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Staphylococcus aureus Alpha-Hemolysin Impairs Corneal Epithelial Wound Healing and Promotes Intracellular Bacterial Invasion

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Abstract

Colonization by *Staphylococcus aureus (S. aureus)* has been implicated in many infectious and wound healing disorders. This study was performed to characterize the pathogenic role of *S. aureus* alpha-hemolysin (alpha-toxin) in corneal epithelial wound healing and infectious keratitis in the setting of a corneal wound. The effect of wild-type and isogenic Hla mutant (α-hemolysin gene deleted) *S. aureus* bacteria and conditioned media on corneal epithelial wound healing was tested *in vitro* using a scratch assay and *in vivo* using a murine epithelial debridement model. The invasiveness of wild-type and Hla mutant *S. aureus* was evaluated *in vitro* in human corneal epithelial cells and *in vivo* in a murine model of infectious keratitis following total epithelial debridement. *S. aureus* and its conditioned media significantly delayed epithelial wound closure both *in vitro* ($P<0.05$) and *in vivo* ($P<0.05$). The effect of *S. aureus* on wound healing was significantly diminished with the Hla mutant strain ($P<0.05$). Likewise, compared to the wild-type strain, the Hla mutant strain demonstrated significantly reduced ability to invade corneal epithelial cells *in vitro* ($P<0.05$) and infect murine corneas following total epithelial debridement *in vivo* ($P<0.05$). In conclusion, *S. aureus* alpha-hemolysin plays a major role in the pathologic modulation of corneal epithelial wound healing and the intracellular invasion of the bacteria.

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None

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Limiting colonization by *S. aureus* and/or blocking alpha-hemolysin may provide a therapeutic approach for corneal wound healing and infectious disorders.

Keywords

Staphylococcus aureus; alpha-toxin; alpha-hemolysin; cornea; keratitis; infection; wound healing

1. Introduction

Staphylococcus aureus (S. aureus) is commonly found as a part of the normal flora of the skin, nasal mucosa and eyelids (Callegan et al., 1994). It is one of the leading causes of infections in soft tissue such as the skin and the eye; in particular, it is a frequent cause of bacterial corneal infections (keratitis) (Callegan et al., 1994; Girgis et al., 2005; Green et al., 2008; Hume et al., 2001; Klevens et al., 2007; O'Callaghan et al., 1997; Salgado-Pabón et al., 2014a). *S. aureus* keratitis can progress rapidly, resulting in corneal scarring, perforation and significant loss of vision (Callegan et al., 1994; Girgis et al., 2005; Green et al., 2008; Hume et al., 2001; O'Callaghan et al., 1997). It is more common in immunocompromised, diabetic and HIV patients (Hume et al., 2006). People using contact lenses are also at a higher risk of developing this problem (Hume et al., 2006). The estimated annual incidence of microbial keratitis is 500,000 patients world-wide and 30,000 patients in the United States (Hayashida et al., 2015; Wilhelmus, 2002). Infection of the cornea often starts with the spreading of bacteria from adjacent skin or lid margin and progresses by penetrating the tissue with the aid of its virulence factors (Hume et al., 2001). Previous studies have demonstrated that alpha-hemolysin (alpha-toxin) plays an important role in this process (Callegan et al., 1994; McCormick et al., 2009; O'Callaghan et al., 1997).

Besides its role in infectious keratitis, *S. aureus* has also been implicated in pathologic corneal wound healing, however, the specific mechanisms related to this process have not been studied extensively (Brothers et al., 2015). An epithelial defect is a major risk factor for the development of infectious keratitis given the critical role of the corneal epithelial barrier (Reim et al., 1997). Corneal epithelial wound healing complications are associated with inability to reform the epithelial attachments of the corneal surface after injury, which leaves deeper cell layers vulnerable (Reim et al., 1997). One study suggested that some *S. aureus* strains inhibit epithelial cell migration in the cornea, which could impact the corneal epithelial wound healing (Brothers et al., 2015). Aside from that study, only a few other studies have been conducted to explore the effect of *S. aureus* on corneal epithelial wound healing.

Currently, there is limited data on the pathogenic role of *S. aureus* and alpha-hemolysin on corneal wound healing and intracellular invasion, especially in vivo (Girgis et al., 2005). Understanding these pathways is critical for developing novel therapies for many infectious and wound healing disorders involving *S. aureus.* Therefore, in this study, we have examined the effect of *S. aureus* and particularly the pathogenic role of secreted alpha-hemolysin in both wound healing and the invasiveness of *S aureus* in the cornea.

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2. Material and Methods

2.1. Human Corneal Epithelial Cell Culture

Telomerase-immortalized human corneal-limbal epithelial (HCLE) cell line (kindly provided by Ilene Gipson) was used for all the in vitro experiments (Gipson et al., 2003). HCLE cells were grown in keratinocyte serum-free medium (KSFM; Invitrogen, Grand Island, NY, USA) supplemented with 5 ng/mL of epidermal growth factor (EGF) and 50 μg/mL of bovine pituitary extract (BPE; Life Technologies, Grand Island, NY, USA).

2.2. Bacterial Cultures

For this study, wild-type *S. aureus* and isogenic Hla mutant *S. aureus* were used. This mutant strain does not express alpha-hemolysin. "The following reagents were provided by the Network on Antimicrobial Resistance in *S. aureus* (NARSA) for distribution by BEI Resources, NIAID, NIH: *S. aureus* subsp. aureus, Strain JE2, NR-46543 and *S. aureus* subsp. aureus, Strain JE2, Transposon Mutant NE1354 (SAUSA300_1058), NR-47896".

Stock cultures of various *S. aureus* strains were stored in 10% glycerol at −80°C. Bacteria were grown overnight in 100 mL of tryptic soy broth (TSB; Difco, Thermo Fisher scientific, USA) at 37°C. The bacterial pellets were washed with phosphate buffered saline (PBS) three times and re-suspended in 10 mL of PBS. This bacterial suspension was aliquoted into 500 μL per tube and stored at −80°C for future use. The bacterial count was measured by serial dilution and plating in tryptic soy agar (TSA; Difco, Thermo Fisher scientific, USA).

2.3. Fluorescein isothiocyanate (FITC) labeling of *S. aureus*

S. aureus strains were cultured overnight in TSB at 37°C in air with shaking. The bacteria were spun and washed one time with PBS and two times with carbonate buffer (pH 9.0). Bacterial pellets were re-suspended in carbonate buffer containing 100 μg/mL FITC conjugated Concanavalin A (Sigma-Aldrich, St. Luis, MO, USA) for one hour at room temperature. The bacteria were vigorously washed with PBS to remove excess FITC and divided in small volumes to keep at −80°C until use. *S. aureus* colony forming units (CFU) were measured on TSA plates incubated overnight at 37°C in air.

2.4. Gram Staining

Gram staining was performed with a slight modification to what was previously described (Brown and Hopps, 1973). Briefly, cryosections were fixed in 10% formalin and washed with PBS two times. The tissue sections were soaked with Hucker ammonium oxalate crystal violet (Carolina Biological Supply Company, Burlington, NC, USA) for 5 minutes at room temperature, and slides were briefly washed in water to remove excess crystal violet. Several drops of Gram iodine solution were applied to the tissue for two minutes and briefly washed in water. To decolorize and to remove any non-specific crystal violet staining, 95% ethanol was applied to the slides until the crystal violet was almost clear and then quickly rinsed in water. The sections were then soaked with several drops of safranin for one minute, followed by dehydration through a course of alcohols (95-100%) to xylene. The slides were then covered with a coverslip.

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2.5. Preparation of Conditioned Media from Bacterial Culture

Briefly, TSB in a 15 mL tube was inoculated with wild-type *S. aureus* (Cat# NR-46543) and isogenic Hla mutant *S. aureus* (Cat# NR-47896) from an agar plate and incubated for three hours at 37°C with shaking. Subsequently, 50 ml of fresh TSB was added and incubated overnight at 37°C with continuous shaking (150 rpm). The next day, the bacterial growth was measured by taking aliquots (OD600). The culture was stopped at late-logarithmic phase (OD600 ≤ 1). Bacteria were removed by centrifugation at 2000 rpm for one hour, and the supernatant was filtered through a 0.2 μm filter. The conditioned media (CM) was divided into small volumes in 15 mL tubes and preserved in −80°C for future experiments.

2.6. Thiazolyl blue tetrazolium bromide (MTT) Assay

Epithelial cell proliferation and viability was measured using a thiazolyl blue tetrazolium bromide (MTT; Sigma-Aldrich, St. Luis, MO, USA) assay as described previously (Mosmann, 1983). HCLE cells were plated in KSFM (basal media without growth factors) at a density of 5×10^3 cells per well in 96-well plates, and treated with wild-type *S. aureus* CM, isogenic Hla mutant *S. aureus* CM, or control (basal KSFM without growth factors) for 16 hours. After removing the media, 20 μL of 5 mg/mL MTT solution were placed in wells and incubated at 37°C for 3.5 hours. The solution was then removed and replaced by 150 μL of MTT solvent (4mM HCL, 0.1% NP-40, in isopropanol, according to the manufacturer's protocol). Plates were placed on a plate shaker for 15 minutes to enhance solubilization and the absorbance was measured at 562 nm with a 96-well plate reader (GENios plate reader; Tecan, Salzburg, Austria).

2.7. Western Blots Analysis

Wild-type *S. aureus* CM and isogenic Hla mutant *S. aureus* CM were subjected to Western blotting as previously described (Movahedan et al., 2013). Briefly, after measuring protein concentration (BCA assay; Pierce, Thermo Scientific, Rockford, IL, USA), equal amounts of each sample were mixed with Laemmli sample buffer (Bio-Rad, Hercules, CA, USA), denatured by heating at 70°C for 10 minutes, and subjected to electrophoresis on NuPAGE 4-12% bis-tris-gel (Invitrogen). After gel electrophoresis, the proteins were transferred to polyvinylidine fluoride (PVDF) membranes using iBlot gel transfer (Invitrogen). The membranes were then incubated in 5% BSA in tris-buffered saline with 0.03% Tween 20 (TBST) for one hour, followed by incubation with primary antibody while shaking in 4°C overnight. The following antibodies were used: mouse anti-Staphylococcal alpha hemolysin toxin mAb (8B7, IBT BioServices) and monoclonal rabbit anti-GAPDH (Cell Signaling 1:5000). After washes with TBST and incubation with respective horseradish peroxidase-conjugated secondary antibodies (anti-mouse 1:10,000 and anti-rabbit 1:20,000, both from Jackson ImmunoResearch) for one hour in room temperature, protein bands were visualized using the SuperSignal West Femto maximum sensitivity substrate (Thermo Scientific, Waltham, MA) with ImageQuant LAS 4000 biomolecular imager (GE Healthcare Life Sciences, Pittsburgh, PA).

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2.8. In Vitro Scratch Assay

HCLE cells were grown to confluence in 6 well plates. A linear scratch was made using a sterile 200 μL pipette tip and the wells were washed three times with PBS. Each well was then exposed to wild-type *S. aureus* CM, isogenic Hla mutant *S. aureus* CM, , or basal KSFM media (without growth factor and additives) as control. Photographs were taken on zero and six hours on Leica bright field microscopy. The remaining wound area was measured using ImageJ software (available in the public domain at www.nih.gov; National Institutes of Health [NIH], Bethesda, MD, USA).

2.9. In Vivo Wound Healing Assay

C57BL/6 mice were placed under anesthesia as described above after which a 2mm area of the central epithelium was marked using a blunt trephine and removed by gentle scraping using a blunt corneal scraper as previously described (Eslani et al., 2014; Movahedan et al., 2012). Following two-time rinses with 5 mL PBS, various treatments were applied to wounded areas. The different treatment groups included wild-type *S. aureus* CM (5 μL), isogenic Hla mutant *S. aureus* CM (5 μL), live wild-type and Hla mutant *S. aureus* bacteria (solution containing one million bacteria/each cornea), PBS (control) and TSB (control – since the live bacteria and bacterial conditioned media were prepared in TSB). A group with no treatment (control) was included as well. The treatments were applied once for each cornea. No further procedure was done afterwards. At 18, 20, 24 and 36 hours after treatments, the remaining wounded areas were stained with 1% fluorescein sodium (Akron, Lake Forest, IL, USA) and photographed under blue filter using Nikon FS-2 photo-slit lamp with Nikon D200 camera (Nikon, Melville, NY, USA). The remaining wound area was measured using ImageJ software (available in the public domain at www.nih.gov; National Institutes of Health [NIH], Bethesda, MD, USA).

2.10. In Vitro *S. aureus* Invasion Assay

To simulate the clinical scenario, where bacteria infect the corneal epithelium and ultimately lead to the loss of corneal epithelial cells, HCLE cells were used to study the invasiveness of *S. aureus.* HCLE cells were seeded in Millicell EZ slide 8-well glass (cat. #: PEZGS0816, Millipore Sigma) and 12 well plate one day before infection. The HCLE cells were then incubated in serum and additive free RPMI (RPMI 1640, Corning) for one hour and then washed three times with PBS. The cells were infected with *S. aureus* at multiplicity of infection (MOI) 100 in serum-free RPMI for one hour. For the cells in the slide 8-well glass, infection with *S. aureus* was performed in a similar fashion, using the FITC labeled bacteria.

Cells were then washed three times with PBS and incubated for one hour in the presence of 100 μg/mL gentamicin to kill the remaining extracellular bacteria. The cells in the 12 well plate were then washed three times with PBS and lysed with 100 μL of 0.5% Triton X-100. The cell lysates were serially diluted 1:10 several times in PBS, plated onto TSA containing Erythromycin (5 μg/mL), and incubated overnight at 37°C in air. Colonies were counted after 24 hours, and CFU per condition was calculated by multiplying the number of colonies grown on the plate by the dilution factor of the cultured lysate to determine the number of intracellular *S. aureus* bacteria. All bacterial cultures were done in triplicate and the results of CFU is presented as mean±SD for all experiments. The cells in the slide 8-well glass were

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then washed three times with PBS and imaged using Cell Observer SD Microscope (Carl Zeiss) with a 100× objective lens to detect the fluorescein labeled bacteria inside the cells.

2.11. In Vivo *S. aureus* Invasion Assay

C57BL/6 mice were used in this experiment and all procedures were conducted in compliance under the recommendations of the Association for Research in Vision and Ophthalmology (ARVO). The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Illinois at Chicago. The C57BL/6 mice were originally purchased from The Jackson Laboratory, and were house bred in the Biologic Resources Laboratory of the University of Illinois at Chicago according to the protocol. Eight to ten week old mice of both genders (with similar distribution in all groups) were used for all of the in vivo experiments, and each group consisted of five mice (one eye per mouse). Mice were anesthetized using injection of ketamine (100mg/kg) and xylazine (5mg/kg). Topical proparacaine 0.5% was applied to the cornea and the whole corneal epithelium was debrided from limbus to limbus using a scraper as previously described (Eslani et al., 2014; Movahedan et al., 2012). The debrided corneas were then rinsed two times with 5 mL PBS and then inoculated with either live wild-type *S. aureus* or Hla mutant *S. aureus* (5 μL solution containing 3 million bacteria/each cornea). The solutions were applied one time for each cornea and left on the eyes. No further procedure was done.

Slit lamp photography was taken after one day. The severity of the infection was assessed using a Bacterial Corneal Infection Scoring System (Table 1) (Cowell et al., 1999). At the end of the experiment mice were euthanized and eye balls were enucleated, washed twice with PBS and 5% gentamycin (to remove possible contamination and outer surface bacteria) and homogenized in 1.0 μL sterile PBS containing 0.01% Triton-x with sterile disposable tissue grinders. A 0.1-mL aliquot of the homogenate were serially diluted 1:10 in PBS and plated onto TSA containing Erythromycin (5 μg/mL) and incubated overnight at 37°C in air. Colonies were counted after 24 hours, and CFU per cornea was calculated by multiplying the number of colonies grown on the plate by the dilution factor of the cultured lysate to determine the number of intracellular *S. aureus* bacteria. All bacterial cultures were done in triplicate and the results of CFU is presented as mean±SD for all experiments.

2.12. Statistical analysis

All the in vitro and in vivo experiments where repeated three times. The in-vivo experiments were done in three independent sets, in groups of five mice, in which one eye was involved per mouse. Results are presented as the mean ± SD of all the experiments. Normality of the data was tested using D'Agostino & Pearson normality test. Based on normality test, Mann-Whitney U-test or two-sided student's t-test was performed to determine significance, which was set at $P<0.05$. For more than two arms comparison, one-way ANOVA with Tukey's post hoc correction was used. All statistics were performed using statistical and spreadsheet software (GraphPad Prism 7.0; GraphPad Software Inc., La Jolla, CA, USA, and Excel; Microsoft Corp., Redmond, WA, USA).

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3. Results

3.1. Live *S. aureus* delays Corneal Epithelial Wound Healing in Vivo via Alpha-Hemolysin

We first examined the effect of live wild-type *S. aureus* bacteria on corneal epithelial wound healing. At 24 and 36 hours after wounding, the degree of wound healing was assessed by fluorescein staining and documented photographically. Comparing the 24 and 36 hours and the zero hour photographs, we found that murine corneas exposed to live wild-type *S. aureus* showed significantly slower wound closure than control (no treatment; Fig 1). Considering previous findings implicating the role of alpha-hemolysin in *S. aureus* infection, we hypothesized that the detrimental effects of *S. aureus* on would healing could be mediated through this secreted toxin. To test our hypothesis, we also included a group exposed to live isogenic Hla mutant *S. aureus* bacteria (alpha-hemolysin gene deleted). At 24 and 36 hours after wounding, murine corneas exposed to isogenic Hla mutant *S. aureus* showed similar wound healing compared to control (no treatment), while this was significantly better than those exposed to wild-type *S. aureus* (Fig 1). We continued to observe the mice until the corneal wounds in the Hla mutant *S. aureus* group were completely closed (36 hours). By this time, the wild-type *S. aureus* group still had notable wound areas.

3.2. *S. aureus* Conditioned Media inhibits Corneal Epithelial Wound Healing in Vitro via Alpha-Hemolysin

To assess the effect of alpha-hemolysin on human epithelial cells, HCLE cells were subjected to scratch assay (as described in the methods) while exposed to either wild-type *S. aureus* (containing alpha-hemolysin gene) CM, isogenic Hla mutant *S. aureus* (alpha-hemolysin gene deleted) CM, or KSFM basal media (without growth factor and additives) as control. Western blotting of bacterial conditioned media confirmed that alpha-hemolysin was present in wild-type *S. aureus* CM and not present in isogenic Hla mutant *S. aureus* CM (Fig 2-A).

After six hours, no significant wound closure was observed in the cells treated with *S. aureus* CM, which was significantly different from those treated with KSFM only (control) ($P<0.001$). The cells treated with isogenic Hla mutant *S. aureus* CM migrated significantly more than those treated with wild-type *S. aureus* CM and had a higher wound closure ratio ($P<0.001$) (Fig 2-B).

MTT assay done on HCLE cells showed that the cells exposed to wild-type *S. aureus* conditioned media showed significantly less viability (47.3±4.6%) compared to those exposed to isogenic Hla mutant *S. aureus* conditioned media (110.1±8.2%), and basal KSFM (control, 100.0±8.5%) ($P<0.05$ for all the comparisons).

3.3. *S. aureus* Conditioned Media inhibits Corneal Epithelial Wound Healing in Vivo via Alpha-Hemolysin

Two-millimeter epithelial debridement wounds were created as described in methods, after which the murine eyes were treated with either wild-type *S. aureus* CM, TSB (control) or PBS (control), or left untreated (control). After 18, 24 and 36 hours, the wounded corneas treated with wild-type *S. aureus* CM showed significantly lower wound closure ratio

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compared to control groups (P<0.001 for 18 hours comparisons, P<0.05 for 24 hours and 36 hours comparisons) (Fig 3-A).

To assess the effect of alpha-hemolysin, we repeated this experiment using wild-type *S. aureus* CM and isogenic Hla mutant *S. aureus* CM. The corneas treated with isogenic Hla mutant *S. aureus* CM showed significantly higher wound closure ratio than those treated with wild-type *S. aureus* CM (P<0.001) (Fig 3-B).

3.4. *S. aureus* intracellular invasion in part depends on alpha-hemolysin

We investigated intracellular invasion of *S. aureus* in corneal epithelial cells and the role of alpha-hemolysin in this process in vitro. Wild-type *S. aureus* showed significantly more invasion into corneal epithelial cells compared to isogenic Hla mutant *S. aureus.* As shown in Figure 4-A, after one hour a higher number of fluorescent-labeled wild-type *S. aureus* bacteria were observed inside the cells in vitro compared to isogenic Hla mutant *S. aureus.* Likewise, the number of wild-type *S. aureus* inside the HCLE cells counted in 1 mL of culture lysate was significantly higher compared to isogenic Hla mutant *S. aureus* (P<0.05) (Fig 4-B).

3.5. Severity of murine *S. aureus* keratitis depends on alpha-hemolysin

Limbus to limbus epithelial debridement wounds were created, after which the murine eyes were exposed to either live wild-type *S. aureus* or live isogenic Hla mutant *S. aureus.* Using the described Bacterial Corneal Infection Scoring System (Table 1) (Cowell et al., 1999), after one day the mouse corneas exposed to wild-type *S. aureus* demonstrated a more severe infection (grade 3-4 for all corneas, mean±SD: 3.6±0.55) compared to those exposed to isogenic Hla mutant *S. aureus* (grade 0-1 for all corneas, mean±SD: 0.8±0.45, P<0.001) (Figure 4-C). Gram staining of the corneas also showed more bacterial colonies in the stroma of those exposed to wild-type *S. aureus* compared to those exposed to isogenic Hla mutant *S. aureus* (Figure 4-D). Consistent with these findings, the number of bacteria inside the ocular tissue counted in 1 mL of eye lysate was significantly higher in mice eyes infected with wild-type *S. aureus* compared to isogenic Hla mutant *S. aureus* (P<0.001) (Figure 4-E).

4. Discussion

The ocular microbiome plays a critical role in modulating many physiologic and pathologic states on the ocular surface. It plays a protective role in preventing colonization of pathogenic bacteria and its alteration by environmental insults, disease states, or contact lenses may trigger ocular inflammation (Fujimoto et al., 2008; Lu and Liu, 2016; Miller and Iovieno, 2009; Zegans and Van Gelder, 2014). On the other hand, the microbiome of the eyelid margin, and conjunctiva can be responsible for many cases of ocular surface infections, including bacterial keratitis and postoperative endophthalmitis (Callegan et al., 2002; Peral et al., 2016).

The most commonly cultured bacteria from the eyelids and conjunctiva are gram positive bacteria, mostly *Staphylococcus* species (Peral et al., 2016; Willcox, 2013). Furthermore, this local microbiome, specifically the *Staphylococcus* species, is well known to have a critical role in blepharitis and meibomian gland dysfunction (Lee et al., 2012; Miller and

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Iovieno, 2009; Watters et al., 2017). *S. aureus* is the most virulent of all *Staphylococcus* species, due to its production of factors that enhance host-adhesion, evasion of the human innate immune system, and cytolytic activity (Chang et al., 2015; Zecconi and Scali, 2013). Among these factors, alpha-hemolysin (alpha-toxin) is one of the major contributors to the pathogenic role of *S. aureus* on the ocular surface (Callegan et al., 1994; McCormick et al., 2009; O'Callaghan et al., 1997). Although topical antibiotics are commonly used to reduce bacterial load and symptom relief in the setting of infection and as prophylaxis in the setting of wounds (Lindsley et al., 2012; Miller and Iovieno, 2009; Watters et al., 2017), clinically, *S. aureus* has become increasingly resistant to common topical antibiotics used on the eye (Asbell et al., 2015; Chang et al., 2015; Vola et al., 2013).

In this study, we showed that wild-type *S. aureus* delays corneal epithelial wound healing both in vitro in human corneal epithelial cells and in vivo using the described murine model. To assess whether this inhibitory effect could be related to the secreted toxins of the bacteria, we investigated the effect of CM in the absence of live bacteria on corneal epithelial wound healing as well and observed that bacterial conditioned media similarly delays corneal epithelial wound healing significantly.

We confirmed that the inhibitory effect of *S aureus* on corneal epithelial wound healing could be attributed largely to alpha-hemolysin. The results suggest that patients colonized with *S. aureus*, are more prone to corneal epithelial wound healing delay and that alpha-hemolysin is the major contributor in this process.

We further investigated the invasiveness of *S. aureus* in vitro and in vivo. For in vivo experiments, we used a novel model of murine *S. aureus* keratitis. Researchers have tried various animal models to induce *S. aureus* keratitis in vivo and found it to be challenging (Marquart, 2011). For instance, topical inoculation of the rabbit cornea cannot induce keratitis without additional manipulations, such as intra-corneal injection of the bacteria or application of bacteria-soaked contact lenses (Marquart, 2011). Girgis et al. reported that mice, particularly C57BL/6, are relatively resistant to *S. aureus* corneal infection as well (Girgis et al., 2003; Marquart, 2011). Other researchers made modifications to the mouse model to induce more representative infection, including breaking the tear film before inoculation (Hume et al., 2005), or making a scar in the cornea using a trephine (Sun et al., 2006). To overcome this challenge, we developed a model where instead of just scratching the corneal epithelium prior to inoculation with *S. aureus,* we debrided the corneal epithelium from limbus to limbus. Using this model, we could consistently establish *S. aureus* infection in the cornea. We proceeded to test the various strains of *S. aureus* with this model. We confirmed previous observations that alpha-hemolysin plays a major role in the invasiveness of *S. aureus* and demonstrated this in our novel model of murine *S. aureus* keratitis (Callegan et al., 1994; Hume et al., 2001; McCormick et al., 2009; O'Callaghan et al., 1997; Salgado-Pabon et al., 2014b; Zecconi and Scali, 2013). Wound healing and infection are closely related clinical entities in the cornea. Bacterial keratitis is much more common in the setting of an epithelial defect. The infection model used for this study involved complete debridement of the epithelium in order to facilitate the invasion of the bacteria in to the stroma. As we have shown, the invasiveness of *S aureus* highly depends on alpha hemolysin, although other virulence factors are likely to be involved as well.

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Alpha-hemolysin is a cholesterol dependent cytolysin, which forms a large pore in the cell membrane, leading to cell lysis (Tweten, 2005). As showed in the viability assay results, alpha-hemolysin is responsible for significant decrease in cell viability. The observed delay in the wound healing could be partially attributed to this mechanism; the decreased number of viable cells limits the ability to heal the wound in a timely manner. On the other hand, it is well-known that an impaired epithelial barrier usually precedes and potentially exacerbates the infection, by making it easier for the pathogen to penetrate the deeper layers. We similarly showed that the pathogen armed with alpha-hemolysin is invasive, both in epithelial cells, and in murine eyes with impaired epithelial barrier. Overall, these results, suggest that treatment strategies that target the toxins and virulence factors of *S. aureus* may provide therapeutic approach for wound healing disorders that involve *S aureus.* Likewise, they may be useful adjunct to antibiotics (Asbell et al., 2015; Chang et al., 2015; Vola et al., 2013) given that antibiotics can only kill the bacteria and do not have any effect on the toxins that have already been released (McCormick et al., 2009).

5. Conclusion

In conclusion, we have demonstrated that *S. aureus* alpha-hemolysin plays a major role in the pathologic modulation of corneal epithelial wound healing and bacterial intracellular invasion. Limiting colonization by *S. aureus* and/or blocking alpha-hemolysin may provide a therapeutic approach for corneal wound healing and other infectious disorders.

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Highlights:

S. aureus α-hemolysin pathologically modulates corneal wound healing in vivo

S. aureus α-hemolysin plays a major role in the intracellular invasion of *S. aureus*

Murine *S. aureus* keratitis can be established after total epithelial debridement

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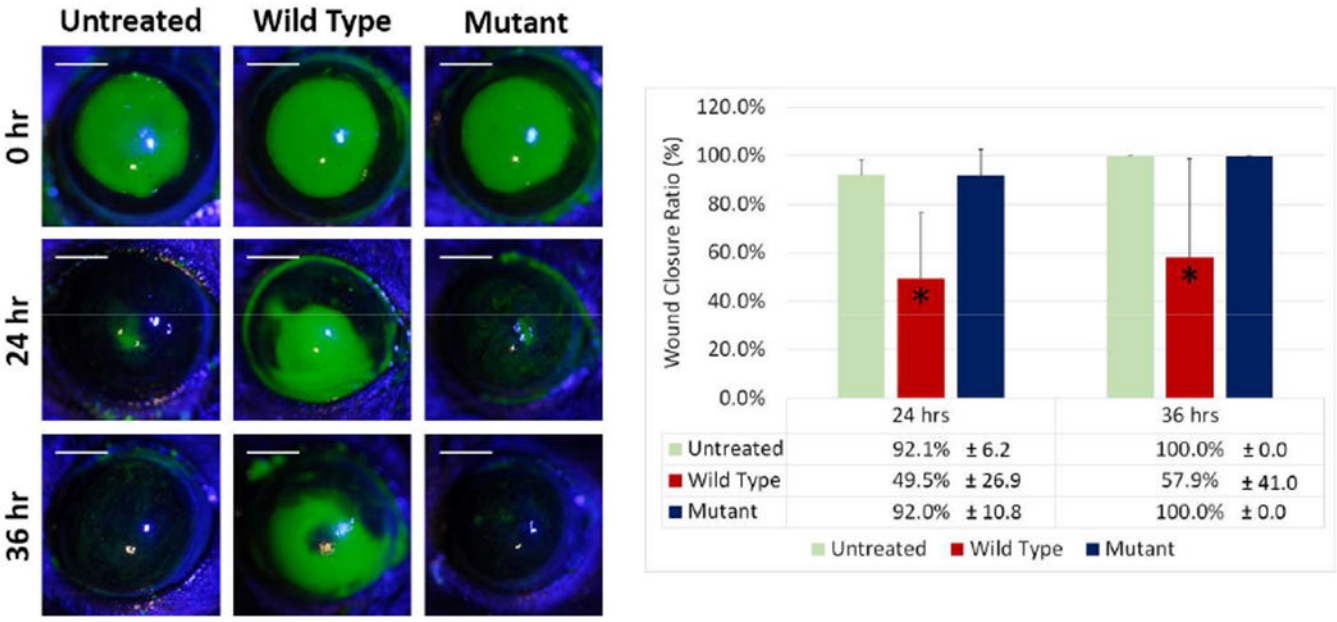

Figure 1:

The effect of live *Staphylococcus aureus* bacteria on corneal epithelial wound healing. Wounded murine corneas exposed to live wild-type *Staphylococcus aureus* showed significantly lower wound closure ratio after 24 and 36 hours compared to control (no treatment; ($P<0.01$ and $P<0.05$, respectively). Those exposed to live isogenic Hla mutant *Staphylococcus aureus* showed similar wound healing to control, and significantly better wound healing than wild-type *Staphylococcus aureus* ($P<0.01$ for 24 hours and $P<0.05$ for 36 hours). Scale bars: 1 mm, n=5.

*: Statistically significant

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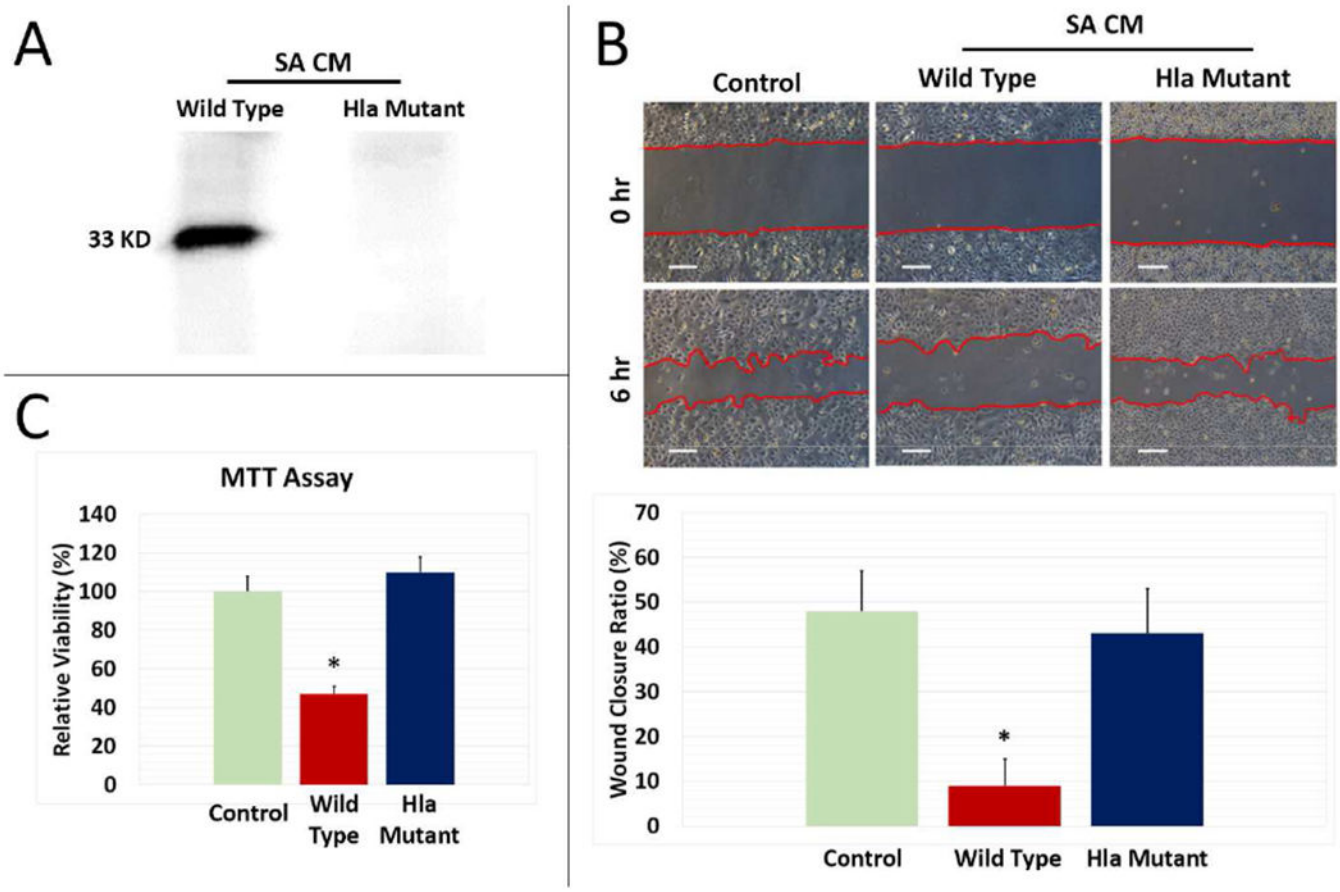

Figure 2:

The effect of wild-type *Staphylococcus aureus* conditioned media and isogenic H1a mutant *Staphylococcus aureus* conditioned media on corneal epithelial wound healing in vitro.

A: Western blotting confirmed that wild-type *Staphylococcus aureus* conditioned media contains alpha-hemolysin, while isogenic Hla mutant *Staphylococcus aureus* conditioned media does not contain alpha-hemolysin.

B: In the scratch assays done on HCLE cells, after 6 hours, wound closure ratio of the cells exposed to wild-type *Staphylococcus aureus* conditioned media was significantly lower than control (KSFM) (9.9±6.1% vs. 48.5±9.5%, P<0.001). HCLE cells exposed to isogenic Hla mutant *Staphylococcus aureus* conditioned media showed significantly higher wound closure ratio compared to those exposed to wild-type *Staphylococcus aureus* conditioned media (43.1±10.1% vs. 9.9±6.1%, respectively; P<0.001). Scale bars: 200 μm, n=3.

C: In the MTT assays, HCLE cells exposed to wild-type *Staphylococcus aureus* conditioned media showed significantly less viability (47.3±4.6%) compared to those exposed to isogenic H1a mutant *Staphylococcus aureus* conditioned media (110.1±8.2%), and basal KSFM (control, 100.0±8.5%) (P<0.05 for all the comparisons, n=8).

SA: *Staphylococcus aureus*

CM: Conditioned Media

*: Statistically significant

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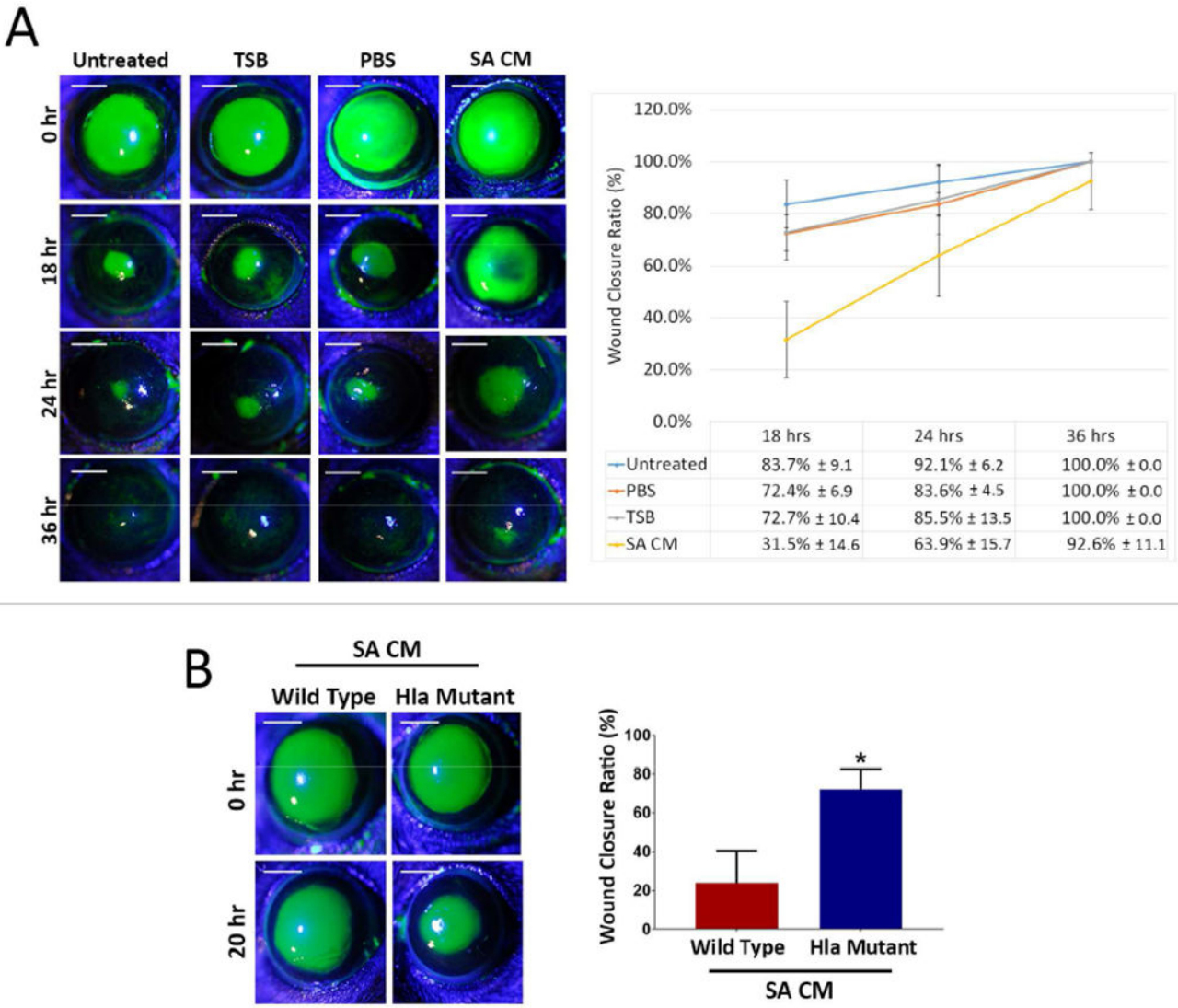

Figure 3:

The effect of wild-type *Staphylococcus aureus* conditioned media and isogenic Hla mutant *Staphylococcus aureus* conditioned media on corneal epithelial wound healing in vivo.

A: In the in vivo experiments, the corneas exposed to *Staphylococcus aureus* conditioned media (SA CM) showed significantly delayed wound healing compared to the control groups, which were exposed to either TSB or PBS, or no treatment (P<0.001 for 18 hours comparisons, P<0.05 for 24 hours and 36 hours comparisons). Scale bars: 1 mm, n=5.

B: Wounded mice corneas exposed to isogenic Hla mutant *Staphylococcus aureus* conditioned media showed significantly higher wound closure ratio compared to those exposed to wild-type *Staphylococcus aureus* conditioned media (72.1±10.2%vs. 23.7±16.8%, respectively; P<0.001). Scale bars: 1 mm, n=5.

SA: *Staphylococcus aureus*

CM: Conditioned Media

TSB: Tryptic soy broth

PBS: Phosphate buffered saline

*: Statistically significant

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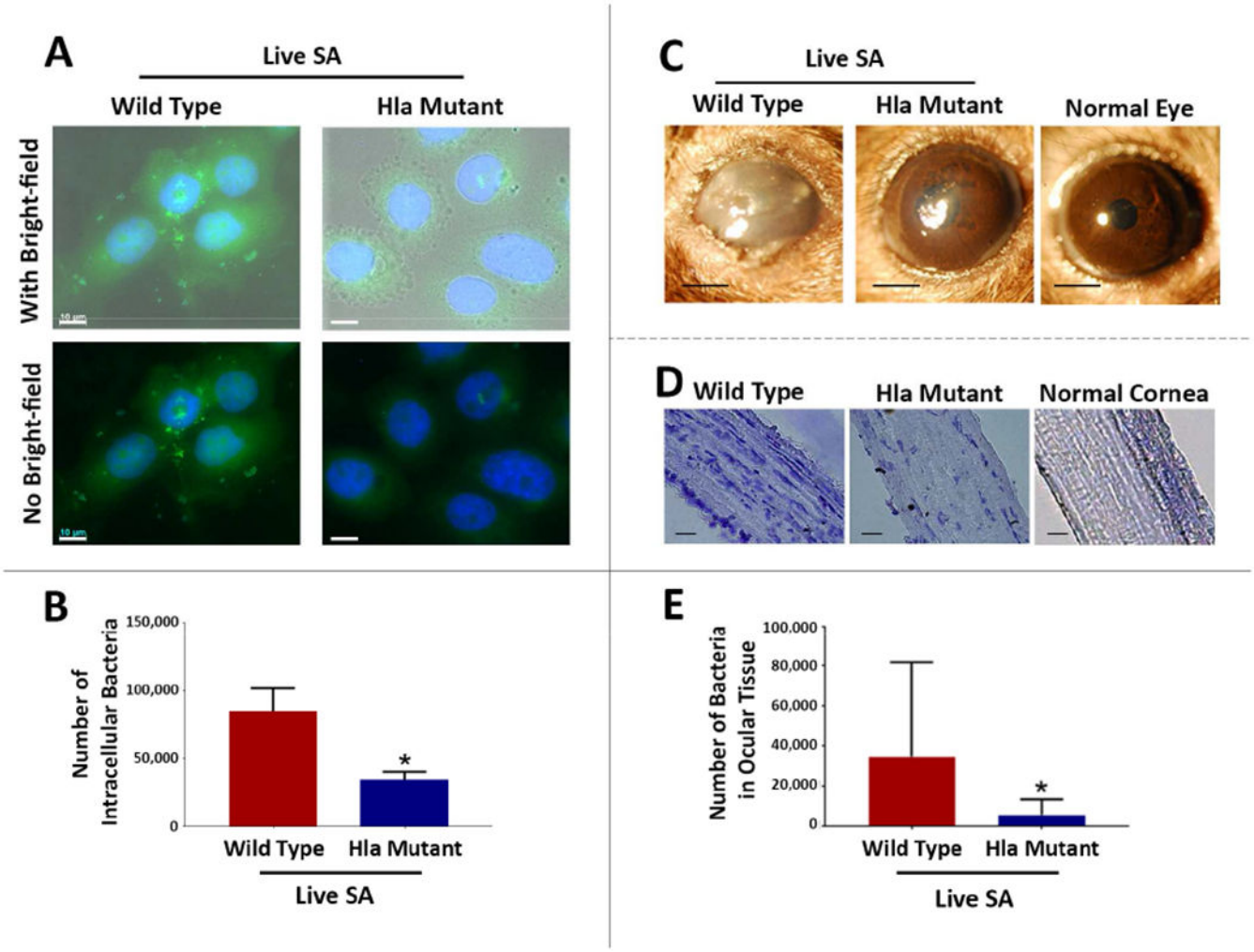

Figure 4:

Intracellular and corneal invasion of wild-type *Staphylococcus aureus* compared to isogenic Hla mutant *Staphylococcus aureus.*

A: Fluorescent imaging of HCLE cells after one hour exposure to FITC labeled wild type or Hla mutant *Staphylococcus aureus* showed that wild type *Staphylococcus aureus* bacteria were able to invade the cells more compared to Hla mutant *Staphylococcus aureus.* Blue color represents DAPI stained nuclei, and green color represents FITC labeled bacteria. Scale bars: 10 μm.

B: To quantify the in vitro intracellular invasion, measuring CFU in HCLE cell lysates demonstrated that the number of bacteria inside HCLE cells was significantly higher for those exposed to wild-type *Staphylococcus aureus* (84750±16660) compared to isogenic Hla mutant *Staphylococcus aureus* (34250±5737) in vitro ($P<0.05$, n=3).

C: The severity of infection measured by Bacterial Corneal Infection Scoring System was greater in the mice eyes exposed to wild type *Staphylococcus aureus* bacteria (grade 3-4 for all corneas, mean±SD: 3.6±0.55) compared to Hla mutant *Staphylococcus aureus* bacteria (grade 0-1 for all corneas, mean±SD: 0.8±0.45, $P<0.001$). Scale bars: 1 mm, n=5.

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D: Gram staining of the mice cornea exposed to either wild type *Staphylococcus aureus* or Hla mutant *Staphylococcus aureus* following epithelial debridement showed more wild-type *Staphylococcus aureus* bacteria in the corneal stroma compared to Hla mutant *Staphylococcus aureus.* Epithelial side is up and right, and endothelial side is down and left. Scale bars: 20 μm.

E: To quantify the in vivo invasion into the cornea and ocular tissues, measuring CFU of eye lysates demonstrated that the number of bacteria was significantly more in mice eyes exposed to wild-type *Staphylococcus aureus* (34805±46611) compared to those exposed to isogenic Hla mutant *Staphylococcus aureus* (6032±7506) ($P<0.05$, n=5).

SA: *Staphylococcus aureus*

*: Statistically significant

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Table 1:

Bacterial Corneal Infection Scoring System.

Grade	**0**	**1**	**2**	**3**	**4**
Area	None	1-25%	26-50%	51-75%	76-100%
Density of opacity	Clear	Slight cloudiness, details of pupil and iris describable	Cloudy, but outline of iris and pupil remains visible	Cloudy, opacity not uniform	Uniform opacity
Surface regularity	Smooth	Slight surface irregularity	Rough surface, some swelling	Significant swelling, crater of descemetocele formation	Perforation or serious descemetocele