# Relationship of Virulence Factors and Clinical Features in Keratitis Caused by *Pseudomonas aeruginosa*

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**Purpose.** To examine bacterial virulence factors in *Pseudomonas aeruginosa* isolates from contact lens (CL) wearers and non–CL wearers with *P. aeruginosa* keratitis, and to investigate relationships between virulence factors and clinical features of keratitis.

**Methods.** The study involved 25 subjects including 18 CL and 7 non–CL-related *P. aeruginosa* keratitis patients. Slit-lamp photographs of all subjects were captured, and the focus occupancy ratio (FOR) was defined as the total focus area/entire cornea area, using image processing software. Twenty-five clinical *P. aeruginosa* isolates from keratitis were assessed for protease production, elastase production, biofilm formation, bacterial swimming and swarming motility, cell surface hydrophobicity, and genes encoding the type III secretion system (TTSS) effectors (ExoU and ExoS).

**Results.** Ring abscess was found in 9 of 18 CL-related *P. aeruginosa* keratitis cases (CL[+] ring[+] group) but not in another 9 cases (CL[+] ring[−] group). Expression or prevalence of virulence factors in *P. aeruginosa* isolates from the CL(+) ring(+) group, CL(+) ring(−) group, and CL(−) group were compared. The FOR for CL(+) ring(+) or CL(−) was higher than for CL(+) ring(−) ($P < 0.05$ and $P < 0.01$, respectively). The rate of positive swimming motility for CL(+) ring(+) or CL(−) was higher than for CL(+) ring(−) ($P < 0.05$), whereas the rate of positive swarming motility for CL(+) ring(+) was higher than for CL(+) ring(−) or CL(−) ($P < 0.05$). Prevalence of an *exoS*+/*exoU*-genotype for CL(+) ring(+) or CL(−) was higher than for CL(+) ring(−) ($P < 0.05$). In the CL-related group, expression of elastase and swarming motility significantly correlated with FOR.

**Conclusions.** Swimming motility, swarming motility, and TTSS ExoS could play a major role in the determination of clinical features of *P. aeruginosa* keratitis.

Keywords: *Pseudomonas aeruginosa*, keratitis, virulence factors, protease, swarming motility

Keratitis caused by *Pseudomonas aeruginosa* can progress rapidly with suppurative infiltration, and can lead to corneal perforation and melt, resulting in the loss of vision.[1] Contact lens (CL) wearers are especially susceptible to development of *P. aeruginosa* keratitis. Ring abscess, which is a ring-shaped accumulation of polymorphonuclear leukocytes surrounding a central corneal lesion, is a well-known hallmark of *P. aeruginosa* keratitis.[2,3] Along with ring abscess, serrated or satellite lesions with keratitis are sometimes found in *P. aeruginosa* keratitis.[4,5] *Pseudomonas aeruginosa* can show various clinical features, but little is known about factors that determine these clinical features.

To understand the mechanism of pathogenesis in keratitis caused by *P. aeruginosa*, the virulence factors as well as extracellular products, including proteases,[6–8] exotoxin A,[9] and biofilms,[10] have been investigated. Of these factors, proteases, including metalloproteases such as alkaline protease, elastase A, and elastase B,[11] are important in virulence. We recently reported that MucD protease inhibits neutrophil recruitment in the cornea, and plays an important role in the pathogenesis of keratitis.[12] *Pseudomonas aeruginosa* elastase activates metalloproteases, inducing the destruction of corneal stroma.[8] Along with these virulence factors, the Type III secretion system (TTSS) is involved in the pathogenesis of keratitis.[13–15] The TTSS transports toxins to host cells, and includes a needle-like apparatus, effector proteins (ExoS and ExoU), and a pore-forming protein. *P. aeruginosa* isolates contain two TTSS genotypes that involve the *exoS* gene in invasive strains and the *exoU* gene in cytotoxic strains.[16] Moreover, *P. aeruginosa*–associated cellular structures, such as the flagella,[17,18] pili,[19] and lipopolysaccharide,[20] also have been studied. Flagella of *P. aeruginosa* assume a motility that is related to adhesion and invasion in the corneal epithelium, and activation of the immune response.[17,21] Specifically, the motility of flagella in liquid media is known as swimming, and the motility of flagella on semisolid surfaces is called swarming, which shows fractal-like patterns of radiating tendril on 0.5% to 0.7% agar plates.[22–24] It is also thought that swarming motility is an important factor in the resistance to antibiotics and the survival of *P. aeruginosa* in tissues associated with biofilm formation.[23]

Few reports have focused on the associations between clinical features and virulence factors in human clinical cases.

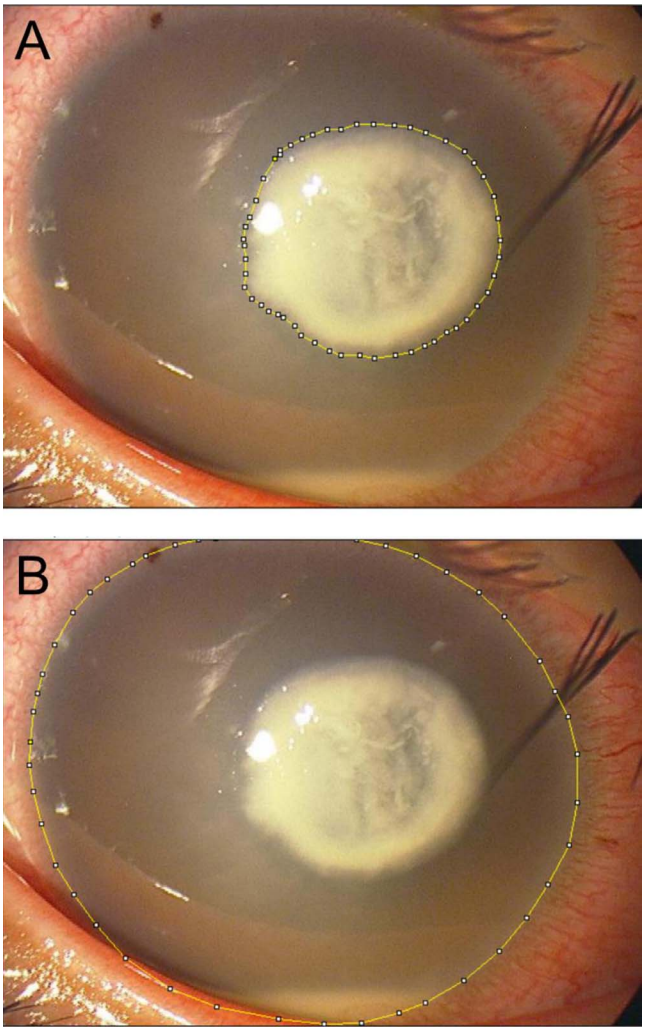


**Figure 1.** Determination of the FOR. (**A**) Tracing the focus, (**B**) tracing the entire cornea; FOR was defined as A/B.

The relationship between clinical features of *P. aeruginosa* keratitis and virulence factors of keratitis isolates is therefore still not completely known.

In the present study, bacterial virulence factors in *P. aeruginosa* isolates from CL wearers or non–CL wearers with *P. aeruginosa* keratitis were examined, to elucidate the relationships between virulence factors and clinical features of keratitis.

## Materials and Methods

### Patients and Clinical Features

The clinical records of *P. aeruginosa* keratitis cases seen at Ehime University Hospital from 2003 to 2014 were retrospectively reviewed, and patients with culture-proven *P. aeruginosa* keratitis were enrolled. The collected data included age, sex, predisposing factors, and days visiting our hospital after complaining of symptoms. The size, shape, and location of corneal infiltrates and the presence of hypopyon or serrated infiltration at first visit were documented. A ring abscess was defined as a ring-shaped opacity with less central opacity. Slit-lamp photographs of patients with keratitis at the first visit were obtained using a slit-lamp microscope equipped with a camera. The photographs were analyzed using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). The outlines of each focus and the entire cornea were manually traced, and the focus area and cornea area were measured using the application tool of the software. The focus occupancy ratio (FOR) of each keratitis case was defined as the total focus area/entire cornea area (Fig. 1).[25] Informed consent was obtained from the patients after explanation of the nature and possible consequences of the study. This study was approved by the Ehime University Review Board and adhered to the tenets of the Declaration of Helsinki.

### Bacterial Strains

Clinical isolates of *P. aeruginosa* obtained from keratitis cases were tested. Our institution has collected and stocked all organisms causing ocular infection, including keratitis and endophthalmitis, from 2003, and used the clinical isolates of *P. aeruginosa* from our collection. All strains were stored in Microbank (Pro-Lab Diagnostics, Ontario, Canada) at −80°C. For investigation of virulence factors, strains were grown on brain heart infusion agar plates, and then cultured in 10 mL of Luria-Bertani (LB) broth and incubated at 37°C for 18 hours, with shaking at 250 rpm. Cells were collected by centrifugation and washed three times with PBS, and continuously used in each assay.

### Determination of Virulence Factors

**Proteolytic Activity.** The modified azocasein assay to assess the proteolytic activity of *P. aeruginosa* was used as previously described.[26] Overnight cultures (500 μL) were transferred to 1.5-mL tubes. The tubes were centrifuged at 7000*g* for 4 minutes, and the culture supernatants were collected, then 75 μL each supernatant and 125 μL 0.2% azocasein (Sigma-Aldrich Corp., St. Louis, MO, USA) were mixed in a 1.5-mL tube. After incubation in a 5% $CO_2$ incubator at 37°C for 30 minutes, 600 μL 10% trichloroacetic acid was added to the mixture, then incubated at room temperature for 30 minutes. The mixture was centrifuged at 10,000*g* for 10 minutes, and the supernatant was transferred to a new 1.5-mL tube. A solution of 10 M NaOH (375 μL) was added to each 1.5-mL tube, and 200 μL mixture was transferred to a 96-well microtiter plate (Costar, Corning, NY, USA). Absorption at 440 nm was measured in a microtiter plate reader (Immuno-Mini NJ-2300; Nalge Nunc International KK, Bunkyo-ku, Tokyo, Japan).

**Elastase Activity.** The elastase activity of *P. aeruginosa* culture fluids was quantitated by elastin Congo Red assays, as previously modified.[27,28] Overnight cell cultures of 5-mL cultures in PTSB (5% peptone; Difco, Detroit, MI, USA) and 0.25% trypticase soy broth (Difco) were washed and resuspended in 10 mL PTSB to an $OD_{600}$ of 0.05. The cultures were incubated at 37°C for 16 hours with rapid shaking, then the culture supernatants were filtered (0.45-μm filters; Millipore, Bedford, MA, USA). Elastin Congo Red (Sigma-Aldrich Corp.) and 0.75 mL reaction buffer (0.05 M Tris, 0.5 mM $CaCl_2$, pH 7.5) was added to 0.25 mL culture filtrates, and the mixtures were incubated at 37°C for 24 hours with shaking. The reactions were centrifuged at 18,000*g* for 15 minutes, and the absorbance of the supernatants was read at 495 nm using a microplate reader.

**Biofilm Assay.** The microtiter plate biofilm assay was performed as previously described.[29] In brief, each diluted culture (200 μL) was incubated at 37°C for 24 hours in 96-well microtiter plates. The planktonic bacteria were removed, and washed three times with PBS. After drying at room temperature, a 200-μL suspension of 0.1% Crystal Violet was added to

**Table 1.** Summary of CL- and Non–CL-Related Keratitis Cases

| | CL-Related Keratitis, $n = 18$ | Non–CL-Related Keratitis, $n = 7$ | Total, $n = 25$ | *P* Value* |
|---|---|---|---|---|
| Age, y | 27.39 ± 11.79 | 75.86 ± 12.33 | 40.96 ± 25.10 | <0.001 |
| Sex, M:F | 1:1 | 1:3 | 11:14 | 0.332 |
| Ring abscess, *n* (%) | 9/18 (50.0) | 0/7 (0) | 9/25 (36.0) | <0.05 |
| Focus size, >1/4 corneal diameter, *n* (%) | 9/18 (50.0) | 7/7 (100) | 16/25 (64.0) | <0.05 |
| Hypopyon, *n* (%) | 6/18 (33.3) | 4/7 (57.1) | 10/25 (40.0) | 0.275 |
| Serrated infiltration, *n* (%) | 8/18 (44.4) | 0/7 (0) | 8/25 (32.0) | 0.057 |

M, male; F, female.

* Comparison between CL- and non–CL-related keratitis cases using the Student's *t*-test or the Fisher's exact test.

each well, and incubated at room temperature for 15 minutes. Then, the wells were rinsed three times with PBS and dried, and 200 μL 95% ethanol was added to solubilize the biofilm. The Crystal Violet/ethanol solution of each well was transferred to another plate for reading the optical density, and absorption at 600 nm was measured using a microtiter plate reader.

**Motility Assays.** Swarming and swimming motility assays with modifications were performed as previously described.[22,30,31] Swarming agar (0.5% [wt/vol] bacto-agar [Difco] with 8 g/L nutrient broth [Difco], with 5 g/L glucose) and swimming agar (Tryptone broth [10 g/L Tryptone and 5 g/L NaCl] containing 0.3% [wt/vol] bacto-agar) were prepared for assays. A single colony of each strain from a fresh LB agar plate was inoculated in the center of a swimming or swarming agar plate with a toothpick, then incubated at 37°C for 12 hours. After photographing the plate, the extent of swimming or swarming was determined by measuring the diameter of the motility using ImageJ software.[32]

**Cell Surface Hydrophobicity.** The technique of microbial adhesion to hydrocarbons (MATH) was used.[33] Bacterial cell suspensions ($OD_{600}$:0.6; 1.2 mL) were then transferred to 1.5-mL tubes and 200 μL n-hexadecane was added. The mix was vortexed for 2 minutes, and allowed to stand undisturbed for 15 minutes at room temperature. The $OD_{600}$ of the aqueous phase was then measured. Hydrophobicity was calculated as percentage using $OD_{600}$ before and after vortexing.

**TTSS Effector Genotyping.** Chromosomal DNA was extracted from bacterial cells with the use of a commercial kit (QIAamp Tissue Kit; Qiagen, Valencia, CA, USA). The TTSS effector genotype of each clinical isolate was determined using multiplex PCR to detect the *exoS* and *exoU* genes in a single reaction as described and optimized by Ajayi et al.[34] Statistical analyses of TTSS gene positivity were performed using indexes (exoS:0, exoU:1) as previously described.[35]

### Statistical Analysis

Student's *t*-test or Fisher's exact test were used to analyze the clinical data as appropriate. The prevalence of virulence factors between groups with different clinical features was compared using the ANOVA test or Kruskal-Wallis test. Student's *t*-test and the ANOVA test were used when the data had normal distribution. The correlations between virulence factors and FOR in CL-associated keratitis were calculated using the Spearman's correlation test. The FOR between the *exoS+/exoU−* group and the *exoS−/exoU+* group was evaluated using the Student's *t*-test. Multivariate analyses were used to evaluate how factors affected each other. A *P* value of less than 0.05 was considered statistically significant. Data were analyzed using JMP statistical software, version 11.2.0 (SAS Institute, Inc., Cary, NC, USA).

## Results

### Clinical Features of Keratitis

In total, 25 cases of *P. aeruginosa* keratitis were evaluated in the present study. The patients' ages ranged from 13 to 94 years (mean, 40.96 ± 25.10 years). There were 11 male patients and 14 female patients. A predisposing condition was identified in 21 patients (84.0%), and the most common condition was CL wearing (18 patients, 72.0%). Other predisposing factors included trauma (two patients, 8.0%), and Descemet's stripping automated endothelial keratoplasty (one patient, 4.0%). The patients were divided into two groups as follows: CL-related (CL[+] group) and non–CL-related keratitis (CL[−] group). Patients' background and prevalence of clinical manifestations for the two groups were compared (Table 1). The mean age of the CL(+) group was younger than the CL(−) group ($P < 0.001$). Ring abscess and serrated infiltration were more frequent in the CL(+) group. All cases in the CL(−) group had more than a quarter corneal diameter focus size. Moreover, all cases with ring abscess in the CL(+) group had more than a quarter corneal diameter focus size. Hypopyon was observed in both groups. Based on these results, patients in the CL(+) group were classified into two groups according to the presence of a ring abscess (CL[+] ring[+]; CL[+] ring[−]), because cases with ring abscesses had a larger focus. Thus, virulence factors were compared among three groups: CL(+) ring(+), CL(+) ring(−), and CL(−) in subsequent analyses.

### Virulence Factors of Keratitis

Virulence factors, FOR, and days to first visit after complaining of symptoms among the three groups were compared using ANOVAs to evaluate which factors influenced clinical manifestations (Table 2). Mean days to first visit after complaining of symptoms for the CL(−) group was significantly longer than the CL(+) ring(−) group ($P < 0.05$), but mean days between the CL(+) ring(+) and CL(+) ring(−) or CL(−) groups were not significant. The FOR, which evaluated the focus size, for the CL(+) ring(−) group was significantly less than the CL(+) ring(+) and CL(−) groups ($P < 0.01$ and $P < 0.001$, respectively). Virulence factors from isolates from each group were analyzed. The extent of swimming motility in the CL(+) ring(+) or CL(−) groups was higher than the CL(+) ring(−) group (both, $P < 0.05$), whereas the extent of swarming motility for the CL(+) ring(+) group was higher than the CL(+) ring(−) or CL(−) groups (both, $P < 0.05$). The prevalence of the *exoS+/exoU−* genotype in the CL(+) ring(+) or CL(−) group was higher than the CL(+) ring(−) group (both, $P < 0.05$). Other virulence factors were not significantly different among the three groups.

Because days to first visit after complaining of symptoms in the CL(−) group were longer than other groups, keratitis

**Table 2.** Comparison of Virulence Factors Among Groups

| | CL(+) Ring(+) | CL(+) Ring(−) | CL(−) | *P* Value* | | |
|---|---|---|---|---|---|---|
| | | | | CL(+) Ring(+) vs. CL(+) Ring(−) | CL(+) Ring(+) vs. CL(−) | CL(+) Ring(−) vs. CL(−) |
| Days† | 2.56 ± 1.51 | 2.11 ± 1.05 | 5.71 ± 3.68 | NS | NS | <0.05 |
| FOR, % | 15.63 ± 10.66 | 1.90 ± 0.98 | 27.01 ± 17.50 | <0.01 | NS | <0.001 |
| Proteolytic activity, OD 440 nm | 0.19 ± 0.08 | 0.12 ± 0.10 | 0.17 ± 0.11 | NS | NS | NS |
| Elastase activity, OD 495 nm | 0.28 ± 0.22 | 0.11 ± 0.10 | 0.28 ± 0.25 | NS | NS | NS |
| Biofilm formation, OD 600 nm | 1.52 ± 0.82 | 2.13 ± 0.87 | 2.08 ± 0.57 | NS | NS | NS |
| Swimming motility, cm | 1.63 ± 0.25 | 1.20 ± 0.57 | 1.82 ± 0.37 | <0.05 | NS | <0.01 |
| Swarming motility, cm | 3.77 ± 2.04 | 1.26 ± 1.46 | 1.75 ± 0.77 | <0.01 | <0.05 | NS |
| Cell surface hydrophobicity, % | 25.22 ± 15.34 | 16.37 ± 13.48 | 19.73 ± 11.76 | NS | NS | NS |
| TTSS, *exoS* = 0, *exoU* = 1 | 0.11 ± 0.33 | 0.67 ± 0.50 | 0.14 ± 0.38 | <0.01 | NS | <0.05 |

Please see Results, Clinical Features of Keratitis, for definitions of each group of cases.
* Comparison using the ANOVA test or Kruskal-Wallis test.
† Days to first visit after complaining of symptoms.

severity in the CL(−) group could progress until the hospital visit. We therefore investigated the correlation between each virulence factor and FOR for only the CL-related group ($n = 18$). As shown in Figure 2, proteolytic activity and swarming motility were positively correlated with FOR (Spearman's correlation coefficient: $r = 0.6326$, $P = 0.0048$; $r = 0.7069$, $P = 0.0010$, respectively). There was no correlation between the other virulence factors and FOR. The FOR for the *exoS*+/*exoU*− genotype group significantly outperformed the *exoS*−/*exoU*+ genotype group ($P < 0.05$), signifying that the focus size was larger in keratitis caused by invasive strains. Furthermore, correlations between each clinical manifestation and virulence factor, or among virulence factors or clinical manifestations, were analyzed using multivariate analysis. Table 3 shows combinations that were statistically significant. The associations between FOR and factors were as follows: age, presence

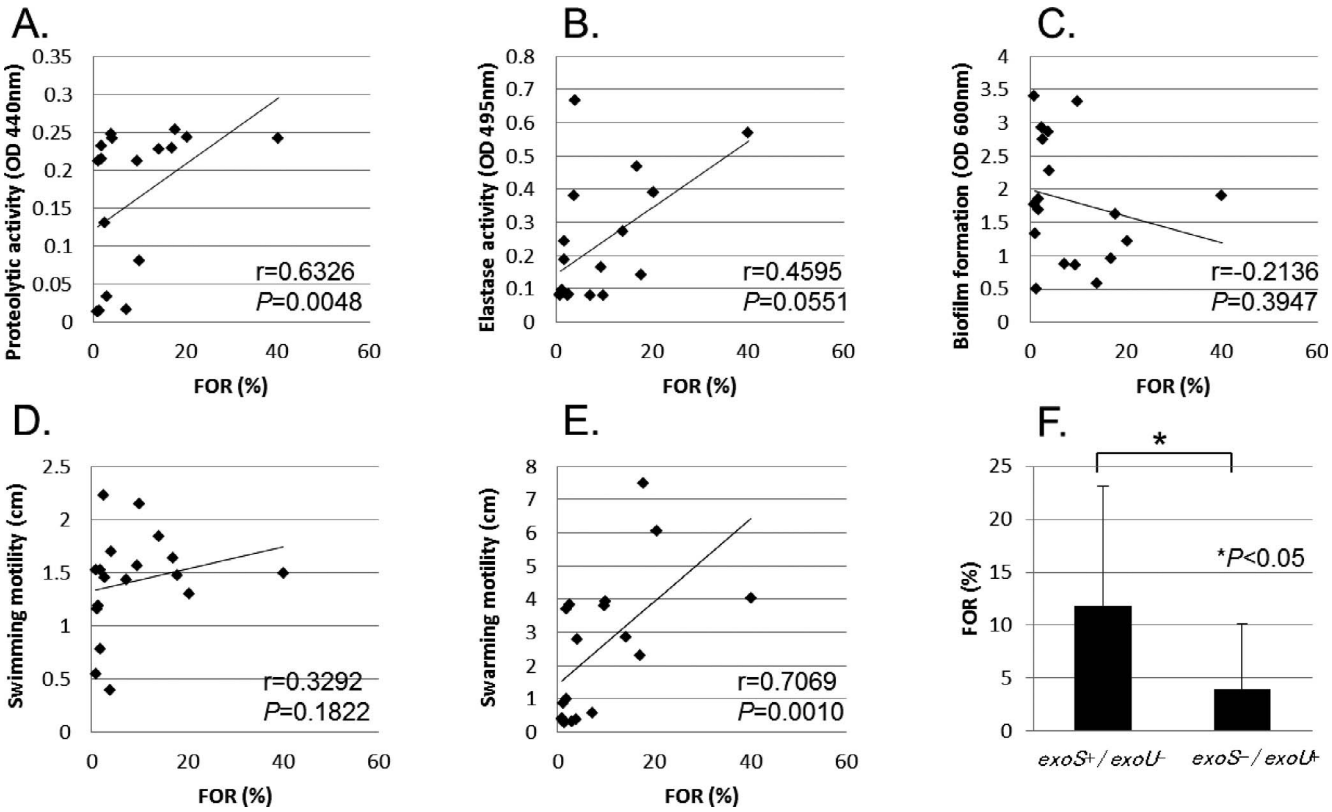


**Figure 2.** Correlation between virulence factor and FOR in CL-related keratitis cases. (**A**) Proteolytic activity, (**B**) elastase activity, (**C**) biofilm formation, (**D**) swimming motility, (**E**) swarming motility, and (**F**) FOR between the *exoS*+/*exoU* group and the *exoS*−/*exoU*+ group. (**A**–**E**) *r* and *P*, Spearman's correlation coefficient, and (**F**) *P*, Student's *t*-test are shown in each graph. A positive correlation was present in proteolytic activity, swarming motility, and *exoS* gene positivity.

**Table 3.** Factors With Statistically Significant Correlations Using Multivariate Analysis

| Covariate | vs. Covariate | Correlation Coefficient | 95% Confidence Interval | *P* Value |
|---|---|---|---|---|
| FOR | Age | 0.477 | 0.013 to 0.7719 | 0.0453 |
| | Ring abscess | 0.6933 | 0.3348 to 0.8765 | 0.0014 |
| | Hypopyon | 0.6402 | 0.2473 to 0.8523 | 0.0042 |
| | Elastase | 0.5415 | 0.0999 to 0.8049 | 0.0203 |
| | Swarming | 0.5874 | 0.1661 to 0.8274 | 0.0104 |
| Ring abscess | Swarming | 0.6014 | 0.187 to 0.8341 | 0.0083 |
| | TTSS (*exoS* = 0, *exoU* = 1) | −0.5698 | −0.8188 to −0.1402 | 0.0136 |
| Hypopyon | Age | 0.5313 | 0.0857 to 0.7998 | 0.0233 |
| | Ring abscess | 0.7071 | 0.3586 to 0.8826 | 0.001 |
| | Protease | 0.5724 | 0.1441 to 0.8201 | 0.013 |
| | Elastase | 0.5299 | 0.0838 to 0.7991 | 0.0237 |
| | Swarming | 0.6767 | 0.3068 to 0.869 | 0.002 |
| Swarming | Protease | 0.5322 | 0.0869 to 0.8002 | 0.023 |
| | Cell surface hydrophobicity | 0.5006 | 0.044 to 0.7842 | 0.0343 |
| | Swimming | 0.4687 | 0.0024 to 0.7676 | 0.0497 |
| Elastase | Protease | 0.6522 | 0.2664 to 0.8578 | 0.0034 |
| TTSS, *exoS* = 0, *exoU* = 1 | Swimming | −0.6868 | −0.8736 to −0.3238 | 0.0016 |

of ring abscess or hypopyon, elastase activity, and swarming motility were significant. Swarming motility and prevalence of an *exoS*+/*exoU*− genotype were significantly associated with ring abscess. The presence of hypopyon correlated significantly with age, proteolytic activity, elastase, and swarming motility. Swarming motility was correlated with all clinical manifestations. Among virulence factors, swarming motility was associated with proteolytic activity, cell surface hydrophobicity, and swimming motility. Elastase activity was correlated with proteolytic activity, and prevalence of *exoS* was associated with swimming motility.

## Discussion

*Pseudomonas aeruginosa* keratitis is generally recognized as a microbial keratitis that exhibits ring abscess. However, little has been reported about the present occurrences of clinical manifestations, such as ring abscess and grading of keratitis severity. In the present study, clinical features of CL-related keratitis differed from non–CL-related keratitis. Non–CL-related keratitis had a larger focus, and had no ring abscess. The mean age of patients with non–CL-related keratitis was older, and days to visit to our hospital after complaining of symptoms were longer. In addition, the non–CL-related group had various predisposing symptoms, such as trauma and keratoplasty. Thus, host conditions such as age and duration of keratitis could influence clinical manifestations, and clinical manifestations of keratitis for non–CL-related keratitis could be progressed. The relationships between clinical manifestations of non–CL-related keratitis and each virulence factor could therefore be complicated. Contact lens–related keratitis showed various clinical manifestations. The focus shapes developed serrated infiltration in 44.4% of CL-related keratitis patients. Ishikawa et al.[5] reported *P. aeruginosa* keratitis cases that had serrated margins of the main focus resembling fungal keratitis. The ring abscess was found in half of patients with CL-related keratitis, and cases with ring abscess had a larger focus. Furthermore, we could classify CL-related keratitis with a ring abscess as severe cases, and could classify mild cases for patients without a ring abscess. Because days to visiting our hospital after complaining of symptoms between both groups were similar, clinical manifestations may be influenced by the virulence of causative agents, and not by keratitis duration.

In CL-related keratitis, isolates from cases with ring abscess had higher swimming or swarming motilities than cases without a ring abscess. Moreover, swarming motility was positively correlated with FOR in CL-related keratitis, and was correlated with the presence of ring abscess or hypopyon using multivariate analyses. These findings indicate the importance of swarming motility in the progress of *P. aeruginosa* keratitis. Fleiszig et al.[17] reported that *P. aeruginosa* flagellum was related to cellular adhesion and invasion in corneal epithelial cells, therefore motility could be related to pathogenesis of *P. aeruginosa* keratitis. There have been no reports describing the relationship between the motility of isolates and clinical severity. Swarming motility is defined as a movement of *P. aeruginosa* flagella on semisolid surfaces.[28] During the course of infection, *P. aeruginosa* adhere on surfaces of tissue, then invade into the tissue to facilitate spread of bacteria. Therefore, if the strain has higher motility, the focus size may be larger in cases of *P. aeruginosa* keratitis. Swarming is also considered to be an early transition step before biofilm formation.[31] In addition, studies have reported that nutritional condition, viscosity, and rhamnolipids contribute to swarming motility.[31,36] *Pseudomonas aeruginosa* cells may adjust to make an environment more favorable, then exert their virulence. The role of flagella involves not only movement for adhesion on corneal epithelium, but may also involve activation of immunity. A protein that constitutes a flagellum is flagellin. This protein is a ligand-activating toll-like receptor 5, which mediates innate immunity.[18,21,37] The recruitment of neutrophil by innate immunity is very important to exclude *P. aeruginosa*; however, the inflammation can cause corneal opacity.[16] Thus, the motility or condition of the flagella could determine clinical features of *P. aeruginosa* keratitis.

In the present study, invasive strains with the *exoS* gene were found in a greater number of isolates from severe cases of keratitis in CL-related keratitis. These strains could induce larger focus areas. Invasive strains producing ExoS encoded by the *exoS* gene can invade corneal epithelial cells.[13] In contrast, cytotoxicity strains producing ExoU encoded by the *exoU* gene can kill host phagocytic cells.[13,16] Angus et al.[38] reported a role for ExoS of *P. aeruginosa* in survival of the corneal epithelium. In clinical cases, Borkar et al.[39] investigated 101 clinical cases of *P. aeruginosa* keratitis from the Steroids for Corneal Ulcers Trial, involving subtyping invasive or cytotoxic strains. They reported that infiltrate sizes in cases caused by invasive strains were larger than cases caused by cytotoxic strains. Furthermore, the improvement of visual acuity for the invasive strains

group was significantly less than the cytotoxic strains group at 3 months after treatment. However, this report included small cases of CL-related keratitis. Recently, Shen et al.[35] also reported an association between clinical features and genotypes of *P. aeruginosa* keratitis. Consistent with the results of the present study, they reported poor prognoses for the final best-corrected visual acuity with invasive strains. The CL-related keratitis cases with invasive strains could therefore develop more severe keratitis than cases resulting from cytotoxic strains. We previously reported that CL-keratitis isolates with *exoU*+ were found to be clustered by multilocus sequence typing analysis.[40] In addition, Shankar et al.[41] reported that strains with certain genetic backgrounds might adapt to survival in environmental water. Several other studies also reported a relationship between *exoU* expression and resistance of *P. aeruginosa* to CL disinfectants, and to multiple antimicrobials.[35,42,43] Thus, *P. aeruginosa exoU*+ strains could survive CL disinfectants and cause keratitis. Together with results of the present study, CL-related keratitis might include mild clinical manifestations related to cytotoxic strains.

Proteolytic and elastase activity are positively correlated with FOR and the presence of hypopyon. Previous studies showed that proteases contribute directly to corneal tissue damage, and *P. aeruginosa* elastase activates corneal matrix metalloprotease-2 to induce in vitro corneal ulceration.[44,45] Moreover, protease IV and *P. aeruginosa* small protease may degrade complement or antimicrobial peptides, and thus escape from host defenses.[46] Lomholt et al.[14] reported that many *P. aeruginosa* keratitis isolates, among the isolates from patients or from the environment, exhibit high elastase and alkaline protease activities. In the present study, we demonstrated a correlation between the clinical severity of *P. aeruginosa* keratitis and protease/elastase activity. Therefore, protease and elastase contribute to the pathogenesis and the severity of keratitis.

Using multivariate analyses, there were correlations among several virulence factors (e.g., swarming motility and protease activity, prevalence of the *ExoS* gene, and swimming motility). Thus, convoluted interactions between each virulence factor may exist. In the present study, it is noteworthy that swarming motility contributes to keratitis severity. Because of swarming, *P. aeruginosa* could also increase the expressions of other virulence factors, and complicated interactions among virulence factors could be possible. It is not clear which virulence factors are predominant, or how they interact in *P. aeruginosa* keratitis. However we should consider the influence of other factors, including patients' age, type of CL, and the condition of handling CL. Because these factors may affect the outcome, we need further investigation using large numbers of isolates. Although the number of patients in each group was limited in the present study, we were able to elucidate some of the relationships between virulence factors and the clinical features of *P. aeruginosa* keratitis by taking an appropriate step of the statistical processing.

In conclusion, the present study provides new insights into the mechanisms of *P. aeruginosa* keratitis. Proteolytic activity, TTSS ExoS, elastase activity, and especially swarming motility could play significant roles in the determination of clinical features in this disorder. To further clarify the mechanisms of *P. aeruginosa* keratitis, further studies of the swarming motility are warranted.

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