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.

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*) (Fleischig 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...
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 filtered, 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 OD600, 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 to planktonic bacterial growth (OD570 for biofilm cells/OD660 for planktonic cells) then classified using the following classes: no biofilm formation OD< ODcontrol (LB medium). The classification was based upon the cut-off optical density (ODc) value defined as three SD values above the mean optical density of the negative control (LB medium). The classifications were: no biofilm formation OD< ODc; weak biofilm formation 2 × ODc ≥ OD > ODc; moderate biofilm formation 4 × ODc ≥ OD > 2 × ODc; and strong biofilm formation OD > 4 × ODc.

**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, nalidixic acid 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 × 108 c.f.u. ml−1.
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 \( \text{exo}U^+ \) clinical isolates and bacterial adhesion in intestinal epithelium has been reported (Zaborina et al., 2006). It is possible that \( \text{exo}U^+ \) strains adhere more strongly under conditions established through contact lens wear, which may in part explain the larger proportion of \( \text{exo}U^+ \) strains in contact lens-related keratitis. Resistance of \( P. \) aeruginosaocular isolates to contact lens disinfection solutions has also been linked to \( \text{exo}U^+ \)-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 \( \text{exo}S^+ \) 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 linked to strains carrying the \( \text{exo}U \) gene; whereas elastase (LasB) activity at 145 kDa was linked with strains carrying the \( \text{exo}S \) gene (Fig. 2). The high production of LasB from \( \text{exo}S^+ \) strains is a feature that maintains the invasive phenotype. LasB acts to degrade \( \text{exo}S \) and \( \text{exo}T \)-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.

### 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 \( \text{exo}U \) and \( \text{exo}S \) confirmed previous findings that type I and II protease profiles associate with \( \text{exo}S \) and \( \text{exo}U \), respectively (Zhu et al., 2006). Both profiles express protease IV \((>200 \text{ kDa})\) and alkaline protease \((51 \text{ kDa})\). 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 \( \text{exo}U \) gene; whereas elastase (LasB) activity at 145 kDa was linked with strains carrying the \( \text{exo}S \) gene (Fig. 2). The high production of LasB from \( \text{exo}S^+ \) strains is a feature that maintains the invasive phenotype. LasB acts to degrade \( \text{exo}S \) and \( \text{exo}T \)-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 \( \text{exo}S^-/\text{exo}U^+ \) genotype was statistically associated with strong biofilm-formation strains, and the \( \text{exo}S^+/\text{exo}U^- \) genotype with moderate and weak biofilm-formation strains \((P<0.05)\). The two isolates with both \( \text{exo}S^+ \) and \( \text{exo}U^+ \) genes showed a strong

![Fig. 2. Protease profiles produced by representative \( P. \) aeruginosa strains with \( \text{exo}S^+ \) (Paer130, 131 and 152) or \( \text{exo}U^+ \) (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 \( >200 \text{ kDa} \) \( \text{protease IV} \), 145 kDa \( \text{elastase B} \), 98 kDa \( \text{modified elastase B} \) or \( P. \) aeruginosa small protease (PASP), 51 kDa \( \text{alkaline protease} \) (Zhu et al., 2006).](image)

<table>
<thead>
<tr>
<th>Biofilm density*</th>
<th>Total</th>
<th>CL</th>
<th>Non-CL</th>
<th>( \text{exo}S^+ )</th>
<th>( \text{exo}U^+ )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strong</td>
<td>36 (65)</td>
<td>19 (68)</td>
<td>17 (63)</td>
<td>17 (49)</td>
<td>17 (94)</td>
</tr>
<tr>
<td>Moderate</td>
<td>17 (31)</td>
<td>8 (29)</td>
<td>9 (33)</td>
<td>16 (46)</td>
<td>1 (6)</td>
</tr>
<tr>
<td>Weak</td>
<td>2 (4)</td>
<td>1 (4)</td>
<td>1 (4)</td>
<td>2 (6)</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>55 (100)</td>
<td>28 (51)</td>
<td>27 (49)</td>
<td>35 (64)</td>
<td>18 (33)</td>
</tr>
</tbody>
</table>

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

*Biofilm density was classified upon the ODc value of 0.09. Strong OD > 0.36 (OD > 4 × ODc); moderate 0.36 OD > 0.18 (4 × ODc > OD > 2 × ODc); weak 0.18 OD > 0.09 (2 × ODc > OD > ODc).
biofilm formation phenotype. The relationship found between the \textit{exoU} \textsuperscript{+} 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 \textit{exoU} \textsuperscript{+} isolates possess a strong abiotic biofilm formation phenotype (17/18, 94 \%) in the present study may partially explain why there were more \textit{exoU} \textsuperscript{+} isolates in contact lens-related keratitis isolates than in non-contact lens-related keratitis isolates. Contact lens-related \textit{P. aeruginosa} keratitis has been associated with biofilms on contact lenses and contact lens storage cases (McLaughlin-Borlace \textit{et al.}, 1998). The results from our studies suggest that contact lenses and contact lens storage cases may facilitate the selection of \textit{exoU} \textsuperscript{+} and strong biofilm formation strains.

**Serotyping**

Smooth lipopolysaccharide with O-antigen serotype appears to be required for corneal infection (Priebe \textit{et al.}, 2004), which makes serotyping useful for studying \textit{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 \textit{P. aeruginosa} isolates. Our observation (Table 2) that strains with serotypes G, A and B were significantly associated with the \textit{exoS} gene (\textit{P}<0.001), and those with serotypes C and E were associated with the \textit{exoU} gene (\textit{P}<0.001), are consistent with previous findings (Berthelot \textit{et al.}, 2003; Faure \textit{et al.}, 2003; Zhu \textit{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 (\textit{P}<0.01, Table 2). The presence of particular O antigens on the surface of \textit{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 \textit{et al.}, 1997; Augustin \textit{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 (Thuruthyil \textit{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 \textit{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 \%) \textit{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>
<thead>
<tr>
<th>O serotype\textsuperscript{*}</th>
<th>Total no. of isolates</th>
<th>Source</th>
<th>Toxin-encoding gene</th>
<th>Biofilm density\textsuperscript{†}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CL</td>
<td>Non-CL</td>
<td>\textit{exoS}\textsuperscript{+}</td>
</tr>
<tr>
<td>A (O:3)</td>
<td>9</td>
<td>3</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>B (O:16)</td>
<td>7</td>
<td>4</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>C (O:7/8)</td>
<td>8</td>
<td>5</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>D (O:9)</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>E (O:11)</td>
<td>8</td>
<td>5</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>F (O:4)</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>G (O:6)</td>
<td>10</td>
<td>3</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>H (O:10)</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>I (O:1)</td>
<td>8</td>
<td>5</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Negative</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>55</td>
<td>28</td>
<td>27</td>
<td>35</td>
</tr>
</tbody>
</table>

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

\textsuperscript{*}Corresponding serotype of \textit{P. aeruginosa} grouped by Liu (Difco) antisera.

\textsuperscript{†}Biofilm density was classified upon the OD\textsubscript{c} value of 0.09. Strong OD>0.36 (OD>4 × OD\textsubscript{c}); moderate 0.36≥OD>0.18 (4 × OD\textsubscript{c}>OD>2 × OD\textsubscript{c}); weak 0.18≥OD>0.09 (2 × OD\textsubscript{c}≥OD≥OD\textsubscript{c}).
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; Wilhelms et al., 1993; O’Brien et al., 1995; Kunimoto 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.

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (μg ml(^{-1}))*</th>
<th>Source</th>
<th>Year of isolation</th>
<th>Toxin-encoding gene</th>
<th>Biofilm density†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paer17</td>
<td>OFX 4 (I) MXF 4 (R)</td>
<td>Non-CL</td>
<td>1992</td>
<td>(exoS)</td>
<td>Moderate</td>
</tr>
<tr>
<td>Paer149</td>
<td>OFX 4 (I) MXF 4 (R)</td>
<td>Non-CL</td>
<td>2004</td>
<td>(exoS)</td>
<td>Strong</td>
</tr>
<tr>
<td>Paer157</td>
<td>OFX 4 (I) MXF 8 (R)</td>
<td>Non-CL</td>
<td>2006</td>
<td>(exoS)</td>
<td>Strong</td>
</tr>
<tr>
<td>Paer171</td>
<td>OFX 4 (I) MXF 2 (I)</td>
<td>Non-CL</td>
<td>2006</td>
<td>(exoS)</td>
<td>Strong</td>
</tr>
<tr>
<td>Paer173</td>
<td>OFX 4 (I) MXF 4 (R)</td>
<td>Non-CL</td>
<td>2006</td>
<td>(exoS)</td>
<td>Moderate</td>
</tr>
<tr>
<td>Paer175</td>
<td>OFX 4 (I) MXF 2 (I)</td>
<td>Non-CL</td>
<td>2006</td>
<td>(exoU)</td>
<td>Strong</td>
</tr>
</tbody>
</table>

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

*The interpretive criteria of MIC breakpoints were: susceptible ≤2 μg ml\(^{-1}\), intermediate=4 μg ml\(^{-1}\) and resistant ≥8 μg ml\(^{-1}\) for ofloxacin (CLSI, 2007); and susceptible ≤1 μg ml\(^{-1}\), intermediate=2 μg ml\(^{-1}\) and resistant ≥4 μg ml\(^{-1}\) for moxifloxacin (Andrews, 2007).

†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\(_c\))

The aminoglycosides were significantly more active than the fluoroquinolones (P<0.05) with only strain Paer17 being resistant to ticarcillin and aztreonam of the \(\beta\)-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.

**ACKNOWLEDGEMENTS**

We gratefully acknowledge Dr Jacqueline Harper, Princess Alexandria Hospital, Brisbane, Australia, and Dr Paul Badenoch, St Vincent’s Hospital, Melbourne, Australia, for providing the P. aeruginosa clinical isolates. We are grateful to Dr Judith Flanagan, Institute for Eye Research, Sydney, Australia, for assistance in the preparation of the manuscript. This work was partially supported by the Australian Federal Government through the Cooperative Research Centre programme.

**REFERENCES**


Willcox, M. D. (2006). Which is more important to the initiation of contact lens related microbial keratitis, trauma to the ocular surface or bacterial pathogenic factors? Clin Exp Ophthalm 89, 277–279.


