Comparison of virulence factors in *Pseudomonas aeruginosa* strains isolated from contact lens- and non-contact lens-related keratitis

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Pseudomonas aeruginosa is one of the common pathogens associated with corneal infection, particularly in contact lens-related keratitis events. The pathogenesis of *P. aeruginosa* in keratitis is attributed to the production of virulence factors under certain environmental conditions. The aim of this study was to determine differences in the virulence factors of *P. aeruginosa* isolated from contact lens- and non-contact lens-related keratitis. Associations were assessed between type III secretion toxin-encoding genes, protease profiles, biofilm formation, serotypes and antibiotic-resistance patterns among 27 non-contact lens- and 28 contact lens-related *P. aeruginosa* keratitis isolates from Australia. Strains with a *exoS*⁺/*exoU*[−] genotype and a type I protease profile predominated in the non-contact lens-related keratitis isolates, whereas the *exoS*[−]/*exoU*⁺ and a type II protease profile was associated with contact lens-related isolates ($P < 0.05$). A strong biofilm formation phenotype was found to be associated with the possession of the *exoU* gene, and serotypes E, I and C. The *exoS* gene was strongly associated with serotypes G, A and B, while *exoU* was associated with serotypes E and C. Six out of fifty-five (11 %) clinical isolates were non-susceptible (intermediate-resistant or resistant) to ofloxacin and moxifloxacin. All resistant isolates were from non-contact lens-related keratitis. The results suggest that *P. aeruginosa* isolates from different infection origins may have different characteristics. A better understanding of these differences may lead to further development of evidence-based clinical guidelines for the management of keratitis.

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INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative, opportunistic pathogen implicated in sight-threatening ocular infectious diseases such as keratitis (Sharma *et al.*, 2006; Willcox, 2007; Green *et al.*, 2008a). *Pseudomonas* keratitis is a serious ocular infection that can lead to corneal scarring and severe visual disability if aggressive and appropriate therapy is not promptly initiated (Keay *et al.*, 2006; Stapleton *et al.*, 2007). Until recently, most cases of bacterial keratitis were associated with ocular trauma, ocular surface disease and prior ocular surgery (Bourcier *et al.*, 2003; Green *et al.*, 2007). However, the widespread use of contact lenses is now recognized as an increasingly common risk factor for development of corneal infection in otherwise healthy eyes (Green *et al.*, 2008b). *P. aeruginosa* has remained the most common cause of contact lens-related keratitis, accounting for 60–70 % of

culture-proven cases (Galentine *et al.*, 1984; Schein *et al.*, 1989; Liesegang, 1997; Cheng *et al.*, 1999). *P. aeruginosa* is also one of the most commonly cultured organisms in non-contact lens-related ocular trauma events that lead to keratitis (Hooi & Hooi, 2005; Parmar *et al.*, 2006; Green *et al.*, 2008b). Other predisposing factors associated with *P. aeruginosa* keratitis are ocular surgery (Sharma *et al.*, 2006) and ocular surface disorders (Green *et al.*, 2008b).

The pathogenesis of *P. aeruginosa* is due to the production of several cell-associated and extracellular virulence factors. The virulence factors most associated with ocular damage in *P. aeruginosa* include cytotoxins ExoU (encoded by *exoU*) and ExoS (encoded by *exoS*) (Fleiszig *et al.*, 1997; Feltman *et al.*, 2001), elastase B (encoded by *lasB*) (Lau *et al.*, 2005; Sadikot *et al.*, 2005), alkaline protease (encoded by *aprA*) (Goodman *et al.*, 2004; Tingpej *et al.*, 2007), protease IV (encoded by *prpL*) (Hobden, 2002) and *P. aeruginosa* small protease (encoded by *pasp*) (Thibodeaux *et al.*, 2007). The type III secretion toxin-encoding genes

Abbreviations: CF, cystic fibrosis; ODc, cut-off optical density.

exoU and *exoS* have conformity to either cytotoxic or invasive phenotypes, respectively, in *P. aeruginosa* isolates (Fleiszig *et al.*, 1997). The presence of type III secretion toxin-encoding genes in clinical isolates from different infections is associated with differences in bacterial virulence (Feltman *et al.*, 2001) and clinical outcomes (Hauser *et al.*, 2002). Proteases contribute to pathogenesis in keratitis through destruction of connective tissue and degradation of host immunological factors (Engel *et al.*, 1998).

P. aeruginosa keratitis is considerably more common in contact lens wearers compared with non-contact lens wearers, presumably because of the altered ocular environment. Biofilms produced by *P. aeruginosa* are thought to be a main cause of persistent ocular infections associated with contact lens wear (Costerton *et al.*, 1999) through attachment to contact lens and contact lens storage case surfaces (McLaughlin-Borlace *et al.*, 1998). Bacterial contamination of lenses and storage cases has been reported even in association with good compliance with care and hygiene regimens. Biofilm-associated *P. aeruginosa* contamination is found in both contact lens cases and disinfectants, with rates varying between 24 and 81% (Liesegang, 1997; Zegans *et al.*, 2002). Phenotypic traits expressed in biofilms are partially responsible for the emerging resistance against antimicrobial therapy (del Pozo & Patel, 2007) of contact lens-related keratitis. In addition, emergence of multi-drug resistance in *P. aeruginosa* strains (Rossolini & Mantengoli, 2005) becomes a major concern when antibiotics such as fluoroquinolones are used as monotherapeutic agents.

In the present study, the genotype (*exoU* and *exoS*) and phenotype (protease profiles, biofilm formation, serotypes and antibiotic-resistance patterns) of *P. aeruginosa* isolates implicated in Australian keratitis cases were determined and compared between isolates, and categorized based on the type of infection (contact lens- and non-contact lens-related keratitis). The hypothesis tested was that *P. aeruginosa* strains isolated from contact lens-related keratitis possess distinguishing virulence characteristics in comparison with isolates from non-contact lens-related keratitis. Evidence that causative bacteria belong to specific subpopulations of *P. aeruginosa* would support further development of strategies for better control of the disease.

METHODS

Bacterial strains and growth conditions. A collection of 55 *P. aeruginosa* clinical isolates were obtained between December 1986 and December 2006 in Australia. The strains were grouped into 28 contact lens-related and 27 non-contact lens-related keratitis isolates for comparative analysis. All strains were stored in tryptic soy broth (TSB) containing 30% glycerol at -80°C . Isolates were inoculated on chocolate agar plates and incubated overnight at 37°C . Resulting single colonies were grown in 10 ml TSB or Luria-Bertani (LB) medium at 37°C for 18 h without shaking. After incubation, centrifugation was carried out, and the supernatant was filter-sterilized and transferred to a new test tube for exo-protease assay,

and the remaining cell pellet was resuspended in 10 ml MilliQ water for PCR amplification. Cells and supernatants were stored at 4°C for further experiments within a week.

Detection of type III secretion toxin-encoding genes. PCR assays were used to determine the distribution of the type III secretion toxin-encoding genes *exoU* and *exoS* for the test strains. Strains were subjected to cell lysis using *microLYSIS* buffer (Microzone) in accordance with the manufacturer's instructions. The sequences of oligonucleotide primers for amplifying *exoU* (428 bp fragment) and *exoS* (1352 bp fragment), and the PCR amplification procedure were based on a previous report (Zhu *et al.*, 2006). *P. aeruginosa* strains 6294 and 6206 were used as positive controls for amplification of *exoS* and *exoU* genes, respectively (Zhu *et al.*, 2006).

Zymography for analysis of the protease profile. Gelatin zymography was conducted using modifications of the procedures described by Zhu *et al.* (2006). Culture supernatants (25 μl aliquots) were separated on 7.5% (w/v) SDS-polyacrylamide gel co-polymerized with 0.1% (w/v) gelatin, using 120 V at 4°C for 3 h. Gels were then soaked in 100 ml 2.5% (v/v) Triton X-100 for 1 h and incubated for 18 h at 37°C in gelatin gel substrate buffer (Zhu *et al.*, 2006). To reveal the area of enzymic degradation, gels were stained in a mixture of 15 ml 0.2% (w/v) Coomassie blue R-250 (4:6 distilled water: methanol) and 100 ml glacial acetic acid: methanol: distilled water (1:3:6) for 1 h.

Biofilm assay. The ability of *P. aeruginosa* to develop biofilm on an abiotic surface was determined using the method described by O'Toole & Kolter (1998). Briefly, *P. aeruginosa* overnight cultures in LB were diluted 1:100 in fresh medium, 200 μl per well suspension was loaded into a flat bottom 96-well polyvinyl chloride microtitre plate and incubated for 15 h at 37°C . The growth of each test strain was measured at OD₆₆₀ following incubation. After discarding planktonic cells, attached cells were stained with an aqueous crystal violet solution (0.25%, w/v). The optical density of crystal violet solubilized in ethanol was then measured at 570 nm. Biofilm density was normalized by planktonic bacterial growth (OD₅₇₀ for biofilm cells/OD₆₆₀ for planktonic cells) then classified using the scheme of Stepanovic *et al.* (2000, 2007). The classification was based upon the cut-off optical density (OD_c) value defined as three SD values above the mean optical density of the negative control (LB medium). The classifications were: no biofilm formation OD $\leq$ OD_c; weak biofilm formation $2 \times \text{OD}_c \geq \text{OD} > \text{OD}_c$; moderate biofilm formation $4 \times \text{OD}_c \geq \text{OD} > 2 \times \text{OD}_c$; and strong biofilm formation OD $> 4 \times \text{OD}_c$.

Serotyping. All test isolates were subjected to O-antigen serotyping by slide agglutination test using a commercially available *P. aeruginosa* antisera kit (Accurate Chemical & Scientific) following the manufacturer's instructions.

Antibiotic susceptibility tests. Antibiotics used in susceptibility tests included β -lactams (aztreonam, ceftazidime, piperacillin and ticarcillin), aminoglycosides (amikacin, gentamicin, netilmicin and tobramycin), and fluoroquinolones (ciprofloxacin, moxifloxacin, norfloxacin and ofloxacin). Bacterial susceptibilities to these antibiotics were determined using the disc diffusion method (Oxoid) in accordance with the CDS (Calibrated Dichotomous Sensitivity) method standard (Bell *et al.*, 2006).

Strains that exhibited resistance to fluoroquinolones in the disc diffusion assay were further assessed for the MICs of antibiotics to which they were resistant using the broth microdilution method in Mueller-Hinton broth (MHB) as recommended by the Clinical and Laboratory Standard Institute (CLSI, 2007) and the British Society for Antimicrobial Chemotherapy (Andrews, 2007). Inocula were prepared by suspending growth from chocolate agar plates in MHB broth to a starting concentration of 5×10^5 c.f.u. ml⁻¹.

Statistical analysis. Statistical analysis was performed using SPSS 14.0 (SPSS). Data were analysed between factors as categorical using 2-sided Pearson chi-square or Fisher's exact tests as appropriate. The non-parametric linear-by-linear association test was used to test for linear trends for bacterial resistance to antimicrobial agents across time. *P* values of ≤ 0.05 were considered significant.

RESULTS AND DISCUSSION

Type III secretion toxin-encoding genes

The type III secretion system in *P. aeruginosa* is known to be a very important virulence factor in acute human infections, but it is less important in maintaining chronic infections, such as cystic fibrosis (CF), in which the expression of type III toxin-encoding genes is down-regulated (Jain *et al.*, 2004; Lee *et al.*, 2005). Documentation of the presence of type III toxin-encoding genes is important for understanding the different pathogenic roles of *P. aeruginosa* during ocular infections. In the current study, of the total 55 keratitis isolates, 64 % (35/55) possessed the *exoS*⁺/*exoU*[−] genotype, whereas 33 % (18/55) were *exoS*[−]/*exoU*⁺, 4 % (2/55) (strain Paer9 from non-contact lens-related keratitis and strain Paer126 from contact lens-related keratitis) were *exoS*⁺/*exoU*⁺ (Fig. 1). The difference in the distribution of type III secretion toxin-encoding genes between contact lens- and non-contact lens-related isolates was significant (*P* < 0.01). This significant difference is reported for what is believed to be the first time. Earlier studies with smaller sample sizes did not observe this distinction in ocular isolates (Cowell *et al.*, 2003b; Zhu *et al.*, 2002, 2006; Pinna *et al.*, 2008). It was anticipated that the presence of *exoS* and *exoU* would conform to the invasive and acute cytotoxic phenotypes, respectively (Fleiszig *et al.*, 1997; Zhu *et al.*, 2006), although it has recently been reported that the presence of a type III-

effector-encoding gene does not necessitate expression and/or secretion of the protein *in vivo* (Wong-Beringer *et al.*, 2008). The *P. aeruginosa* type III secretion system consists of 43 co-ordinately regulated genes encoding type III secretion and translocation machinery and regulatory functions (Frank, 1997). *P. aeruginosa* uses this complex set of signalling pathways, both to activate and to repress the expression of the type III secretion system in response to extracellular and intracellular triggers (Yahr & Wolfgang, 2006; Willcox *et al.*, 2008).

The *exoS*⁺/*exoU*[−] genotype predominated in the non-contact lens isolates, accounting for 85 % (23/27) of isolates, compared with 43 % (12/28) of isolates from contact lens-related keratitis (Fig. 1). The selection for the *exoS* genotype in non-contact lens-related keratitis isolates could be attributed to the association between *exoS*/invasiveness and ocular trauma. In the absence or presence of contact lens, damage to the corneal epithelium (i.e. trauma to the corneal surface and the disruption of the tight inter-cellular junctions) is perhaps required to establish infection caused by *exoS*⁺ *P. aeruginosa* strains, which allows *exoS*⁺ and invasive *P. aeruginosa* to internalize within the exposed basolateral surface of corneal epithelial cells and initiate keratitis (Willcox, 2006). The observation of a high rate (85 %) of *exoS*⁺ isolates in non-contact lens-related keratitis events is consistent with the findings from other human infections. Rumbaugh *et al.* (1999) reported 96 % of *P. aeruginosa* strains isolated from urinary, wound and tracheal infections carried *exoS*. Similarly, a study evaluating 115 environmental and non-corneal clinical isolates reported that 72 % of specimens carried *exoS* (Feltman *et al.*, 2001). Wareham & Curtis (2007) demonstrated that 84 % of *P. aeruginosa* strains from CF patients were *exoS*⁺. A recent Australian study reported that 98 % (43/44) of CF isolates, including both clonal and non-clonal *P. aeruginosa* strains, carried an *exoS* gene (Tingpej *et al.*, 2007).

Conversely, the *exoS*[−]/*exoU*⁺ genotype was found in 54 % (15/28) of contact lens isolates versus 11 % (3/27) in non-contact lens isolates (Fig. 1). The possible genotypic selection of *exoU*⁺ isolates in contact lens-related keratitis is intriguing. The higher proportion of contact lens-related *P. aeruginosa* strains processing *exoU* genes than non-contact lens-related strains may suggest that *exoU*-mediated cytotoxicity is a much more important virulence factor in contact lens-related ocular infection than other types of *P. aeruginosa* ocular infections and other infections where the invasive phenotype predominates. The type III effector ExoU (encoded by *exoU*) has been reported to mediate pathogenicity of *P. aeruginosa* in experimental keratitis based on its capacity to induce rapid lysis of epithelial cells and macrophages (Fleiszig *et al.*, 1997; Lomholt *et al.*, 2001). Cytotoxic (*exoU*⁺) strains can damage epithelia on an uninjured corneal surface providing there is prolonged bacterial contact (Fleiszig *et al.*, 1998). Stagnation of cytotoxic bacteria against the corneal surface may contribute to the pathogenesis of infection

Fig. 1. The distribution of type III secretion system-associated toxin-encoding genes in *P. aeruginosa* isolates from contact lens-related and non-contact lens-related keratitis. Black bars, *exoS*⁺/*exoU*[−]; hatched bars, *exoS*[−]/*exoU*⁺; spotted bars, *exoS*⁺/*exoU*⁺.

associated with the use of soft contact lenses (Fleiszig *et al.*, 1998). A positive correlation between *exoU*⁺ clinical isolates and bacterial adhesion in intestinal epithelium has been reported (Zaborina *et al.*, 2006). It is possible that *exoU*⁺ strains adhere more strongly under conditions established through contact lens wear, which may in part explain the larger proportion of *exoU*⁺ strains in contact lens-related keratitis. Resistance of *P. aeruginosa* ocular isolates to contact lens disinfection solutions has also been linked to *exoU*⁺-encoded cytotoxic activity (Lakkis & Fleiszig, 2001). On the other hand, the presence of corneal damage and changes to the corneal epithelia (such as disruption of tight junctions) with contact lens wear can be conceived as the predisposing factors allowing *P. aeruginosa* infections to be established, especially for *exoS*⁺ strains. Contact lens materials and designs are regarded as possible contributors to disrupt tight junctions of epithelial cells and expose basolateral cell surfaces (Willcox, 2006). A recent study demonstrated that the frequent use of a multipurpose disinfecting solution with high cytotoxicity may result in breakdown of corneal epithelial tight junctions in hydrogel lens wearers (Imayasu *et al.*, 2008).

Protease production

Gelatin zymography of the culture supernatants showed two different protease profiles among the ocular *P. aeruginosa* strains (Fig. 2). The results of relationships between the expression of exoproteases and *exoU* and *exoS* confirmed previous findings that type I and II protease profiles associate with *exoS* and *exoU*, respectively (Zhu *et al.*, 2006). Both profiles express protease IV (>200 kDa)

Fig. 2. Protease profiles produced by representative *P. aeruginosa* strains with *exoS*⁺ (Paer130, 131 and 152) or *exoU*⁺ (Paer115, 116 and 156) in a gelatin zymograph. Two Indian isolates, Paer39 and 34, were used as positive controls for type I and type II protease profiles, respectively (Zhu *et al.*, 2006); the bands >200 kDa ≈ protease IV, 145 kDa ≈ elastase B, 98 kDa ≈ modified elastase B or *P. aeruginosa* small protease (PASP), 51 kDa ≈ alkaline protease (Zhu *et al.*, 2006).

and alkaline protease (51 kDa). Similar to previous findings (Zhu *et al.*, 2006), the modified elastase activity at 98 kDa (or *P. aeruginosa* small protease, PASP) was linked to strains carrying the *exoU* gene; whereas elastase (LasB) activity at 145 kDa was linked with strains carrying the *exoS* gene (Fig. 2). The high production of LasB from *exoS*⁺ strains is a feature that maintains the invasive phenotype. LasB acts to degrade *exoS*- and *exoT*-encoded exotoxins that can suppress invasion of epithelial cells (Cowell *et al.*, 2003a). Not surprisingly, the type I protease phenotype was detected in 23/27 (85 %) non-contact lens isolates versus 14/28 (50 %) contact lens isolates, while a type II protease phenotype was detected in 13/28 (46 %) contact lens versus 3/27 (11 %) of non-contact lens isolates (Fig. 2). The type I protease phenotype was significantly associated with non-contact lens-related keratitis isolates and a type II phenotype significantly associated with contact lens-related isolates ($P < 0.01$). The protease profiles in two isolates (Paer174 from contact lens-related keratitis and Paer149 from non-contact lens-related keratitis) were too weak to be detected.

Biofilm formation

Biofilm production has been considered to be an important determinant of pathogenicity in *P. aeruginosa* infections. Table 1 displays the ability to form biofilms among ocular isolates of *P. aeruginosa*. The majority of isolates (65 %, 36/55) showed strong biofilm formation, 31 % (17/55) of isolates formed moderate biofilms, while weak biofilm formation was found in 4 % (2/55) of isolates. The frequency of clinical isolates that produced specific biofilm density levels was equally distributed between contact lens- and non-contact lens-related keratitis groups ($P > 0.05$). When type III toxin-encoding genes were compared with each biofilm density group, the *exoS*[−]/*exoU*⁺ genotype was statistically associated with strong biofilm-formation strains, and the *exoS*⁺/*exoU*[−] genotype with moderate and weak biofilm-formation strains ($P < 0.05$). The two isolates with both *exoS*⁺ and *exoU*⁺ genes showed a strong

Table 1. Biofilm-formation ability of *P. aeruginosa* keratitis isolates

Biofilm density*	No. of isolates (%)				
	Total	CL	Non-CL	<i>exoS</i> ⁺	<i>exoU</i> ⁺
Strong	36 (65)	19 (68)	17 (63)	17 (49)	17 (94)
Moderate	17 (31)	8 (29)	9 (33)	16 (46)	1 (6)
Weak	2 (4)	1 (4)	1 (4)	2 (6)	0
Total	55 (100)	28 (51)	27 (49)	35 (64)	18 (33)

CL, Contact lens-related isolates; non-CL, non-contact lens-related isolates.

*Biofilm density was classified upon the OD_c value of 0.09. Strong OD > 0.36 (OD > 4 × OD_c); moderate 0.36 ≥ OD > 0.18 (4 × OD_c ≥ OD > 2 × OD_c); weak 0.18 ≥ OD > 0.09 (2 × OD_c ≥ OD > OD_c).

biofilm formation phenotype. The relationship found between the *exoU*⁺ genotype and strong biofilm formation in ocular isolates to the best of our knowledge has not been reported before. The findings that significant numbers of *exoU*⁺ isolates possess a strong abiotic biofilm formation phenotype (17/18, 94 %) in the present study may partially explain why there were more *exoU*⁺ isolates in contact lens-related keratitis isolates than in non-contact lens-related keratitis isolates. Contact lens-related *P. aeruginosa* keratitis has been associated with biofilms on contact lenses and contact lens storage cases (McLaughlin-Borlace *et al.*, 1998). The results from our studies suggest that contact lenses and contact lens storage cases may facilitate the selection of *exoU*⁺ and strong biofilm formation strains.

Serotyping

Smooth lipopolysaccharide with O-antigen serotype appears to be required for corneal infection (Priebe *et al.*, 2004), which makes serotyping useful for studying *P. aeruginosa* isolates from eye infections. It was possible to assign 54 of the 55 ocular isolates into 9 different serotypes using the O-antigen antisera kit. The most frequent serotypes were G, A, C, E, I and B (Table 2). The overall serotype distributions between the two keratitis isolate groups were not significantly different. There have been many investigations on the associations between serotype and type III secretion toxin-encoding gene distributions amongst *P. aeruginosa* isolates. Our observation (Table 2) that strains with serotypes G, A and B were significantly associated with the *exoS* gene ($P<0.001$), and those with serotypes C and E were associated with the *exoU* gene ($P<0.001$), are consistent with previous findings (Berthelot *et al.*, 2003; Faure *et al.*, 2003; Zhu *et al.*, 2006). There were

more strains with serotype E, I or C forming strong biofilm compared with strains of serotype B, G, A or others ($P<0.01$, Table 2). The presence of particular O antigens on the surface of *P. aeruginosa* is known to affect the overall charge and physico-chemistry of the bacterial cell; strains lacking the B-band O antigen have demonstrated greater ability to adhere to abiotic hydrophobic surfaces (Beveridge *et al.*, 1997; Augustin *et al.*, 2007). Serotypes E, I and G have been shown to occur frequently in contact lens wearers and induce higher adhesion to contact lens than other serotype isolates (Thuruthiyil *et al.*, 2001). Therefore, it is highly likely that certain serotypes can adhere more strongly or develop strong biofilm on contact lens and case surfaces, and thus be overrepresented in contact lens-related keratitis. However, distribution of serotypes between contact lens- and non-contact lens-related isolates was similar in the current study. The lack of statistical significance between the serotype of *P. aeruginosa* and the isolation source in our study may be due to the relatively small sample size in each serogroup and deserves further investigation.

Antibiotic resistance

Fluoroquinolones are commonly used as topical monotherapy for corneal infections. Six out of fifty-five (11 %) *P. aeruginosa* isolates tested in the present study were intermediate-resistant or resistant to both ofloxacin and moxifloxacin (Table 3). These six isolates displayed resistance to ofloxacin and/or moxifloxacin by the agar diffusion method and were also non-susceptible (intermediate-resistant or resistant) to either or both of the two fluoroquinolones in the MIC test. Since the introduction of second-generation fluoroquinolones ciprofloxacin and

Table 2. Serotype distribution of *P. aeruginosa* isolates in different groups

O serotype*	Total no. of isolates	Source		Toxin-encoding gene		Biofilm density†		
		CL	Non-CL	<i>exoS</i> ⁺	<i>exoU</i> ⁺	Strong	Moderate	Weak
A (O:3)	9	3	6	8	1	5	4	0
B (O:16)	7	4	3	7	0	2	5	0
C (O:7/8)	8	5	3	2	6	6	1	1
D (O:9)	1	0	1	1	0	0	1	0
E (O:11)	8	5	3	1	6	8	0	0
F (O:4)	1	0	1	0	0	1	0	0
G (O:6)	10	3	7	10	0	4	5	1
H (O:10)	2	2	0	0	2	2	0	0
I (O:1)	8	5	3	6	2	7	1	0
Negative	1	1	0	0	1	1	0	0
Total	55	28	27	35	18	36	17	2

CL, Contact lens-related isolates; non-CL, non-contact lens-related isolates.

*Corresponding serotype of *P. aeruginosa* grouped by Liu (Difco) antisera.

†Biofilm density was classified upon the OD_c value of 0.09. Strong OD>0.36 (OD>4 × OD_c); moderate 0.36 ≥ OD>0.18 (4 × OD_c ≥ OD>2 × OD_c); weak 0.18 ≥ OD>0.09 (2 × OD_c ≥ OD>OD_c).

Table 3. Fluoroquinolone resistance in keratitis isolates of *P. aeruginosa* and characteristics of the isolates

Strain	MIC ($\mu\text{g ml}^{-1}$)*		Source	Year of isolation	Toxin-encoding gene	Biofilm density†
	OFX	MXF				
Paer17	4 (I)†	4 (R)	Non-CL	1992	<i>exoS</i>	Moderate
Paer149	4 (I)	4 (R)	Non-CL	2004	<i>exoS</i>	Strong
Paer157	4 (I)	8 (R)	Non-CL	2006	<i>exoS</i>	Strong
Paer171	4 (I)	2 (I)	Non-CL	2006	<i>exoS</i>	Strong
Paer173	4 (I)	4 (R)	Non-CL	2006	<i>exoS</i>	Moderate
Paer175	4 (I)	2 (I)	Non-CL	2006	<i>exoU</i>	Strong

I, Intermediate resistant; MXF, moxifloxacin; non-CL, non-contact lens-related isolate; OFX, ofloxacin; R, resistant.

*The interpretive criteria of MIC breakpoints were: susceptible $\leq 2 \mu\text{g ml}^{-1}$, intermediate = $4 \mu\text{g ml}^{-1}$ and resistant $\geq 8 \mu\text{g ml}^{-1}$ for ofloxacin (CLSI, 2007); and susceptible $\leq 1 \mu\text{g ml}^{-1}$, intermediate = $2 \mu\text{g ml}^{-1}$ and resistant $\geq 4 \mu\text{g ml}^{-1}$ for moxifloxacin (Andrews, 2007).

†Biofilm density was classified upon the ODc value of 0.09. Strong $\text{OD} > 0.36$ ($\text{OD} > 4 \times \text{ODc}$); moderate: $0.36 \geq \text{OD} > 0.18$ ($4 \times \text{ODc} \geq \text{OD} > 2 \times \text{ODc}$); weak: $0.18 \geq \text{OD} > 0.09$ ($2 \times \text{ODc} \geq \text{OD} > \text{ODc}$).

ofloxacin in the 1990s, the reported incidence of *in vitro* resistance to these antibiotics among bacteria isolated from bacterial keratitis and endophthalmitis has been steadily increasing in the USA (Kowalski *et al.* 2001, 2003; Hwang, 2004) and India (Smitha *et al.*, 2005). In the current study, 11% of all keratitis isolates in Australia were non-susceptible to ofloxacin, which is higher than the previous report (Zhu *et al.*, 2006) in which the strains used were isolated through the years 1986 to 2004. The current findings may reflect an increasing trend of fluoroquinolone resistance in non-contact lens-related isolates, as the resistance rate increased from 8% (2/24) before year 2005 to 24% (4/17) from year 2006. However, due to the small sample size in each year, this trend was not statistically significant. It must be also noted that the present study and other studies referenced above are based on *in vitro* results, which do not necessarily mirror the clinical response to an antibiotic and could differ to the drug efficacy demonstrated *in vivo* (Leibowitz, 1991; Wilhelmus *et al.*, 1993; O'Brien *et al.*, 1995; Kunitomo *et al.*, 1999; Smitha *et al.*, 2005). Nevertheless, this *in vitro* study supports the concern about emerging fluoroquinolone-resistant *P. aeruginosa* strains in ocular infections, and highlights the need for continuous monitoring of emerging bacterial resistance.

It was noticed that non-susceptible strains were all isolated from the non-contact lens-related keratitis group (6/27, 22%), and the number was significantly higher than that from the contact lens-related keratitis group (0/28, $P < 0.05$). Five out of six (83%) non-susceptible isolates were *exoS*⁺, and three of these *exoS*⁺ isolates also produced strong biofilm (Table 3). However, the association between antibiotic resistance and type III toxin-encoding genes or the degree of biofilm formation was not significant.

The aminoglycosides were significantly more active *in vitro*, with no *P. aeruginosa* isolates showing resistance to any of the aminoglycosides. Both β -lactams and aminoglycosides

were significantly more efficacious than fluoroquinolones ($P < 0.05$) with only strain Paer17 being resistant to ticarcillin and aztreonam of the β -lactam class. All isolates were susceptible to both the fluoroquinolones norfloxacin and ciprofloxacin.

In summary, this study reports what is believed to be the first comprehensive evaluation of virulence factors between ocular isolates of *P. aeruginosa* isolated from contact lens- and non-contact lens-related keratitis. The data suggest that type III secreted proteins, protease profiles, biofilm formation and fluoroquinolone resistance may be important traits that play a role in creating highly virulent strains involved in specific keratitis. Adverse outcomes associated with the keratitis caused by these clinical strains may be attributed to the associations between virulence characteristics, which may function co-operatively. Further investigations are required to understand the mechanisms involved in *P. aeruginosa* virulence, which in effect provide the tools to rapidly monitor for newly virulent strains and provide better strategies to contain the disease.

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