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The Mouse Model for Ocular Surface *Staphylococcus aureus* Infection

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Abstract

To find an appropriate animal model that accurately reflects the disease and host defenses immune response to bacterial infection in humans is a major challenge in ocular surface infection research. Due to the variety of genetic backgrounds and targeted defects, the relatively low cost and ready availability, and the immunologic reagents available, mice are widely believed to be the ideal small animal model for ocular surface infection research for decades. By using of different combinations of mouse and bacterial strains, murine infection models can be used to explore a complete picture of bacterial infection and innate immunity in ocular surface. Studies using a murine model of *S. aureus* infection under normal ocular circumstances serve as a convenient and tractable model system that appears to be representative of mammalian host responses to pathogens.

Keywords

infection; cornea; *Staphylococcus aureus*; host defense; eye

INTRODUCTION

The protocols in this unit describe a murine model of ocular surface *S. aureus* infection for studying the responses of host defense and innate immune factors on the bacterial incursion and the impairment of the bacteria factors on ocular innate immunity. Scratch of the central corneal epithelium with a needle is the traditional model for studying bacterial keratitis which does not allow host defense resistant factors to be directly examined (Tam *et al.* 2007; Tang *et al.* 2013; Zaidi *et al.* 2013). The present protocols are easy to be used to address a variety of research questions related to ocular innate immunity that protects the eye under normal circumstances, and the factors that compromise ocular clearance of bacteria. The protocols illustrate preparation of bacterial working stocks for ocular surface infection (basic

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Conflict of Interest

The authors have declared no conflicts of interest for this article.

protocol 1), processing inoculation of murine ocular surface with *S. aureus* to induce an eye infection model (basic protocol 2), observation of clearance of bacteria in ocular surface (support protocol 1), assessment of the bacterial invasion index of epithelial cells (support protocol 2), analysis of phagocytic index of neutrophils (support protocol 3), and determination of corneal injury by fluorescein staining for infection mice (support protocol 4). The present protocols can be applied to study different aspects of ocular surface innate immunity after infection with different pathogens.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee and must follow officially approved procedures for care and use of laboratory animals.

NOTE: *S. aureus* is a biosafety level-2 (BSL-2) pathogen. All works with *S. aureus* should be conducted in a Class II biological safety cabinet (BSC). Appropriate precautions should be followed when handling this microorganism.

BASIC PROTOCOL 1. Ocular surface of mice inoculated with *S. aureus*

The traditional model for studying bacterial keratitis (abrading the central corneal epithelium with a needle) has been used in numerous laboratories. However, the host factors caused by innate immunity are not able to be directly studied by the model. Our present model provides a more straightforward way to examine the host factors that protect the eye under normal circumstances (Mun *et al.* 2009). Furthermore, the potential bacterial impairment factors can also be detected.

Materials

C57BL/6 mice, 8 to 12 week old, or other suitable strains of laboratory mice

Animal scale

Heated pads for surgical bed and recovery

Non-fenestrated sterile field drape

Sterile 1-ml plastic syringes and 25-G needles.

Pipette with sterile tip (appropriate for 5 µl volume)

Additional reagents and equipment for (ketamine/xylazine) anesthesia of the mouse

1. Ensure that all instruments are autoclaved prior to the procedure.
2. Set up the surgical bed with a heating pad covered by a non-fenestrated sterile field drape.
3. Weigh the mouse prior to the procedure.
4. Hold a mouse by grabbing the scruff of the neck and tail in one hand. Tilt the mouse so the abdomen is exposed and the head is pointed downward.
5. Insert the needle into the lower left or right quadrant of the abdomen just so the tip of the needle is in the mouse. Aspirate gently to ensure correct needle

placement. No fluid should be aspirated when the needle is correctly positioned. Inject with ketamine/xylazine (90 mg/kg ketamine, 10 mg/kg xylazine).

6. Once the mouse is adequately anesthetized (as assessed by complete suppression of pedal reflexes), the mouse is placed on its side on surgical bed. Using a pipette, slowly inoculate into the ocular surface of the mouse with a 5 μ l/eye suspension containing 10^7 – 10^8 CFUs of bacteria (Fig. 1).
7. Monitor animals on the heated recovery bed until they are fully awake before returning them back to their cage. Monitor the animals hourly after inoculation.

SUPPORT PROTOCOL1. Preparation of bacteria working stocks

The antibiotic-resistant strains of *S. aureus* such as Methicillin-resistant *S. aureus* (MRSA) are a worldwide problem in clinical medicine. *S. aureus* is also one of the most common causative organisms in patients presenting with sight-threatening corneal ulcers. Pathogenic strains of *S. aureus* often promote infections by producing potent proteintoxins, and expressing cell-surface proteins that bind and inactivate antibodies. We present the model using the *S. aureus* strain (ATCC 25923) since numerous laboratories studying innate immune response of ocular surface to the gram-positive coccobacterium utilizing this strain (Callegan *et al.* 1992; Hume *et al.* 1999; Oguz *et al.* 2005). Besides, a wide variety of solid and liquid media can support the growth of *S. aureus*, including tryptic soy broth or agar, as well as Dulbecco's modified Eagle's medium broth. Meanwhile, the inoculation of *S. aureus* in mice should approach a single colony.

Materials

S. aureus (ATCC 25923)

Tryptic soy agar plates

Tryptic soy broth

Phosphate-buffered saline (PBS), sterile

1. Culture *S. aureus* over night (16–18 h) on tryptic soy agar plates at 37°C.
2. Pick a typical isolated colony for further expansion.
3. Inoculate *S. aureus* bacteria into a 10 ml Tryptic soy broth.
4. Incubate broth cultures at 37°C in incubator with shaking at 250 rpm until they reach early-to-mid-log phase (an optical density (OD)₆₀₀ of 0.4 to 0.6; 12–14 h).
5. Sediment bacterial cells by centrifugation at 4°C at $1000 \times g$ for 10 min and washed three times with cold PBS followed by sedimentation.
6. Re-suspend the final suspension in 1 ml of PBS suspension to a bacterial concentration of 2×10^9 colony-forming units (CFU)/ml.
7. Perform 1:10 serial dilutions of the lysates in PBS, and plate 100 μ l of a range of dilutions as needed to achieve countable numbers of bacterial colonies (<100 CFU per plate section). Incubate plates for 18–24 h at 37°C in an incubator. The

CFU per milliliter value in final suspension is determined by counting the number of colonies on solid agar plates.

8. Use the bacterial suspension with appropriate CFUs for inoculation. (The final bacterial suspension must be kept on wet ice or at 4°C, and stored short than 1 h after it is ready for use.)

SUPPORT PROTOCOL2. Bacterial clearance from the ocular surface of mice

Clearance of *S. aureus* from the surface indicates the capability to eliminate bacteria in eyes of healthy mice. For this study, the concentration of *S. aureus* working stocks (e.g., 10^7 – 10^8 CFU in present experiments) is used, the innate immunity of the mouse ocular surface may rapidly scavenge the bacterial cells (Kreger 1983).

Materials

C57BL/6 mice or other suitable strains of laboratory mice inoculated with *S. aureus*

Tryptic soy agar plates

Pipette with sterile tip (appropriate for 5 µl volume)

Animal scale

Heated pads for surgical bed and recovery

Non-fenestrated sterile field drape

Sterile 1-ml plastic syringes and 25-G needles.

Additional reagents and equipment for (ketamine/xylazine) anesthesia of the mice

1. At 3 h post-inoculation, the mice are reanesthetized as described in basic protocol 1 and set up a surgical bed with a heating pad covered by a non-fenestrated sterile field drape is used.
2. Once the mice are anesthetized and no longer responds to a toe pinch, place the mouse on its side on the surgical bed.
3. Using a pipette with a sterile tip, administer 5 µl of PBS to the ocular surface.
4. After PBS was added for 30 second, collect tear fluid by the same pipette with another sterile tip from the lateral canthus of mouse (Fig. 1).
5. Assess viable bacteria in tear fluids using quantitative plating as described in support protocol 1, steps 7.
6. At 6, 12 and 24 hours post-inoculation, determine the number of viable bacteria within tear fluid by counting the number of colonies on solid agar plates.

NOTE: When quantitative plating, depending on the time point, dilutions ranging from 10^{-1} to 10^{-3} may be needed.

NOTE: To determine the sterile conditions in mice that have not been inoculated, a control experiment is needed by assessing the number of viable bacteria within tear fluid at certain time.

NOTE: To determine the influence of PBS on *S. aureus* survival, a control experiment is needed by analyzing the bacteria incubated in PBS for certain time.

SUPPORT PROTOCOL 3. Bacterial invasion index of epithelial cells

Expressing adhering and invading ability of *S. aureus* indicate the invasive characteristics of bacteria, and may produce keratoconjunctivitis in eyes (Vallas *et al.* 1999; Gadjeva *et al.* 2010). The invasive properties can be observed by the bacterial burden in an individual epithelial cell. We quantify bacterial invasion in epithelial cells using invasion index that represent the proportion of cells containing bacteria in total cell population.

Materials

C57BL/6 mice or other suitable strains of laboratory mice inoculated with *S. aureus*

Pipette with sterile tip (appropriate for 5 µl volume)

Animal scale

Heated pads for surgical bed and recovery

Non-fenestrated sterile field drape

Sterile 1-ml plastic syringes and 25-G needles.

Additional reagents and equipment for (ketamine/xylazine) anesthesia of the mouse

Cytospin centrifuge

Hema-3 Stain Kit

Light research microscope

1. At 3, 6, 12 and 24 hours post-inoculation, collect tear fluid from the ocular surface as described in support protocol 2, steps 1 to 5.
2. sediment the cells by centrifugation at 4°C at 250xg for 10 min and wash three times with cold PBS followed by sedimentation.
3. Resuspend the final suspension in 200 µl of PBS.
4. Mount the cells on slides by cytospin centrifugation.
5. Stain the slides using the Hema-3 Stain Kit.
6. Using light microscopy, randomly select one hundred epithelia per slide at ×1,000 magnification using oil immersion lens.
7. Count bacteria at adherence and internalization as invasion bacteria. Calculate the invasion index as the ratio of invasion bacteria to total bacterium-laden epithelial cells. Calculate the percentage of epithelial cells invaded by *S. aureus*.

(e.g., if there are 50 bacterium-laden epithelia in randomly select one hundred epithelia, and total invasion bacteria number in the 50 epithelial cells is 1000, the invasion index and percentage of epithelial cells invaded by *S. aureus* are 20 and 50%, respectively.)

SUPPORT PROTOCOL 4. Phagocytic index of neutrophils

Neutrophil, which forms an essential part of the innate immune system, is one of the first-responders of inflammatory cells to migrate towards the site of inflammation within minutes following trauma, and are the hallmark of acute inflammation. During the acute phase of *S. aureus* keratitis, intensive neutrophil infiltration is the main characteristic of the corneal inflammation. Neutrophils are phagocytes which ingest microorganisms, and internalize and kill many microbes. To indicate the phagocytic capability of neutrophils, phagocytic index is used in numerous studies.

Materials

C57BL/6 mice or other suitable strains of laboratory mice inoculated with *S. aureus*

Pipette with sterile tip (appropriate for 5 µl volume)

Animal scale

Heated pads for surgical bed and recovery

Non-fenestrated sterile field drape

Sterile 1-ml plastic syringes and 25-G needles.

Additional reagents and equipment for (ketamine/xylazine) anesthesia of the mouse

Cytospin centrifuge

Hema-3 Stain Kit

Light research microscope

1. At 3, 6, 12 and 24 hours post-inoculation, collect tear fluid from the ocular surface and stain the cells as described in support protocol 3, steps 1 to 5.
2. With light microscopy, analyze one hundred randomly selected neutrophils per slide at $\times 1,000$ magnification.
3. Cells that contain at least one bacterium count as bacteria-positive neutrophils. Calculate the phagocytic index (PI) as the percent of bacteria-positive neutrophils multiplied by the average number of bacteria per bacteria-positive neutrophil. (e.g., if there are 50 bacteria-positive neutrophils in randomly select one hundred neutrophils, and total invasion bacterial number in the 50 bacteria-positive neutrophils is 1000, the average number of bacteria per bacteria-positive neutrophil and percentage of epithelial cells invaded by *S. aureus* are 20 and 50%, respectively. PI value is 1000%, which results from $20 \times 50\%$.)

SUPPORT PROTOCOL 5. Corneal fluorescein staining

Fluorescein is thought to stain corneal lesion by dyeing devitalized and living epithelial cells. For this reason, corneal fluorescein staining is widely used in clinical practice and research as an indicator of damage to the corneal epithelium. In the present protocol, corneal fluorescein staining is used to evaluate the ocular injury caused by *S. aureus* on the ocular surface. Besides, there are varieties of methods to investigate the pattern of corneal fluorescein staining including overall corneal staining which is graded using a scale from 0 to 4 in one-half steps, and five corneal zones staining which divided cornea as superior, inferior, nasal, temporal, and central zone (Wei *et al.* 2011; Xiao *et al.* 2015; Zhang *et al.* 2015). However, the latter method is used more widely.

Materials

C57BL/6 mice or other suitable strains of laboratory mice inoculated with *S. aureus*

Pipette with sterile tip (appropriate for 5 µl volume)

Animal scale

Heated pads for surgical bed and recovery

Non-fenestrated sterile field drape

Sterile 1-ml plastic syringes and 25-G needles.

Additional reagents and equipments for (ketamine/xylazine) anesthesia of the mouse

1% sodium fluorescein

slit-lamp biomicroscope and camera

1. At 3, 6, 12 and 24 hours post-inoculation, prepare, anesthetize and place mouse on the surgical bed as described in support protocol 2, steps 1 to 2.
2. Using a pipette with a sterile tip, administer 1 µl of 1% sodium fluorescein to the inferior-lateral conjunctival sac of the mouse (Fig. 2).
3. 3 minutes after staining, photograph the mouse corneas with slit-lamp biomicroscope under cobalt blue light.
4. Staining for each of the five corneal zones (i.e., superior, inferior, temporal, nasal, and central) was scored for each zone as 0 (absent), 1 (regional or diffuse punctuate staining and moderate stipple staining), 2 (heavy stippling, dense coalesced staining), and 3 (diffuse loss of epithelium).

COMMENTARY

Background Information

Ocular surface infections include keratitis, conjunctivitis, and corneoscleral ulcers which affect the external part of the eye (Wilhelmus 1988; Limberg 1991; Miyazaki *et al.* 2008). As a cause of potentially sight-threatening condition, microbial corneal infection is one of the most widely studied diseases among ocular surface infections. *S. aureus* is a leading

cause of the potentially blinding microbial keratitis, especially in immunocompromised individuals, aged populations and users of daily wear contact lenses (Aristimuno *et al.* 1993; Cruz *et al.* 1993; Pachigolla *et al.* 2007; Chou & Prabhu 2011; Ji *et al.* 2015). Although antibiotic therapies may succeed in reducing or eliminating the bacterial load, scarring, loss of visual acuity and even blindness still result. In addition, *S. aureus* is particularly difficult to treat due to its ability to acquire resistance to many antibiotics. Keratitis caused by methicillin-resistant *S. aureus* (MRSA) has been documented for resistance to vancomycin, which is the drug of choice for MRSA (Lichtinger *et al.* 2012; Ni *et al.* 2015). A more in-depth understanding is a crucial step in developing specific drugs to treat infection. Consequently, the number of animal models of *S. aureus*-induced ocular surface diseases studies concerning about pathogenesis, prevention, and treatment of the infection has increased markedly in recent years.

This protocol introduces a murine model in which *S. aureus* is inoculated in normal ocular surface for the study of host–pathogen interactions. This situation is similar to the environment that eyes face daily. Bacteria adhere and invade the integrated ocular surface, and the ocular surface immune system removes the pathogenic microbes to keep the eye healthy. Due to the simplicity, speed, and reproducibility of the procedure, the protocol is helpful to study the host factors that protect the eye under normal circumstances and bacterial factors with the potential to compromise clearance. This model is also well suited for studies assessing basic antibiotic molecular processes of pathological ocular surface (e.g., dry eye disease) host defense system. Establishing a corneal stroma infection or corneal perforation mouse model is difficult because the host defense of murine ocular surface is very strong, and may rapidly scavenge the bacteria. The advantage of the protocol includes decreasing the difficulty of inducing the murine ocular surface infection mode, lowering the experimental cost and saving the experimental time. The protocol studies the severity of infection by collection of bacteria in ocular surface after inoculating with 5µl of PBS. This approach simplifies the experimental procedure, reducing the number of steps, providing more robust and reproducible outcomes.

Critical Parameters and Troubleshooting

The strain of mice and virulence of the strain of bacteria will significantly affect the outcome. Thus, these parameters should be carefully considered for the design of animal models of bacterial ocular surface infection. The difference of various strains of mice in their sensitivity to corneal challenge with bacteria has been demonstrated (Kreger 1983). For example, Swiss-Webster mice are more resistant to the development of experimental bacterial ocular surface disease than is C57BL/6 mice. In addition, selection of inoculum volume of bacteria is critical since variations in the dose of bacteria administered can significantly affect the experimental results. An excess volume of inoculums will spill from the conjunctival sac, while too little of the liquid leads to an insufficient amount of bacteria. The volume of 5µl inoculums is appropriate for the experiment. As unexpected head movements of mice may affect the exact amount of inoculum that reaches the ocular surface, the level of anesthesia can influence the outcome of bacterial infection in mice. The level of anesthesia may be assessed by lightly squeezing the mouse's forepaw with the finger tips. If the mouse tries to retract its paw, retest in a minute to ensure complete induction.

For ensuring a sufficient quantity of bacteria in the inoculum, the concentration of bacterial suspension used for inoculation should reach 1×10^8 CFU/ml. Meanwhile, the bacterial suspension must be kept on wet ice or at 4°C at all times. Sterile pipette tips should be changed at each inoculation of bacteria suspension and collection of tear fluid from the lateral can thus. In order to minimize the potential for cross-contamination, each mouse should be maintained in a separate cage under pathogen-free housing conditions and fed rodent chow and autoclaved water *ad libitum*. Each hour after inoculation, the animals should be monitored. Tissues harvested for infection evaluation should be kept on wet ice or refrigerated and processed as soon as possible.

For bacterial infection in ocular surface assays, corresponding samples from all of the experimental groups in the study should be tested at the same time.

Anticipated Results

This protocol is to study host–pathogen interactions in ocular surface with bacterial infection, e.g., assessing regulated mechanisms of host defense and impairment factors of bacterium for resistance to host innate immunity. The most common outcome measures that can be used in the protocol include counting of viable bacteria in tear fluids using quantitative plating to study the clearance of bacteria in ocular surface, western blotting analysis to examine the level of SP-D in murine tear fluid, the use of immunohistochemistry to observe the SP-D expression in the ocular tissues, evaluation of bacterial invasion index of epithelial cells and phagocytic index of neutrophils to determine the bacterial burden in an individual epithelial cell and phagocytosis of bacteria by neutrophils, respectively.

It is often preferable to select multiple time points 48–72 hours after inoculation to evaluate the clearance of bacteria from ocular surface and the response of host defense system. It is also optimal to use two or more methods to approach the assessment of every kind of ocular surface changing after inoculation (e.g., investigate the changes of certain proteins in ocular surface by using immunohistochemistry, Western blotting analysis and enzyme linked immunosorbent assay). As in all experimental research, the inclusion of appropriate controls is critical. After inoculation, the level of different factors in ocular surface innate immune system must be compared with a control group.

Time Considerations

To avoid cross-infection among different groups of mice, one experimental group should be finished before performing anesthesia and ocular intervention on the other group. The clearance of bacteria is often assayed for 48–72 hours post-inoculation. Phagocytosis of bacteria by neutrophils is detectable in ocular surface as early as ~ 6 h after inoculation. Corneal fluorescein staining is observed 12–24 hours after infection. Immunohistochemistry evaluation requires about 3 days. Measurement of protein concentrations by western blotting can be completed in 1 or 2 days. Mounting the cells in tear fluid on slides by cytospin centrifugation, and subsequently histopathological assessment can be completed in 4 hr. Finally, preparation of bacteria for inoculation requires 18–20 hours.

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Figure 1.
S. aureus inoculation and tear fluid collection in ocular surface of mice. (A) inoculate in right eye. (B) inoculate in left eye. (C) collect tear fluid from the lateral canthus of right eye. (D) collect tear fluid from the lateral canthus of left eye.

Figure 2. Corneal fluorescein staining. (A) administer 1 μ l of 1% sodium fluorescein to the inferior-lateral conjunctival sac of right eye. (B) administer 1 μ l of 1% sodium fluorescein to the inferior-lateral conjunctival sac of left eye.