POIREL, L., LIARD, A., RODRIGUEZ-MARTINEZ, J. M. & NORDMANN, P. 2005c. Vibrionaceae as a possible source of Qnr-like quinolone resistance determinants. *J Antimicrob Chemother,* 56**,** 1118-21.

POIREL, L., NORDMANN, P., LAGRUTTA, E., CLEARY, T. & MUNOZ-PRICE, L. S. 2010b. Emergence of KPC-Producing Pseudomonas aeruginosa in the United States. *Antimicrobial Agents and Chemotherapy,* 54**,** 3072-3072.

POIREL, L., RONCO, E., NAAS, T. & NORDMANN, P. 1999. Extended-spectrum beta-lactamase TEM-4 in Pseudomonas aeruginosa. *Clinical Microbiology and Infection,* 5**,** 651-652.

POIREL, L., WELDHAGEN, G. F., NAAS, T., DE CHAMPS, C., DOVE, M. G. & NORDMANN, P. 2001b. GES-2, a class A beta-lactamase from Pseudomonas aeruginosa with increased hydrolysis of imipenem. *Antimicrob Agents Chemother,* 45**,** 2598-2603.

POLLINI, S., ANTONELLI, A., VENTURELLI, C., MARADEI, S., VEGGETTI, A., BRACCO, S., RUMPIANESI, F., LUZZARO, F. & ROSSOLINI, G. M. 2013. Acquisition of plasmid-borne bla IMP-19 gene by a VIM-1-positive Pseudomonas aeruginosa of the sequence type 235 epidemic lineage. *J Antimicrob Chemother,* 68**,** 722-724.

POOLE, K. 2001. Multidrug efflux pumps and antimicrobial resistance in Pseudomonas aeruginosa and related organisms. *J Mole Microbiol Biotechnol,* 3**,** 255-264.

POOLE, K. 2011. Pseudomonas aeruginosa: resistance to the max. *Front Microbiol,* 2**,** 65.

POOLE, K., GOTOH, N., TSUJIMOTO, H., ZHAO, Q., WADA, A., YAMASAKI, T., NESHAT, S., YAMAGISHI, J.-I., LI, X.-Z. & NISHINO, T. 1996a. Overexpression of the mexC–mexD–oprJ efflux operon in nfxB-type multidrug-resistant strains of Pseudomonas aeruginosa. *Mole Microbiol,* 21**,** 713-725.

POOLE, K., KREBES, K., MCNALLY, C. & NESHAT, S. 1993. Multiple antibiotic resistance in Pseudomonas aeruginosa: evidence for involvement of an efflux operon. *J Bacteriol,* 175**,** 7363-7372.

POOLE, K., TETRO, K., ZHAO, Q., NESHAT, S., HEINRICHS, D. E. & BIANCO, N. 1996b. Expression of the multidrug resistance operon mexA-mexB-oprM in Pseudomonas aeruginosa: mexR encodes a regulator of operon expression. *Antimicrob Agents Chemother,* 40**,** 2021-2028.

POONSUK, K., TRIBUDDHARAT, C. & CHUANCHUEN, R. 2013. Aminoglycoside resistance mechanisms in Pseudomonas aeruginosa isolates from non-cystic fibrosis patients in Thailand. *Can J Microbiol,* 59**,** 51-6.

PORTER, G. & GRILLS, N. 2015. Medication misuse in India: a major public health issue in India. *Journal of Public Health,* 38**,** e150-e157.

POURNARAS, S., KÖCK, R., MOSSIALOS, D., MELLMANN, A., SAKELLARIS, V., STATHOPOULOS, C., FRIEDRICH, A. W. & TSAKRIS, A. 2013. Detection of a phylogenetically distinct IMP-type metallo-β-lactamase, IMP-35, in a CC235 Pseudomonas aeruginosa from the Dutch–German border region (Euregio). *J Antimicrob Chemother,* 68**,** 1271-1276.

PUIGBÒ, P., BRAVO, I. G. & GARCIA-VALLVE, S. 2008. CAIcal: a combined set of tools to assess codon usage adaptation. *Biol Direct,* 3**,** 38.

QING, Y., CAO, K.-Y., FANG, Z.-L., HUANG, Y.-M., ZHANG, X.-F., TIAN, G.-B. & HUANG, X. 2014. Outbreak of PER-1 and diversity of β-lactamases among ceftazidime-resistant Pseudomonas aeruginosa clinical isolates. *J Med Microbiol,* 63**,** 386-392.

QU, T.-T., ZHANG, J.-L., WANG, J., TAO, J., YU, Y.-S., CHEN, Y.-G., ZHOU, J.-Y. & LI, L.-J. 2009. Evaluation of phenotypic tests for detection of metallo-β-lactamase-producing Pseudomonas aeruginosa strains in China. *J Clin Microbiol,* 47**,** 1136-1142.

QUALE, J., BRATU, S., GUPTA, J. & LANDMAN, D. 2006. Interplay of efflux system, ampC, and oprD expression in carbapenem resistance of Pseudomonas aeruginosa clinical isolates. *Antimicrob Agents Chemother,* 50**,** 1633-1641.

QUINONES-FALCONI, F., GALICIA-VELASCO, M., MARCHIARO, P., MUSSI, M. A., BALLERINI, V., VILA, A. J., VIALE, A. M., BERMEJO-MORALES, K. & LIMANSKY, A. S. 2010. Emergence of Pseudomonas Aeruginosa Strains Producing Metallo-β-lactamases of the IMP-15 and VIM-2 Types in Mexico. *Clin Microbiol Infect,* 16**,** 126-131.

RAMIREZ-DIAZ, M. I., DIAZ-MAGANA, A., MEZA-CARMEN, V., JOHNSTONE, L., CERVANTES, C. & RENSING, C. 2011. Nucleotide sequence of *Pseudomonas aeruginosa* conjugative plasmid pUM505 containing virulence and heavy-metal resistance genes. *Plasmid,* 66**,** 7-18.

RAMÍREZ, D. G., NICOLA, F., ZARATE, S., RELLOSO, S., SMAYEVSKY, J. & ARDUINO, S. 2013. Emergence of Pseudomonas aeruginosa with KPC-type carbapenemase in a teaching hospital: an 8-year study. *J Med Microbiol,* 62**,** 1565-1570.

RAMIREZ, M. S., NIKOLAIDIS, N. & TOLMASKY, M. 2013. Rise and dissemination of aminoglycoside resistance: the aac(6′)-Ib paradigm. *Frontiers in Microbiology,* 4.

RANELLOU, K., KADLEC, K., POULOU, A., VOULGARI, E., VRIONI, G., SCHWARZ, S. & TSAKRIS, A. 2011. Detection of Pseudomonas aeruginosa isolates of the international clonal complex 11 carrying the blaPER-1 extended-spectrum β-lactamase gene in Greece. *J Antimicrob Chemother,* 67**,** 357-361.

RASMUSSEN, T. B. & GIVSKOV, M. 2006. Quorum-sensing inhibitors as anti-pathogenic drugs. *Int J Med Microbiol,* 296**,** 149-161.

RAY, K. J., PRAJNA, L., SRINIVASAN, M., GEETHA, M., KARPAGAM, R., GLIDDEN, D., OLDENBURG, C. E., SUN, C. Q., MCLEOD, S. D., ACHARYA, N. R. & LIETMAN, T. M. 2013. Fluoroquinolone Treatment and Susceptibility of Isolates From Bacterial Keratitis. *JAMA Ophthalmology,* 131**,** 310-313.

RAYSSIGUIER, C., THALER, D. S. & RADMAN, M. 1989. The barrier to recombination between Escherichia coli and Salmonella typhimurium is disrupted in mismatch-repair mutants. *Nature,* 342**,** 396-401.

REHMAN, A., PATRICK, W. M. & LAMONT, I. L. 2019. Mechanisms of ciprofloxacin resistance in Pseudomonas aeruginosa: new approaches to an old problem. *J Med Microbiol,* 68**,** 1-10.

RELLO, J., OLLENDORF, D. A., OSTER, G., VERA-LLONCH, M., BELLM, L., REDMAN, R. & KOLLEF, M. H. 2002. Epidemiology and outcomes of ventilator-associated pneumonia in a large US database. *Chest,* 122**,** 2115-2121.

RESNIKOFF, S., PASCOLINI, D., ETYA'ALE, D., KOCUR, I., PARARAJASEGARAM, R., POKHAREL, G. P. & MARIOTTI, S. P. 2004. Global data on visual impairment in the year 2002. *Bull World Health Organ,* 82**,** 844-51.

RICE, L. B. 2008. Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE. The University of Chicago Press.

RICHARDS, M. J., EDWARDS, J. R., CULVER, D. H. & GAYNES, R. P. 1999. Nosocomial infections in medical intensive care units in the United States. National Nosocomial Infections Surveillance System. *Crit Care Med,* 27**,** 887-92.

RIETVELD, R. P., TER RIET, G., BINDELS, P. J., SLOOS, J. H. & VAN WEERT, H. C. 2004. Predicting Bacterial Cause in Infectious Conjunctivitis: Cohort study on Informativeness of Combinations of Signs and Symptoms. *Bmj,* 329**,** 206-210.

ROBICSEK, A., JACOBY, G. A. & HOOPER, D. C. 2006a. The worldwide emergence of plasmid-mediated quinolone resistance. *Lancet Infect Dis,* 6**,** 629-40.

ROBICSEK, A., STRAHILEVITZ, J., JACOBY, G. A., MACIELAG, M., ABBANAT, D., PARK, C. H., BUSH, K. & HOOPER, D. C. 2006b. Fluoroquinolone-modifying enzyme: a new adaptation of a common aminoglycoside acetyltransferase. *Nature medicine,* 12**,** 83.

RÖMLING, U., GREIPEL, J. & TÜMMLER, B. 1995. Gradient of Genomic Diversity in the Pseudomonas Aeruginosa Chromosome. *Mole Micro,* 17**,** 323-332.

ROMLING, U., GROTHUES, D., KOOPMANN, U., JAHNKE, B., GREIPEL, J. & TUMMLER, B. 1992. Pulsed-Field Gel Electrophoresis Analysis of a Pseudomonas Aeruginosa Pathovar. *Electrophoresis,* 13**,** 646-8.

RÖMLING, U., WINGENDER, J., MÜLLER, H. & TÜMMLER, B. 1994. A Major Pseudomonas Aeruginosa Clone Common to Patients and Aquatic Habitats. *Appl. Environ. Microbiol.,* 60**,** 1734-1738.

ROSENTHAL, R. A., SUTTON, S. V. & SCHLECH, B. A. 2002. Review of standard for evaluating the effectiveness of contact lens disinfectants. *PDA journal of pharmaceutical science and technology,* 56**,** 37-52.

ROWE-MAGNUS, D. A. & MAZEL, D. 2002. The role of integrons in antibiotic resistance gene capture. *Int J Med Microbiol,* 292**,** 115-125.

ROY, P. H., TETU, S. G., LAROUCHE, A., ELBOURNE, L., TREMBLAY, S., REN, Q., DODSON, R., HARKINS, D., SHAY, R., WATKINS, K., MAHAMOUD, Y. & PAULSEN, I. T. 2010. Complete genome sequence of the multiresistant taxonomic outlier Pseudomonas aeruginosa PA7. *PLoS One,* 5**,** e8842.

ROZWANDOWICZ, M., BROUWER, M. S. M., FISCHER, J., WAGENAAR, J. A., GONZALEZ-ZORN, B., GUERRA, B., MEVIUS, D. J. & HORDIJK, J. 2018. Plasmids carrying antimicrobial resistance genes in Enterobacteriaceae. *J Antimicrob Chemother,* 73**,** 1121-1137.

RUIZ-LINARES, M., FERRER-LUQUE, C. M., ARIAS-MOLIZ, T., DE CASTRO, P., AGUADO, B. & BACA, P. 2014. Antimicrobial activity of alexidine, chlorhexidine and cetrimide against Streptococcus mutans biofilm. *Annals of Clinical Microbiology and Antimicrobials,* 13**,** 41.

RUIZ, J. 2019. CrpP, a passenger or a hidden stowaway in the Pseudomonas aeruginosa genome? *J Antimicrob Chemother,* 74**,** 3397-3399.

RUSSELL, A. D. 1999. Bacterial resistance to disinfectants: present knowledge and future problems. *Journal of Hospital Infection,* 43**,** S57-S68.

RUTHERFORD, S. T. & BASSLER, B. L. 2012. Bacterial quorum sensing: its role in virulence and possibilities for its control. *Cold Spring Harbor perspectives in medicine,* 2**,** a012427.

SAINI, J. S., JAIN, A. K., KUMAR, S., VIKAL, S., PANKAJ, S. & SINGH, S. 2003. Neural network approach to classify infective keratitis. *Curr Eye Res,* 27**,** 111-116.

SALYERS, A. A. & AMÁBILE-CUEVAS, C. F. 1997. Why are antibiotic resistance genes so resistant to elimination? *Antimicrob Agents Chemother,* 41**,** 2321-2325.

SÁNCHEZ, M. & SIVARAMAN, S. 2010. News Media Reporting of Antimicrobial Resistance in Latin America and India. *In:* SOSA, A. D. J., BYARUGABA, D. K., AMÁBILE-CUEVAS, C. F., HSUEH, P.-R., KARIUKI, S. & OKEKE, I. N. (eds.) *Antimicrobial Resistance in Developing Countries.* New York, NY: Springer New York.

SANDOVAL‐MOTTA, S. & ALDANA, M. 2016. Adaptive resistance to antibiotics in bacteria: a systems biology perspective. *Wiley Interdisciplinary Reviews: Systems Biology and Medicine,* 8**,** 253-267.

SATO, H., FRANK, D. W., HILLARD, C. J., FEIX, J. B., PANKHANIYA, R. R., MORIYAMA, K., FINCK-BARBANCON, V., BUCHAKLIAN, A., LEI, M., LONG, R. M., WIENER-KRONISH, J. & SAWA, T. 2003. The mechanism of action of the Pseudomonas aeruginosa-encoded type III cytotoxin, ExoU. *Embo j,* 22**,** 2959-69.

SAUER, A., MEYER, N. & BOURCIER, T. 2016. Risk Factors for Contact Lens-Related Microbial Keratitis: A Case-Control Multicenter Study. *Eye Contact Lens,* 42**,** 158-62.

SAWA, T., SHIMIZU, M., MORIYAMA, K. & WIENER-KRONISH, J. P. 2014. Association between Pseudomonas aeruginosa type III secretion, antibiotic resistance, and clinical outcome: a review. *Crit Care,* 18**,** 668.

SCHEIN, O. D., MCNALLY, J. J., KATZ, J., CHALMERS, R. L., TIELSCH, J. M., ALFONSO, E., BULLIMORE, M., O'DAY, D. & SHOVLIN, J. 2005. The incidence of microbial keratitis among wearers of a 30-day silicone hydrogel extended-wear contact lens. *Ophthalmol,* 112**,** 2172-9.

SCHEIN, O. D., ORMEROD, L. D., BARRAQUER, E., ALFONSO, E., EGAN, K. M., PATON, B. G. & KENYON, K. R. 1989. Microbiology of contact lens-related keratitis. *Cornea,* 8**,** 281-285.

SEAL, D. V., KIRKNESS, C. M., BENNETT, H. G. B. & PETERSON, M. 1999. Population-based cohort study of microbial keratitis in Scotland: incidence and features. *Cont Lens Anterior Eye,* 22**,** 49-57.

SEEMAN, T. 2015. Snippy: Fast Bacterial Variant Calling from NGS Reads

https://github.com/tseemann/snippy.

SEEMANN, T. 2014. Prokka: Rapid Prokaryotic Genome Annotation. *Bioinformatics,* 30**,** 2068-9.

SETHI, S., SETHI, M. J. & IQBAL, R. 2010. Causes of microbial keratitis in patients attending an eye clinic at Peshawar. *GJMS,* 8.

SEVILLANO, E., GALLEGO, L. & GARCÍA-LOBO, J. 2009. First detection of the OXA-40 carbapenemase in P. aeruginosa isolates, located on a plasmid also found in A. baumannii. *Pathologie Biologie,* 57**,** 493-495.

SHANKAR, J., SUEKE, H., WIEHLMANN, L., HORSBURGH, M. J., TUFT, S., NEAL, T. J., KAYE, S. B. & WINSTANLEY, C. 2012. Genotypic analysis of UK keratitis-associated Pseudomonas aeruginosa suggests adaptation to environmental water as a key component in the development of eye infections. *FEMS Microbiol letters,* 334**,** 79-86.

SHARMA, N., SINHA, R., SINGHVI, A. & TANDON, R. 2006. Pseudomonas keratitis after laser in situ keratomileusis. *J Cataract Refract Surg,* 32**,** 519-21.

SHARMA, R., JHANJI, V., SATPATHY, G., SHARMA, N., KHOKHAR, S. & AGARWAL, T. 2013. Coinfection with Acanthamoeba and Pseudomonas in Contact Lens–Associated Keratitis. *Optometry & Vision Science,* 90**,** e53-e55.

SHARMA, S., GOPALAKRISHNAN, S., AASURI, M. K., GARG, P. & RAO, G. N. 2003. Trends in contact lens–associated microbial keratitis in Southern India. *Ophthalmology,* 110**,** 138-143.

SHARP, P. M. & LI, W. H. 1987. The codon Adaptation Index--a measure of directional synonymous codon usage bias, and its potential applications. *Nucleic Acids Res,* 15**,** 1281-95.

SHAW, K., RATHER, P., HARE, R. & MILLER, G. 1993. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. *Microbiol Mol Biol Rev,* 57**,** 138-163.

SHEN, E. P., CHU, H. S., HSIEH, Y. T., CHEN, W. L., CHANG, S. C. & HU, F. R. 2020. Analysis of P. aeruginosa disinfectant sensitivity and microbial adhesions to worn cosmetic contact lenses. *Cont Lens Anterior Eye,* 43**,** 338-344.

SHEN, E. P., HSIEH, Y.-T., CHU, H.-S., CHANG, S.-C. & HU, F.-R. 2015. Correlation of Pseudomonas aeruginosa Genotype With Antibiotic Susceptibility and Clinical Features of Induced Central KeratitisAssociation of T3SS With Pseudomonal Keratitis. *Investigative ophthalmology & visual science,* 56**,** 365-371.

SHEN, E. P., TSAY, R.-Y., CHIA, J.-S., WU, S., LEE, J.-W. & HU, F.-R. 2012. The role of type III secretion system and lens material on adhesion of Pseudomonas aeruginosa to contact lenses. *Invest Ophthalmol Vis Sci,* 53**,** 6416-6426.

SHI, G.-S., BOOST, M. V. & CHO, P. 2016. Does the presence of QAC genes in staphylococci affect the efficacy of disinfecting solutions used by orthokeratology lens wearers? *Br J Ophthalmol,* 100**,** 708-712.

SIEVERT, D. M., RICKS, P., EDWARDS, J. R., SCHNEIDER, A., PATEL, J., SRINIVASAN, A., KALLEN, A., LIMBAGO, B. & FRIDKIN, S. 2013. Antimicrobial-Resistant Pathogens Associated with Healthcare-Associated Infections Summary of Data Reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2009–2010. *Infection Control & Hospital Epidemiology,* 34**,** 1-14.

SIGUIER, P., PEROCHON, J., LESTRADE, L., MAHILLON, J. & CHANDLER, M. 2006. ISfinder: the reference centre for bacterial insertion sequences. *Nucleic Acids Res,* 34**,** D32-6.

SKOV, R. L. & MONNET, D. L. 2016. Plasmid-mediated colistin resistance (mcr-1 gene): three months later, the story unfolds. *Eurosurveillance,* 21.

SMITH, A., PENNEFATHER, P. M., KAYE, S. B. & HART, C. A. 2001. Fluoroquinolones: place in ocular therapy. *Drugs,* 61**,** 747-61.

SMITHA, S., LALITHA, P., PRAJNA, V. N. & SRINIVASAN, M. 2005. Susceptibility trends of *Pseudomonas* species from corneal ulcers. *Indian J Med Microbiol,* 23**,** 168-71.

SMITS, T. H. M. 2019. The importance of genome sequence quality to microbial comparative genomics. *BMC Genomics,* 20**,** 662.

SPIERER, O., MILLER, D. & O'BRIEN, T. P. 2018. Comparative activity of antimicrobials against Pseudomonas aeruginosa, Achromobacter xylosoxidans and Stenotrophomonas maltophilia keratitis isolates. *Br J Ophthalmol,* 102**,** 708-712.

SRIKUMAR, R., LI, X. Z. & POOLE, K. 1997. Inner membrane efflux components are responsible for beta-lactam specificity of multidrug efflux pumps in Pseudomonas aeruginosa. *J Bacteriol,* 179**,** 7875-7881.

SRIKUMAR, R., PAUL, C. J. & POOLE, K. 2000. Influence of mutations in the mexR repressor gene on expression of the MexA-MexB-oprM multidrug efflux system of Pseudomonas aeruginosa. *J Bacteriol,* 182**,** 1410-1414.

SRIKUMAR, R., TSANG, E. & POOLE, K. 1999. Contribution of the MexAB-OprM multidrug efflux system to the β-lactam resistance of penicillin-binding protein and β-lactamase-derepressed mutants of Pseudomonas aeruginosa. *J Antimicrob Chemother,* 44**,** 537-540.

SRINIVASAN, M., GONZALES, C. A., GEORGE, C., CEVALLOS, V., MASCARENHAS, J. M., ASOKAN, B., WILKINS, J., SMOLIN, G. & WHITCHER, J. P. 1997. Epidemiology and aetiological diagnosis of corneal ulceration in Madurai, south India. *Br J Ophthalmol,* 81**,** 965-971.

STALDER, T. & TOP, E. 2016. Plasmid transfer in biofilms: a perspective on limitations and opportunities. *NPJ biofilms and microbiomes,* 2**,** 16022.

STAPLETON, F. 2020. Contact lens-related corneal infection in Australia. *Clin Exp Optom,* 103**,** 408-417.
STAPLETON, F., EDWARDS, K., KEAY, L., NADUVILATH, T., DART, J. K. G., BRIAN, G. & HOLDEN, B. 2012. Risk Factors for Moderate and Severe Microbial Keratitis in Daily Wear Contact Lens Users. *Ophthalmology,* 119**,** 1516-1521.
STAPLETON, F., EDWARDS, K., KEAY, L., NADUVILATH, T., DART, J. K. G., BRIAN, G., SWEENEY, D. & HOLDEN, B. A. 2005. The incidence of contact lens related microbial keratitis in Australia. *Invest Ophthalmol Vis Sci,* 46**,** 5025-5025.
STAPLETON, F., KEAY, L., EDWARDS, K., NADUVILATH, T., DART, J. K. G., BRIAN, G. & HOLDEN, B. A. 2008a. The Incidence of Contact Lens–Related Microbial Keratitis in Australia. *Ophthalmology,* 115**,** 1655-1662.
STAPLETON, F., KEAY, L., EDWARDS, K., NADUVILATH, T., DART, J. K. G., BRIAN, G. & HOLDEN, B. A. 2008b. The incidence of contact lens–related microbial keratitis in Australia. *Ophthalmol,* 115**,** 1655-1662.
STAPLETON, F., KEAY, L. J., SANFILIPPO, P. G., KATIYAR, S., EDWARDS, K. P. & NADUVILATH, T. 2007. Relationship between climate, disease severity, and causative organism for contact lens–associated microbial keratitis in Australia. *Am J Ophthalmol,* 144**,** 690-698.e1.
STEIN, C., MAKAREWICZ, O., BOHNERT, J. A., PFEIFER, Y., KESSELMEIER, M., HAGEL, S. & PLETZ, M. W. 2015. Three dimensional checkerboard synergy analysis of colistin, meropenem, tigecycline against multidrug-resistant clinical klebsiella pneumonia isolates. *PLoS One,* 10**,** e0126479.
STEWART, L., FORD, A., SANGAL, V., JEUKENS, J., BOYLE, B., KUKAVICA-IBRULJ, I., CAIM, S., CROSSMAN, L., HOSKISSON, P. A., LEVESQUE, R. & TUCKER, N. P. 2014. Draft genomes of 12 host-adapted and environmental isolates of Pseudomonas aeruginosa and their positions in the core genome phylogeny. *Pathog Dis,* 71**,** 20-5.
STEWART, R. M. K., WIEHLMANN, L., ASHELFORD, K. E., PRESTON, S. J., FRIMMERSDORF, E., CAMPBELL, B. J., NEAL, T. J., HALL, N., TUFT, S., KAYE, S. B. & WINSTANLEY, C. 2011. Genetic characterization indicates that a specific subpopulation of Pseudomonas aeruginosa is associated with keratitis infections. *J Clin Microbiol,* 49**,** 993-1003.
STICKLAND, H. G., DAVENPORT, P. W., LILLEY, K. S., GRIFFIN, J. L. & WELCH, M. 2010. Mutation of nfxB causes global changes in the physiology and metabolism of Pseudomonas aeruginosa. *J Proteome Res,* 9**,** 2957-2967.
STOKES, H. W. & HALL, R. M. 1989. A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons. *Mol Microbiol,* 3**,** 1669-83.
SUBEDI, D., KOHLI, G. S., VIJAY, A. K., WILLCOX, M. & RICE, S. A. 2019. Accessory genome of the multi-drug resistant ocular isolate of *Pseudomonas aeruginosa* PA34. *PLoS One,* 14**,** e0215038.
SUBEDI, D., VIJAY, A. K., KOHLI, G. S., RICE, S. A. & WILLCOX, M. 2018a. Association between possession of ExoU and antibiotic resistance in Pseudomonas aeruginosa. *PLoS One,* 13**,** e0204936.
SUBEDI, D., VIJAY, A. K., KOHLI, G. S., RICE, S. A. & WILLCOX, M. 2018b. Comparative genomics of clinical strains of Pseudomonas aeruginosa Strains isolated from different Geographic Sites. *Sci Rep,* 8**,** 15668.
SUBEDI, D., VIJAY, A. K., KOHLI, G. S., RICE, S. A. & WILLCOX, M. 2018c. Nucleotide sequence analysis of NPS-1 β-lactamase and a novel integron (In 1427)-carrying transposon in an MDR Pseudomonas aeruginosa keratitis strain. *J Antimicrob Chemother,* 73**,** 1724-1726.
SUBEDI, D., VIJAY, A. K. & WILLCOX, M. 2018d. Overview of mechanisms of antibiotic resistance in Pseudomonas aeruginosa: an ocular perspective. *Clin Exp Optom,* 101**,** 162-171.

SULLIVAN, E., BENSMAN, J., LOU, M., AGNELLO, M., SHRINER, K. & WONG-BERINGER, A. 2014. Risk of developing pneumonia is enhanced by the combined traits of fluoroquinolone resistance and type III secretion virulence in respiratory isolates of Pseudomonas aeruginosa. *Crit Care Med,* 42**,** 48-56.

SULLIVAN, M. J., PETTY, N. K. & BEATSON, S. A. 2011. Easyfig: a genome comparison visualizer. *Bioinformatics,* 27**,** 1009-10.

SUNDIN, G. W. & BENDER, C. L. 1996. Dissemination of the strA-strB streptomycin-resistance genes among commensal and pathogenic bacteria from humans, animals, and plants. *Mol Ecol,* 5**,** 133-43.

SZCZOTKA-FLYNN, L. B., IMAMURA, Y., CHANDRA, J., YU, C., MUKHERJEE, P. K., PEARLMAN, E. & GHANNOUM, M. A. 2009. Increased resistance of contact lens related bacterial biofilms to antimicrobial activity of soft contact lens care solutions. *Cornea,* 28**,** 918-926.

TABATA, A., NAGAMUNE, H., MAEDA, T., MURAKAMI, K., MIYAKE, Y. & KOURAI, H. 2003. Correlation between resistance of Pseudomonas aeruginosa to quaternary ammonium compounds and expression of outer membrane protein OprR. *Antimicrob Agents Chemother,* 47**,** 2093-2099.

TADA, T., MIYOSHI-AKIYAMA, T., SHIMADA, K., SHIMOJIMA, M. & KIRIKAE, T. 2013. IMP-43 and IMP-44 metallo-β-lactamases with increased carbapenemase activities in multidrug-resistant Pseudomonas aeruginosa. *Antimicrob Agents Chemother,* 57**,** 4427-4432.

TADA, T., SHIMADA, K., MYA, S., ZAN, K. N., KUWAHARA, K., KIRIKAE, T. & TIN, H. H. 2018. A New Variant of 16S rRNA Methylase, RmtD3, in a Clinical Isolate of Pseudomonas Aeruginosa in Myanmar. *Antimicrob Agents Chemother,* 62**,** e01806-17.

TANIMOTO, K., TOMITA, H., FUJIMOTO, S., OKUZUMI, K. & IKE, Y. 2008. Fluoroquinolone enhances the mutation frequency for meropenem-selected carbapenem resistance in Pseudomonas aeruginosa, but use of the high-potency drug doripenem inhibits mutant formation. *Antimicrob Agents Chemother,* 52**,** 3795-3800.

TAUCH, A., SCHLUTER, A., BISCHOFF, N., GOESMANN, A., MEYER, F. & PUHLER, A. 2003. The 79,370-bp conjugative plasmid pB4 consists of an IncP-1beta backbone loaded with a chromate resistance transposon, the strA-strB streptomycin resistance gene pair, the oxacillinase gene bla(NPS-1), and a tripartite antibiotic efflux system of the resistance-nodulation-division family. *Mol Genet Genomics,* 268**,** 570-84.

TAYLOR, P., SCHOENKNECHT, F., SHERRIS, J. & LINNER, E. 1983. Determination of minimum bactericidal concentrations of oxacillin for Staphylococcus aureus: influence and significance of technical factors. *Antimicrob Agents Chemother,* 23**,** 142-150.

TEIXEIRA, B., RODULFO, H., CARREÑO, N., GUZMÁN, M., SALAZAR, E. & DE DONATO, M. 2016. AMINOGLYCOSIDE RESISTANCE GENES IN Pseudomonas aeruginosa ISOLATES FROM CUMANA, VENEZUELA. *Revista do Instituto de Medicina Tropical de Sao Paulo,* 58**,** 13-13.

TENOVER, F. C. 2006. Mechanisms of antimicrobial resistance in bacteria. *Am J Med,* 119**,** S3-S10.

THEODORE, F. H., JAKOBIEC, F. A., JUECHTER, K. B., MA, P., TROUTMAN, R. C., PANG, P. M. & IWAMOTO, T. 1985. The diagnostic value of a ring infiltrate in acanthamoebic keratitis. *Ophthalmol,* 92**,** 1471-1479.

THIBODEAUX, B. A., DAJCS, J. J., CABALLERO, A. R., MARQUART, M. E., GIRGIS, D. O. & O'CALLAGHAN, R. J. 2004. Quantitative comparison of fluoroquinolone therapies of experimental gram-negative bacterial keratitis. *Curr Eye Res,* 28**,** 337-42.

THOMAS, P., LECK, A. & MYATT, M. 2005. Characteristic clinical features as an aid to the diagnosis of suppurative keratitis caused by filamentous fungi. *Br J Ophthalmol,* 89**,** 1554-1558.

TIAN, G.-B., ADAMS-HADUCH, J. M., BOGDANOVICH, T., WANG, H.-N. & DOI, Y. 2011. PME-1, an extended-spectrum β-lactamase identified in Pseudomonas aeruginosa. *Antimicrob Agents Chemother,* 55**,** 2710-2713.

TIRABY, J. G. & FOX, M. S. 1973. Marker discrimination in transformation and mutation of pneumococcus. *Proc Natl Acad Sci U S A,* 70**,** 3541-5.

TOLEMAN, M. A., ROLSTON, K., JONES, R. N. & WALSH, T. R. 2003. Molecular and biochemical characterization of OXA-45, an extended-spectrum class 2d′ β -lactamase in Pseudomonas aeruginosa. *Antimicrob Agents Chemother,* 47**,** 2859-2863.

TRAN, J. H. & JACOBY, G. A. 2002. Mechanism of plasmid-mediated quinolone resistance. *Proc Natl Acad Sci U S A,* 99**,** 5638-42.

TRAN, Q. T., NAWAZ, M. S., DECK, J., FOLEY, S., NGUYEN, K. & CERNIGLIA, C. E. 2011. Detection of type III secretion system virulence and mutations in *gyrA* and *parC* genes among quinolone-resistant strains of *Pseudomonas aeruginosa* isolated from imported shrimp. *Foodborne Pathog Dis,* 8**,** 451-3.

TREANGEN, T. J., ONDOV, B. D., KOREN, S. & PHILLIPPY, A. M. 2014. The Harvest suite for rapid core-genome alignment and visualization of thousands of intraspecific microbial genomes. *Genome Biol,* 15**,** 524.

TROUILLET, J. L., VUAGNAT, A., COMBES, A., KASSIS, N., CHASTRE, J. & GIBERT, C. 2002. Pseudomonas aeruginosa ventilator-associated pneumonia: comparison of episodes due to piperacillin-resistant versus piperacillin-susceptible organisms. *Clin Infect Dis,* 34**,** 1047-54.

TSAKRIS, A., POURNARAS, S., WOODFORD, N., PALEPOU, M.-F. I., BABINI, G. S., DOUBOYAS, J. & LIVERMORE, D. M. 2000. Outbreak of infections caused by Pseudomonas aeruginosa producing VIM-1 carbapenemase in Greece. *J Clin Microbiol,* 38**,** 1290-1292.

TUMMANAPALLI, S. S. & WILLCOX, M. D. P. 2020. Antimicrobial resistance of ocular microbes and the role of antimicrobial peptides. *Clin Exp Optom,* n/a.

UEMURA, S., YOKOTA, S.-I., MIZUNO, H., SAKAWAKI, E., SAWAMOTO, K., MAEKAWA, K., TANNO, K., MORI, K., ASAI, Y. & FUJII, N. 2010. Acquisition of a transposon encoding extended-spectrum beta-lactamase SHV-12 by Pseudomonas aeruginosa isolates during the clinical course of a burn patient. *Antimicrob Agents Chemother,* 54**,** 3956-3959.

UNG, L., ACHARYA, N. R., AGARWAL, T., ALFONSO, E. C., BAGGA, B., BISPO, P. J., BURTON, M. J., DART, J. K., DOAN, T., FLEISZIG, S. M., GARG, P., GILMORE, M. S., GRITZ, D. C., HAZLETT, L. D., IOVIENO, A., JHANJI, V., KEMPEN, J. H., LEE, C. S., LIETMAN, T. M., MARGOLIS, T. P., MCLEOD, S. D., MEHTA, J. S., MILLER, D., PEARLMAN, E., PRAJNA, L., PRAJNA, N. V., SEITZMAN, G. D., SHANBHAG, S. S., SHARMA, N., SHARMA, S., SRINIVASAN, M., STAPLETON, F., TAN, D. T., TANDON, R., TAYLOR, H. R., TU, E. Y., TULI, S. S., VAJPAYEE, R. B., VAN GELDER, R. N., WATSON, S. L., ZEGANS, M. E. & CHODOSH, J. 2019. Infectious corneal ulceration: a proposal for neglected tropical disease status. *Bulletin of the World Health Organization,* 97**,** 854-856.

UPADHYAY, M. P., KARMACHARYA, P. C., KOIRALA, S., SHAH, D. N., SHAKYA, S., SHRESTHA, J. K., BAJRACHARYA, H., GURUNG, C. K. & WHITCHER, J. P. 2001. The Bhaktapur eye study: ocular trauma and antibiotic prophylaxis for the prevention of corneal ulceration in Nepal. *Br J Ophthalmol,* 85**,** 388-92.

UPADHYAY, M. P., SRINIVASAN, M. & WHITCHER, J. P. 2015. Diagnosing and managing microbial keratitis. *Community eye health,* 28**,** 3-6.

VAHABOGLU, H., OZTÜRK, R., AYGÜN, G., COŞKUNKAN, F., YAMAN, A., KAYGUSUZ, A., LEBLEBICIOGLU, H., BALIK, I., AYDIN, K. & OTKUN, M. 1997. Widespread detection of PER-1-type extended-spectrum beta-lactamases among nosocomial Acinetobacter and Pseudomonas aeruginosa isolates in Turkey: a nationwide multicenter study. *Antimicrob Agents Chemother,* 41**,** 2265-2269.

VAKULENKO, S. B. & MOBASHERY, S. 2003. Versatility of aminoglycosides and prospects for their future. *Clin Microbiol Rev,* 16**,** 430-450.

VALOT, B., GUYEUX, C., ROLLAND, J. Y., MAZOUZI, K., BERTRAND, X. & HOCQUET, D. 2015. What It Takes to Be a Pseudomonas aeruginosa? The Core Genome of the Opportunistic Pathogen Updated. *PloS one,* 10**,** e0126468-e0126468.

VAN BOECKEL, T. P., BROWER, C., GILBERT, M., GRENFELL, B. T., LEVIN, S. A., ROBINSON, T. P., TEILLANT, A. & LAXMINARAYAN, R. J. P. O. T. N. A. O. S. 2015. Global trends in antimicrobial use in food animals. *PNAS,* 112**,** 5649-5654.

VAN BOECKEL, T. P., GANDRA, S., ASHOK, A., CAUDRON, Q., GRENFELL, B. T., LEVIN, S. A. & LAXMINARAYAN, R. 2014. Global antibiotic consumption 2000 to 2010: an analysis of national pharmaceutical sales data. *Lancet Infect Dis,* 14**,** 742-750.

VAZIRANI, J., WURITY, S. & ALI, M. H. 2015. Multidrug-resistant Pseudomonas aeruginosa keratitis: Risk factors, clinical characteristics, and outcomes. *Ophthalmol,* 122**,** 2110-2114.

VENTOLA, C. L. 2015. The antibiotic resistance crisis: part 1: causes and threats. *Pharmacy and Therapeutics,* 40**,** 277.

VERANI, J. R., LORICK, S. A., YODER, J. S., BEACH, M. J., BRADEN, C. R., ROBERTS, J. M., CONOVER, C. S., CHEN, S., MCCONNELL, K. A. & CHANG, D. C. 2009. National outbreak of Acanthamoeba keratitis associated with use of a contact lens solution, United States. *Emerging infectious diseases,* 15**,** 1236.

VERSPORTEN, A., BOLOKHOVETS, G., GHAZARYAN, L., ABILOVA, V., PYSHNIK, G., SPASOJEVIC, T., KORINTELI, I., RAKA, L., KAMBARALIEVA, B. & CIZMOVIC, L. 2014. Antibiotic use in eastern Europe: a cross-national database study in coordination with the WHO Regional Office for Europe. *The Lancet infectious diseases,* 14**,** 381-387.

VIDAL, F., MENSA, J., MARTINEZ, J., ALMELA, M., MARCO, F., GATELL, J., RICHART, C., SORIANO, E. & DE ANTA, M. J. 1999. Pseudomonas aeruginosa bacteremia in patients infected with human immunodeficiency virus type 1. *Eur J Clin Microbiol Infect Dis,* 18**,** 473-477.

VIJAY, A. K., WILLCOX, M., ZHU, H. & STAPLETON, F. 2015. Contact lens storage case hygiene practice and storage case contamination. *Eye Contact Lens,* 41**,** 91-7.

WALSH, T. R., TOLEMAN, M. A., POIREL, L. & NORDMANN, P. 2005. Metallo-beta-lactamases: the quiet before the storm? *Clin Microbiol Rev,* 18**,** 306-325.

WANG, C., CAI, P., CHANG, D. & MI, Z. 2006. A Pseudomonas aeruginosa isolate producing the GES-5 extended-spectrum β-lactamase. *J Antimicrob Chemother,* 57**,** 1261-1262.

WANG, Y., WANG, X., SCHWARZ, S., ZHANG, R., LEI, L., LIU, X., LIN, D. & SHEN, J. 2014. IMP-45-producing multidrug-resistant Pseudomonas aeruginosa of canine origin. *J Antimicrob Chemother,* 69**,** 2579-2581.

WATANABE, K., ZHU, H. & WILLCOX, M. 2014. Susceptibility of Stenotrophomonas maltophilia Clinical Isolates to Antibiotics and Contact Lens Multipurpose Disinfecting Solutions. *Investigative Ophthalmology & Visual Science,* 55**,** 8475-8479.

WATSON, S., CABRERA-AGUAS, M., KHOO, P., PRATAMA, R., GATUS, B. J., GULHOLM, T., EL-NASSER, J. & LAHRA, M. M. 2018. Keratitis antimicrobial resistance surveillance program, Sydney, Australia: 2016 Annual Report. *Clin Exp Ophthalmol,* 47**,** 20-25.

WERNER, N. L., HECKER, M. T., SETHI, A. K. & DONSKEY, C. J. 2011. Unnecessary use of fluoroquinolone antibiotics in hospitalized patients. *BMC Infect Dis,* 11**,** 187.

WHITE, R. L., BURGESS, D. S., MANDURU, M. & BOSSO, J. A. 1996. Comparison of three different in vitro methods of detecting synergy: time-kill, checkerboard, and E test. *Antimicrob Agents Chemother,* 40**,** 1914-1918.

WHO 1973. *Prevention of blindness-Report of a WHO study group*.

WI, Y. M., CHOI, J.-Y., LEE, J.-Y., KANG, C.-I., CHUNG, D. R., PECK, K. R., SONG, J.-H. & KO, K. S. 2017. Antimicrobial effects of β-lactams on imipenem-resistant ceftazidime-

susceptible Pseudomonas aeruginosa. *Antimicrob Agents Chemother,* 61**,** e00054-17.
WICK, R. R., JUDD, L. M., GORRIE, C. L. & HOLT, K. E. 2017. Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. *PLoS computational biology,* 13**,** e1005595.
WILHELMUS, K. R., ABSHIRE, R. L. & SCHLECH, B. A. 2003. Influence of fluoroquinolone susceptibility on the therapeutic response of fluoroquinolone-treated bacterial keratitis. *Arch Ophthalmol,* 121**,** 1229-33.
WILLCOX, M. D. P. 2011. Review of resistance of ocular isolates of Pseudomonas aeruginosa and Staphylococci from keratitis to ciprofloxacin, gentamicin and cephalosporins. *Clin Exp Optom,* 94**,** 161-168.
WINSTANLEY, C., LANGILLE, M. G., FOTHERGILL, J. L., KUKAVICA-IBRULJ, I., PARADIS-BLEAU, C., SANSCHAGRIN, F., THOMSON, N. R., WINSOR, G. L., QUAIL, M. A. & LENNARD, N. 2009. Newly introduced genomic prophage islands are critical determinants of in vivo competitiveness in the Liverpool Epidemic Strain of Pseudomonas aeruginosa. *Genome research,* 19**,** 12-23.
WOLFGANG, M. C., KULASEKARA, B. R., LIANG, X., BOYD, D., WU, K., YANG, Q., MIYADA, C. G. & LORY, S. 2003. Conservation of genome content and virulence determinants among clinical and environmental isolates of Pseudomonas aeruginosa. *PNAS,* 100**,** 8484-8489.
WOLTER, D. J., KHALAF, N., ROBLEDO, I. E., VÁZQUEZ, G. J., SANTÉ, M. I., AQUINO, E. E., GOERING, R. V. & HANSON, N. D. 2009a. Surveillance of carbapenem-resistant Pseudomonas aeruginosa isolates from Puerto Rican Medical Center Hospitals: dissemination of KPC and IMP-18 beta-lactamases. *Antimicrob Agents Chemother,* 53**,** 1660-1664.
WOLTER, D. J., KURPIEL, P. M., WOODFORD, N., PALEPOU, M.-F. I., GOERING, R. V. & HANSON, N. D. 2009b. Phenotypic and enzymatic comparative analysis of the novel KPC variant KPC-5 and its evolutionary variants, KPC-2 and KPC-4. *Antimicrob Agents Chemother,* 53**,** 557-562.
WONG-BERINGER, A., WIENER-KRONISH, J., LYNCH, S. & FLANAGAN, J. 2008. Comparison of type III secretion system virulence among fluoroquinolone-susceptible and -resistant clinical isolates of Pseudomonas aeruginosa. *Clinical Microbiology and Infection,* 14**,** 330-336.
WOODFORD, N., TURTON, J. F. & LIVERMORE, D. M. 2011. Multiresistant Gram-Gegative Bacteria: The role of high-risk clones in the dissemination of antibiotic resistance. *FEMS Microbiol Rev,* 35**,** 736-755.
WORLD HEALTH ORGANIZATION (WHO) 2017. WHO priority pathogens list for R&D of new antibiotics.
XAVIER, B. B., LAMMENS, C., RUHAL, R., KUMAR-SINGH, S., BUTAYE, P., GOOSSENS, H. & MALHOTRA-KUMAR, S. 2016. Identification of a novel plasmid-mediated colistin-resistance gene, mcr-2, in Escherichia coli, Belgium, June 2016. *Eurosurveillance,* 21.
XIONG, J., HYNES, M. F., YE, H., CHEN, H., YANG, Y., M'ZALI, F. & HAWKEY, P. M. 2006. blaIMP-9 and its association with large plasmids carried by Pseudomonas aeruginosa isolates from the People's Republic of China. *Antimicrob Agents Chemother,* 50**,** 355-358.
YAHR, T. L., HOVEY, A. K., KULICH, S. M. & FRANK, D. W. 1995. Transcriptional analysis of the Pseudomonas aeruginosa exoenzyme S structural gene. *J Bacteriol,* 177**,** 1169-1178.
YAMANO, Y., NISHIKAWA, T., FUJIMURA, T., YUTSUDOU, T., TSUJI, M. & MIWA, H. 2006. Occurrence of PER-1 producing clinical isolates of Pseudomonas aeruginosa in Japan and their susceptibility to doripenem. *J Antibiotics,* 59**,** 791-796.
YAN, J.-J., HSUEH, P.-R., KO, W.-C., LUH, K.-T., TSAI, S.-H., WU, H.-M. & WU, J.-J. 2001. Metallo-β-lactamases in clinical Pseudomonas isolates in Taiwan and identification of

VIM-3, a novel variant of the VIM-2 enzyme. *Antimicrob Agents Chemother,* 45**,** 2224-2228.

YAN, J.-J., HSUEH, P.-R., LU, J.-J., CHANG, F.-Y., KO, W.-C. & WU, J.-J. 2006. Characterization of acquired β-lactamases and their genetic support in multidrug-resistant Pseudomonas aeruginosa isolates in Taiwan: the prevalence of unusual integrons. *Journal of antimicrobial chemotherapy,* 58**,** 530-536.

YANG, H., CHEN, H., YANG, Q., CHEN, M. & WANG, H. 2008. High prevalence of plasmid-mediated quinolone resistance genes qnr and aac(6')-Ib-cr in clinical isolates of Enterobacteriaceae from nine teaching hospitals in China. *Antimicrob Agents Chemother,* 52**,** 4268-73.

YAYAN, J., GHEBREMEDHIN, B. & RASCHE, K. 2015. Antibiotic resistance of *Pseudomonas aeruginosa* in pneumonia at a single University hospital center in Germany over a 10-year period. *PLoS One,* 10**,** e0139836.

YOKOYAMA, K., DOI, Y., YAMANE, K., KUROKAWA, H., SHIBATA, N., SHIBAYAMA, K., YAGI, T., KATO, H. & ARAKAWA, Y. 2003. Acquisition of 16S rRNA methylase gene in Pseudomonas aeruginosa. *The lancet,* 362**,** 1888-1893.

YOO, J. S., YANG, J. W., KIM, H. M., BYEON, J., KIM, H. S., YOO, J. I., CHUNG, G. T. & LEE, Y. S. 2012. Dissemination of genetically related IMP-6-producing multidrug-resistant Pseudomonas aeruginosa ST235 in South Korea. *Int J Antimicrob Agents,* 39**,** 300-304.

YOSHIDA, H., BOGAKI, M., NAKAMURA, M. & NAKAMURA, S. 1990. Quinolone resistance-determining region in the DNA gyrase gyrA gene of Escherichia coli. *Antimicrob Agents Chemother,* 34**,** 1271-1272.

YOUSEFI-AVARVAND, A., KHASHEI, R., EBRAHIM-SARAIE, H. S., EMAMI, A., ZOMORODIAN, K. & MOTAMEDIFAR, M. 2015. The frequency of exotoxin A and exoenzymes S and U genes among clinical isolates of Pseudomonas aeruginosa in Shiraz, Iran. *International journal of molecular and cellular medicine,* 4**,** 167.

YU, Y.-S., QU, T.-T., ZHOU, J.-Y., WANG, J., LI, H.-Y. & WALSH, T. R. 2006. Integrons Containing the VIM-2 Metallo-β-lactamase Gene Among Imipenem-Resistant Pseudomonas Aeruginosa Strains from Different Chinese Hospitals. *J Clin Microbiolo,* 44**,** 4242-4245.

ZANKARI, E., HASMAN, H., COSENTINO, S., VESTERGAARD, M., RASMUSSEN, S., LUND, O., AARESTRUP, F. M. & LARSEN, M. V. 2012. Identification of acquired antimicrobial resistance genes. *J Antimicrob Chemother,* 67**,** 2640-2644.

ZHANG, C., LIANG, Y., DENG, S., WANG, Z., LI, R. & SUN, X. 2008. Distribution of bacterial keratitis and emerging resistance to antibiotics in China from 2001 to 2004. *Clin Ophthalmol (Auckland, N.Z.),* 2**,** 575-579.

ZHAO, W.-H., CHEN, G., ITO, R. & HU, Z.-Q. 2009. Relevance of resistance levels to carbapenems and integron-borne blaIMP-1, blaIMP-7, blaIMP-10 and blaVIM-2 in clinical isolates of Pseudomonas aeruginosa. *J Med Microbiol,* 58**,** 1080-1085.

ZHU, H., CONIBEAR, T. C., BANDARA, R., ALIWARGA, Y., STAPLETON, F. & WILLCOX, M. D. 2006. Type III secretion system-associated toxins, proteases, serotypes, and antibiotic resistance of Pseudomonas aeruginosa isolates associated with keratitis. *Curr Eye Res,* 31**,** 297-306.

ZUG, K. A., WARSHAW, E. M., FOWLER, J. F., JR., MAIBACH, H. I., BELSITO, D. L., PRATT, M. D., SASSEVILLE, D., STORRS, F. J., TAYLOR, J. S., MATHIAS, C. G., DELEO, V. A., RIETSCHEL, R. L. & MARKS, J. 2009. Patch-test results of the North American Contact Dermatitis Group 2005-2006. *Dermatitis,* 20**,** 149-60.

APPENDIX 1 CONFERENCE PRESENTATIONS FROM THE THESIS

Conference presentations

Khan M, Rice S, Stapleton F, Willcox MDP. (Accepted). Resistance and virulence in the ocular isolates of *Pseudomonas aeruginosa*. American Society for Microbiology conference Chicago USA, June 2020 (Poster).

Khan M, Rice S, Stapleton F, Willcox MDP. (Accepted). Virulence genes possessed by multidrug resistant *P. aeruginosa* ocular isolates. Gordon Research Conference Italy, February 2020 (Presentation).

Khan M, Rice S, Stapleton F, Willcox MDP. Acquired resistance genes in the ocular isolates of Pseudomonas aeruginosa. Mobile Genetic Elements Conference, Melbourne, 2019 (Presentation).

Khan M, Stapleton F, Willcox MDP. Sensitivity of contact lens-related *Pseudomonas aeruginosa* keratitis isolates to multipurpose disinfecting solution, disinfectants and antibiotics. Association for research in vision and ophthalmology conference Canada, 2019 (Poster).

Khan M. Whole genome sequence analysis. Bioinformatics connect UNSW Sydney, 2018 (Poster).