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Pathogenic Phenotype and Genotype of *Pseudomonas aeruginosa* Isolates from Spontaneous Canine Ocular Infections

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Abstract

Purpose—This study was designed to determine whether the ability to adversely affect corneal epithelial cell health is a factor common to *Pseudomonas aeruginosa* keratitis strains and to assess the prevalence of each pathogenic phenotype and genotype in a canine model of naturally-acquired *P. aeruginosa* ocular infection.

Methods—*P. aeruginosa* ocular isolates were collected by sampling 100 dogs without disease (six isolates collected) and by sampling dogs with conjunctivitis (two isolates), endophthalmitis (one isolate), active keratitis (12 isolates), and resolved *P. aeruginosa* keratitis (four isolates). Phenotype was determined in vitro by quantifying corneal epithelial cell invasion by gentamicin survival assays, and cytotoxic activity by Trypan blue exclusion assays. Genotyping was performed for genes encoding the type III secreted effectors.

Results—The ratio of invasive to cytotoxic strains with 95% confidence intervals (CI) was 0.83 (CI, 0.42–0.99) for conjunctival microflora isolates, 0.80 (CI, 0.54 – 0.94) for ocular infection isolates, and 1.0 (CI, 0.45–1.0) for strains isolated post-resolution of keratitis. Among ocular infection isolates, invasive and cytotoxic strains were significantly ($P \leq 0.02$) associated with older and younger dogs, respectively. Visible adverse effects on epithelial cells were significantly ($P \leq 0.03$) more frequent for keratitis strains (6/12) than other strains (1/13), but only three of these keratitis strains and the single non-keratitis strain possessed ExoU.

Conclusions—Invasive strains predominated in the dogs of this study. Only keratitis strains had visible adverse effects on epithelial cells without overt cytotoxicity, suggesting virulence strategies affecting live corneal epithelial cell health are selected for among keratitis strains.

The Gram-negative bacillus *Pseudomonas aeruginosa* is a frequent cause of opportunistic ocular infection.¹ *P. aeruginosa* has evolved a multitude of diverse virulence mechanisms and factors that permit efficient colonization of compromised ocular tissues and subsequent destructive disease.^{2–5} A diverse array of ocular and adnexal lesions are attributable to *P. aeruginosa* infection, including blepharitis, conjunctivitis, dacryocystitis, keratitis, scleritis, chorioretinitis, endophthalmitis, and orbital cellulitis.^{6–10} Ulcerative keratitis associated with *P. aeruginosa* infection is characterized by extensive dissolution of the corneal stroma and rapid progression of clinical signs.^{11,12}

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Historically, *P. aeruginosa* has been regarded as an extra-cellular pathogen. More recently, however, it was reported that some strains of *P. aeruginosa* are capable of invading and residing within corneal epithelial cells.¹³ Intracellular multiplication assays confirm that bacteria remain viable and multiply within the invaded epithelial cells.¹⁴ Other studies reported that *P. aeruginosa* is capable of toxin-mediated killing of epithelial cells.¹⁵ When strains of *P. aeruginosa* that induced acute cytotoxicity were compared with strains that invade epithelial cells, a significant inverse correlation was found between these two properties.¹⁶ This demonstrated that there are two distinct phenotypes of *P. aeruginosa* corneal isolates, one that is acutely cytotoxic and one that can enter corneal epithelial cells and survive intracellularly without killing the host cell. Experimental infection of mouse corneas with *P. aeruginosa* isolates of known pathogenic phenotype produced distinct corneal pathologies.¹⁷ Infection with both cytotoxic and invasive strains produced severe keratitis; however, the predominant pathologic response with cytotoxic strains was corneal edema and the predominant response with invasive strains was corneal ulceration. A strain that was neither cytotoxic nor invasive produced minimal keratitis.

The invasive and cytotoxic phenotypes of *P. aeruginosa* correspond to distinct genotypes.¹⁸ The molecular basis of these phenotypic properties is bacterial effector proteins secreted into host cell cytoplasm by a type III secretion system and controlled by the transcriptional activator ExsA.¹⁹ Currently, four bacterial effector proteins have been identified: ExoS, ExoU, ExoT, and ExoY. Among human corneal isolates, both phenotypes have been associated with a classic genotype. *P. aeruginosa* invasive strains typically possess genes encoding for ExoS, ExoT, and ExoY, but lack the gene for ExoU.¹⁸ Cytotoxic strains typically possess genes for ExoU, ExoT, and ExoY, but lack the gene for ExoS.¹⁸ The effectors ExoS, ExoY, and ExoT inhibit invasion and ExoU confers cytotoxicity; therefore, the invasive phenotype is considered the default if bacterial effectors are not present.^{20–23}

The purpose of this study was to determine whether the ability to adversely affect corneal epithelial cell health was a factor common to *P. aeruginosa* ulcerative keratitis strains and to characterize the relationship between *P. aeruginosa* pathogenic phenotype and ocular disease in a canine model of naturally-acquired *P. aeruginosa* ocular infection. A specific objective of this study was to determine whether the same two broad classes of *P. aeruginosa* genotypes are associated with ocular infection in dogs, as has been demonstrated for humans. Exploring the similarities and differences between results of studies examining different *P. aeruginosa* strain types in spontaneous human infection, induced animal models of infection, and spontaneous animal infection models increases our knowledge of the validity of experimental animal models of ocular infection and their role in *P. aeruginosa* ocular disease research.

Materials and Methods

Animals and Microbiologic Sample Collection

All protocols were approved by the Animal Care and Use Committee of Cornell University and were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Conjunctival swabs were collected from 200 canine eyes (100 dogs) meeting the following inclusion criteria: no clinical or historical evidence of external ocular disease, no history of receiving systemic antimicrobials in the preceding 30 days, and no history of receiving topical ophthalmic medications in the preceding 30 days. Samples were collected from dogs representing 27 separate canine breeds. Slit lamp biomicroscopic examination (Kowa SL-14; Kowa Co., Tokyo, Japan) was performed for each eye before sample collection. Samples were collected before the instillation of any ophthalmic solutions. A separate rayon-tipped swab and culture transport system (BBL Culture-Swab Plus; Becton Dickinson, Sparks, MD) was used for each eye. The swab was gently brushed against the dorsal and ventral conjunctival fornices, avoiding contact with eyelid skin and cilia.

All dogs meeting inclusion criteria presented to the Cornell University Hospital for Animals during the 24-month study period, with naturally-acquired intra- or extraocular infection in which *P. aeruginosa* was isolated from ocular samples, were included in the study. Inclusion criteria for dogs with ocular infection included no history of receiving systemic or topical ophthalmic antimicrobials in the preceding 30 days. Samples were collected from dogs with ulcerative keratitis, conjunctivitis, and endophthalmitis by corneal scraping, conjunctival swab, or vitreous aspirate, respectively. Conjunctival swabs were also collected, as described for dogs without external ocular disease, from culture-confirmed *P. aeruginosa* ulcerative keratitis eyes 2 to 3 months after corneal ulcer resolution and the discontinuation of ophthalmic antimicrobials. Dogs examined at the Cornell University Hospital for Animals included both primary and referred cases; however, most ocular infections were either presented primarily or referred before medical intervention.

Clinical Data Collection

Signalment information, determined by physical examination and client supplied information, was recorded for each dog included in the infection group of the study. Complete ocular examination, including slit lamp biomicroscopy, indirect ophthalmoscopy (Heine EN20-01 indirect ophthalmoscope; Heine Optotechnik, Herrsching, Germany), fluorescein staining (Fluor-I-Strips; Schering-Plough Animal Health Co., Union, NJ), and Schirmer I tear testing (Schirmer tear test strips; Schering-Plough Animal Health Co.) were performed on each dog with ocular disease. For eyes with ulcerative keratitis, a corneal ulcer scoring system was used to quantify biomicroscopy findings. The following variables were scored for each eye: corneal ulcer area (defined as area of fluorescein retention relative to total corneal area): 0 = <25%, 1 = 26% to 50%, 2 = 51% to 75%, 3 = >76%; corneal ulcer depth (defined as deepest point of the ulceration relative to total corneal depth): 0 = < 25%, 1 = 26% to 50%, 2 = 51% to 75%, 3 = >76%; anterior chamber reaction: 0 = none, 1 = mild aqueous flare, 2 = moderate-to-marked aqueous flare, 3 = hypopyon; corneal edema area relative to total corneal area: 0 = <25%, 1 = 26% to 50%, 2 = 51% to 75%, 3 = >76%; corneal leukocyte infiltration: 0 = none, 1 = mild, 2 = moderate, 3 = severe; and keratomalacia: 0 = none, 1 = mild, 2 = moderate, 3 = severe.

Bacteria Identification

Direct cultures on solid media (trypticase soy agar with 5% sheep blood, chocolate agar, Levine eosin methylene blue agar, and Columbia colistin-nalidixic acid agar) and an enrichment broth (brain heart infusion broth) were performed for each sample. Direct cultures and enrichment subcultures were incubated at 37°C in 6% CO₂ and read at 24 and 48 hours. All microorganism identification and antimicrobial susceptibility determinations were performed with an automated system (Sensititre; Trek Diagnostic Systemic Inc, Cleveland, OH).

Preparation of Bacteria

Bacteria were grown on trypticase soy agar plates overnight at 37°C. Immediately before infection, bacteria were suspended in minimal essential Eagle medium (50% [vol/vol]) in Hams F-12 (BioWhittaker, Walkersville, MD) tissue culture medium (EMEM/Hams F-12) to a final approximate concentration of 5×10^6 CFU/mL. *P. aeruginosa* clinical isolates 6294 and 6206 were used as positive controls for invasion and cytotoxicity assays, respectively.

Invasion and Cytotoxicity Assays

Rabbit corneal epithelial cells (RCEs) were grown in SHEM medium as previously described¹⁶ and seeded onto 96-well flat-bottom microtiter plates until cells reached 50% to 75% confluency. At the time of infection, RCEs were washed with pre-warmed phosphate-buffered saline (PBS) and 100 μ L of pre-warmed diluted bacteria was added to each well in triplicates. EMEM/Hams F-12 medium was used as a negative control. Bacterial exposure

continued for 3 hours at 37°C, 5.0% CO₂. RCEs were washed with pre-warmed PBS and then treated with 100 µL of gentamicin (0.4% [vol/vol]; 200 µg/mL; BioWhittaker) in EMEM/Hams F-12 medium for 1 hour. After another PBS wash, 100 µL of Trypan blue (10% [vol/vol]) in Hams F-12 was added to the cells and incubation continued for 15 minutes. Trypan blue was removed and 100 µL of pre-warmed Hams F-12 medium was added to each well. Brightfield and phase contrast images were taken on an inverted microscope (Olympus IX-70; Olympus America Inc., Center Valley, PA) attached to a video camera (Optronics, Goleta, CA) at ×200 magnification.

To assay for bacterial invasion, the RCEs were lysed in Triton X-100 (0.25% [vol/vol]; LabChem Inc., Pittsburgh, PA) in PBS for 15 minutes. The cells were scraped vigorously to ensure complete lysis. Undiluted and 10⁻¹ dilutions of the lysates were plated in duplicates onto Mac-Conkey agar plates and incubated for 14 to 16 hours at 37°C. Isolates were considered cytotoxic if they exhibited Trypan blue staining with low invasion numbers, invasive if they exhibited low or no Trypan blue staining and high invasion numbers, and neither phenotype if they exhibited low or no Trypan blue staining and low invasion numbers.

Genotyping of *P. aeruginosa*

Genotyping of bacterial isolates was performed by the polymerase chain reaction (PCR) on target loci for exotoxins of the type III secretory system in *P. aeruginosa*. The following primers were used to amplify *P. aeruginosa* exotoxins: ExoS (sense, 5'-TCA GGT ACC CGG CAT TCA CTA CGC GG-3'; antisense, 5'-TCA CTG CAG GTT CGT GAC GTC TTT CT-3'), ExoT (sense, 5'-AAT CGC CGT CCA ACT GCA TGC G-3'; antisense, 5'-TGT TCG CCG AGG TAC TGC TC-3'), ExoU (sense, 5'-AGC GTT AGT GAC GTG CG-3'; antisense, 5'-GCG CAT GGC ATC GAG TAA CTG-3'), ExoY (sense, 5'-TCC AAG CTT ATG CGT ATC GAC GGT CA-3'; antisense, 5'-CGT ATC GAT CCG AGG GGG GTG TAT CT-3'). All PCR reactions were performed in 50 µL volumes containing 0.2 µM of each primer pair in 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl₂, 200 µM of each dNTP, 0.1% TritonX-100, 0.5 U *Taq* Polymerase (Promega, Madison, WI) and with approximately 10⁵ bacteria. All reactions except for ExoU were performed at 1 cycle at 94°C for 5 minutes, 30 cycles at 94°C for 1 minute, 58°C for 1 minute, 72°C for 1 minute, and 1 cycle at 72°C for 5 minutes. ExoU reactions were performed at an annealing temperature of 55°C. All amplified targets were resolved on 1.2% agarose at 10 V/cm for 30 minutes to give product sizes of 565bp (ExoS), 956bp (ExoT), 1572bp (ExoU), and 749bp (ExoY).

Statistical Analysis

The distribution of *P. aeruginosa* invasive and cytotoxic phenotype isolates was compared between dogs with ocular infections and dogs without clinical evidence of extraocular disease using the Fisher's exact test. The distribution of phenotypes for dogs with all types of ocular infections together and dogs with ulcerative keratitis specifically were analyzed separately. For signalment and clinical data analysis, dogs were placed into two groups: dogs with *P. aeruginosa* invasive phenotype isolates and dogs with *P. aeruginosa* cytotoxic phenotype isolates. Continuous variables (i.e., age and corneal ulcer scores) were compared with the Wilcoxon rank sum test. Categorical variables (i.e., sex and skull conformation) were compared between the groups using the Fisher's exact test. For the purpose of analysis, dog breeds were grouped by skull conformation (i.e., brachycephalic versus mesatacephalic/dolichocephalic). Data analyses were performed separately for all dogs with ocular infections and dogs with ulcerative keratitis specifically. The occurrence of in vitro adverse effects on corneal epithelial cell health as observed with light microscopy was compared between keratitis stains and non-keratitis strain using the Fisher's exact test. Statistical significance was defined by $P \leq 0.05$ for all comparisons.

Results

P. aeruginosa was isolated from conjunctival samples of six eyes (3.0%) sampled without clinical evidence of ocular disease. *P. aeruginosa* was isolated from 15 dogs with ocular infections, including 12 eyes with ulcerative keratitis, two eyes with bacterial conjunctivitis associated with keratoconjunctivitis sicca, and one eye with exogenous infectious endophthalmitis resulting from penetrating ocular trauma. Eight of the dogs with *P. aeruginosa* ulcerative keratitis were available for repeat sampling 2 to 3 months post-resolution of the corneal lesion and *P. aeruginosa* was again isolated from four of these eyes. These *P. aeruginosa* isolates differed by two to six in vitro antimicrobial susceptibilities from their respective initial isolate, but all were susceptible to the antimicrobial used during treatment of the original infection. All dogs with ulcerative keratitis were successfully treated (i.e., globe and vision preservation) with topical ciprofloxacin 0.3% ophthalmic solution administered with ($n = 6$) or without ($n = 6$) additional surgical procedures (e.g., lamellar keratoplasty, corneconjunctival transposition, or conjunctival pedicle graft). Selected in vitro antimicrobial susceptibility determinations for all 25 isolates are listed in Table 1.

P. aeruginosa isolates from the conjunctival flora of dogs without clinical extraocular disease were phenotypically invasive ($n = 5$) and cytotoxic ($n = 1$; Table 2). Isolates from dogs with ocular infections were phenotypically invasive ($n = 12$), cytotoxic ($n = 2$), or displayed neither invasiveness nor cytotoxicity ($n = 1$). The ratio of the prevalence of invasive to cytotoxic strains with 95% confidence intervals (CI) was 0.83 (CI, 0.42– 0.99) for normal conjunctival flora isolates and 0.80 (CI, 0.54 – 0.94) for ocular infection isolates. There was no significant difference in the distribution of each phenotype between dogs with or without ocular infections. All *P. aeruginosa* isolates from the conjunctival flora of dogs post-resolution of ulcerative keratitis were invasive ($n = 4$), but only two of these isolates shared the same phenotype and genotype as the original isolate.

The genotype of 21 isolates was congruent with the classic genotype-phenotype pattern reported for human corneal isolates (Table 2). Discordance between phenotype and the classic genotype was identified in four isolates, including one normal conjunctival microflora strain, one conjunctivitis strain, and two ulcerative keratitis strains. The conjunctival microflora strain was phenotypically cytotoxic, but the genotype was unusual as it encoded all four known effectors. The conjunctivitis strain displayed neither phenotype, but a classic invasive genotype. The ulcerative keratitis strains were both phenotypically invasive, but unusual because one had a cytotoxic genotype without ExoY and one encoded none of the type III secretion effectors.

Microscopically visible adverse effects on corneal epithelial cell health (e.g., cell rounding, sloughing from plates, loss) were observed significantly ($P \leq 0.03$) more frequently for keratitis strains (6/12) than other strains (1/13; Fig. 1). Of the six keratitis strains adversely affecting cell health, three were invasive, two were cytotoxic, and one was neither genotype (lacked all four known effectors). The one non-keratitis strain affecting cell health was cytotoxic, but unusual in that it encoded all four known effectors. Therefore, only keratitis strains had visible adverse effects on live host cells in the absence of overt cytotoxicity (4/12 keratitis strains vs. 0/13 non-keratitis strains).

Other notable findings included that all normal conjunctival microflora strains encoded ExoS, including an ExoU+ strain that was phenotypically cytotoxic. Of the keratitis strains negatively impacting cell health, two strains did not encode ExoU and were both phenotypically and genotypically invasive. The three strains with atypical genotypes had visible adverse effects on corneal epithelial cell health. All strains isolated from ocular infections had at least one of

the following effects on corneal epithelial cells: invasion, cytotoxicity, or the induction of abnormal morphology.

The clinical appearance of corneal lesions in dogs with ulcerative keratitis was highly variable; however, all corneal ulcerations were unilateral, severe, and rapidly progressive (Fig. 2). In all dogs sampled, clinical findings were typical for canine *P. aeruginosa* ulcerative keratitis.¹² No significant differences were found between dogs infected with each phenotype and the clinical biomicroscopic corneal ulcer scoring system characteristics evaluated (data not shown). Dogs with ocular infections included nine brachycephalic and six mesaticephalic/dolichocephalic skull types. Breeds represented included Shih Tzu ($n = 5$ dogs), Pekingese ($n = 3$), Jack Russell Terrier ($n = 2$), mixed breed ($n = 2$), Pug ($n = 1$), American Cocker Spaniel ($n = 1$), and Miniature Schnauzer ($n = 1$). There were four spayed females, seven intact females, two castrated males, and two intact males. There were no significant differences for sex and skull type between dogs with invasive and cytotoxic isolates. Preexisting ocular disease identified during ophthalmological examination in dogs with ulcerative keratitis included keratoconjunctivitis sicca ($n = 4$ dogs), medial canthal trichiasis ($n = 2$), facial nerve paralysis ($n = 1$), and pigmentary keratitis ($n = 1$).

For dogs with all types of ocular infections, the median age was 65 months (range: 14 to 180) in dogs with the invasive phenotype and the median age was 7 months (all dogs 7 months old) in dogs with the cytotoxic phenotype. This difference in dog ages between the invasive and cytotoxic phenotype groups for dogs with all types of ocular infections was statistically significant ($P \leq 0.02$). For dogs with ulcerative keratitis specifically, the median age for dogs with the invasive phenotype was 61.5 months (range, 1–180) and the median age was 7 months (all dogs 7 months old) for dogs with the cytotoxic phenotype. This difference in dog ages between the invasive and cytotoxic phenotype ulcerative keratitis groups was statistically significant ($P \leq 0.03$).

Discussion

In contrast to other animal models requiring experimental infection induction,^{24–26} the dogs of this report are a naturally-occurring *P. aeruginosa* ulcerative keratitis model. This large-animal model of infection, occurring without experimental corneal wounding or bacterial inoculation, may more closely replicate conditions in natural human *P. aeruginosa* ulcerative keratitis. *P. aeruginosa* corneal infections occur spontaneously, subsequent to compromise of anatomic or immunologic corneal protective barriers, with a high frequency in dogs and produce clinical lesions similar to those observed in humans.^{27–29}

The invasive phenotype of *P. aeruginosa* predominated in the dogs of this study, accounting for 80% of isolates from dogs with ocular infections and 84% of all isolates. The ability to invade and replicate within epithelial cells may protect invasive strains from host immune responses and certain antimicrobials while still stimulating release of inflammatory mediators and inducing tissue damage.¹⁴ Invasive strains of *P. aeruginosa* may also directly kill epithelial cells after prolonged incubation in a bacterial density-dependent manner.³⁰ Aminoglycosides, and other poorly cell-permeable antimicrobials, may be suboptimal therapeutics against invasive strains of *P. aeruginosa*.^{31–33} A murine model of *P. aeruginosa* keratitis has confirmed tobramycin, a non cell-permeable antibiotic, is less effective at eradicating bacteria from corneas infected with invasive strains than ofloxacin, a cell-permeable antibiotic.³⁴ Furthermore, that study demonstrated several aminoglycosides and fluoroquinolones were capable of killing cytotoxic and invasive strains when not cell-associated, but only cell-permeable fluoroquinolones killed invasive strains within corneal epithelial cells.

Invasive and cytotoxic *P. aeruginosa* strains were isolated in approximately equal proportion from human clinical cases of ulcerative keratitis.³⁵ A significant relationship was present between *P. aeruginosa* phenotype and patient age, with cytotoxic strains associated with patients <50 years of age and invasive strains associated with patients >50 years of age.³⁵ The authors of that report suggested impairment of immune function with age might reduce the host's ability to respond to intracellular pathogens, increasing the prevalence of invasive strains in older individuals. Similar to humans with *P. aeruginosa* ulcerative keratitis, a significant correlation between age and phenotype was found in the ulcerative keratitis group of the present study, with invasive strains associated with older dogs and cytotoxic strains with younger dogs. This finding may reflect age-related immunologic changes similar to those proposed for humans. The canine immune system undergoes a process of age-related compromise in function, with declines in leukocyte proliferation, mitogen stimulation, chemotaxis, phagocytosis, and various other immunologic parameters.^{36–38} These age-associated changes in immunity are similar between dogs and humans.^{39,40} Age-related changes of polymorphonuclear cell function impair *P. aeruginosa* phagocytosis, delay corneal clearance, and increase corneal bacterial loads.^{41–43}

The prevalence of *P. aeruginosa* isolation from dogs without clinically apparent extraocular disease in this study was comparable to previous reports in which *P. aeruginosa* was a consistent, but relatively infrequent, inhabitant of the canine extraocular microflora and was isolated from 1% to 14% of eyes.^{44–50} The relative importance of conjunctival versus environmental sources of *P. aeruginosa* during initiation of canine ocular infections is unknown, but the similar prevalence of invasive and cytotoxic strains among extraocular microflora and infection isolates suggests that the conjunctival flora may be an important bacterial reservoir and both phenotypes are capable of inducing opportunistic ocular infection during appropriate circumstances. The consistent presence of *P. aeruginosa* in the normal conjunctival microflora of dogs contrasts with humans where *P. aeruginosa* is most often reported to be a rare and sporadic isolate in the absence of predisposing extraocular disease.^{51–53} It is partially on the basis of these reports that, in humans, *P. aeruginosa* ocular infections are frequently believed to originate from environmental bacterial sources.^{54–56} In disagreement with most studies of the human extraocular microflora; however, sporadic large human conjunctival microflora surveys have isolated *P. aeruginosa* from 5.8% to 6.7% of eyes; a prevalence similar to that reported for dogs.^{57,58} Among individuals or populations where *P. aeruginosa* is present in the extraocular microflora, the importance of this endogenous source of bacteria in the pathogenesis of ocular infections and the predisposition toward infection resultant from its presence is currently unknown.

P. aeruginosa was isolated from the conjunctival flora of 50% of dogs sampled post-resolution of ulcerative keratitis. This frequency is notably higher than the 3% prevalence of *P. aeruginosa* within the conjunctival flora of dogs without ocular disease. All isolates collected from dogs after keratitis resolution had different in vitro antimicrobial susceptibility profiles, and only 50% shared the same phenotype and genotype as the original corneal isolate. It is unknown whether *P. aeruginosa* was present in the conjunctival flora before development of ulcerative keratitis; however, these results suggest that some dogs may be prone to the chronic presence of *P. aeruginosa* in the extraocular microflora. It does not appear that the same bacterial strain is responsible for this presence, but that bacterial mutation or, more likely, conjunctival recolonization from environmental sources or other endogenous flora is occurring. The reasons for this recolonization are currently unclear, but could include a variety of bacterial, environmental, or host factors.

Discordance between phenotype and the classic genotype was identified in four canine isolates. This may have resulted from production of effectors that were not secreted into host cells or activity of unidentified effectors modulating invasion and cytotoxicity. Only ulcerative

keratitis strains had visible adverse effects on corneal epithelial cell health in the absence of overt cytotoxicity, suggesting that virulence strategies that affect live epithelial cells are selected for among ulcerative keratitis strains. The results of this study further suggest *P. aeruginosa* possesses virulence factors, other than invasiveness and cytotoxicity, which impact cell health. These virulence factors were not identified in the present study, but may include one or more specific proteases or exoenzymes. Study of the canine model of spontaneous *P. aeruginosa* ocular infection has the potential to advance the understanding of both human and animal infection pathogenesis, improve therapeutic and management approaches for human infection, and contribute to the development of animal models that more closely resemble spontaneous human infection.

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Figure 1.

Brightfield (BF) and phase contrast (PC) photomicrographs of Trypan blue stained rabbit corneal epithelial cell cultures infected with *Pseudomonas aeruginosa*. (A–N) Clinical isolates adversely affecting epithelial cell health in vitro. Epithelial cell rounding, sloughing from plates, and loss is visible. (A, B) Cytotoxic ulcerative keratitis strain #5; (C, D) cytotoxic ulcerative keratitis strain #6; (E, F) cytotoxic normal conjunctival flora strain #25; (G, H) invasive ulcerative keratitis strain #7; (I, J) invasive ulcerative keratitis strain #10; (K, L) invasive ulcerative keratitis strain #11; (M, N) invasive ulcerative keratitis strain #12. (O, P) Standard cytotoxic strain 6206. (Q, R) Standard invasive strain 6294. (S, T) Media control without bacteria. Original magnification, $\times 200$. Strain numbers correspond with numbers in Table 2.

Figure 2. Representative external photographs of eight dogs with spontaneous, naturally-acquired *Pseudomonas aeruginosa* ulcerative keratitis. (A–F) Dogs infected with invasive phenotype strains; (G, H) dogs infected with cytotoxic phenotype strains.

Table 1In Vitro Antimicrobial Susceptibly Results for 25 Canine Ocular *Pseudomonas aeruginosa* Isolates

Antimicrobial	MIC ($\mu\text{g/ml}$)	Susceptible (No. Isolates)	Intermediate (No. Isolates)	Susceptible or Intermediate (% Isolates)
Amikacin	≤ 32	25	0	100
Amoxicillin/clavulanic acid	> 32	0	0	0
Ampicillin	> 16	0	0	0
Bacitracin	≤ 2	3	0	12
Cefazolin	> 16	0	0	0
Cefoxitin	> 16	0	0	0
Cefpodoxime	> 16	0	0	0
Cephalothin	> 16	0	0	0
Ciprofloxacin	≤ 2	23	1	96
Clindamycin	> 2	0	0	0
Chloramphenicol	≤ 16	0	6	24
Enrofloxacin	≤ 1	21	4	100
Erythromycin	< 4	5	0	20
Gentamicin	≤ 8	25	0	100
Imipenem	≤ 8	23	2	100
Marbofloxacin	≤ 0.25	25	0	100
Neomycin	≤ 8	24	0	96
Orbifloxacin	≤ 4	19	6	100
Oxytetracycline	≤ 8	5	1	24
Tetracycline	≤ 8	1	5	24
Ticarcillin	≤ 64	25	0	100
Ticarcillin/clavulanic acid	≤ 32	25	0	100
Tobramycin	≤ 8	23	2	100
Trimethoprim/sulphamethoxazole	≤ 2	5	0	20

Table 2
Pathogenic Phenotype and Genotype Assay Results for 25 Canine Ocular *Pseudomonas aeruginosa* Isolates

Isolate	Lesion/Site	ExoS	ExoU	ExoT	ExoY	Trypan Blue *	Invasion Assay †	Visible Adverse Effects on Epithelial Cell Health
1	Ulcerative keratitis	+	-	+	+	-	37.5%	Absent
2	Ulcerative keratitis	+	-	+	+	-	77.3%	Absent
3	Ulcerative keratitis	+	-	+	+	-	62.5%	Absent
4	Ulcerative keratitis	+	-	+	+	-	16.9%	Absent
5	Ulcerative keratitis	-	+	+	+	++	12.7%	Present
6	Ulcerative keratitis	-	+	+	+	++++	0.04%	Present
7	Ulcerative keratitis	+	-	+	+	-	90.8%	Present
8	Ulcerative keratitis	+	-	+	+	-	68.2%	Absent
9	Ulcerative keratitis	+	-	+	+	-	31.2%	Absent
10	Ulcerative keratitis	+	-	+	+	-	37.7%	Present
11	Ulcerative keratitis	-	+	+	-	-	TNTC	Present
12	Ulcerative keratitis	-	-	-	-	-	2.8%	Present
13	Resolved ulcerative keratitis	+	-	+	+	-	2.3%	Absent
14	Resolved ulcerative keratitis	+	-	+	+	-	80.1%	Absent
15	Resolved ulcerative keratitis	+	-	+	+	-	30.3%	Absent
16	Resolved ulcerative keratitis	+	-	+	+	-	31.2%	Absent
17	Conjunctivitis	+	-	+	+	-	6.5%	Absent
18	Conjunctivitis	+	-	+	+	-	1.6%	Absent
19	Endophthalmitis	+	-	+	+	-	62.8%	Absent
20	Normal conjunctival flora	+	-	+	+	-	4.0%	Absent
21	Normal conjunctival flora	+	-	+	+	-	5.0%	Absent
22	Normal conjunctival flora	+	-	+	+	-	6.3%	Absent
23	Normal conjunctival flora	+	-	+	+	-	11.5%	Absent
24	Normal conjunctival flora	+	-	+	+	-	50.9%	Absent
25	Normal conjunctival flora	+	+	+	+	++++	0.2%	Present
6206	Standard cytotoxic isolate	-	+	+	+	+++	1.3%	Absent
6294	Standard invasive isolate	+	-	+	+	-	100%	Absent

* Trypan Blue cytotoxicity assay results presented as cytotoxicity scores assigned by estimating the percentage of corneal epithelial cells that retained stain (-, 0%; +, <1%; ++, 1% to 5%; +++, 5% to 20%; +++, >20%), normalized to the standard cytotoxic strain 6206.

† Invasion assay results presented as % of corneal epithelial cells invaded normalized to standard invasive strain 6294.