Association Between Cytotoxic and Invasive *Pseudomonas aeruginosa* and Clinical Outcomes in Bacterial Keratitis

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**Objectives:** To determine whether cytotoxic and invasive *Pseudomonas aeruginosa* strains differentially influence clinical presentation, outcomes, or therapeutic response in bacterial keratitis.

**Methods:** *Pseudomonas aeruginosa* isolates from the National Eye Institute–funded Steroids for Corneal Ulcers Trial were subtyped as cytotoxic or invasive strains. The main outcome measure compared between the 2 subtypes was change in visual acuity at 3 months using Huber robust regression, adjusting for topical corticosteroid treatment.

**Results:** Of 101 confirmed *P aeruginosa* isolates from the Steroids for Corneal Ulcers Trial, 74 had a classically cytotoxic or invasive genotype. While corneal ulcers caused by genotypically invasive *P aeruginosa* strains were associated at presentation with significantly better visual acuity than corneal ulcers caused by genotypically cytotoxic *P aeruginosa* strains when adjusting for the effect of ulcer location (*P* = .008), invasive ulcers had improved significantly less than cytotoxic ulcers at 3 months (0.35; 95% CI, 0.04-0.66 logMAR; *P* = .03 [3.5-line difference]). Compared with topical moxifloxacin alone, adjunctive treatment with topical corticosteroids was associated with significantly more improvement in visual acuity in the invasive subgroup (*P* = .04) but was associated with less improvement in visual acuity in the cytotoxic subgroup (*P* = .07).

**Conclusions:** Rational profiling of differentially expressed virulence determinants (eg, cytotoxicity and invasiveness for *P aeruginosa*) could be used as a tool for decision making in the management of infections to optimize outcomes.


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**PEUDEMONAS AERUGINOSA** is a significant cause of bacterial keratitis in the United States and South India, accounting for 8% to 29% and 11% to 48% of cases, respectively. However, not all *P aeruginosa* strains affect host cells in the same manner. Two important virulence determinants are invasiveness and cytotoxicity. Invasive strains encode exoS and can sequester themselves intracellularly, replicating and stimulating membrane bleb formation within host cells. Cytotoxic strains lack exoS and instead encode the acute cytotoxin exoU, which can quickly kill cells. Both ExoS and ExoU are effectors of the *P aeruginosa* type III secretion system. Effector proteins are injected into eukaryotic cells via the type III secretion system apparatus, activated within the targeted cell, and then trafficked to specific organelles, where they mediate various phenotypic changes that ultimately result in cell death.

Research in cultured cells and in mouse models has compared cytotoxic and invasive strains. Cell culture investigations show an inverse relationship between the capacity for cell killing and the capacity for invasiveness, while mouse models show different effects on pathology, immune responses, and response to antibiotics. Although some studies have shown differences in corneal ulcer presentation between these 2 subtypes of *P aeruginosa*, a larger study in humans investigating differences in functional outcomes and therapeutic response is lacking to date.

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The Steroids for Corneal Ulcers Trial (SCUT; trial registration: clinicaltrials.gov identifier:NCT00324168) was a National Eye Institute–funded, randomized, placebo-controlled trial investigating
the effect of topical corticosteroids as adjunctive treatment with antibiotics for bacterial keratitis. The use of topical corticosteroids for bacterial keratitis has been controversial, and before the SCUT, insufficient evidence was available to guide clinical practice. The SCUT found no overall benefit or harm with adjunctive topical corticosteroid treatment across all types of bacterial corneal ulcers, but the results suggested a benefit for patients with the most severe ulcers. The large sample size from the SCUT allows for further investigation by subgroup, including by organism type. The objective of this prespecified study was to determine whether the cytotoxic and invasive subtypes of *P. aeruginosa* have different clinical signs at baseline and result in a different response to therapy, which may allow for a more tailored treatment approach.

**METHODS**

In the SCUT, patients with culture-confirmed bacterial keratitis were randomized to receive adjunctive treatment either with topical prednisolone phosphate, 1%, or with placebo after 48 hours of topical moxifloxacin, 0.5%. Specific methods of the trial, including inclusion and exclusion criteria, as well as examination and treatment protocols, are described in detail elsewhere.26

Patients were evaluated at enrollment, at 3 weeks, and at 3 months by certified refractionists and ophthalmologists, who performed visual acuity and slitlamp examinations, respectively. Best spectacle-corrected visual acuity (BSCVA) was measured in logMAR units using a tumbling E chart at 4 m, with 0.1 logMAR being approximately 1 line of visual acuity. Slit-lamp examination was used to measure infiltrate/scar size, epithelial defect size, hypopyon size, and ulcer depth, if present, as well as to assess for ocular adverse events. Infiltrate/scar size and epithelial defect size were evaluated to the nearest 0.1 mm by taking the geometric mean of the longest diameter and the longest perpendicular to the first measurement. Reepithelialization was defined as the absence of an epithelial defect with administration of fluorescein. Ulcer depth was measured in thirds (0%-33%, >33%-67%, or >67%-100%). Hypopyon size was measured to the nearest 0.5 mm. Ulcer location was assessed using photographs with an artificial 4-mm pupil superimposed by cornea-specific software (Optscore; Dartmouth-Hitchcock Medical Center). Ulcer location was graded as completely central, partially central, or completely peripheral. Further details of how these assessments were obtained have been previously described.26

Informed consent was obtained for all participants enrolled in the SCUT. Institutional review board approval was granted by the Aravind Eye Care System (Madurai, Tamil Nadu, India) Institutional Review Board; by the University of California, San Francisco, Committee on Human Research; and by the Dartmouth-Hitchcock Medical Center (Lebanon, New Hampshire) Committee for Protection of Human Subjects.

**MICROBIOLOGY**

All corneal isolates from the SCUT with growth morphology and Gram stain characteristics consistent with *P. aeruginosa* were strain typed after confirming speciation by growth on cetrimide agar. Laboratory personnel (S.M.J.F., C.L., A.A.G., C.T., and W.Y.L.) were masked to clinical data (both baseline data and treatment outcomes) until strain typing was completed. Bacteria were grown on trypticase soy agar overnight, and rabbit corneal epithelial cells were exposed to bacterial strain suspensions. Corneal epithelial cells were washed and treated with gentamicin to kill extracellular bacteria. Isolates that were resistant to gentamicin were also treated with amikacin. Rabbit corneal epithelial cells were washed and lysed. Lysate was plated on MacConkey agar and incubated. Percentage invasiveness was determined by comparing growth from the intracellular lysate of each isolate to growth from *P. aeruginosa* clinical isolate 6294, which was used as a positive control. Because the assay for invasion depends on sensitivity to gentamicin or amikacin, percentage invasiveness was not measured for gentamicin-resistant and amikacin-resistant strains. However, *P. aeruginosa* speciation was confirmed for these strains with an analytical profile index kit (API 20E; bioMerieux, Inc).

Percentage cytotoxicity for each corneal isolate was determined by measuring the amount of lactate dehydrogenase released into the media by dead or dying host cells after bacterial exposure using a cytotoxicity detection kit (LDH Cytotoxicity Detection Kit; Roche Diagnostics). Values were compared with a positive control (*P. aeruginosa* clinical isolate 6206). In some experiments, invasive control 6294 had slightly lower lactate dehydrogenase values than the media (baseline) control. Negative values for percentage cytotoxicity corresponded to strains that were not cytotoxic and had negative lactate dehydrogenase values after the media control had been included in the calculation. Values greater than 1 corresponded to cytotoxicity or invasiveness greater than the respective positive control.

Each bacterial isolate was genotyped using polymerase chain reaction (PCR). The PCR was specifically performed on target loci for 4 effectors (exoU, exoS, exoT, and exoY) of the Type III secretion system for *P. aeruginosa*. Strains 6206 and 6294 were used as positive controls for exoU and exoS amplification, respectively. Both strains were used as positive controls for exoY and exoT amplification. Negative controls were also used for amplification of each effector protein sequence. Strains that were positive for exoU or exoS, but not both, were considered typical strains. Within this group, exoU exoS strains were classified as classically cytotoxic, while exoU exoS strains were classified as classically invasive on the basis of genotype. Strains that were positive or negative for both exoU and exoS were classified as atypical strains. More detailed methods for the cytotoxicity and invasion assays, as well as the PCR protocol with specific primers, are described elsewhere.24,27

**STATISTICAL ANALYSIS**

Univariate analyses for genotype were performed using Fisher exact test for categorical variables or 2-group mean comparison t test for continuous variables. Multivariate analyses were performed using Huber robust regression to assess the relationship between strain type (either genotype or phenotype) and clinical outcomes. In these models, strain genotype was used as a dichotomous predictor variable (either classically cytotoxic or classically invasive). Strains with atypical genotypes were not used. Phenotype was used as a continuous predictor variable, measured as percentage invasiveness or as percentage cytotoxicity. All confirmed isolates were used in phenotype analyses except when percentage invasiveness could not be measured. Baseline characteristics used were age, ulcer location, contact lens wear, hypopyon size, BSCVA at enrollment, ulcer depth at enrollment, infiltrate/scar size at enrollment, and epithelial defect size at presentation. Clinical outcomes were measured by change in BSCVA at 3 months. Ulcer location was added as a covariate in analyses involving BSCVA to control for possible confounding. In addition, treatment arm was added as a covariate to control for the possible effect of corticosteroid use on change in BSCVA at 3 months. An inter-
action term (treatment arm × strain genotype) was added to the model assessing genotype and clinical outcomes to test if a differential effect of corticosteroid use on visual acuity was present for cytotoxic vs invasive P aeruginosa ulcers. Analyses were performed using STATA version 11.0 (StataCorp LP). P values reported for all analyses are nominal values and have not been adjusted for multiple comparisons.

RESULTS

Of 500 patients in the SCUT enrolled between September 1, 2006, and February 22, 2010, a total of 111 corneal bacterial isolates were strain-typed based on growth morphology and Gram stain characteristics consistent with P aeruginosa. One hundred one of these isolates were confirmed P aeruginosa based on the description in the “Microbiology” subsection of the “Methods” section. Of 101 confirmed P aeruginosa isolates, 27 were determined to have atypical genotypes defined as exoU′/exoS′ or exoU′/exoS′. The remaining 74 isolates had typical genotypes; 56 were classically invasive, and 18 were classically cytotoxic. Percentage invasiveness, expressed as a decimal, ranged from 0.03 to 3.32. Percentage cytotoxicity ranged from −0.72 to 2.94. A significant difference was observed between the genotypically cytotoxic and invasive strains for percentage invasiveness and for percentage cytotoxicity (Figure 1).

Baseline demographic features and clinical examination findings were compared between the 2 genotype groups (Table 1). The mean infiltrate/scar sizes of 4.66 and 3.61 mm for invasive and cytotoxic isolates, respectively, were significantly different (P = .049). The difference in BSCVA at enrollment between the 2 genotype groups was not statistically significant (P = .80). Phenotypically, no significant association was observed between percentage cytotoxicity and BSCVA at enrollment (0.10; 95% CI, −0.18 to 0.38 logMAR; P = .47). However, a statistically significant correlation was found between percentage invasiveness and BSCVA at enrollment; regression analysis showed that 100% invasiveness was associated with an approximately 2.5-line better visual acuity at enrollment compared with 0% invasiveness (−0.26; 95% CI, −0.51 to −0.01 logMAR; P = .04).

When controlling for ulcer location, P aeruginosa ulcers caused by genotypically invasive strains were associated with significantly better visual acuity at enrollment (approximately 3.5 lines) than those caused by genotypically cytotoxic strains (−0.36; 95% CI, −0.63 to −0.10 logMAR; P = .008) (Table 2). Phenotypically, increasing percentage invasiveness was associated with significantly better visual acuity at enrollment when controlling for ulcer location. One hundred percent invasiveness was associated with an approximately 3-line better visual acuity at enrollment than 0% invasiveness (−0.32; 95% CI, −0.49 to −0.15 logMAR; P < .001). Increasing percentage cytotoxicity was associated with worse visual acuity at enrollment, however, this relationship was not significant (0.19; 95% CI, −0.002 to 0.38 logMAR; P = .52).

Change in BSCVA at 3 months was compared between patients with ulcers caused by cytotoxic vs invasive P aeruginosa strains. Phenotypically invasive strains were associated with an approximately 3.5-line less improvement in visual acuity compared with genotypically cytotoxic strains (0.33; 95% CI, 0.04-0.66 log-
MAR; *P* = .03). When controlling for the effect of ulcer location on change in BSCVA at 3 months, genotypically invasive strains were associated with an approximately 4-logMAR less invasive improvement in visual acuity compared with genotypically cytotoxic strains (0.40; 95% CI, 0.09-0.70 logMAR; *P* = .01) (Table 3). Sensitivity analyses demonstrated that treatment arm (corticosteroid or placebo) did not affect these results; the corticosteroid treatment difference, approximately 1 line, was not significant (−0.09; 95% CI, −0.36 to 0.17 logMAR; *P* = .49). However, an interaction term between treatment arm and strain genotype added to the model was significant (*P* = .005) (Table 4), suggesting a differential effect of corticosteroid use on ulcers caused by cytotoxic and invasive *P. aeruginosa* strains (Figure 2).

Analyses of phenotypic properties of each corneal isolate supported these findings. One hundred percent invasiveness was associated with an approximately 2-line smaller improvement in BSCVA at 3 months vs 0% invasiveness (0.22; 95% CI, 0.03-0.40 logMAR; *P* = .02). Similarly, 100% cytotoxicity was associated with an approximately 3-line greater improvement in BSCVA at 3 months compared with 0% cytotoxicity (−0.29; 95% CI, −0.50 to −0.08 logMAR; *P* = .007). Treatment arm was added to these models as a covariate but was not significant. However, an interaction term between percentage invasiveness and treatment arm was significant (−0.37; 95% CI, −0.744 to −0.004 logMAR; *P* = .047) (Table 4), supporting a differential effect of corticosteroid use on change in visual acuity as percentage invasiveness varied.

### COMMENT

Several studies8,9,10 have compared the effect of cytotoxicity and invasiveness of *P. aeruginosa* on the cornea in mouse models and have shown a difference in pathogenesis. The few studies20,21 performed in humans with...
The SCUT demonstrated no overall difference in visual acuity at 3 months with adjunctive topical corticosteroid treatment. Analysis of the data from all patients with confirmed P aeruginosa infection demonstrated the same result. In the current study, we found a statistically significant difference between the 2 treatment arms in the invasive P aeruginosa subgroup. On average, patients with invasive ulcers in the corticosteroid treatment arm had a 2.5-line greater improvement in BSCVA from enrollment to 3 months compared with the placebo treatment arm. Phenotypic analyses supported this finding; increasing percentage invasiveness was associated with a greater difference in visual acuity improvement between the corticosteroid and placebo arms.

Prior studies have shown that neutrophilic infiltration into the center of the cornea, not just damage caused by bacteria, is the hallmark of a pathologic response to invasive P aeruginosa corneal infection in mouse models. In contrast, cytotoxic strains suppress neutrophil infiltration as a direct result of exoU expression.

The improved outcomes we observed in the corticosteroid treatment arm of the invasive subgroup may reflect the local inhibition of polymorphonuclear leukocyte infiltration due to topical corticosteroid exposure. However, modification of other host responses may also have a role in the effect of topical corticosteroid use on P aeruginosa corneal ulcers. We found that patients with cytotoxic ulcers in the corticosteroid arm had an approximately 5.3-line less improvement in visual acuity at 3 months compared with those in the placebo arm; however, this result did not reach significance. A prior study showed delayed clearance of cytotoxic, but not invasive, P aeruginosa strains from the ocular surface in mice deficient in surfactant protein D, an immunologic protein present in the tear film. Systemic literature has shown

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**Table 4. Multivariate Analyses Predicting logMAR Change in BSCVA at 3 Months, Controlling for Treatment Arm**

<table>
<thead>
<tr>
<th>Covariate</th>
<th>Coefficient (SE)</th>
<th>95% CI</th>
<th>P Valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Invasive vs cytotoxic</td>
<td>0.850 (0.229)</td>
<td>0.392 to 1.309</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Corticosteroid vs placebo</td>
<td>0.594 (0.262)</td>
<td>0.070 to 1.119</td>
<td>.03</td>
</tr>
<tr>
<td>Treatment arm × strain genotype</td>
<td>−0.876 (0.298)</td>
<td>−1.472 to −0.280</td>
<td>.005</td>
</tr>
</tbody>
</table>

**Association Between Percentage Invasiveness and BSCVA at Enrollment (n = 96)c**

| Percentage invasiveness              | 0.378 (0.131)    | 0.116 to 0.639 | .005    |
| Corticosteroid vs placebo            | 0.315 (0.202)    | −0.086 to 0.716 | .12      |
| Percentage invasiveness × strain genotype interaction | −0.374 (0.186) | −0.744 to −0.004 | .047      |

**Association Between Percentage Cytotoxicity and BSCVA at Enrollment (n = 101)d**

| Percentage cytotoxicity             | −0.317 (0.107)   | −0.529 to −0.105 | .004    |
| Corticosteroid vs placebo            | −0.182 (0.109)   | −0.399 to 0.034  | .10      |

Abbreviation: BSCVA, best spectacle–corrected visual acuity.

a Obtained by Huber robust regression.
b Atypical genotypes were excluded.
c Five of the genotypically cytotoxic strains were gentamicin and amikacin resistant; therefore, percentage invasiveness was not measured for these 5 strains.
d All confirmed Pseudomonas aeruginosa isolates were included.

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Figure 2. Change in logMAR best spectacle–corrected visual acuity at 3 months by treatment arm (placebo vs corticosteroid) for each Pseudomonas aeruginosa strain type. The median for each group is represented by the line in the middle of each box. The interquartile range (the span of the 25th to 75th percentiles) is denoted by the lower to upper bounds of each box. The whiskers extend from the smallest visual acuity change within 1.5 times the interquartile range below the 25th percentile to the largest visual acuity change within 1.5 times the interquartile range above the 75th percentile. Individual data points denote values outside this range. P values shown were obtained by 2-group mean comparison t test. The P value for the interaction term between treatment arm and strain genotype was .005 and was obtained using Huber robust regression.
that inhaled corticosteroid use decreases serum surfactant protein D levels. This raises the question of whether there is a negative effect of topical corticosteroid use on immunologic proteins such as surfactant protein D that are important for clearance of cytotoxic P aeruginosa at the level of the ocular surface.

We examined differences in several other clinical characteristics between these 2 P aeruginosa subtypes. Specifically, we found that cytotoxic ulcers were associated at presentation with an approximately 1-mm smaller infiltrate size on average compared with invasive ulcers in our sample. While previous studies have found that ulcers caused by cytotoxic strains are more common in patients younger than 50 years, we did not find a significant difference in the age of patients with cytotoxic vs invasive ulcers. However, the young age of patients in our sample may have made it hard to assess the association between strain type and older age. In addition, a prior study found that corneal isolates from contact lens wearers were more likely to be cytotoxic. We were unable to observe such a relationship, but only 3 contact lens wearers were included in the study.

A few potential limitations of our study should be considered. This study focused on 2 specific pathogen virulence determinants of P aeruginosa, without considering differences in host factors or other pathogen virulence determinants that might also affect corneal ulcer healing. For instance, factors, such as tear film composition and the presence of specific surfactant proteins at the ocular surface, have been shown to affect the capacity to recover from P aeruginosa keratitis. In comparison, a study of 55 human corneal P aeruginosa isolates from Australia reported only 2 isolates with atypical genotypes. Moreover, previous studies have found an approximately even distribution between cytotoxic and invasive genotypes. In this study, only 18 of 74 corneal isolates with typical genotypes were cytotoxic. This difference in distribution may be attributed to the fact that cytotoxic strains have been reported more commonly among infections associated with contact lens wear, which was rare in the SCUT patient population. A notable finding is that the proportion of cytotoxic strains isolated from the SCUT study participants resembled the distribution found among canines with P aeruginosa keratitis. In addition, a large sample of clinical nonocular P aeruginosa isolates had a similar proportion of cytotoxic strains. While the small sample of cytotoxic strains could have affected our findings, particularly for subgroup analyses of the cytotoxic strains, the clinical data were collected prospectively in a standardized manner at set time points, increasing the ability to detect relationships.

The microbiological methods used in this study were performed in a basic science laboratory setting. However, the distinction between cytotoxic and invasive P aeruginosa genotypes can be made using PCR, which is inexpensive and is available in many clinical microbiology laboratory settings. In addition, the time needed to obtain PCR results may be acceptable given that corticosteroid treatment could be added after 48 hours of antibiotic treatment, as was done in the SCUT.

In summary, the results of this study show that P aeruginosa bacterial keratitis caused by genotypically invasive strains is associated at presentation with significantly better visual acuity than genotypically cytotoxic ulcers but had less improvement in visual acuity at 3 months. Our findings also reveal that adjunctive treatment with topical corticosteroids has a differential effect on cytotoxic and invasive ulcers. These results suggest the potential to guide management decisions using existing therapies such as corticosteroid treatment based on these specific virulence determinants. Additionally, they illustrate the concept that not all infections caused by pathogens of a single species present or respond to treatment similarly. Further studies to elucidate differences in clinical presentation and therapeutic response based on specific virulence determinants for other pathogens may be warranted.

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Author Contributions: Dr Acharya had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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