Assessment of Keratitis Severity Using Quantitative Image Analysis in an In Vivo Murine Model of *Staphylococcus aureus* Bacterial Keratitis

Tomas E. Meijome¹, Rachel Wozniak², Linda Kang¹, Lyna Azzouz¹, Leslie M. Niziol¹, William L. Johnson², Matthias Kriegel¹,³, and Maria A. Woodward¹

¹ Department of Ophthalmology and Visual Sciences, University of Michigan, Ann Arbor, MI, USA
² Department of Ophthalmology, University of Rochester School of Medicine and Dentistry, Rochester, NY, USA
³ Augenzentrum am St. Franziskus Hospital, Muenster, Germany

Correspondence: Maria A. Woodward, Department of Ophthalmology and Visual Sciences, W.K. Kellogg Eye Center, University of Michigan, Ann Arbor, Michigan, 1000 Wall Street, Ann Arbor, Michigan 48105, USA. e-mail: mariawoo@med.umich.edu

Received: May 15, 2022
Accepted: October 14, 2022
Published: November 16, 2022

Keywords: Cornea; Microbial keratitis; Image analysis

Citation: Meijome TE, Wozniak R, Kang L, Azzouz L, Niziol LM, Johnson WL, Kriegel M, Woodward MA. Assessment of keratitis severity using quantitative image analysis in an in vivo murine model of *Staphylococcus aureus* bacterial keratitis. Transl Vis Sci Technol. 2022;11(11):12, https://doi.org/10.1167/tvst.11.11.12

Purpose: Bacterial keratitis (BK) severity in murine models has traditionally been measured by subjective clinical grading or quantification of ocular bacterial burden. This investigation explores an objective and repeatable quantification of slit lamp photography (SLP) images to measure BK severity.

Methods: BALB/c strain mice underwent three parallel scratches of the right cornea with subsequent inoculation of $10^7$ *Staphylococcus aureus* cells. SLP imaging and clinical severity grading were performed at 48 hours post-infection. Stromal infiltrate (SI) area on SLP images were quantified. Bacterial burden was determined after enucleation and homogenization. Spearman rank correlations ($r_s$) were used to estimate associations between SI area, clinical severity grades, and bacterial burden.

Results: BALB/c strain mice ($n = 14$) were evaluated with an average SI area of $0.92 \text{ mm}^2$ (standard deviation, SD = 0.65) and average bacterial burden of $3.16 \times 10^5 \text{ colony forming units per milliliter (CFU/mL)}$ (SD = $8.3 \times 10^5$). Clinical severity grade positively correlated with SI area ($r_s = 0.59, p = 0.0276$) and bacterial burden ($r_s = 0.66, p = 0.0106$). There was a trend towards positive association between SI area and bacterial burden ($r_s = 0.51, p = 0.0543$).

Conclusions: SLP annotation of SI area is correlated with clinical severity and may provide an objective, quantitative, and repeatable assessment of BK disease severity.

Translational Relevance: SLP annotation of SI area is a novel quantitative method to evaluate bacterial keratitis severity longitudinally in mouse models which may be a powerful tool to better understand BK pathogenesis and response to treatments.

Introduction
Bacterial keratitis (BK) is an infection of the cornea that is responsible for nearly 2 million new cases of blindness annually worldwide.¹ *Staphylococcus aureus* (S. aureus) is among the most common causes in BK and a significant contributor to the worldwide burden of corneal vision loss and blindness.²,³ In well-established murine models of BK, disease severity has classically been evaluated using a subjective clinical grading score. Hazlett et al.⁴ described a clinical severity grading scheme of BK disease severity that involved macroscopic evaluation of the density of corneal opacity and amount of cornea occupied by the opacity. This grading system ranged from a grade of 0, meaning clear or slight opacity partially covering the pupil, to a grade of 4, meaning corneal perforation or phthisis. More recent studies have utilized similar severity grading schemes using slit lamp to evaluate the cornea at higher magnification. Hume et al.⁵ developed a more extensive 24-point clinical severity grading scheme using slit lamp to evaluate 6 parameters...
(exudate, epithelial defect, corneal infiltrate, corneal edema, conjunctival hyperemia, and chemosis).

These grading tools have provided a method to measure BK disease severity, helping to explore associations of clinical severity with the host and pathogen response. However, these grading systems rely on subjective measurement of BK severity and are likely subject to bias and inconsistency. There is limited data on inter-grader and intra-grader reliability of keratitis severity grading systems, though the concept of low grader reliability has been well established in other ocular diseases. This is likely due to the difficulty of having two graders available to grade at the same time (testing inter-grader reliability) or the practical limitations of one grader grading the mouse severity twice (intra-grader reliability).

Quantitative metrics to assess BK severity do exist. Determination of bacterial burden in ocular tissues is a quantitative method used as a surrogate for BK disease severity in some studies. However, not surprisingly, clinical severity does not always correlate with bacterial burden. Bacterial burden does not account for the host immune response nor organism virulence, which both influence keratitis severity.

Since current severity scores are subjective and bacterial burden is unique from the clinical endpoint of pathologic changes on the cornea, an opportunity exists to create quantitative measures of BK disease severity based on murine BK morphology. This investigation explores a component of such a model by quantifying the corneal stromal infiltrate (SI) using image annotation from slit lamp photography (SLP) images. Manual annotation of SLP images has previously shown to be a reliable method to quantify keratitis morphological features. By comparing SLP-quantified stromal infiltrate area to clinical grading scores and bacterial burden, we aim to explore the utility of quantitative SLP image analysis as an objective alternative to measure BK severity. A quantitative method utilizing SLP images may enhance murine keratitis models by improving reliability and precision and allowing severity grading to be evaluated longitudinally and compared across investigations.

**Methods**

**Murine Model of Bacterial Keratitis**

4 to 6-week-old female BALB/c mice were obtained from Charles River Laboratories (Washington, MA) and housed according to a protocol approved by the University of Rochester Council on Animal Research. This study adhered to guidelines outlined in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The mice were anesthetized with 100 mg/kg ketamine (Par Pharmaceutical, Chestnut Ridge, NY) and 10 mg/kg xylazine (Akorn, Inc., Lake Forest, IL), and 0.5% proparacaine (Akorn, Inc., Lake Forest, IL) was applied to the right eye of each mouse. A 27-gauge needle was used to create three parallel 1-mm scratches across the central right corneal epithelium and anterior stroma. Eyes were subsequently inoculated with 5ul of \textit{S. aureus} bacterial culture containing $10^7$ colony forming units (CFU). At 48 hours post infection, slit lamp biomicroscopy (Topcon SL-8Z with attached Nikon D80 digital camera) was used to evaluate each infection and a post-doctoral student (WJ) assigned a clinical severity score using the Hazlett et al. grading scheme: 0, no corneal opacity; 1, faint corneal opacity; 2, dense corneal opacity overlying pupil; 3, dense corneal opacity covering entire cornea; 4, corneal perforation. SLP images were obtained for quantitative analysis. A certified ophthalmic photographer performed all imaging with defined camera settings (camera setting mode: manual, shutter speed setting: 60, optimize image setting: professional, image quality setting: normal, image file size setting: normal, while balance setting: automatic, sensor sensitivity setting: ISO 1000, noise reduction setting: off, sensor sensitivity sub setting: normal, multiple exposure setting: off) and under diffuse slit lamp lighting. Mice with grade 0 infections were excluded due to the lack of SI to annotate, and grade 4 infections were not observed. Animals were subsequently euthanized, and eyes enucleated for quantification of ocular bacterial burden.

**Bacterial Culture**

\textit{S. aureus} strains were grown overnight at 37°C on both Mueller-Hinton agar (Fisher Scientific, Hampton, NH) and in Mueller-Hinton broth. Broth cultures were centrifuged at 2,000 \times g for 10 minutes at 4°C, and bacterial cell pellets were resuspended in phosphate-buffered saline (PBS) in a volume equivalent to the starting volume.

**Corneal Image Analysis**

The SI was annotated on SLP images using ImageJ software (National Institute of Health, Bethesda, MD) by each of two study team members (LA, LK) masked to the clinical severity grading. Horizontal corneal diameter was also annotated to assess the relative size of the SI. Features were first quantified using pixels for the diameter and squared pixels for the SI area. Measures were then converted to millime-
ters and squared millimeters by using the previously published mean murine corneal diameter conversion of 2.6 mm.16

**Evaluation of Bacterial Burden**

After the animals were euthanized and enucleated, the whole eyes were homogenized and CFU were enumerated to determine the bacterial burden (CFU/mL). To do so, samples were suspended in tubes containing 1.4 mm ceramic beads (Fisher Scientific, Hampton, NH) and 0.5 ml phosphate-buffered saline (PBS) and homogenized using the Fisher brand Bead Mill homogenizer. Aliquots (0.1 ml) were then removed, serially diluted in 0.8% sodium chloride, and plated on mannitol salt agar plates (Fisher Scientific, Hampton, NH) for enumeration after incubating at 37°C for 16 hours.

**Statistical Methods**

BK characteristics (clinical severity grade, SI area for each grader, and bacterial burden) were summarized with descriptive statistics including mean, standard deviation (SD), median, frequency, and percentage. Scatterplots were used to display the relationship between pairs of BK characteristics. Spearman rank correlations were used to assess the association between BK characteristics. SI area was assessed for inter-grader reliability with the Dice similarity coefficient (DSC).17 SI area and bacterial burden were compared between clinical severity grades with Kruskal-Wallis (KW) testing and post-hoc pairwise comparisons using the Dwass, Steel, Critchlow-Fligner (DSCF) multiple comparison test method. All statistical analysis was performed using SAS version 9.4 statistical software (SAS institute, Cary, NC).

**Results**

A total of 14 mice with *S. aureus* BK were studied. Table 1 presents descriptive statistics of BK characteristics for the sample. Specifically, 28.6% of infections were clinical severity grade 1 (n = 4), 42.9% were grade 2 (n = 6), and 28.6% were grade 3 (n = 4). Bacterial burden was on average 315,511 CFU/mL (SD = 830,192, median = 4,750).

SI area was on average 0.92 mm² (SD = 0.65, median = 0.83) when annotated by grader 1 and 1.03 mm² when annotated by grader 2. SI area showed fair inter-grader reliability (DSC = 0.69, 95% confidence interval: 0.58–0.81). Example SLP images and respective annotations from mice with *S. aureus* BK are shown in Supplementary Figure S1.

**Clinical Severity and Stromal Infiltrate Area**

A significant positive correlation was observed between clinical severity grade and SI area (grader 1: Spearman rank correlation = 0.59, p = 0.028, Fig. A; grader 2: Spearman rank correlation = 0.77, p = 0.001, Fig. B). As clinical severity grade increased, mean SI area also increased from annotations from grader 1 (severity grade 1: 0.55 mm² [SD = 0.49], severity grade 2: 0.75 mm² [SD = 0.61], severity grade 3: 1.53 mm² [SD = 0.47]) and grader 2 (severity grade 1: 0.36 mm² [SD = 0.29], severity grade 2: 0.70 mm² [SD = 0.36], severity grade 3: 2.17 mm² [SD = 0.16]). However, these group differences did not achieve statistical significance (Table 2, grader 1: KW p = 0.087,

| Continuous Variable | Mean (SD) | Min, Max | Median |
|---------------------|-----------|----------|--------|
| Stromal Infiltrate Area (mm²) - Grader 1 | 0.92 (0.65) | 0.07, 1.93 | 0.83 |
| Stromal Infiltrate Area (mm²) - Grader 2 | 1.03 (0.98) | 0.04, 3.44 | 0.75 |
| Bacterial Burden (CFU/mL) | 315511 (830192) | 10, 3000000 | 4750 |
| Categorical Variable | Frequency (Percentage) |
| Grade | 1 | 4 (28.6) |
| | 2 | 6 (42.9) |
| | 3 | 4 (28.6) |

SD, Standard Deviation; Min, Minimum; Max, Maximum; CFU, Colony Forming Units; mm, millimeter; mL, milliliter.
Figure. Relationship between bacterial keratitis characteristics including (A) stromal infiltrate area as annotated by grader 1 and BK severity grade, (B) stromal infiltrate area as annotated by grader 2 and BK severity grade, (C) bacterial burden and stromal infiltrate area as annotated by grader 1, (D) bacterial burden and stromal infiltrate area as annotated by grader 2, and (E) bacterial burden and BK severity grade. Best fit linear regression line is also plotted for each pair of bacterial keratitis measures. BK, Bacterial Keratitis; CFU, Colony Forming Units; mm, millimeters; mL, milliliter.
**Table 2. Comparison of Bacterial Keratitis Characteristics by Clinical Severity Grade.**

| BK Characteristic | Severity Grade 1 (n = 4) | Severity Grade 2 (n = 6) | Severity Grade 3 (n = 4) | P-Value* |
|-------------------|--------------------------|--------------------------|--------------------------|----------|
|                   | Mean (SD) | Min, Max | Median | Mean (SD) | Min, Max | Median | Mean (SD) | Min, Max | Median |                       |
| Stromal Infiltrate Area (mm²) - Grader 1 | 0.55 (0.49) | 0.07, 1.15 | 0.48 | 0.75 (0.61) | 0.08, 1.51 | 0.60 | 1.53 (0.47) | 0.94, 1.93 | 1.63 | 0.0871 |
| Stromal Infiltrate Area (mm²) - Grader 2 | 0.36 (0.29) | 0.04, 0.74 | 0.33 | 0.70 (0.36) | 0.29, 1.28 | 0.68 | 2.17 (1.16) | 0.88, 3.44 | 2.18 | 0.0190* |
| Bacterial Burden (CFU/mL) | 3788 (2834) | 1500, 3075 | 3750 | 6502 (9251) | 10, 25000 | 3750 | 1090750 (1365504) | 45000, 659000 | 659000 | 0.0180* |

**Quantitative Image Analysis of Bacterial Keratitis**

In this study, quantitative image analysis of SI area, one key component of BK disease severity, was explored to develop an alternative objective measure of keratitis severity. Our results show that worse clinical severity grading was significantly correlated with increased SI area, but group comparisons did not achieve statistical significance. There are a few possible reasons that there was not a statistically significant association for group comparisons between SI area and the clinical severity grade. First, the clinical severity score grades more features than just SI area. Second, the sample was small so the intrinsic error in both methods could have masked a statistically significant correlation in group comparisons. Third, severity of BK is due to several factors, including host immune response and pathogen virulence, which may have a differential impact on clinical severity grade versus infiltrate area. For example, density of corneal opacity and location compared to visual axis are part of the Hazlett et al. grading scheme but is not evaluated in our described quantification of SI area. A quantitative method to evaluate BK SLP images that includes centration and density of corneal opacity may improve upon subjective methods for clinical grading of BK.

**Discussion**

Increased SI area showed a trend towards increased bacterial burden but did not achieve statistical significance. It is unclear if these measures are not related or if this is a factor of the small sample size. Bacte-
rial burden in ocular tissues is a quantitative but indirect measure of keratitis severity as severity is also affected by other host and pathogen factors (e.g., immune host response). \[^{14,18,19}\] Practically, bacterial burden requires animals be sacrificed, so it precludes this measure of severity to be used longitudinally in the same mouse. SLP image analysis can be obtained in vivo, providing greater utility by allowing for longitudinal studies and evaluation of rate of change in SI area. Use in longitudinal studies also allows comparing morphologic changes over time in response to interventions.

Though quantification of SI area may improve objectivity as compared to a severity grading scale, it is still subject to error. SLP has shown utility in many clinical studies. \[^{8}\] However, SLP acquisition may introduce variability due to differences in positioning, lighting, and image quality. SLP annotation completed by different people may also introduce variability due to differences in interpreting the location of the SI border. A standardized protocol for SLP acquisition and annotation could minimize variability. SI area does not account for other morphologic features such as depth or density of corneal opacity, both of which may be important prognostic indicators in BK. Grading of peripheral or discontinuous infiltrates may have differences in grading and SI quantification. Future plans include refining SLP annotation interpretation by subtracting areas of clearing within infiltrate and to quantify degree of opacification. Other imaging modalities such as ocular coherence tomography (OCT) \[^{20}\] may provide quantification of infiltrate depth or total infiltrate volume. As compared to OCT, SLP imaging is more widely available, requires less technical training, is a lower cost, and may provide better imaging of other features besides depth.

This study has several limitations. First, although it is typical to have small sample sizes in animal studies, this contributes to low power to find significant results. However, this study can provide initial estimates to form the basis of power and sample size calculations for detecting significant associations and group differences for futures studies. Second, bacterial burden showed a sizeable range of values (10 to 3,000,000 CFU/ml). Non-parametric statistical methods were used to account for this wide range, but the two large outlying values (bacterial burden >10\(^6\) CFU/ml) were highly influential and sensitivity analyses excluding these cases showed non-significant associations of bacterial burden with SI area and severity grade. However, these values are within the previously published range of bacterial burden for this model. \[^{21}\] Future work with a larger sample size will help explore if these values are typical or true outliers. It is important to note that the current grading system uses four levels for severity, so there must be a visible difference between each grade level. SI area is quantified as a continuous variable it may improve detection in smaller effect sizes between experimental groups. Another limitation of the study was the use of a previously published standard corneal diameter of 2.6mm. In the future, the white-to-white distance should be measured on individual mice to obtain more precise estimate of SI area. Lastly, this study did not include grade 4 infections as none were observed. This is potentially due to the relatively short 48-hour period at which clinical grades and bacterial burden were assessed. Inclusion of grade 4 infections in future studies may provide a more accurate measure of the associations between SI area, clinical severity, and bacterial burden.

The association of slit lamp quantified SI area with bacterial burden and clinical severity scores indicates this is a potentially new method to objectively measure BK disease severity. Further, image-based analysis would provide a quantitative, not categorical, assessment of severity, which may be able to discern smaller differences between experimental groups. Image-analysis can be expanded to quantify other morphologic features on SLP such as neovascularization, evaluate the distribution and degree of opacification, or may be applied to other animal models. Using the annotated SLP images and the current gold-standard clinical grading system, deep-learning image classification algorithms could be developed.

There is potential for quantified image-based methods to measure disease severity in animal models. This will require validation in larger data sets, but if proven, may provide researchers with greater precision in measuring keratitis severity. This could allow hypothesis testing on smaller samples thus serving as a building block to better understand host and pathogen responses and the efficacy of interventions.

### Acknowledgments

This project was supported in part by a gift by Susan Lane and by the National Institutes of Health R01EY031033 (MAW), Research to Prevent Blindness Career Advancement Award (MAW), National Institutes of Health K08EYE29012 (RW); Research to Prevent Blindness Career Development Award (RW). The funding organizations had no role in study design or conduct, data collection, management, analysis, interpretation of the data, decision to publish, or preparation of the manuscript. MAW had full access
to the data and takes responsibility for the integrity and accuracy of the data analysis.

Discloser: T.E. Meijome, None; R. Wozniak, None; L. Kang, None; L. Azzouz, None; L.M. Niziol, None; W.L. Johnson, None; M. Kriegel, None; M.A. Woodward, None

References

1. Whitcher JP, Srinivasan M, Upadhyay MP. Corneal blindness: a global perspective. Bull World Health Organ. 2001;79:214–221.
2. Peng MY, Cevallos V, McLeod SD, et al. Bacterial keratitis: Isolated organisms and antibiotic resistance patterns in San Francisco. Cornea. 2018;37:84–87.
3. Orlans HO, Hornby SJ, Bowler ICJW. In vitro antibiotic susceptibility patterns of bacterial keratitis isolates in Oxford, UK: A 10-year review. EYE. 2011;25:489–493.
4. Hazlett LD, Moon MM, Strejc M, et al. Evidence for N-acetylmannosamine as an ocular receptor for P. aeruginosa adherence to scarified cornea. Invest Ophthalmol Vis Sci. 1987;28:1978–1985.
5. Hume EBH, Cole N, Khan S, et al. A Staphylococcus aureus mouse keratitis topical infection model: cytokine balance in different strains of mice. Immunol Cell Biol. 2005;83:294–300.
6. Patel TP, Prajna NV, Farsiu S, et al. Novel Image-Based Analysis for Reduction of Clinician-Dependent Variability in Measurement of the Corneal Ulcer Size. Cornea. 2018;37:331–339.
7. Kriegel MF, Loo J, Farsiu S, et al. Measurement Reliability for Keratitis Morphology. Cornea. 2020;39:1503–1509.
8. Wilhelmus KR, Mitchell BM, Dawson CR, et al. Slitlamp biomicroscopy and photographic image analysis of herpes simplex virus stromal keratitis. Arch Ophthalmol. 2009;127:161–166.
9. Chiang MF, Quinn GE, Fielder AR, et al. International classification of retinopathy of prematurity. Ophthalmology. 2021;128:e51–e68.
10. Varma R, Steinmann WC, Scott IU. Expert agreement in evaluating the optic disc for glaucoma. Ophthalmology. 1992;99:215–221.
11. Powell DR, Nichols JJ, Nichols KK. Inter-examiner reliability in meibomian gland dysfunction assessment. Invest Ophthalmol Vis Sci. 2012;53:3120–3125.
12. Yang K, Wu M, Li M, et al. miR-155 Suppresses Bacterial Clearance in Pseudomonas aeruginosa–Induced Keratitis by Targeting Rheb. J Infect Dis. 2014;210:89–98.
13. Me R, Gao N, Dai C, et al. IL-17 Promotes Pseudomonas aeruginosa Keratitis in C57BL/6 Mouse Corneas. J Immunol. 2020;204:169–179.
14. Girgis DO, Sloop GD, Reed JM, et al. Effects of toxin production in a murine model of Staphylococcus aureus keratitis. Invest Ophthalmol Vis Sci. 2005;46:2064–2070.
15. El Feghaly RE, Stauber JL, Deych E, et al. Markers of intestinal inflammation, not bacterial burden, correlate with clinical outcomes in Clostridium difficile infection. Clin Infect Dis. 2013;56:1713–1721.
16. Henriksson JT, McDermott AM, Bergmanson JGP. Dimensions and morphology of the cornea in three strains of mice. Invest Ophthalmol Vis Sci. 2009;50:3648–3654.
17. Zou KH, Warfield SK, Bharatha A, et al. Statistical validation of image segmentation quality based on a spatial overlap index. Acad Radiol. 2004;11:178–189.
18. Hobden JA, Masinick SA, Barrett RP, et al. Proinflammatory cytokine deficiency and pathogenesis of Pseudomonas aeruginosa keratitis in aged mice. Infect Immun. 1997;65:2754–2758.
19. O’Brien TP. Management of bacterial keratitis: beyond exorcism towards consideration of organism and host factors. Eye. 2003;17:957–974.
20. Boote C, Du Y, Morgan S, et al. Quantitative assessment of ultrastructure and light scatter in mouse corneal debridement wounds. Invest Ophthalmol Vis Sci. 2012;53:2786–2795.
21. Chojnacki M, Philbrick A, Wucher B, et al. Development of a Broad-Spectrum Antimicrobial Combination for the Treatment of Staphylococcus aureus and Pseudomonas aeruginosa Corneal Infections. Antimicrob Agents Chemother. 2018;63(1):e01929–e02018, doi:10.1128/AAC.01929-18.